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Ionophore A23187: The Effect of H^+ Concentration on Complex Formation with Divalent and Monovalent Cations and the Demonstration of K^+ Transport in Mitochondria Mediated by A23187[†]

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ABSTRACT: The two-phase extraction technique has been used to study the equilibrium between A23187, metal cations, and H^+. Under these conditions the ionophore forms charge neutral isostoichiometric complexes with divalent cations in which both carboxylate groups of the 2:1 A23187:M^{2+} complexes are deprotonated. In ethanol, however, the methyl ester of A23187 also binds divalent cations indicating that protonated complexes between A23187 and cations should also exist. With monovalent cations, A23187 forms two charge-neutral complexes of stoichiometries and relative stabilities: $A_2HM > AM$. Examination of energy utilization, K^+ and H^+ movements, and light scattering capacity of mitochondria in the presence of divalent cation chelators, A23187, and valinomycin demonstrates that A23187 can act as a nigericin type K^+ ionophore under appropriate conditions. Formation constants for the A_2HM complexes with monovalent cations indicate that with appropriate conditions transport of Li^+ and Na^+ mediated by A23187 would also be expected. The binding constant data and associated free energies of complex formation are compared as a function of ionic radius and of cation charge. The data indicate that lack of conformational mobility in A23187 is responsible for the high cation size selectivity of this compound. To explain the transport selectivity of A23187 for divalent cations, it is proposed that this ionophore forms a family of five complexes, isostoichiometric between cations of different valence but of which only charge-neutral species are permeant to membranes. The charge of a given complex is in turn determined by that of the cation. The concept is consistent with the divalent cation transport specificity of A23187, explains the observed monovalent cation transport, and is useful in rationalizing the differences in charge selectivity between A23187 and X-537A.

The carboxylic acid ionophore A23187 is now used widely to investigate the involvement of Ca^{2+} in the control of cellular functions. Most of the available literature on chemical studies of A23187 has been summarized in a recent manuscript from this laboratory (Deber and Pfeiffer, 1976). The available data are largely consistent with the original hypothesis that A23187 catalyzes an electroneutral exchange of $2H^+$ for M^{2+} across biological membranes (Reed and Lardy, 1972a). To accomplish this exchange, it is believed that the cation crosses the membrane complexed by two molecules of A23187 which are present as the carboxylate anion and so are available to carry 2 equiv of H^+ back across the membrane after releasing the transported divalent cation. Less certain at this time are the underlying chemical features of A23187 and its complexes which impart the unique ability of this ionophore to transport selectively divalent over monovalent cations. The extent of this selectivity has also not been ascertained.

In this report, we demonstrate the requirement for deprotonation of both carboxylic acid functions during the formation of the electroneutral A23187-Ca^{2+} complex. Binding constants that account for the equilibrium $AH \rightleftharpoons A^- + H^+$ are obtained for a number of divalent cations and compared as a function of ionic radius.[1] In addition, two charge-neutral complexes of A23187 with monovalent cations are identified. Using isolated mitochondria, the transport of K^+ mediated by A23187 is demonstrated. The data demonstrate that while A23187 remains the most selective divalent cation ionophore available, its charge selectivity is not complete and caution is required in interpretation of physiological data obtained by use of this compound.

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[1] Throughout this paper, AH refers to the protonated form of A23187 and A^- to the carboxylate anion. M^{n+} refers to a metal cation of charge $n+$. In combination, these symbols indicate a cation complex of A23187 of the indicated stoichiometry and net charge. A23187:M^{n+} is used to indicate complex(es) without regard to stoichiometry.

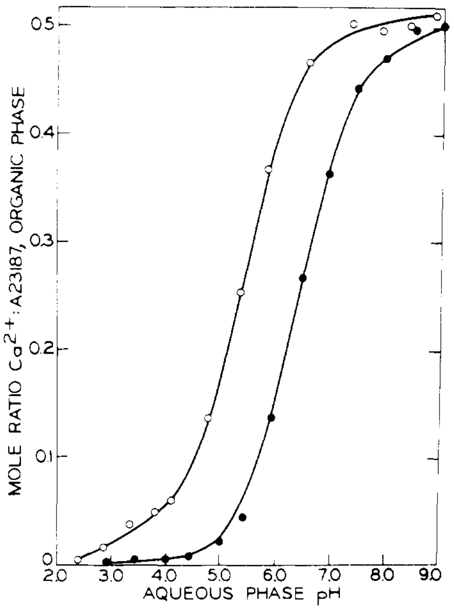

FIGURE 1: The effect of aqueous phase H^+ and Ca^{2+} concentrations on the A23187-dependent extraction of Ca^{2+} from a bulk aqueous to a bulk organic phase. The experiments were performed as described under Materials and Methods, at an organic phase A23187 concentration of 100 μM: (○) the aqueous phase $CaCl_2$ concentration was 500 mM; (●) the aqueous phase $CaCl_2$ concentration was 5.0 mM.

Materials and Methods

The A23187-Dependent Extraction of Metal Ions from a Bulk Aqueous to a Bulk Organic Phase. Solutions of A23187 (4.0 ml) in 70% toluene-30% butanol were mixed (Vortex rotor) with an aqueous solution (2.0 ml) containing metal ion chlorides at the concentrations and pH values indicated. For divalent cations, the pH of the aqueous phase was varied between 3 and 9 by buffering the solution with 50 mM β,β′-dimethylglutaric acid (pH 3-7) or 50 mM Tris (pH 7.5-9) using the opposite member of this buffer pair for final pH adjustment. For monovalent cations, the aqueous phase was buffered with 50 mM Tris-Cl. After separating the phases by centrifugation, the metal ion concentration in the organic phase was determined and taken as the extent of complex formation between A23187 and the metal in question. The values presented were obtained at room temperature from duplicate samples and were corrected for the organic phase metal ion concentration present in the absence of A23187. In most cases experiments were performed at least twice and were readily reproducible. The organic phase metal ion concentration was determined by scintillation counting of $^{45}Ca^{2+}$ or by atomic absorption for the other metals. Samples for atomic absorption were prepared either by acid digestion as described previously (Pfeiffer et al., 1974) or by extracting the metal ion directly from organic phase residue with 0.5 N HCl. Since equilibration of the aqueous and organic phases produced some alterations of the aqueous phase pH, this value was redetermined with a pH electrode after separating the phases to obtain the final value used for calculation of binding constants.

The difference spectra between the methyl ester derivative of A23187 and its Mn^{2+} complex as well as other spectroscopic determinations were recorded with a Cary 15 spectrophotometer.

Transport of K^+ Mediated by A23187 in Mitochondria. Rat liver mitochondria were prepared according to the procedure of Johnson and Lardy (1967). The homogenizing medium contained, in addition, EGTA (0.1 mM) and bovine serum albumin (low fatty acid type) (0.5 mg/ml) which were absent from the washing medium. All mannitol-sucrose solutions were deionized with a mixed bed resin (amberlite MB-3) prior to use. Protein was determined by the Biuret method in the presence of 1% deoxycholate.

The mitochondrial respiration rates and their derivatives were determined simultaneously with K^+ and H^+ movement and the 180° light scattering capacity using a multiparameter apparatus designed by Pressman (1967). The Beckman K^+ selective electrode No. 39047 and combination H^+ electrode No. 39030, respectively, were utilized to monitor the medium concentration of these cations. Experiments were performed at 25 °C. When indicated, antibiotics were added to the mitochondrial suspensions in negligible volumes of EtOH-dimethylformamide (3:1). Other components of the media are described in the legend to Figure 6.

All metal ion chlorides and other reagents were commercially available and reagent grade or better. The divalent metal chloride solutions were standardized by titration with EDTA, and the organic solvents were distilled before use. A23187, its methyl ester derivative, and the carboxylic acid K^+ ionophore, septamycin, were gifts from Dr. Robert Hamill of Eli Lilly and Company.

Results

The Equilibrium between A23187, H^+, and Ca^{2+} in the Two-Phase Extraction System. The two-phase extraction technique (Pressman et al., 1967; Pressman, 1969; Eisenman et al., 1969) has been used in our laboratory (Deber and Pfeiffer, 1976) and by others (Puskin and Gunter, 1975) to prepare metal ion complexes of A23187 for study by nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) spectroscopy and for determining metal ion to ionophore stoichiometries and relative binding constants (Pfeiffer et al., 1974; Reed and Lardy, 1972a; Caswell and Pressman, 1972). The protonation equilibrium of A23187 has not been considered directly in these studies. The existence of A23187 as a carboxylate anion in metal ion complexes formed by this method has been inferred from: (1) analogy to other carboxylic acid ionophores (see Pressman et al., 1967); (2) the failure of lipophilic anions to enhance metal cation extraction by A23187 (Reed and Lardy, 1972a; Puskin and Gunter, 1975); and (3) the largely electrically silent increase in bilayer permeability produced by this compound (Case et al., 1974; Kafka and Holz, 1975; Hyono et al., 1975). Data bearing more directly on this problem are shown in Figure 1. The A23187 dependent extraction of Ca^{2+} (complex formation) from a bulk aqueous to a bulk organic phase is strongly dependent on the pH of the aqueous phase in the more acid region. This effect indicates a Ca^{2+}-H^+ extraction competition and so the probable deprotonation of the carboxylate functions in the calcium complex. These data further allow a more quantitative demonstration of the overall equilibrium between Ca^{2+}, H^+, and A23187 as follows. When the Ca^{2+} concentration in the aqueous phase was decreased from 500 to 5 mM (two orders of magnitude), the dependence of Ca^{2+} complex formation on aqueous phase pH was displaced by one unit (one order of magnitude in H^+ concentration) toward the basic region. This demonstrates that the order of the reaction with respect to H^+ is twice that with respect to Ca^{2+} and together with the present and previous

Table I: A Comparison of the Overall Binding Constants of A23187 for Selected Divalent Cations Obtained by Various Techniques.

Divalent Cation	Overall Binding Constant[a]	−Log Overall Binding Constant[a]	Rel Binding Constant, Mg^{2+} = 1[b]			
			These Data	Pfeiffer et al. (1974)	Puskin and Gunter (1975)	Caswell and Pressman (1972)
Mg^{2+}	1.3×10^{-7}	6.9	1.0	1.0	1.0	1.0
Ca^{2+}	3.7×10^{-7}	6.5	2.8	2.6	2.3	8.3×10^{-1}
Sr^{2+}	1.1×10^{-9}	9.0	1.2×10^{-2}	1.2×10^{-2}	1.8×10^{-1}	1.1×10^{-1}
Ba^{2+}	1.7×10^{-12}	11.8	1.3×10^{-5}			
Mn^{2+}	1.3×10^{-4}	3.9	1.0×10^{3}	2.1×10^{2}	2.5×10^{2}	
Co^{2+}	3.5×10^{-4}	3.5	2.7×10^{3}			
Ni^{2+}	6.1×10^{-5}	4.2	4.7×10^{2}			
Cu^{2+}					1.2×10	
Zn^{2+}	1.3×10^{-3}	2.9	1.0×10^{4}			

[a] Values are calculated according to eq 2 using the points of half-saturation (mole ratio M^{2+}:A23187 = 0.25) in Figure 3. [b] Values are calculated from the work referenced by dividing the published binding constants by the value reported for Mg^{2+}.

demonstration of a 2:1 stoichiometry between A23187 and Ca^{2+} establishes the overall equilibria under these conditions as:

$$2AH_{org} + Ca^{2+}{}_{aq} \rightleftharpoons A_2Ca_{org} + 2H^{+}{}_{aq} \qquad (1)$$

The validity of the subscripts was verified by examining the aqueous and organic phases spectroscopically after equilibrating 100 μM organic phase solutions of A23187 with aqueous phases buffered between pH 3 and 9 and in the absence of metal cations. These data (not shown) revealed no significant extraction of A23187 into the aqueous phase (limit of detection about 0.5 μM) and that A23187 in the organic phase remained protonated over the pH region employed.

Equation 1 therefore closely describes the reaction for complex formation by this technique and since all components of the two-phase system are in equilibrium with each other, an overall formation constant, or bulk extraction constant, for the Ca^{2+} complex may be calculated as:

$$K_f = \frac{[A_2Ca]_{org}[H^+]_{aq}{}^2}{[Ca^{2+}]_{aq}[AH]_{org}{}^2} \qquad (2)$$

Using the data of Figure 1, the value for the Ca^{2+} complex is 3.7×10^{-7}.

Overall Formation Constants for Complexes of A23187 with Selected Divalent Cations. When the dependence on aqueous phase pH of complex formation with other alkaline earth and some transition metal divalent cations was examined (Figure 2) results analogous to those for Ca^{2+} were obtained. For some members of this set of cations, the requirements for maintaining the desired oxidation state and avoiding the formation of insoluble metal hydroxides prohibited examination of a broad aqueous phase pH range. However, within these limitations all cations appeared to form analogous complexes with A23187. Using these data and eq 2, overall formation constants for complexes with these cations were calculated and are shown in Table I. In the table, these values are compared to earlier determinations of relative binding constants obtained by ourselves and others. The values of relative affinity obtained by various techniques are in good agreement considering the different solvents and conditions employed in these studies. Some quantitative variations which exist probably reflect solvent effects on conformational equilibria of the ionophore and solvation energies of the cations, particularly when comparing values obtained by the two-phase technique to those obtained in a single phase. We were unable to obtain a value for Cu^{2+} as it appeared to form a complex of near 1:1 stoichiometry and to be relatively insensitive to aqueous phase pH under the present conditions. A mixed complex of Cu^{2+} with A23187 and the dicarboxylate buffer ions is suspected as the probable cause of this result. The existence of 1:1 and 2:1 A23187:Cu^{2+} complexes which depended on conditions was also reported by Puskin and Gunter (1975). The predominance of the 1:1 complex with Cu^{2+} probably reflects the preference of this ion for the square planar liganding geometry compared to the nearly octahedral array presented to the cation by A23187 in dimeric complexes (Deber and Pfeiffer, 1976; Puskin and Gunter, 1975).

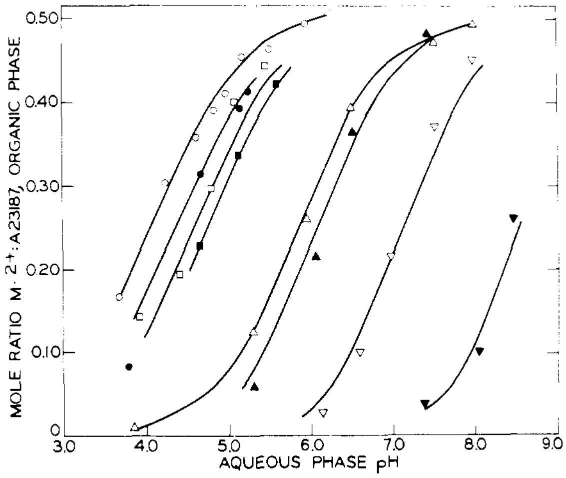

FIGURE 2: The effect of aqueous phase H^+ concentration on the A23187 dependent extraction of selected divalent cations from a bulk aqueous to a bulk organic phase. The experiments were performed as described under Materials and Methods at an organic phase A23187 concentration of 100 μM. The aqueous phase MCl_2 concentration was 50 mM for all salts: (○) Zn^{2+}; (●) Co^{2+}; (□) Mn^{2+}; (■) Ni^{2+}; (△) Ca^{2+}; (▲) Mg^{2+}; (▽) Sr^{2+}; and (▼) Ba^{2+}.

In Figure 3, the negative logarithms of the overall formation constants are plotted as a function of ionic radius. Included for comparison are values obtained by Degani and Friedman (1974) for X-537A using a similar procedure. The values for the two ionophores are not strictly comparable quantitatively since the solvents used as organic phase

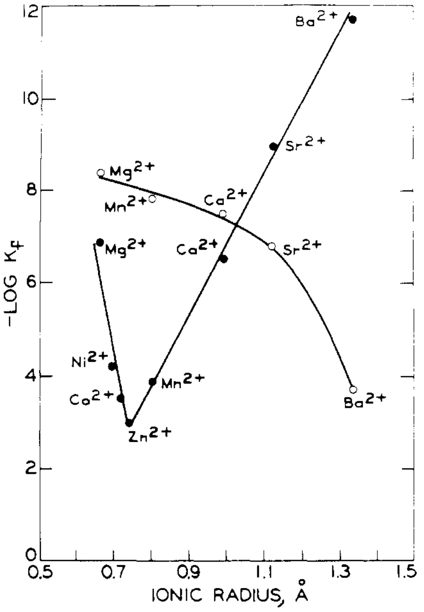

FIGURE 3: The negative logarithms of overall binding constants for divalent cations with ionophores A23187 and X-537A as a function of ionic radius. The values are overall binding constants calculated as per eq 2: (●) data for A23187 taken from Table I; (○) data for X-537A taken from Degani and Friedman (1974). Decreasing values of $-\log K$ represent increasing complex stability.

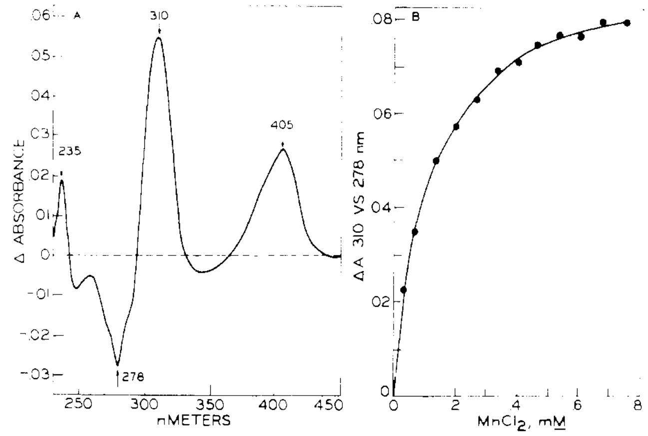

FIGURE 4: Complex formation between the methyl ester of A23187 and Mn^{2+}. In both panels the cuvettes contained 4.0 μM A23187 methyl ester in ethanol. Added in the sample cuvette was 3.3 mM $MnCl_2$ in panel A or the indicated concentration in panel B. The indicated $MnCl_2$ concentrations were produced by a single addition of the requisite amount of salt in 5 μl of H_2O, added to 1.5 ml of derivative solution.

were not the same. However, the complexes with both ionophores are of analogous stoichiometries and it is useful to compare the overall patterns displayed by these ionophores. A23187 shows a linear increase in complex stability (decreasing values of $-\log K_F$) for divalent cations whose size is intermediate between that of Ba^{2+} and Zn^{2+}. This trend is abruptly reversed for ions smaller than Zn^{2+} resulting in a nearly symmetrical V-shaped pattern. If the relative formation constant for the Cu^{2+} complex in Table I is used to obtain a value for that ion, it also conforms to the pattern as does the fact that we have not been able to detect complex formation with Be^{2+} (radius = 0.31 Å) by criteria of fluorescence or difference spectroscopy. In contrast, X-537A shows little selectivity between divalent cations whose size is intermediate between Mg^{2+} and Sr^{2+} whereas stability is considerably enhanced for the larger Ba^{2+}. Some possible indications from these variances are discussed below.

Complexes of the Methyl Ester Derivative of A23187 with Divalent Cations. The deprotonated state of the carboxylate moieties in A_2M complexes demonstrated in Figures 1 and 2 is in agreement with available data on other carboxylic acid ionophores. According to x-ray crystallographic studies, in most compounds the free carboxylate serves both as a cation liganding group and in hydrogen bonding to the opposite end of the molecule, thus stabilizing the cyclic conformation present in the cation complex. We have proposed a similar state for the carboxylates in the 2:1 A23187:Ca^{2+} complex based on NMR studies of the complex in chloroform solution. The free carboxylates bind to the divalent cation and are also hydrogen bonded to the hydrogen of the pyrrole nitrogen from the second ionophore molecule, thus stabilizing the dimeric structure (Deber and Pfeiffer, 1976). With such a structure it is obvious that deprotonation of carboxyls would strengthen these interactions and so stabilize the complex. It remains possible, however, that in a single, more polar solvent, complexes structurally analogous to the charge-neutral species (required by this two-phase system) could exist while retaining one or both carboxylic acid protons. Data supporting that possibility are shown in Figure 4. Figure 4A shows the difference spectrum between the methyl ester of A23187 and that compound in the presence of a saturating concentration of Mn^{2+}. The general features of the difference spectrum (including the positions of isosbestic points, maxima and minima, and the difference extinction coefficients) are very similar to those induced by Mn^{2+} (or other divalent cations) in the spectrum of the parent compound (Pfeiffer et al., 1974). The only substantial deviation is in the peak at 405 nm (due to the benzoxazole portion of the molecule), which is inverted and of lesser extinction in metal ion complexes with the parent compound. Similar spectra (not shown) are induced by other divalent cations. These observations indicate that the nonanionic derivative can complex divalent cations and that the complexes are rather similar to those formed by the free acid. The dependence of complex formation between the methyl ester of A23187 and Mn^{2+} on metal ion concentration is shown in panel B of Figure 4. The curve is hyperbolic and demonstrates a substantial reduction in stability (2–3 orders of magnitude) compared to the Mn^{2+} complex with the free acid in ethanol (Pfeiffer et al., 1974). It seems probable therefore that under appropriate conditions complexes between A23187 and divalent cations could exist with one or both carboxylates protonated although they would be of lower stability than the charge-neutral species.

Complexes of A23187 with Monovalent Cations. The ability of A23187 to transport selectively divalent over monovalent cations constitutes the primary utility of this ionophore for physiological studies. Surprisingly, the chemical basis or the extent of selectivity has not been pursued extensively to date. Using difference spectral and fluorescence titrations, we demonstrated the ability of A23187 to complex monovalent cations with the selectivity order $Li^+ > Na^+ > K^+$ (Pfeiffer et al., 1974). Under the conditions of that work these complexes were substantially less stable than those with the alkaline earth series. At an aqueous pH of 7.4, Li^+ was extracted by A23187 into a bulk organic phase; no extraction of Na^+ or K^+ was detected. At an aqueous phase pH 9.8 extraction of K^+ had been observed (Reed and Lardy, 1972a). These data are extended in Figures 5 and 6. Figures 5A and B show the dependence of A23187:Li^+ complex formation on the aqueous phase Li^+

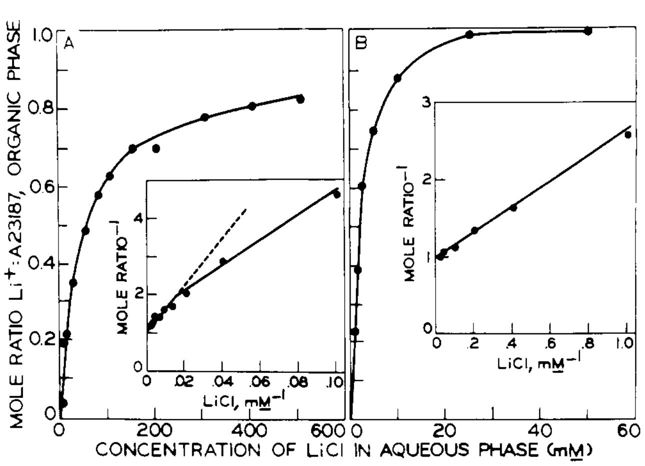

FIGURE 5: The effect of aqueous phase H^+ and Li^+ concentrations on the A23187-dependent extraction of Li^+ from a bulk aqueous to a bulk organic phase. The experiments were performed as described under Materials and Methods at an organic phase A23187 concentration of 50 μM. In panels A and B, the aqueous phases were buffered at pH 7.6 and 8.9, respectively. The inserts in these panels are double reciprocal plots of portions of the same data.

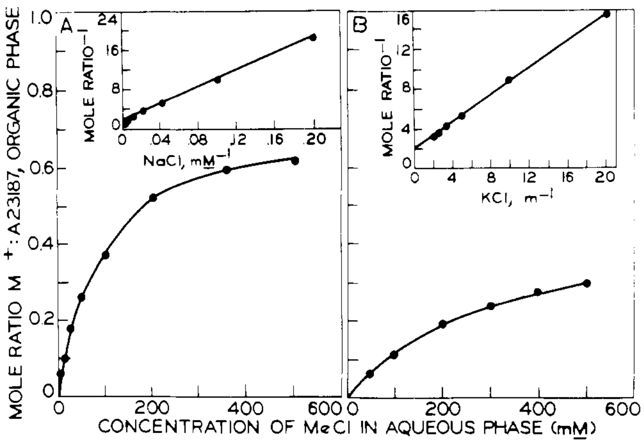

FIGURE 6: The effect of aqueous phase Na^+ and K^+ concentration on the A23187-dependent extraction of these cations from a bulk aqueous to a bulk organic phase. The experiments were performed as described under Materials and Methods at an organic phase A23187 concentration of 50 μM and an aqueous phase pH of 8.9. Panels A and B show data for Na^+ and K^+, respectively. The inserts in these panels are double reciprocal plots of portions of the same data. The insert of B reports KCl concentration as the reciprocal of molarity, (not m^{-1}).

concentration at pH values of 7.6 and 8.9, respectively. At the more acid pH, the curve is of an apparent biphasic nature which would be consistent with the existence of two types of complexes of the ionophore with Li^+. The biphasic nature of the curve results in a nonlinear double reciprocal plot indicating that both 1:1 and 2:1 A23187:Li^+ complexes are present with the dimeric form being the most stable. By contrast, at the more basic pH, the curve is a hyperbola resulting in a linear double reciprocal plot reflecting the predominance of the 1:1 complex. Figures 6A and B were obtained at an aqueous phase pH of 8.9 for Na^+ and K^+, respectively. Like the Li^+ data at pH 7.6, Na^+ gave a biphasic curve which indicated the predominance of the 2:1 complex at the lower Na^+ concentrations with subsequent formation of a less stable 1:1 complex as the aqueous phase Na^+ concentration was increased. Only the 2:1 complex was formed with K^+ under the same conditions of aqueous phase pH and metal ion concentration.

Since the two-phase system employed here contains no lipophilic cations or anions, a charge-neutral condition in the two complexes (required for extraction to the bulk organic phase) must result from the carboxylate moieties of A23187 and the balancing charge of the monovalent cation. Accordingly, reactions for formation of the two types of complexes apparent in Figures 5 and 6 may be written as:

$$\underset{(\text{org})}{2\text{AH}} + \underset{(\text{aq})}{\text{M}^+} \overset{K_f}{\rightleftharpoons} \underset{(\text{org})}{\text{A}_2\text{HM}} + \underset{(\text{aq})}{\text{H}^+} \tag{3}$$

and

$$\underset{(\text{org})}{\text{AH}} + \underset{(\text{aq})}{\text{M}^+} \overset{K_f}{\rightleftharpoons} \underset{(\text{org})}{\text{AM}} + \underset{(\text{aq})}{\text{H}^+} \tag{4}$$

Formation constants, analogous to those obtained from eq 2 for divalent cations, can be calculated for the A_2HM complexes and ALi complex from the double reciprocal plots in Figures 5 and 6 and are shown in Table II.

Transport of K^+ Mediated by A23187 in Rat Liver Mitochondria. Either type of complex between A23187 and the monovalent cations could potentially catalyze an electroneutral exchange of the cation for H^+ across a biological membrane. A K^+ loss from isolated mitochondria and other membrane bounded systems has been observed previously following the addition of A23187 (Reed and Lardy, 1972a,b; Selinger et al., 1974; Reed et al., 1975). In the case of mitochondria, these K^+ movements were interpreted as being secondary to divalent cation depletion since the presence of EDTA was required to produce the effect and since A23187 fails to extract K^+ to a bulk organic phase at pH 7.4. By an alternative interpretation, the K^+ loss could be a result of transport mediated by A23187. EDTA could be required to prevent the divalent cations from tying up the free A23187, and the basic nature of the mitochondrial matrix space (relative to the bulk medium) plus high matrix K^+ concentration might be allowing formation of an A23187:K^+ complex sufficient to account for the observed rates of depletion. Data favoring the latter interpretation are presented in Figure 7.

Table II: Binding Constants of A23187 for Monovalent Cations.[a]

Monovalent Cation	K_f, A_2HM	K_f, AM
Li^+	4.9×10^{-2} M^{-1}	7.7×10^{-7}
Na^+	6.0×10^{-4} M^{-1}	
K^+	7.3×10^{-5} M^{-1}	

[a] Values of formation constants for complexes of the type A_2HM and AM are calculated from eq 3 and 4, respectively. Data are taken from the appropriate portions of the double reciprocal plots in Figures 4 and 5.

In Figure 7, panel A, EDTA was present in the medium prior to the addition of mitochondria. In agreement with the earlier reports, subsequent addition of A23187 failed to release state 4 respiration since free Ca^{2+} is not available to establish the energy dissipating uptake and release cycle (Reed and Lardy, 1972a,b). The concomitant slow release of K^+ is indicated by the K^+ electrode tracing which occurs without significant alteration in light scattering (mitochondrial swelling). The apparent H^+ release indicated by the H^+ electrode tracing upon addition of A23187 results from a combination of effects (an electrode artifact due to A23187, chelation of M^{2+} by EDTA, and a complex pattern of cation and anion movements across the mitochon-

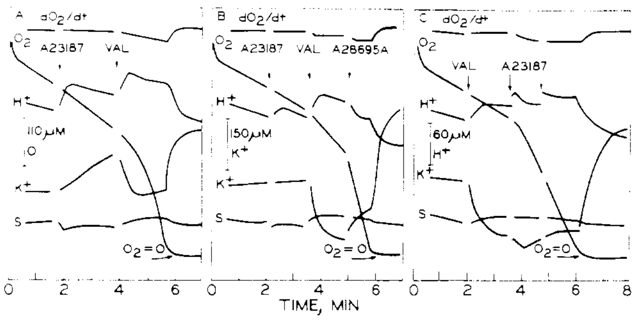

FIGURE 7: Transport of K^+ mediated by A23187 in rat liver mitochondria. The medium contained 0.22 M mannitol, 0.07 M sucrose, 3 mM triethylammonium chloride (pH 7.4), 10 mM sodium succinate plus rotenone (0.3 nmol per mg of protein), 1.0 mM KCl, and 2.5 mg of mitochondrial protein per ml. Reactions were initiated at time 0 by the addition of mitochondria. Downward deflections of the oxygen, hydrogen ion, and potassium ion traces represent a decrease in the medium concentration of that component. An upward deflection of the 180° light scattering trace (S) would indicate mitochondrial swelling. The electrode calibrations refer to all three panels. In addition to the above components, the media contained EDTA (1 mM) in panel A or EGTA (15 nmol per mg of mitochondrial protein) in panels B and C added before mitochondria. Further additions where indicated were: A23187, 1.0 nmol per mg of protein (except the second addition in panel C which was 2.0 nmol per mg); valinomycin, 0.3 nmol per mg of protein; and A28695A (identical with septamycin), 1 μg. A28695A is a potent K^+ ionophore of the nigericin type.

drial membrane (D. R. Pfeiffer et al., to be presented elsewhere)) for which the electrode reports only the sum. That observation does not necessarily indicate therefore that K^+ is not being exchanged for H^+. The subsequent addition of valinomycin produced the prompt reuptake of K^+, release of H^+, and time-dependent acceleration of respiration, indicating cyclic uptake and release of K^+ was occurring. Under these conditions (low external K^+ concentration and absence of permeant anion) the addition of valinomycin alone does not produce a sustained elevation of respiration (see Figure 7, panel C) since mitochondrial swelling, and so the rate of passive K^+ loss, is limited. These data do not support the earlier interpretation of K^+ loss due to a generalized increase in membrane permeability occurring secondarily to the severe Ca^{2+} and Mg^{2+} depletion produced by A23187 and EDTA. With such a leaking membrane, the retention of state 4 respiration (energized state) prior to valinomycin addition would not be expected nor would the ability to perform the energy-dependent uptake of K^+ in the presence of valinomycin. Indeed, the presence of valinomycin to establish K^+ membrane permeability for energy dependent uptake should not be required. Instead, these observations indicate that A23187 plus valinomycin can establish an energy dissipating flux of K^+ analogous to nigericin plus valinomycin (see Pressman et al., 1967; Henderson, 1971). In panel B, the dependence of this phenomenon on severe mitochondrial Mg^{2+} depletion and chelation was examined. The medium contained a low concentration of EGTA which was sufficient to chelate the endogenous mitochondrial Ca^{2+} and so prevent uncoupling but not to decrease the level of free Mg^{2+}. In agreement with the earlier work and in contrast to the effect of nigericin-like ionophores, the loss of mitochondrial K^+ was largely eliminated. However, the uptake of K^+ and release of H^+ occurring subsequent to valinomycin addition were again accompanied by enhanced respiration like that produced by nigericin plus valinomycin. The further addition of septamycin (a potent nigericin-like ionophore) partially reversed the valinomycin induced K^+ uptake and H^+ extrusion and further accelerated respiration to a maximal rate. Panel C shows that in the presence of a low EGTA concentration, the nigericin plus valinomycin like enhancement of respiration is not affected by the order of ionophore addition. Shown further is that, in contrast to other carboxylic ionophores, the submaximal respiration rate established by the first addition of A23187 (1.0 nmol per mg of protein) was not enhanced further by a second addition of A23187 (2.0 nmol per mg of protein) nor was the valinomycin-induced K^+ uptake reversed. Even with a second addition of A23187 as high as 10 nmol per mg of protein, we have not observed reversal of the K^+ uptake produced originally by valinomycin, providing a coupled state is maintained.

Thus, these data represent somewhat of a paradox. Like the monocarboxylic acid-K^+ ionophores, A23187 in combination with valinomycin establishes an apparent energy dissipating uptake and release of K^+, even in the presence of free Mg^{2+}. However, unlike that class of compound, high levels of A23187 fail to reverse readily the uptake of K^+ produced by previous addition of valinomycin. Yet when EDTA is present, some apparent A23187-mediated depletion of K^+ does occur. Some possible explanations for these complex phenomena are discussed below.

Discussion

The Divalent Cation Binding Selectivity of A23187. Ionophore A23187 is a member of the carboxylic acid class of ionophores (Lardy et al., 1967) of which about 25 compounds are presently known (see Westley, 1975). These compounds are in general less well characterized chemically than the macrocyclic ionophores. Hundreds of naturally occurring and synthetic macrocyclic compounds are available and in some cases their complexation chemistry has been studied in great detail. A major obstacle in solution studies of carboxylic acid ionophores by spectroscopic techniques and in the preparation of synthetic analogues is a general lack of symmetry in the structures compared to macrocyclic compounds. A second complicating factor is a general increase in conformational mobility in the carboxylic acid ionophores resulting from their open chain structures. Compared to the other ionophores in the class, A23187 is well suited to chemical investigation since, in spite of an open chain structure, its conformational mobility is greatly reduced by steric hindrance in the spiroketal portion of the molecule and by the highly aromatic nature of the compound (Chaney et al., 1974). The aromatic substituents must have a planar conformation and are coplanar with those liganding groups that are conjugated to them. The orientations of the liganding groups are therefore fixed by the orientation of the aromatic "arms" of the molecule, both for the free ionophore and in metal ion complexes. The arms in turn possess conformational mobility of available rotational freedom in the "hinge regions" of the molecule which link the arms to the rigid spiroketal backbone of the compound. Thus, conformation variants of A23187 resulting from altered intramolecular hydrogen bonding or solvent interactions or when accommodating variably sized cations in complexes are essentially confined to those obtainable by rotations about a small number of carbon–carbon bonds. This situation is somewhat in contrast to other carboxylic acid ionophores where more regions of the molecules are mobile, making the deduction of conformations more difficult. The conformationally rigid nature of A23187, together with the small number of potential liganding groups available in a single molecule of A23187 (Pfeiffer et al., 1974; Deber and Pfeiffer, 1976), further suggests that the ionophore molecules in complexes with different cations will be largely isosteric and will utilize the same liganding groups, thus simplifying the interpretation of observed selectivity properties.

A further consideration prompting interest in chemical studies of A23187 is its transport selectivity for divalent over monovalent cations. This property of the ionophore makes it particularly useful in physiological work, and, based on available data, is in sharp contrast to the majority of carboxylic acid ionophores which are normally highly specific for monovalent cations in both binding and transport properties. Only Dianemycin (see Pressman, 1972) and X-537A are also able to complex divalent cations to an appreciable extent and of these two, only X-537A produces significant transport of the divalent cations (Pressman, 1972; Caswell and Pressman, 1972; Scarpa and Inesi, 1972). That ionophore, however, also produces comparable or greater rates of monovalent cation transport.

To gain insight into the basis of charge selectivity, a comparison of the complexation chemistries of A23187 and X-537A should then be useful. At present the available data, particularly for A23187, are too limited to allow rigorous discussion of cation binding by A23187 based on thermodynamic parameters or bonding theory. Nevertheless, some useful qualitative interpretation can be made.

In terms of the basis of binding selectivity for divalent cations displayed by A23187 the available data are shown in Figure 3 and Table I. Together with the data of Figure 2 and the above discussion, it appears that the complexes with divalent cations are both isostoichiometric with respect to A23187, M^{2+}, and H^+ and that individual ionophore molecules in the different complexes are largely isosteric as well. This assumption was also indicated by ultraviolet and fluorescence spectra reported previously and is currently under further study by NMR. The binding constants obtained by the two-phase technique may be used to calculate values for the free energy of complex formations ΔG_f under those conditions. These values may be further examined according to the relationship:

$$\Delta G_f = \Delta G_{ml} - \Delta G_m{}^s + \Delta(\Delta G^s)_l{}^{ml} \qquad (5)$$

where ΔG_{ml} is the gas phase or "inherent stability" of the complex, $\Delta G_m{}^s$ is the energy of solvation of the free cation, and $\Delta(\Delta G^s)_l{}^{ml}$ is the difference in solvation energies between the free and complex ionophore (see Ovchinnikov et al., 1974). Similarly, the first term of the expression ΔG_{ml} can be further represented as:

$$\Delta G_{ml} = \Delta G_{conf} + \Delta G_{ml}' \qquad (6)$$

where ΔG_{conf} is the free energy required to convert the ionophore's conformation in the free state to the conformation present in the complex and $\Delta G_{ml}'$ is the interaction energy between the ion and the ligand in that conformation. One may then inquire which of the component terms is most likely to be producing the overall high selectivity displayed by A23187 and why the isostoichiometric complexes of X-537A are more nearly equivalent energetically (Figure 3). It seems improbable that the third term in eq 5, $\Delta(\Delta G^s)_l{}^{ml}$, is of great significance in determining selectivity (but not absolute stability) for A23187. A difference in this value would not be expected for a series of complexes between an ionophore and cations unless there were substantial differences in the ability of the compound to shield cations from solvent interaction. Since the complexes are isostoichiometric and probably isosteric, substantial differences in shielding are not expected. Also, importance of the third term would not be consistent with the inflection in the stability trend occurring for cations smaller than Zn^{2+} since it is the larger cations for which poorer shielding would be anticipated. The difference in the magnitude of the second term in eq 5, $\Delta G_m{}^s$, for complexes with two ions is much larger than the overall difference in stability and so an important contribution to selectivity might be present. For instance, while the Zn^{2+} complex is more stable by 12.1 kcal/mol than the Ba^{2+} complex, the difference in the solvation energies of these two ions is about 375 kcal/mol. However, the trend in complex stability as a function of ionic size and type of ion argues that differences in this term for complexes with different cations are also not dominant in determining selectivity. First of all there is no obvious trend between complex stability and ion hydration energy. Passing from Ba^{2+} to Zn^{2+} the stability of the complexes improves markedly even though the much larger changes in the solvation energies of the cations would predict a reversed stability pattern. Then, for ions smaller than Zn^{2+}, the decreasing complex stability while agreeing with the decreasing ion solvation energy is smaller by at least two orders of magnitude than the change in overall stability. Furthermore, the transition metal cation complexes with Ni^{2+}, Co^{2+}, and Cu^{2+} conform to the size pattern established by the alkaline earth cations and Mn^{2+} and Zn^{2+}. These ions (Ni^{2+}, Co^{2+}, and Cu^{2+}) are both better hydrated and form more stable complexes due to the increased stabilization from ligand field effects, yet they are not anomalous to patterns for cations not showing ligand field effects. It seems reasonable, therefore, that as with many ionophores, the magnitudes of $\Delta G_{ml}'$ and $\Delta G_m{}^s$ are very similar (see Ovchinnikov et al., 1974). These considerations then point to ΔG_{conf} in eq 5 as being a dominant component resulting in the high selectivity of A23187 indicated in Figure 3. The same conclusion is arrived at intuitively based on the conformationally hindered nature of A23187, and the preceding discussion of the component free-energy terms, while speculative, does fail to disclose any obvious flaws in the intuitive considerations. Apparently, the size of the cation binding cavity formed by the six liganding groups (four oxygens and two heterocyclic nitrogens) is best suited to bind the cation Zn^{2+}. The expansion of the cavity required for binding of larger cations is accompanied by unfavorable strain in other parts of the molecules which cannot readily be relieved by conformational adjustment. The decreasing stability of complexes with cations smaller than Zn^{2+} could be accounted for by inability of six liganding groups to simultaneously contact the smaller cations due to prior contact between themselves (see Simon and Morf, 1973). This would be equivalent to a reduction in the term $\Delta G_{ml}'$. Unfavorable contacts in other areas of the two A23187 molecules as they are drawn closer together in complexes with small cations might also contribute to weaker complexes for the smaller cations.

The same kinds of arguments suggest that a conformationally more mobile structure of X-537A accounts for its relative lack of selectivity as compared to A23187. Based on x-ray data, this ionophore utilizes nine oxygens as liganding groups (six from one and three from the other molecule of the 2:1 complexes) in its most stable dimeric complex with Ba^{2+} (Johnson et al., 1970). The resultant large binding cavity is well suited to the large cations. Interligand repulsions between these groups in complexes with smaller cations could be avoided by conformational adjustment elsewhere in the molecules without unfavorable strains.

A Proposed Basis of the Charge Selective Transport by A23187. If differences in conformational mobility between A23187 and X-537A can explain their different abilities to discriminate between cations of different size, it is not apparent how the same criteria could account for the divalent transport specificity of A23187. Like the case of cation selectivity, extensive studies of the overall transport process are available for some better studied ionophores and some thermodynamic treatments have been presented. However, again, since available data for A23187 are limited more intuitive considerations must be relied upon at present.

For ionophorous cation transport to occur at a significant rate, the binding constant of the ionophore must be sufficient to allow complex formation under existing conditions, the rate constants for the complexing and decomplexing reactions must not be prohibitively slow, and properties of the complex must allow it to move through the hydrophobic membrane at a significant rate. It does not seem probable that complexing and decomplexing rate constants are responsible for the divalent cation selectivity of A23187 since ligand substitution reactions with alkaline cations are normally much faster than corresponding reactions with alkaline earth cations. We previously suggested (Pfeiffer et al., 1974) that insufficient affinity of A23187 for monovalent cations could account for the divalent cation transport selectivity of this compound. Weak binding in turn was attributed to a small number of available complexing ligands in a single molecule of A23187. Figures 5 and 6, however, demonstrate that more stable 2:1 complexes also occur. The stabilities of the A_2HM complexes with monovalent cations cannot be compared directly to those with divalent cations in Figure 3 due to an inconsistency of units. The ALi complex, however, can be compared and its formation constant (Table II) indicates a stability similar to the A_2Mg complex under the two-phase conditions. Since appreciable formation of A_2HM complexes occurs under similar conditions and since transport of K^+ (most weakly bound of the present set) mediated by A23187 is indicated in Figure 7, further explanation of the normally divalent cation selective transport is required.

Previous work has shown that both 2:1 (Reed and Lardy, 1972a; Pfeiffer et al., 1974; Puskin and Gunter, 1975) and 1:1 (Caswell and Pressman, 1972; Puskin and Gunter, 1975) A23187:M^{2+} complexes exist under appropriate conditions. The data in Figure 4 suggest that deprotonation of the carboxylates is not absolutely required to form the divalent cation complexes so that these complexes could be considered to be in further equilibrium with H^+. Similarly, Figures 5 and 6 show that both 2:1 and 1:1 complexes can form with monovalent cations which also may be protonated (in the case of the 2:1) to result in a charge neutral species. Together, the data indicate that there is a set of five dominant complexes with both monovalent and divalent cations (and probably higher valences as well) which are in rank order of inherent stability:

$$\underset{(a)}{(A_2M^{n+})^{n-2}} > \underset{(b)}{(A_2M^{n+}H)^{+n-1}} > \underset{(c)}{(A_2M^{n+}H_2)^{n+}}$$

$$\text{or } \underset{(d)}{(AM^{n+})^{+n-1}} > \underset{(e)}{(AM^{n+}H)^{n+}}$$

The overall charge of a given isostoichiometric complex is then determined by the charge of the metal cation. For the series a–e, formed with a monovalent cation, the complexes would have net charges of −1, 0, +1, 0, and +1, while with a divalent cation the charges would be 0, +1, +2, +1, and +2 and so forth with metals of higher charge. If those complexes bearing net charge do not cross the membrane at a significant rate, then the normal divalent cation transport specificity of A23187 can be explained together with the data in Figure 7 without implicating insufficient affinity as the major factor.

Based again on the NMR studies (Deber and Pfeiffer, 1976), the surface area of the complex composed of the aromatic moieties from the two molecules does not shield the cation binding site from solvent interaction as effectively as does the opposite complex surface which is composed of the highly aliphatic spiroketal portions of the molecule. This condition is probably required for the efficient complexation and decomplexation reaction rates involving stepwise solvent substitutions. However, a net charge at the binding site would not be totally shielded from bulk solvent, thus resulting in a diminished rate constant for the diffusion of complex across the membrane. That situation is avoided in charge-neutral complexes since the anionic carboxylates contact the cation closely so that areas of net charge are minimized. Also supporting the proposed slow transmembrane diffusion of A23187 complexes which carry net charge is the failure of the compound to produce uncoupling in mitochondria without involvement of cations. That observation (according to the chemiosmotic hypothesis) indicates the failure of the compound to act as a protonophore or that the anionic form of free A23187 does not readily cross the inner mitochondrial membrane.

The above hypothesis is also useful for rationalizing the altered charge selectivity of A23187 compared to X-537A. Degani and Freidman (1974) have shown that X-537A also forms complexes of both 1:1 and 2:1 stoichiometries between ionophore and cation, and that with some cations these complexes are in further equilibrium with H^+. They differ in rank order of inherent stability compared to A23187 in that for the cations smaller than Cs^+ or Ba^{2+}, the 1:1 complexes are preferred. Apparently this difference reflects the larger number of liganding groups available in a single molecule of X-537A compared to A23187. Thus, X-537A is well suited to form 1:1 charge neutral complexes with monovalent cations which in turn can result in nigericin-like monovalent cation transport. The analogous complexes with divalent cations are positively charged and presumably not able to cross a membrane readily. When divalent cation transport mediated by X-537A does occur, the less favored but charge-neutral 2:1 complex must be responsible.

Conditions Affecting the Electroneutral Transport of K^+ Mediated by A23187 in Mitochondria. According to the above discussion, the magnitude of the pH gradient across the inner mitochondrial membrane should be a dominant factor controlling the rate of K^+ transport mediated by A23187 in Figure 7. Without sufficient ΔpH (basic in the matrix space) the level of A23187-K^+ complexes is not sufficient to produce a significant rate of K^+ depletion. This requirement also exists with other nigericin-like ionophores depending on the pK_a of the compound in question. In contrast to the case with nigericin-like ionophores, large values of ΔpH favor a complex A_2K^- which does not readily cross the inner membrane. Indeed, were that complex present and permeable, an energy-dependent depletion of mitochondrial K^+ would commence; no such phenomenon is indicated in

Figure 7. Thus, there should exist a range of matrix space pH where the complex A_2HK could form and result in nigericin-like depletion of mitochondrial K^+. The aspects of K^+ transport mediated by A23187 (Figure 7) which are not analogous to transport by other carboxylic acid ionophores can be explained by the requirements for forming the A_2HK complex in the matrix space. The failure of high levels of A23187 to reverse valinomycin-induced K^+ uptake is a result of this self-limiting H^+ for K^+ exchange which, by continuing, acidifies the matrix space and so decreases further transport until ΔpH is recovered by electrogenic K^+ uptake (with hydrogen extrusion) produced by valinomycin. Similarly, the magnitude of Mg^{2+} depletion produced by A23187 is larger when EDTA is present in the media (Reed and Lardy, 1972a). With Mg^{2+}:$2H^+$ exchange occurring during loss of Mg^{2+}, the final value of matrix pH should be higher in panel B (low EGTA) compared to panel A (excess EDTA) which in turn can explain the diminished K^+ depletion (prior to valinomycin addition) as well as the reduced rate of K^+ cycling (following valinomycin addition) in panel B.

Clearly, further work is required to verify the present hypothesis and to allow prediction of the extent of monovalent cation transport mediated by A23187 under a given set of conditions. In the meantime, it is important to point out that since the formation constant in Table II indicates that A_2HM complexes with Na^+ and Li^+ are more stable than the A_2HK complex, significant transport of those cations should also occur in some conditions and could interfere with interpretation of physiological data obtained by the use of A23187 in those cases.

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