Ultraviolet and Fluorescent Spectral Properties of the Divalent Cation Ionophore A23187 and Its Metal Ion Complexes†

Douglas R. Pfeiffer, Peter W. Reed,‡ and Henry A. Lardy*

ABSTRACT: The antibiotic ionophore A23187 and its metal ion complexes have been studied by spectrophotometric methods and by organic phase extraction techniques. The free acid in ethanol displays absorption peaks at 378, 278, 225, and 204 nm with respective molar extinction coefficients of 8,200, 18,200, 26,200, and 28,200. Ultraviolet and fluorescent spectral properties are dramatically altered by metal ion complexing. Difference spectral titrations in ethanol and organic phase extraction experiments show a uniform stoichiometry, A23187:Me^{2+}, of 2:1 for Mn^{2+} and alkaline earth cations. These complexes appear also to be otherwise structurally analogous by spectophotometric criteria. The stability of these complexes decreases with increasing solvent polarity. The relative complex stabilities determined by an organic phase extraction technique are Mn^{2+} (210) $\gg$ Ca^{2+} (2.6) $\simeq$ Mg^{2+} (1.0) $\gg$ Sr^{2+} (0.012) > Ba^{2+}. In addition, an H^+-Ca^{2+} binding competition is indicated. This high selectivity indicates the binding of unsolvated metal ions. A23187 binds monovalent cations weakly in the order $Li^+ > Na^+ > K^+ \simeq 0$. Of these, only Li^+ is transported into a bulk organic phase by the antibiotic. Spectral studies further indicate the formation of antibiotic complexes with the Ca^{2+} transport inhibitors La^{3+} and ruthenium red. The two affinity series for mono- and divalent cations suggest that the specificity for divalent ions is a consequence of both charge interactions and of the small number of complexing ligands available from a single antibiotic molecule.

Ionophorous antibiotics that reduce membrane permeability barriers to biologically important alkali cations have provided a powerful experimental tool for investigating the roles of transmembrane monovalent cation distributions in regulating complex biological phenomena (see Henderson (1971) and Lardy and Ferguson (1969) for reviews). The high specificity that ionophores such as valinomycin display toward alkali metal ions (see Kinsky (1970) and Pressman and Haynes (1969) for reviews) has also been considered as a model system which may help explain the specificity of ion pumps in natural membranes (Grell *et al.*, 1972, 1974). More recently ionophorous antibiotics active toward divalent cations have become available. The known compounds are X537A (Pressman, 1972; Scarpa and Inesi, 1972; Caswell and Pressman, 1972), A23187 (Reed, 1972; Reed and Lardy, 1972a,b; Caswell and Pressman, 1972; Reed *et al.*, 1974; Wong *et al.*, 1973), beauvericin (Roeske *et al.*, 1974), and possibly even avenaciolide (Harris and Wimhurst, 1973). A23187 is potentially the most useful of these ionophores for specifically perturbing the transmembrane distribution of divalent cations since it does not act as a monovalent cation ionophore (Reed, 1972; Reed and Lardy, 1972a,b; Reed *et al.*, 1974), whereas the other compounds lack this specificity. Of the available compounds, it might also be expected to provide the most appropriate specificity model, both because of divalent over monovalent specificity, and because it displays maximum affinity for divalent ions of physiological importance whereas X537A is bound most tightly by Ba^{2+} (Johnson *et al.*, 1970; Pressman, 1972).

It is apparent that the usefulness of this ionophore will be further enhanced by a more detailed understanding of its chemical properties and mechanism of ion transport. In this report we present ultraviolet and fluorescence spectral properties of A23187 as the free acid and as metal ion complexes. In addition, the order of binding selectivity of the compound for a number of ions of biological interest has been determined.

Materials and Methods

Absorption and fluorescence spectra were recorded on the Cary 15 spectrophotometer and the Aminco-Bowman spectrophotofluorometer, respectively. The absorbance scale of the spectrophotometer was calibrated against standard potassium dichromate solutions according to the National Bureau of Standards letter circular LC-1017. *N,N*-Dimethylformamide–ethanol (1:3) was used as solvent for stock solutions of A23187 (Reed and Lardy, 1972a). However, due to the basicity of dimethylformamide and to its producing spectral interferences below 240 nm, aliquots from the stock solution of antibiotic were dried in a stream of N_2 and redissolved in ethanol before recording spectral data. The solubility of A23187 in pure ethanol was in excess of 200 μM. The addition of small amounts of H_2O to A23187 in ethanol resulted in minor alterations in the spectral properties of the antibiotic. Therefore, when titrating A23187 with metal ions, each point was obtained by the addition of the indicated amount of metal chloride in a single aliquot of H_2O (5 μl/1.50 ml of antibiotic solution) rather than by repetitive additions.

The extraction of metal ion from aqueous to organic phases induced by A23187 was performed as previously described (Reed and Lardy, 1972a) with minor modifications of the sample preparation technique. For all cations except sodium, after evaporation *in vacuo* of organic phase aliquots, the residue was ashed in Pyrex tubes with 0.1 ml of H_2SO_4. Heating at about 400° was continued to dryness. The ashed material was extracted with 0.5 M HCl and the ion content measured with a Perkin-Elmer Model 403 atomic absorption spectrophotome-

† From the Department of Biochemistry and Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706. *Received April 3, 1974.* This work was supported by grants from the National Institutes of Health (AM-10334) and from the Graduate School Research Committee.

‡ Established investigator of the American Heart Association. Present address: Department of Physiology, University of Massachusetts Medical Center, Worcester, Mass. 01605.

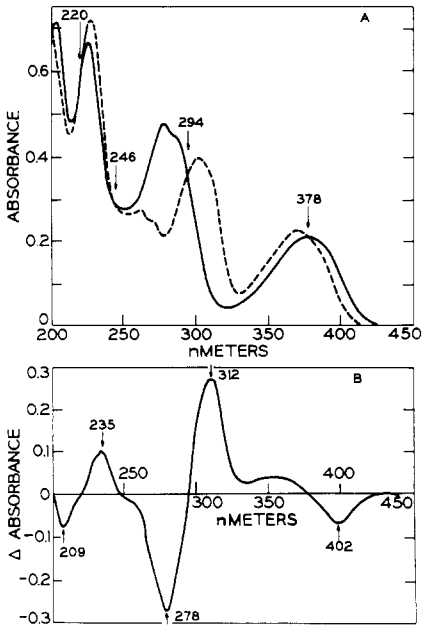

FIGURE 1: The effect of $MgCl_2$ on the absorption spectrum of A23187 in ethanol: (A) solid line, the spectrum of 25.7 μM A23187 in ethanol; dashed line, the spectrum after the addition of 133 μM $MgCl_2$; (B) the sample cuvet contained 25.7 μM A23187, 133 μM $MgCl_2$, and 2 μl of H_2O in 1.50 ml of ethanol. The reference cuvet lacked $MgCl_2$.

ter. For sodium, evaporation was performed in plastic tubes and the residue dissolved without digestion in 0.5 N HCl. Appropriate standards indicated that the presence of the antibiotic did not interfere with the sodium determinations.

The relative binding affinity of A23187 for various metal ions was determined by measuring the amounts extracted by the antibiotic into a bulk organic phase from an aqueous solution that contained two metal chloride salts, each at a concentration higher than required individually to saturate the antibiotic in the organic phase. The volume of the aqueous phase was such that the cation concentrations were not significantly decreased by A23187-dependent extraction to the organic phase. After evaporation and digestion, the concentration ratio of the two cations in organic phase aliquots was determined by atomic absorption. The observed concentration ratio multiplied by the initial ratio in the aqueous phase was then taken as the relative binding affinity for the two cations. The technique does not account for potential difference in the activity coefficients of the cations in the aqueous phase; however, it should closely simulate the specific transport of metal ions into the nonpolar regions of membranes induced by A23187.

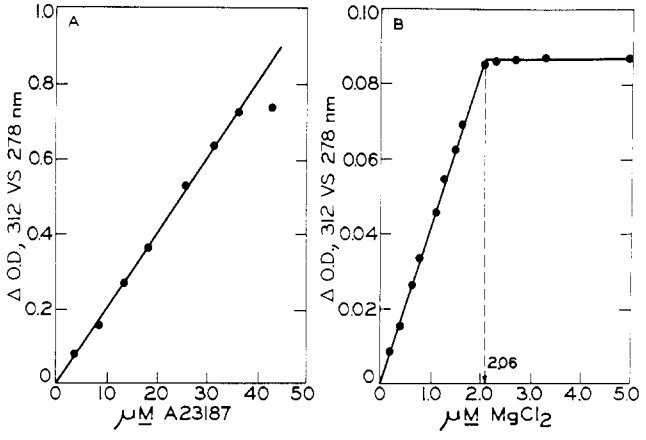

FIGURE 2: The magnitude of $MgCl_2$-induced absorbance changes as a function of A23187 and Mg^{2+} concentrations. (A) Difference spectra with ethanol as solvent were recorded as described in the legend to Figure 1B, at concentrations of A23187 as indicated. The concentration of $MgCl_2$ in the sample cut only was 133 μM. (B) Each point was obtained from the difference spectra of 4.25 μM A23187 plus the indicated concentration of $MgCl_2$ *vs.* 4.25 μM antibiotic as the free acid.

TABLE I: Properties of A23187–Me^{2+} Complexes.[a]

Metal Ion	Max/Min (nm)	$\Delta\Sigma_{max-min}$	Stoichiometry (A23187: Me^{2+})
Mg^{2+}	312/278	20,200	2.06
Ca^{2+}	308/279	23,100	2.10
Sr^{2+}	308/279	19,400	2.08
Ba^{2+}	304/277	15,300	2.11
Mn^{2+}	310/279	20,600	1.94

[a] The difference extinction coefficients and antibiotic to metal ion stoichiometries were obtained from data analogous to those shown in Figures 2A and B, respectively. All divalent metals were present as chloride salts.

A23187, as the free acid, was a gift of Dr. Robert Hamill, Eli Lilly and Co., Indianapolis, Ind. Since the available quantities are limited, recrystallization to constant spectra was not attempted. This may necessitate later minor revisions in the spectral properties or extinction coefficients reported below. Ruthenium red was purchased from K&K Laboratories and used without further purification. Standard metal chloride solutions were prepared from reagent grade salts dissolved in distilled deionized water. All experiments were conducted at room temperature.

Results

Spectral Properties. The ultraviolet absorption spectrum of A23187 as the free acid in ethanol is shown in Figure 1A (solid line). The compound displays four resolvable maxima located at 204, 225, 278, and 378 nm with respective extinction coefficients of 28,200, 26,200, 18,200, and 8,200. In addition, the 278-nm peak has a prominent shoulder at about 290 nm. The absorption at these maxima was a linear function of antibiotic concentrations to as high as 60 μM A23187. The addition of excess $MgCl_2$ to produce the magnesium complex of A23187 was accompanied by substantial alteration of the absorption spectrum (Figure 1A, dashed line). Four isosbestic points were present as indicated. The recorded difference spectrum of the magnesium complex *vs.* the free acid is shown in Figure 1B.

Stoichiometry of A23187–Me^{2+} Complexes. Difference spectra measured during titrations of the antibiotic with metal ion were used to study the complexes of A23187 with various metal ions. With ethanol as solvent the difference in extinction of the free acid *vs.* the Mg^{2+} complex (saturating $MgCl_2$) between the peak at 312 nm and the trough at 278 nm was a linear function of A23187 concentration to at least 35 μM (Figure 2A). At constant antibiotic and variable Mg^{2+} concentrations (Figure 2B), the magnitude of this difference increased linearly with the metal ion concentration until apparent saturation of the antibiotic. Increasing the metal ion concentration to as high as 333 μM produced no further alterations in this region of the spectrum. From these data, a molar difference extinction coefficient ($\Delta\Sigma_{312\ vs.\ 278}$) of 20,200 and a stoichiometry

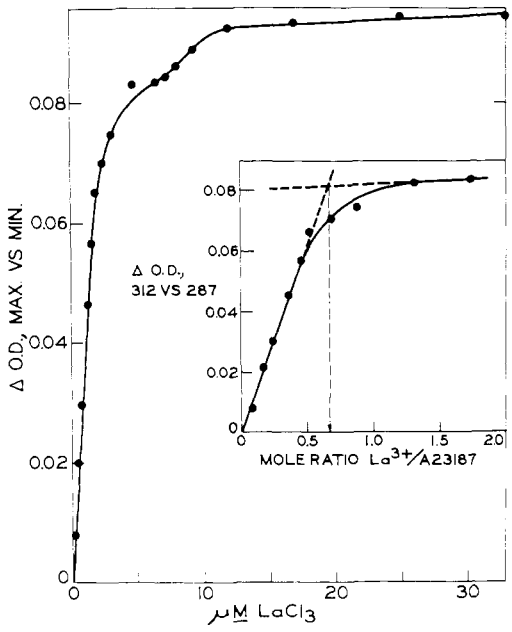

FIGURE 3: The difference spectral titration of A23187 in ethanol by $LaCl_3$. The A23187 concentration was 3.82 μM in sample and reference cuvets with $LaCl_3$ as indicated in the sample cuvet. The insert in this figure is a replot of the data to the first equivalence point of the titration.

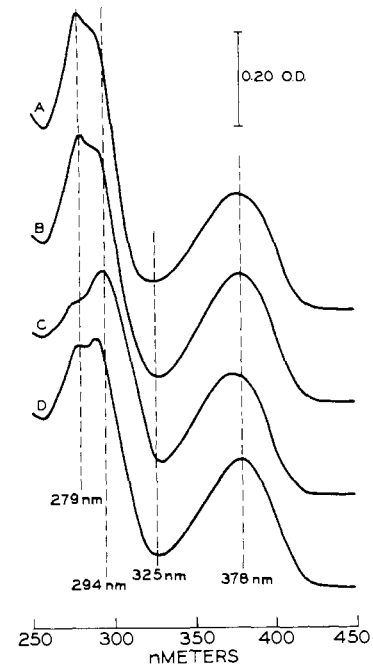

FIGURE 4: Effects of ruthenium red on the spectrum of A23187 in the presence or absence of $MgCl_2$. The four absorption spectra were obtained with 31.8 μM A23187 in 90% ethanol–10% water as solvent. When present in the sample or reference cuvets, the concentrations of ruthenium red and $MgCl_2$ were 133 and 333 μM, respectively: (A) A23187 *vs.* blank; (B) A23187 plus ruthenium red *vs.* ruthenium red; (C) A23187 plus $MgCl_2$ *vs.* $MgCl_2$; (D) A23187 plus ruthenium red and $MgCl_2$ *vs.* ruthenium red and $MgCl_2$. The presence of $MgCl_2$ did not alter the spectrum of ruthenium red.

(A23187:Mg^{2+}) at the equivalence point of 2.06 may be calculated. The lack of curvature at the region of the equivalence point in Figure 2B also indicates that Mg^{2+} is bound very tightly by A23187 when ethanol is the solvent. Assuming 95% saturation at the equivalence point (a minimum estimate), an association constant of 4×10^{14} M^{-2} may be calculated.

With other divalent metal ions, experiments analogous to Figure 2B gave very similar results that are summarized in Table I. However, in the case of Ba^{2+} there was a degree of "S-shaped" character to the titration curve, probably indicating a cooperativity in the binding of the first and second antibiotic molecules to this ion. The similarity in difference spectra and the uniform stoichiometries, A23187:Me^{2+} of nearly 2:1, however, indicate that the complexes are analogous structurally for this series of divalent cations.

The Binding of La^{3+} and Ruthenium Red by A23187. Because La^{3+} and ruthenium red are used widely as inhibitors of Ca^{2+} translocation across a variety of subcellular and cellular membranes, knowledge of any interaction between these inhibitors and A23187 would be of value in interpreting data obtained in the presence of both types of agents. The titration of A23187 with $LaCl_3$ as observed by difference spectroscopy is shown in Figure 3. In contrast to the divalent cations, La^{3+} appeared to form more than one type of complex. The first apparent equivalence point (insert in Figure 3) was attained at a mole ratio of La^{3+}/A23187 = 0.66, which could indicate the presence of a 2:3 complex, 1:1 and 2:1 mixtures or the formation of higher order aggregates with this cation. With La^{3+} concentrations below those required to reach this first equivalence point, the difference spectrum peak and trough occurred at 312 and 287 nm, respectively. Increasing the $LaCl_3$ concentration shifted the position of the peak and the trough to 318 and 289 nm, respectively, and produced a further increase in absorption difference. The presence of at least two complexes of A23187 with La^{3+} was also detected by fluorescence properties which are presented below.

Because ruthenium red absorbs in the spectral region of the antibiotic which displays alterations upon complexing with metal ions (Luft, 1971), difference spectra of A23187 plus ruthenium red *vs.* ruthenium red were recorded. A comparison of spectra A and B in Figure 4 shows that even at 135 μM ruthenium red, the spectrum of A23187 was not appreciably altered. Ruthenium red also failed to produce significant spectral alteration of A23187 in water or 30 and 60% ethanol–water mixtures. However, it did prevent the Mg^{2+}-induced spectral changes when both cations were present together with the antibiotic (compare spectra C and D, Figure 4). Thus, both of these inhibitors of Ca^{2+} transport would be expected to interfere directly with the action of A23187 as an ionophore unless the presence of excess ionophore is assured.

Binding and Transport Properties of A23187 for Monovalent Cations. Although A23187 decreases the permeability barrier to K^+ in mitochondria, erythrocytes, epididymal sperm (Reed and Lardy, 1972a,b), and parotid slices (Selinger *et al.*, 1974), this effect is thought to be secondary to redistribution of Ca^{2+} and Mg^{2+} since the compound does not appear to act as an ionophore for K^+ at pH 7.4 (Reed and Lardy, 1972a). Figure 5 extends this previous result to other monovalent cations. Only Li^+ produced difference spectrum alterations similar to those of the divalent ions or La^{3+} (Figure 5A). These data also reveal that Li^+ was required in at least a 100-fold higher concentration than Mg^{2+} to produce apparent saturation (compare Figure 2A). Na^+ was somewhat less effective than Li^+ and the K^+-induced alterations were negligible. Although A23187 did extract Li^+ from an aqueous to a bulk organic phase, no transport of either Na^+ or K^+ was detected by this technique (Figure 5B).

Additional bulk phase extraction experiments (Table II) indicated that A23187 would not function as an ionophore for

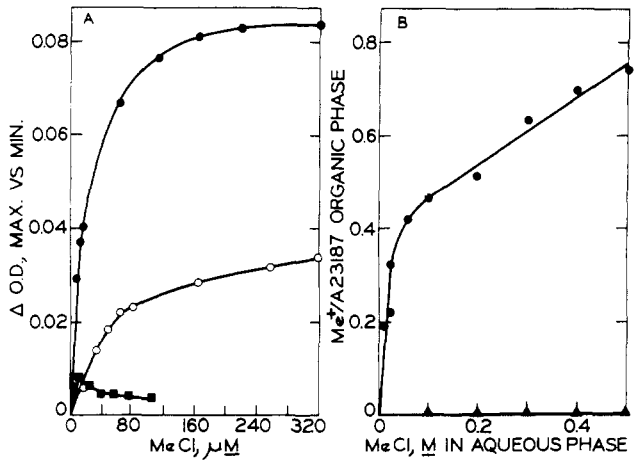

FIGURE 5: Complex formation between A23187 and monovalent cations. (A) The difference spectral titrations with monovalent cations were performed in ethanol as described in the legend to Figure 2B. The concentration of A23187 was 6.18 μM: (●) LiCl; (○) NaCl; and (■) KCl. The KCl data were obtained with the use of 5-cm path length cells although the Δ optical density (OD) for all ions is plotted as per centimeter path length. (B) A23187-induced extraction of monovalent ions from an aqueous to organic phase and the determination of ion content by atomic absorption were performed as described under Materials and Methods. The complete system contained 4.0 ml of organic phase (toluene, 70%; butanol, 30%) and 2.0 ml of aqueous phase containing 40 mM Tris-HCl (pH 7.4); (●) LiCl; (▲) NaCl or KCl. It is assumed that all of the A23187 (472 nmol) partitioned in the organic phase. After mixing and separation of the two-phase system, no antibiotic could be detected in the aqueous phase when aliquots were examined spectrally from 350 to 450 nm. The limit of detection for the absorption peak in this region was about 0.5 μM. The more intense peaks at shorter wavelengths were not utilized due to the presence of interfering components in the aqueous phase.

Na^+ or K^+ under physiological conditions. These data demonstrate that the presence of the divalent ion Ca^{2+} or the absence of Tris ions in the aqueous phase did not facilitate the extraction of monovalent cations. Thus, as expected, complexed divalent cations do not confer the ability to extract monovalent cation but instead further diminish it (compare the transport of Li^+ in the presence and absence of Ca^{2+}) and in addition the failure of A23187 to extract Na^+ or K^+ in this system is not the result of a competition for binding with Tris ions. The data also show that the absence of buffering in the aqueous phase was inhibitory to Ca^{2+} extraction and that under these conditions or in the absence of metal ions in the aqueous phase, essentially all of the antibiotic partitioned into the organic phase of the two-phase system. These data are discussed further below.

TABLE II: Effects of $CaCl_2$ and Tris Ions on the A23187-Induced Transport of Monovalent Ion into a Bulk Organic Phase.[a]

Sample No.	MeCl, 0.5 M, Aq Phase	$CaCl_2$, 20 mM, Aq Phase	Tris-HCl, 40 mM (pH 7.4), Aq Phase	[Me^+], Org Phase (μM)	[Ca^{2+}], Org Phase (μM)	[A23187], Aq Phase[b] (μM)
1	LiCl		+	79		<0.5
2	LiCl	+	+	<1	57	<0.5
3	LiCl	+	−	<1	6	<0.5
4	LiCl	−	−	<1		<0.5
5	NaCl	+	+	<1	58	<0.5
6	NaCl	+	−	<1	6	<0.5
7	NaCl	−	−	<1		<0.5
8	KCl	+	+	<1	58	<0.5
9	KCl	+	−	<1	5	<0.5
10	KCl	−	−	<1		<0.5
11		−	+			<0.5
12		−	−			<0.5

[a] The system was composed of 4.0 ml of organic phase (toluene, 70%; butanol, 30%) containing 118 μM A23178 in 50 μl of dimethylformamide–ethanol (1:3) with a 2.0-ml aqueous phase. The aqueous phase contained additional components as indicated in the table. In the cases where 40 mM Tris-HCl was absent, the pH of the aqueous phase was adjusted to approximately 7.4 by the addition of minimal amounts of Tris base. The metal ion concentrations in the organic phase were determined by atomic absorption as described under Materials and Methods. [b] At the completion of the experiment aliquots of the aqueous phase were examined spectrally for the presence of A23187 as described in the legend to Figure 5.

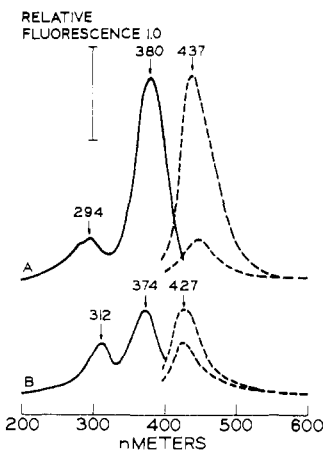

FIGURE 6: The fluorescence excitation and emission spectrum of A23187 and its Mg^{2+} complex in ethanol. The concentration of A23187 was 4.25 μM: (A) solid line, the excitation spectrum of the free acid, emission at 437 nm; dashed lines, the emission spectrum of the free acid; upper trace, excitation at 380 nm; lower trace, excitation at 294 nm; (B) solid line, the excitation spectrum after the addition of 333 μM $MgCl_2$ with emission at 427 nm; dashed lines, the emission spectra in the presence of 333 μM $MgCl_2$; upper trace, excitation at 374 nm; lower trace, excitation at 312 nm.

Fluorescent Properties. A23187 is a fluorescent compound (Figure 6A). The excitation spectrum of the free acid in ethanol displays two maxima at 380 and 294 nm with an additional shoulder at 280 nm. All excitation peaks produce emission at a single maximum located at 437 nm. In addition, the fluorescence is depressed and the position of the emission peak is shifted to longer wavelengths upon increasing the solvent dielectric constant (data not shown). The Mg^{2+} complex of A23187 displayed altered fluorescence properties as shown in Figure 6B. When compared to the free acid, the Mg^{2+} complex shows a shift in the position of the emission maximum to shorter wavelengths, a decreased fluorescence intensity, and alteration of the excitation spectrum as shown. Since the extinction coefficient of the absorption peak corresponding to the major excitation peak is not greatly altered by the complexing of Mg^{2+} (Figure 1A), the changes in the emission properties prob-

TABLE III: Effects of Several Cations on the Fluorescence of A23187 in Ethanol.[a]

Ion	Excitation Peak I	Excitation Peak II	Peak I: Peak II	Emission Peak	Rel Intensity (% of Free Acid)
None	294	380	0.22	437	100
Mg^{2+}	312	374	0.62	427	42
Ca^{2+}	308	374	0.81	427	44
Sr^{2+}	308	374	0.79	427	41
Ba^{2+}	304	374	0.68	427	39
Mn^{2+}	310	374	0.28	423	0.8
La^{3+} (a)	312	381	0.31	438	42
La^{3+} (b)	318	392	0.85	442	38
Li^{+}	304	371	0.66	427	36
Na^{+}	305	373	0.39	431	36
K^{+}	301	379	0.26	436	54

[a] The data were obtained from spectra recorded as described in the legend to Figure 6. In all cases except La^{3+}, the concentration of metal chlorides was 333 μM. Concentrations of $LaCl_3$ were: (a) 5.0 μM; (b) 33 μM.

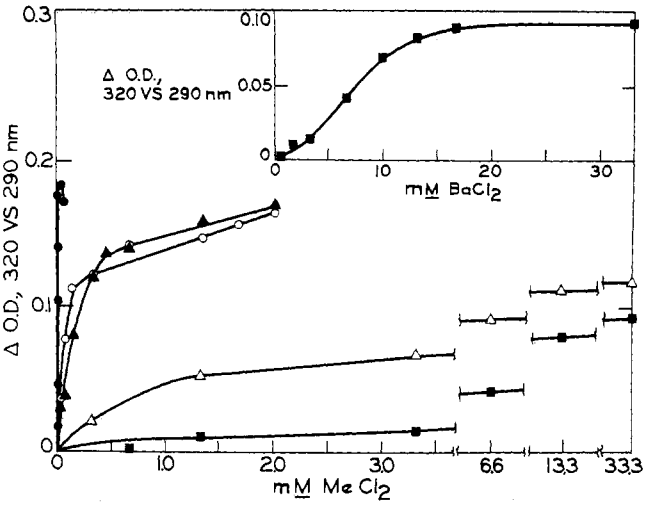

FIGURE 7: Difference spectral titrations of A23187 in aqueous media by divalent cations. Cuvets contained A23187 (25.3 μM, added in 20 μl of dimethylformamide + ethanol, 1:3) in 1.50 ml of 150 mM choline chloride–5 mM Tris-HCl (pH 7.4). Spectra were read before and after addition of metal chloride in a constant volume (25 μl). The absorption difference between 320 and 290 nm (maximum and minimum, respectively, in the difference spectrum) is plotted against divalent cation chlorides: (●) Mn^{2+}; (○) Ca^{2+}; (▲) Mg^{2+}; (△) Sr^{2+}; (■) Ba^{2+}.

ably indicate an alteration in the environment of the fluorescent chromophore upon metal ion complexing. Fluorescence properties of A23187 and some cation complexes have also been reported by Caswell and Pressman (1972) and by Case *et al.* (1974) and are similar to these results.

The effects of other ions on the fluorescent properties of A23187 in ethanol are summarized in Table III and further support Table I in indicating analogous structures for complexes of this ionophore with divalent cations. For all cations, the position of the minor excitation peak corresponds to the difference spectrum maximum of metal ion–antibiotic complex *vs.* the free acid. With divalent cations, the major excitation peak appeared uniformly at 374 nm. With the exception of Mn^{2+}, these ions also uniformly shifted the emission peak to 427 nm and produced a comparable decrease in fluorescence intensity. The approximately 50-fold greater quenching of fluorescence produced by Mn^{2+} when compared to the other divalent cations is probably a consequence of the paramagnetic properties of this ion and would indicate a close spatial association between Mn^{2+} and the fluorescent chromophores in the complex.

The effects of La^{3+} on A23187 fluorescence supported the spectral evidence for the existence of more than one complex. At a La^{3+}:A23187 mole ratio near the position of the first equivalence point in Figure 3 (La^{3+}, (a) in Table III), the position of the emission maxima coincided with that of the free acid. Increasing the La^{3+}:A23187 ratio beyond the second equivalence point (La^{3+}, (b) in Table III) then produced a red shift in both the second excitation peak and the emission peak. This behavior was not seen with mono- or divalent cations. All monovalent cations tested also gave evidence of some interaction with the antibiotic.

Determination of the Binding Affinity Sequence: The binding of divalent cations to A23187 in ethanol was too tight to allow the calculation of association constants. When titrations of A23187 were performed in a buffered aqueous medium (Figure 7), however, considerable disparity was observed in the spectral alterations induced by divalent cations. The "S-shaped" titration curve for the formation of the Ba^{2+}–A23187 complex in ethanol, noted briefly above, is exaggerated in aqueous media (insert in Figure 7). These data indicate the binding affinity sequence of the antibiotic for divalent cations in aqueous media has the order: $Mn^{2+} \gg Ca^{2+} \simeq Mg^{2+} \gg Sr^{2+} > Ba^{2+}$. The affinity sequence for these divalent ions has also been estimated by the independent technique of competitive bulk phase extraction. The data, presented in Table IV, are in agreement with Figure 8. Based on an assigned affinity of A23187 for Mg^{2+} of 1.0, the relative affinities of the ionophore for other divalent cations were: Mn^{2+}, 210; Ca^{2+}, 2.6; Sr^{2+}, 0.012. The extent to which the antibiotic was saturated by Li^{+} at 0.5 M in the aqueous phase (see Figure 5B) indicates that the relative affinity of A23187 for Li^{+} is less than that for Sr^{2+} but greater than that for Ba^{2+}, which was not extracted to an appreciable extent under these conditions.

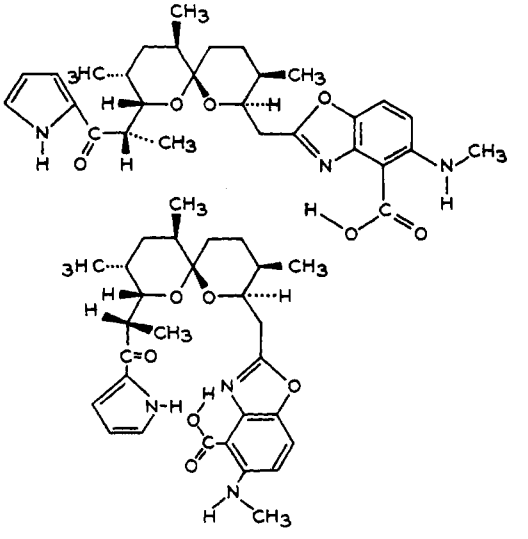

FIGURE 8: The structure of A23187 as the free acid. The structure was determined by Chaney *et al.* (1974). A and B show the structure in "open and closed" conformations, respectively.

TABLE IV: Relative Binding Affinity of A23187 for Various Divalent Metal Ions.[a]

Sample No.	Ion(s)	Concn (mM)		Total Me^{2+}, Org Phase (μM)	Rel[b] Affinity, Me^{2+}: Mg^{2+}
		Aq Phase	Org Phase		
1	Mg^{2+}	20	56	56	1.0
2	Mg^{2+}	500	54	54	1.0
3	Mn^{2+}	20	54	54	
4	Ca^{2+}	20	57	57	
5	Sr^{2+}	500	53	53	
6	Ba^{2+}	500	3.5	3.5	
7	Mn^{2+}:Mg^{2+}	20:500	50:6	56	210
8	Ca^{2+}:Mg^{2+}	20:20	41:16	57	2.6
9	Sr^{2+}:Mg^{2+}	500:20	14:45	59	0.012
10	Ba^{2+}:Mg^{2+}	500:20	0:55	55	

[a] The system was composed of a 4.0 ml organic phase (toluene, 70%; butanol, 30%) containing 118 μM A23187 in 50 μl of dimethylformamide–ethanol (1:3) with 2.0 ml of aqueous phase. The aqueous phase contained 40 mM Tris-HCl (pH 7.4) and MCl_2(s) as indicated in the table. Metal ion concentrations in the organic phase were determined by atomic absorption as described under Materials and Methods. [b] The affinity of A23187 for Mg^{2+} is defined as 1.0. The relative affinities for the other ions are calculated as described under Materials and Methods. No value is presented for Ba^{2+} since this ion did not saturate the antibiotic even at an aqueous phase concentration of 0.5 M.

Discussion

From data obtained with isolated rat liver mitochondria it was postulated (Reed and Lardy, 1972a) that A23187 produces a transmembrane electroneutral exchange of Ca^{2+} and Mg^{2+} for protons analogous to nigericin-induced transport of K^+ (Lardy *et al.*, 1967; Pressman *et al.*, 1967; Mitchell, 1968; Pressman, 1969; Henderson *et al.*, 1969), and the present data support that postulate. The spectral titrations in ethanol and the bulk phase extraction data demonstrate that A23187 can form 2:1 metal ion complexes with the divalent alkaline earth and manganese ions. That the complexes are uncharged is indicated by their ready extractability into organic solvents in the absence of lipophilic anions (Table IV; see also Reed and Lardy, 1972a). The carboxylic acid group of A23187 permits the formation of neutral 2:1 complexes with divalent cations and would mediate the proton antiport required to maintain electroneutrality across the membrane, providing this group is deprotonated in metal ion complexes. The data in Table II support this mechanism, assuming that the anion form of A23187 does not enter the organic phase of the two-phase system. Under all conditions tested, including the absence of metal and/or buffer ions in the aqueous phase, essentially all of the antibiotic partitioned in the organic phase indicating a strong tendency to transport protons from these media. In addition, the absence of buffering in the aqueous phase reduced the apparent affinity of the antibiotic for Ca^{2+}. This result supports deprotonation of the carboxylic groups upon complexing Ca^{2+} by indicating an H^+–Ca^{2+} binding competition. According to this interpretation, the absence of buffering in the aqueous phase favors proton transport since initial Ca^{2+} transport would significantly increase the H^+:Ca^{2+} concentration ratio in the aqueous phase and so progressively decrease additional Ca^{2+} transport until an equilibrium is established.

The present spectrophotometric and fluorescence data do provide evidence for some interaction of A23187 with monovalent cations, in the order $Li^+ > Na^+ > K^+ \simeq 0$; however, even the binding of Li^+ is clearly of a weaker nature than that observed with divalent cations. Of these ions, only Li^+ was transported to an organic phase by the antibiotic when the aqueous phase was buffered at pH 7.4 (Figure 6B). These data further indicate that with biological systems, A23187 should not perturb Na^+ or K^+ distribution across membranes by direct transport. However, the antibiotic will transport K^+ in bulk phase experiments when the aqueous phase is adjusted to pH 9.8 (Reed and Lardy, 1972a). It seems probable that high pH allows Me^+ transport through the formation of lipid-soluble carboxylate salts. At physiological pH values the A23187-induced transport of monovalent cations by salt formation would then not be expected to occur in systems where divalent ions were being released from solutions bounded by a membrane (with subsequent uptake of protons) providing that the bound volume was small compared to the bulk media. Under those conditions any increase of the pH in the bulk media would be comparatively small while the bound volume was being acidified. In the opposite case, if the antibiotic was being used to produce a divalent cation movement from a large into a small space, with subsequent removal of protons in the opposite direction, the pH of the bound volume might be raised sufficiently to allow Na^+ or K^+ present to be released by antibiotic mediated transport involving the formation of lipophilic salts. This potential transport of Na^+ or K^+ by A23187 should not interfere with the use of this compound as a divalent cation specific ionophore when adequate internal buffering capacity is assured, or in the presence of an independent system to reequilibrate internal pH.

At present, the structure of A23187–Me^{2+} complexes has not been determined. The structure of the crystalline antibiotic as free acid has recently been reported (Chaney *et al.*, 1974) and is reproduced in Figure 8. The significance of the two conformational arrangements shown is further discussed below. The carbonyl group of the α-ketopyrrole portion of the molecule is probably responsible for the shoulder in the absorption spectrum occurring at 290 nm since pyrrole-2-carboxaldehyde was found to display an absorption maximum of similar extinction at this wavelength (data not shown). The red shift observed in this portion of the spectrum in the presence of a complexable metal ion would suggest electron withdrawal from the group and its probable involvement as a coordinating ligand. By comparison to known properties of benzoxazoles, it is also apparent that this portion of the molecule is responsible for the absorption peak at 378 nm and for the fluorescent properties of the molecule.

The present data offer additional insights into the structure of A23187–metal ion complexes and into the basis of binding specificity for the compound. Although A23187 is somewhat unique among the known ionophores, the basis of specific ion binding by this compound is expected to be consistent with what is known from more extensive studies with other compounds. Like many monovalent cation ionophores, ion binding by A23187 is highly selective with respect to ionic radius. The difference in affinity for the tightly bound Mn^{2+} compared to the weakly bound Sr^{2+} is of the order of 10^4 and is even greater when compared to Ba^{2+}. In addition, comparing the binding of similar sized alkali and alkaline earth cations shows high specificity for divalent over monovalent ions, which is in contrast to other ionophores. Thus, A23187 is unique among ionophores in

that its specificity pattern is similar to that of divalent cation pumps in natural membranes. To attain high binding selectivity with respect to ionic size it is necessary for the liganding groups to replace solvent molecules during the binding reaction (Grell *et al.*, 1972; Simon and Morf, 1973; Eigen and Winkler, 1970; Diebler *et al.*, 1969). Thus, metal ions complexed with A23187 most likely are unsolvated or largely so. The fact that in aqueous media the smaller ions having higher hydration energies are bound much tighter than larger more poorly hydrated ions indicates that with respect to relative complex stability, ion size is of greater importance than is the ion's hydration energy.

To attain the rapid rates of complexing and decomplexing reactions required for efficient ion transport by an ionophore transporting unhydrated metal ions, the molecule must also be flexible to allow a stepwise substitution of solvent molecules (Eigen and Winkler, 1970; Diebler *et al.*, 1969). From examining space filling molecular models of A23187 constructed according to the structure shown in Figure 8, we feel that A23187 possesses the required flexibility due to rotation of methylmethylene and methylene carbon–carbon bonds which link the α-carbonylpyrrole and benzoxazole derivatives, respectively, to the spiral ring portion of the molecule. These rotations result in the "open and closed" conformations of the antibiotic depicted in Figure 8. The position of polar groups in the two conformations suggests that a closed structure is most likely present in metal ion complexes or with the free acid in solvents of low polarity whereas the open structure would be favored in aqueous solutions. The formation of complexes of A23187 in aqueous media would then be less favorable compared to complexing in solvents of low polarity, since additional energy would be required to form the closed conformation. These interpretations partially explain the large increase in complex stability in ethanol compared to aqueous solvents for the weakly bound Sr^{2+} and Ba^{2+} ions, although a decrease in the ions' solvation energies must also be a contributing factor. The stepwise substitution of solvent molecules would also be facilitated by the formation of an intermediate 1:1 antibiotic–metal ion complex followed by addition of a second antibiotic to yield the transporting 2:1 species. The existence of a 1:1 intermediate complex is probable since extinction *vs.* [A23187] data (not shown) revealed no evidence of dimerization in the absence of Me^{2+} and since the rapid transport rate produced by low levels of A23187 could not be explained by a one-step complexing mechanism involving a trimolecular collision.

Factors other than ionic radii and ion solvation energies must also be important in determining complex stability as seen from a comparison of the relative affinities of A23187 for Li^+ and Mg^{2+}. While these ions are of similar radius (0.60 and 0.65 Å, respectively) and while Mg^{2+} solvation energy is considerably greater than that of Li^+, both the titrations in ethanol and the competitive bulk phase transport data revealed that Mg^{2+} is preferred over Li^+ by at least two orders of magnitude. The reasons for this selectivity may be related only to charge neutralization between the antibiotic and metal ions or the charge to radius ratio of the cations but may be further related to the stoichiometry of the complexes as follows. Interpreting the binding specificity sequence for divalent cations reported here in terms of the "minimal cavity radius" which contains the complexed metal ion would indicate that this cavity is composed of seven or eight liganding groups from the two antibiotic molecules as the radius of such a cavity would most nearly match that of the Mn^{2+} ion (Simon and Morf, 1972). Inspecting the structure and molecular models of A23187 for potential liganding groups suggests that not more than four groups would be able to participate in complexes of 1:1 stoichimetry (steric considerations seem to preclude both spiral ring oxygens and probably both oxygens of the carboxylic acid moiety from participating simultaneously which is in agreement with known liganding groups of monensin, nigericin and grisorixin (see Simon and Morf, 1973)). Thus, if A23187–Me^+ complexes are predominantly 1:1 as is suggested in Figure 5B, metal ion complexes of this antibiotic with monovalent cations would be expected to decrease in stability as ionic size increased due to the presence of a small cavity radius. This interpretation is in agreement with the observed order $Li^+ > Na^+ > K^+ \simeq O$ (Figure 5) and may be an important factor in accounting for the charge specificity displayed by this ionophore.

Finally, the nature of the more complex interactions of A23187 with $LaCl_3$ and the reasons for the unexpected ability of ruthenium red to block Mg^{2+} complexing by the antibiotic are not clear. Although the present data indicate that A23187 forms predominantly 2:1 complexes with divalent cations, other investigators have indicated that the 1:1 complex may be significant under some conditions (Caswell and Pressman, 1972; Case *et al.*, 1974; Puskin and Gunter, 1974) and further work is clearly required to deduce the detailed mechanism of ion transport by this compound.

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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.