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Lipid bilayer permeation by neutral aluminum citrate and by three α-hydroxy carboxylic acids

Mark A. Akeson and Donald N. Munns

Department of Land, Air, and Water Resources, University of California, Davis, CA (U.S.A.)

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Several groups have proposed that aluminum (Al) may permeate biological membranes as a neutral complex with citrate. We tested this hypothesis by measuring aluminum citrate flux across unilamellar phospholipid vesicles (liposomes). Results from two independent procedures show that lipid bilayer permeation by the neutral aluminum-citrate complex is slow ($P \cong 1 \cdot 10^{-11}$ cm · s^{-1}). We then compared aluminum-citrate permeation with permeation by a series of α-hydroxy carboxylic acids and by trimethylcitrate. This comparison showed that the aluminum-citrate flux is limited by diffusion across the water/lipid interface. This is due to hydrogen bonding between water and the citrate carboxyl groups, and by hydration of the bound metal in the aqueous phase. By analogy with citric acid, steric hindrance of diffusion within the bilayer does not affect the permeation rate of aluminum citrate. Elevated tissue levels of Al in subjects fed a diet supplemented with citric acid and $Al(OH)_3$ cannot be explained by lipid bilayer permeation of the neutral complex.

Introduction

Aluminum toxicity has recently been implicated in human disease, and it has long been known to limit crop production on vast areas of arable land [1]. Despite these problems, the molecular mechanisms of aluminum (Al) toxicity are not known. This is due in part to our limited understanding of how Al is absorbed across biological membranes. One possible mechanism is lipid bilayer permeation by neutral aluminum citrate [1–3], a complex which can occur in acid surface waters, and which is believed to constitute a significant mole fraction of total solution Al in the upper intestinal tract of humans [2]. Indirect evidence for neutral aluminum-citrate permeation of biological membranes includes elevated Al in brains of rats fed a diet of aluminum citrate [4] and elevated Al in blood plasma of humans who consumed citric acid simultaneously with $Al(OH)_3$ [3].

Biophysical evidence both supports and opposes a neutral aluminum-citrate permeation mechanism. Low molecular weight neutral acids are known to rapidly permeate lipid bilayers. For example, hydrofluoric acid (HF) and nitric acid (HNO_3) have permeability coefficients (P) of $1 \cdot 10^{-4}$ cm · s^{-1} and $4 \cdot 10^{-4}$ cm · s^{-1}, respectively [5]. Their anionic forms, F^- and NO_3^-, are far less permeant with coefficients of about $5 \cdot 10^{-11}$ cm · s^{-1} and $1 \cdot 10^{-10}$ cm · s^{-1} [5]. These differences are due to the significantly lower activation energy required to move neutral solutes from an aqueous phase to a low dielectric lipid bilayer since P is proportional to $\exp^{-(\Delta G^{\ddagger}/RT)}$ [6]. Thus, it is reasonable to propose that permeation by neutral aluminum citrate could be rapid [1–3].

Evidence against rapid permeation by this complex can be inferred from work with nonelectrolytes. Poznansky et al. [7] have shown that permeability of amides is controlled by resistance to solute diffusion within the bilayer and by resistance to solute movement across the water/bilayer interface. The former depends on size of the solute, therefore aluminum citrate and citric acid (molar volumes of about 115 cm^3 · mol^{-1}) would be significantly less permeable than HF (molar volume = 17 cm^3 · mol^{-1}) or HNO_3 (molar volume = 45 cm^3 · mol^{-1}). The latter depends on the energy required to move the solute from water to the low dielectric bilayer. Stein [8] was one of the first to argue that hydrogen bonding can play a central role in this process. This hypothesis was supported by Cohen [9] who demonstrated a high correlation between the activation

Correspondence: D.N. Munns, Department of Land, Air, and Water Resources, University of California, Davis, CA 95616, U.S.A.

energy for lipid bilayer permeation by nonelectrolytes and the number of hydrogen bonds they form with water. Using Stein's system to assign hydrogen bonds (H-bonds) [8], aluminum citrate (8 H-bonds) would be measurably more permeant than citric acid (11 H-bonds) but both compounds would be significantly less permeant than HF (2H-bonds) and HNO_3 (4 H-bonds).

The objectives of this study were to establish a lipid bilayer permeability coefficient for aluminum citrate, and to determine what properties control permeation by comparison with similar compounds. Our results indicate a low rate of permeation ($P = 1 \cdot 10^{-11}$ cm $\cdot s^{-1}$) due to hydrogen bonding with the citrate ligand and hydration of the bound metal in the aqueous phase. We conclude that lipid bilayer permeation by neutral aluminum citrate cannot explain the high tissue Al observed in subjects fed diet supplements of $Al(OH)_3$ and citrate.

Methods

Liposome preparation

Liposomes were prepared as described previously [10]. Egg phosphatidylcholine (PC) and egg phosphatidic acid (PA) (Avanti Polar Lipids) were combined at a PC/PA mole ratio of 9:1. Organic solvents were removed by evaporation under nitrogen gas, and the lipid was redissolved in equal volumes of buffer and diethyl ether. The suspension was sonicated for 1 min to produce a homogeneous dispersion, and then the diethyl ether phase was removed by rotary evaporation under reduced pressure for about one hour. The large phospholipid vesicles produced by this method were filtered ten times through 0.2 μm Nuclepore filters to produce predominantly unilamellar vesicles of 180 ± 55 nm diameter [11].

Typically, a liposome suspension prepared in this way contained 20 mM phospholipid with identical buffer solutions inside and outside of the vesicles (details on buffer compositions are described under the individual experiments). To replace the external buffer, an aliquot of the liposome suspension was passed over a Sephadex G-50 column that was equilibrated with the desired solution. This step resulted in a 2-fold dilution of the suspension which gave a liposome stock of 10 mM phospholipid. Phospholipid content of stock solutions was checked periodically by digestion and analysis of inorganic phosphate by a standard colorimetric procedure [12].

Measurement of neutral acid permeation by the pH-sensitive dye Pyranine

Our assay was a modification of a technique described by Barchfeld and Deamer [10]. Liposomes were prepared as above in a pH 7.1 solution that contained 10 mM Pipes, 10 mM aspartate, 0.25 M K_2SO_4, and 0.5 mM pyranine, a pH-sensitive fluorescent dye which is impermeant across lipid bilayers [13]. The external phase was identical but for the absence of pyranine. At time zero, 0.2 ml of the liposome suspension were added to a cuvette that contained 1.8 ml of the external buffer. Fluorescence of the entrapped pyranine was stabilized (excitation 430 nm, emission 515 nm), and the pH of the external solution was dropped to 3.5 by the addition of 36 μl of 0.88 M H^+ as H_2SO_4. After 1 min, an aliquot of carboxylic acid in identical buffer at pH 3.5 was added to the cuvette. Final total concentration of the acid and its conjugate base was 5 mM unless otherwise noted. Entry of the weak acid into the slightly alkaline internal phase resulted in a slight decrease in pH and thus a decline in pyranine fluorescence, from which the rate of permeation could be calculated as shown in the results.

Measurement of aluminum-citrate permeation by graphite furnace atomic absorption spectroscopy

Pure egg PC liposomes were prepared as above except that the reverse-phase evaporation step was replaced by a freeze-thaw cycle, and the filter pore size in the sizing step was 0.1 μm rather than 0.2 μm [11]. The entrapped solution contained 0.12 M KNO_3, and 1 mM aluminum citrate. To ensure against formation of Al polymers, the aluminum citrate was prepared by slowly adding Al stock (10 $\mu g \cdot l^{-1}$ in 1% HNO_3) to a pH 3.5 solution that contained citrate. The pH was subsequently raised to 7.1 by the addition of fresh 0.05 M NaOH at 15 $ml \cdot h^{-1}$ [14]. No pH buffer was included in this preparation to ensure against formation of undefined complexes. Prior to flux experiments, the external solution was replaced by a solution that contained 1 mM EDTA, and 0.12 M KNO_3 at pH 7.1 in the absence of Al. The suspension was maintained CO_2-free under argon.

At time zero, 1 ml of the liposome suspension was added to 1 ml of external solution in a Spectrapore 2 dialysis membrane (M_r cutoff 14000) that was suspended in a 10 ml Teflon jar that contained 8 ml of external solution. Thus, the total volume of the system was 10 ml with a PC concentration of 2 mM. The solutions inside and outside of the dialysis membrane were rapidly stirred using a setup similar to one described earlier [15]. The system was maintained CO_2-free under a stream of argon, and temperature was maintained at 25°C by a circulating water bath. At 6-min intervals, 0.5-ml aliquots were removed from the solution outside of the dialysis membrane and stored for Al analysis in HF-washed polypropylene sample containers. The aliquot was immediately replaced by Al-free solution to maintain a constant volume of 10 ml. The samples were digested overnight in 0.1% HNO_3 at 50°C, and then assayed for Al content by graphite furnace atomic absorption spectroscopy using drying, ashing,

and ramping steps outlined previously [16]. Standard additions revealed no interference from EDTA, citrate, nor from PC at the concentrations used in this experiment. Flux across the dialysis membrane was found to be much more rapid than flux across the lipid bilayer and was ignored.

Results

Aluminum citrate and carboxylic acid permeabilities measured by pyranine fluorescence

Typical fluorescence recordings are shown in Fig. 1. The decline in fluorescence at the top-left is due to H^+/OH^- flux driven by the pH 3.5 (out), pH 7.1 (in) gradient, which causes slow acidification of the vesicle interior. Under the conditions of this experiment, fluorescence of the deprotonated form was measured. Consequently, acidification resulted in a reduced signal. Upon addition of 0.5 mM lactate, the fluorescence signal dropped abruptly due to permeation of lactic acid and deprotonation of the acid in the relatively alkaline vesicle interior. Curves for malic acid, citric acid and aluminum citrate were acquired in the same manner. Acetic acid (a known protonophore) was added at the end of the three latter experiments to demonstrate the existence of a pH gradient.

We used Eqn. 1 to calculate neutral acid permeability coefficients

$$P = J/(A \cdot \Delta C) \tag{1}$$

where P is the permeability coefficient in $cm \cdot s^{-1}$, J is flux in $mol \cdot s^{-1}$ per unit area of lipid bilayer surface, A is the total surface area of lipid in cm^2, and ΔC is the neutral acid concentration difference across the bilayer in $mol \cdot cm^{-3}$. Assuming 70 $Å^2$ surface area per phospholipid, the total liposome surface area A in 1 ml of suspension is 2100 cm^2. ΔC values for each of the neutral acids are shown in Table I. These values were calculated from the known total concentration of the solutes added and their pK_a values corrected to 0.5 M ionic strength at room temperature. Table I also shows the flux J for each neutral acid. We calculated J from the rate of fluorescence change per second multiplied by a calibration factor of $5.83 \cdot 10^{-13}$ protons per fluorescence unit per cm^2 surface area derived from Fig. 1, and by the number of protons per molecule that would dissociate in the alkaline vesicle interior. The last column in Table I is the permeability coefficient P calculated according to Eqn. 1.

There are three important points in Table I. First, our permeability coefficients for HF ($7.2 \cdot 10^{-5}$ $cm \cdot s^{-1}$) and HNO_3 ($9 \cdot 10^{-4}$ $cm \cdot s^{-1}$) are similar to values reported earlier ($3.1 \cdot 10^{-4}$ and $9.2 \cdot 10^{-4}$ $cm \cdot s^{-1}$, respectively) using PC black-lipid membranes [5]. Thus, our procedure gives reasonable results. Second, the permeability coefficient for neutral aluminum citrate is at most $2 \cdot 10^{-10}$ $cm \cdot s^{-1}$, which is comparable to coefficients for monovalent cations and is about seven orders of magnitude less than coefficients for HF and HNO_3. On this basis, we conclude that lipid bilayer permeation by aluminum citrate is slow. Third, the permeability coefficients for the series of α-hydroxy carboxylic acids from lactic acid to citric acid decrease by about 500-fold

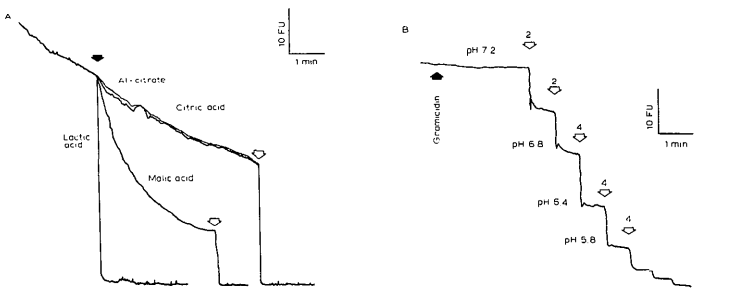

Fig. 1. (A) Change in liposome-entrapped pyranine fluorescence upon addition of neutral acids to the external solution. The entrapped solution contained 0.5 mM pyranine, 10 mM Pipes, 10 mM aspartate, and 0.25 M K_2SO_4 at pH 7.1. The external solution was composed of the same buffer at pH 3.5 except for the absence of pyranine. Neutral acids were added to the external solution at the black arrow in the following final concentrations: lactic acid (31 μM); malic acid (2 mM); citric acid (6 mM); aluminum citrate^0 (1.8 mM); 5 mM acetic acid (a known protonophore) was added at the open arrows to demonstrate the existence of a pH gradient. (B) A calibration curve in which the liposomes were rendered permeable by the addition of gramicidin. Pyranine fluorescence was then varied by the addition of 0.88 M H^+ as H_2SO_4 in μl aliquots as shown at the arrows. The pH values shown are for H^+ activities inside the vesicles over the range of fluorescence intensities used in our assays.

TABLE I

Values used to calculate permeability coefficients for the neutral acids

pK values for the acids at 0.5 M ionic strength were the following: lactic acid, 3.72; malic acid, 4.65 and 3.32; citric acid, 5.72, 4.36, and 2.99; aluminum citrate, 3.26; HF, 2.9; and HNO_3, −1.2.

Compound	Neutral acid conc. (mM)	Mean neutral solute flux, J (mol·cm^{-2}·s^{-1})	P (cm·s^{-1})	
			mean	S.D.
Lactic acid	0.03	$4.9 \cdot 10^{-13}$	$1.4 \cdot 10^{-6}$	$1.5 \cdot 10^{-6}$
Malic acid	2.0	$6.5 \cdot 10^{-14}$	$1.2 \cdot 10^{-8}$	$1.1 \cdot 10^{-8}$
Citric acid [a]	6.00	$9.9 \cdot 10^{-16}$	$3.1 \cdot 10^{-11}$	$3.4 \cdot 10^{-11}$
Al-citrate [b]	1.82	$3.9 \cdot 10^{-16}$	$2.0 \cdot 10^{-11}$	$1.0 \cdot 10^{-11}$
HF	0.008	$5.6 \cdot 10^{-13}$	$7.2 \cdot 10^{-6}$	$1.4 \cdot 10^{-6}$
HNO_3	$7.0 \cdot 10^{-6}$	$6.0 \cdot 10^{-15}$	$9.0 \cdot 10^{-4}$	$1.4 \cdot 10^{-4}$

[a] This estimate of citric acid permeability is equal to or greater than the actual value.

[b] The permeability coefficient shown for aluminum citrate is the mean value for the graphite furnace experiment and the pyranine experiment where P was $3.1 \cdot 10^{-11}$ or less.

per additional carboxyl group. This is a key to understanding the rate limiting process for aluminum-citrate permeation as discussed later.

Measurement of aluminum-citrate permeability by graphite furnace atomic absorption spectroscopy

We corroborated the data for aluminum-citrate permeation using an assay that measured Al flux directly rather than relying on the indirect measure above. Fig. 2 shows the rate of Al movement into an external medium

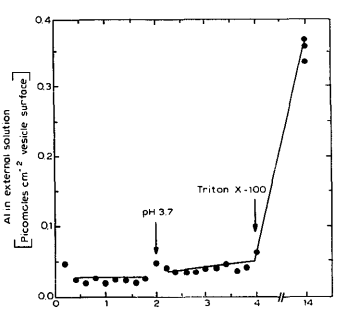

Fig. 2. Aluminum-citrate efflux from liposomes as measured by graphite furnace atomic absorption spectroscopy. The entrapped solution contained 1 mM aluminum-citrate and 0.12 M KNO_3, and the external solution contained 1 mM K_2EDTA in the same electrolyte. From $t = 0$ to 2 h measured efflux is the aluminum-citrate anion at pH 7.1 in the presence of 1 μM valinomycin. From 2 h to 4 h efflux is driven by aluminum citrate^{-1} (0.63 mM entrapped concentration) and by aluminum citrate^{0} (0.27 mM entrapped concentration) at pH 3.7. At 4 h the liposomes were lysed by the addition of Triton X-100 (1% final concn.). The function for the line from 2 to 4 h is $Y = 0.01 + 0.0086)X$, $r^2 = 0.61$.

from a 1 mM aluminum-citrate pool entrapped in PC liposomes. At time zero, the internal pH was 7.1, and 1 μM valinomycin was added to the suspension to allow rapid permeation of K^+, thus preventing generation of a diffusion potential. Under those conditions, the measured aluminum-citrate efflux was of the monovalent anion. At two hours, a pulse of acidity (as H_2SO_4) was added to the suspension driving the pH of the external medium down to 3.7. In the absence of a diffusion potential, it is known that H^+/OH^- permeability of lipid bilayers is extremely fast [17], which leads to equilibration of the entrapped solution at pH 3.7 in a matter of seconds. At pH 3.7, the entrapped concentration of neutral aluminum citrate was 0.27 mM, which caused a modest increase in efflux as can be seen in Fig. 2. Using Eqn. 1, we calculated a neutral aluminum-citrate permeability coefficient of $8.8 \cdot 10^{-12}$ cm·s^{-1} for this experiment, which is about 20-fold less than the value for the pyranine experiment shown above. This supports our conclusion that lipid bilayer permeation by the aluminum-citrate complex is slow.

Comparison of membrane permeation by aluminum citrate, three α-hydroxy carboxylic acids, and trimethylcitrate

Our objective in this series of experiments was to distinguish between mechanisms that could limit lipid bilayer permeation by citric acid and neutral aluminum citrate. The two proposed options are breakage of hydrogen bonds during passage from the aqueous phase to the bilayer and resistance to diffusion within the bilayer.

Fig. 3 shows that the relationship between hydrogen-bond number and lipid bilayer permeability is approximately log-linear for the α-hydroxy carboxylic acids tested. That is, each additional carboxyl group (three additional hydrogen bonds) results in a 500-fold decrease in the permeability coefficient. This supports the postulate that hydrogen-bond breakage is the rate-limiting step for citric acid permeation; however, the

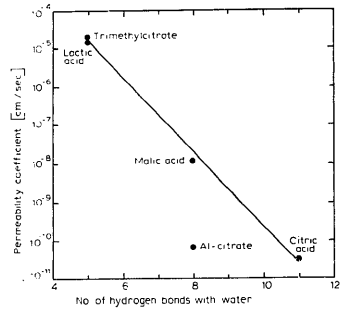

Fig. 3. The relationship between hydrogen bond number and lipid bilayer permeability. Hydrogen bonds per molecule were assigned according to Stein [8]. P for lactic acid, malic acid, and citric acid are the means from Table 1. P for neutral aluminum citrate is from the graphite furnace assay. P for trimethylcitrate was calculated from data of Cohen and Bangham [18] and from data of Sha'afi et al. [28]. The function for the line through the α-hydroxy carboxylic acids is $\log Y = -0.22 - 0.9X$. $r^2 = 0.99$.

correlation is not convincing because it cannot discount the effect of each additional carboxyl group on diffusion within the bilayer. Fig. 4, a log-linear graph of molar volume against permeability coefficient, demonstrates that the change in molar volume associated with each additional carboxyl group within the homologous series also correlates with the reduction in P predicted by steric hindrance.

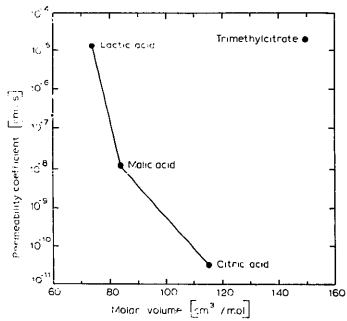

Fig. 4. The relationship between molar volume and lipid bilayer permeability. Permeability coefficients are the same as in Fig. 3. Molar volumes for lactic acid, malic acid, and citric acid were calculated by dividing their densities (in $g \cdot cm^{-3}$) by their molecular weights (in $g \cdot mol^{-1}$). The value assigned to trimethylcitrate is a low estimate based on its molecular weight divided by the density of citric acid. Its true molar volume is probably greater.

To distinguish between the two possible rate-controlling mechanisms, we have included permeability values for trimethylcitrate from Cohen and Bangham [18]. Trimethylcitrate can form five hydrogen bonds with water (like lactic acid) but it has a molar volume larger than citric acid. If hydrogen bonding controls permeation, then trimethylcitrate should have a permeability coefficient similar to that of lactic acid; alternatively, if molar volume limits permeation then trimethylcitrate should have a coefficient like citric acid. Figs. 3 and 4 show that the permeability of trimethylcitrate is predicted by hydrogen-bond number, not by molar volume. We conclude that lipid bilayer permeability of the carboxylic acids is limited by hydrogen bonding in the aqueous phase.

Fig. 3 also includes a point for neutral aluminum citrate. The hydrogen bond number for this complex should be three less than that of citric acid because Al^{3+} displaces a proton from each of two coordinating carboxyl groups and from a coordinating hydroxyl group [19]. It is clear from the figure that aluminum-citrate permeability is significantly lower than predicted by hydrogen-bond number.

Discussion

Our data show that the lipid bilayer permeability of aluminum citrate is slow with a P value of about $1 \cdot 10^{-11}$ cm · s^{-1}. The mechanisms which limit permeation can be understood by comparison with a series of α-hydroxy carboxylic acids from lactic to citric acid. Within that series the rate of permeation is controlled by hydrogen bonding between the solutes and water in the aqueous phase, which limits transfer across the water/lipid interface. Resistance to diffusion in the bilayer did not influence permeation in contrast to the results of Poznansky et al. [7] for a homologous series of amides. This can be explained by the nature of the two series. Acetamide through valeramide are primary monoamines that form three hydrogen bonds with water. The activation energies for permeation by these compounds differ by only 3 kcal · mol^{-1}, therefore, modest increases in activation energy due to steric hindrance (e.g. 1.1 kcal · mol · mol^{-1} from formamide to valeramide [7]), can be observed. By comparison, within the carboxylic acid series, three hydrogen bonds with water are added per carboxyl group. Assuming an activation energy of 1.8 kcal · mol^{-1} per hydrogen bond [18], this results in a 5.4 kcal · mol^{-1} increase in the activation energy required to cross the water/lipid interface per functional group. The difference between the two series is emphasized by the range of permeability coefficients

associated with them. Among the amides (fixed number of hydrogen bonds), the coefficient varies less than 10-fold from $1 \cdot 10^{-5}$ cm $\cdot$ s^{-1}; among the carboxylic acids (5–11 hydrogen bonds per molecule) the permeability coefficient varies by seven orders of magnitude. We conclude that the added steric hindrance associated with each carboxyl group cannot be measured because the activation energy due to hydrogen bonding at the water/lipid interface has a much greater influence on permeation.

Hydrogen bonding at the interface cannot alone explain the permeability of aluminum citrate relative to the carboxylic acid series as shown in Fig. 3. If the proposed model were adequate, aluminum citrate would have a permeability coefficient like malic acid because Al displaces three protons from citric acid and reduces the number of possible hydrogen bonds from 11 to 8. We believe that the difference is due to waters of hydration associated with the metal. In its dissociated forms, Al^{3+} is in 6-fold coordination with water. Formation of the citrate complex involves displacement of three waters of hydration from the ion, but three waters of hydration are presumably retained [20]. There are no known values for the free energy of hydration of the metal within the aluminum-citrate complex. A conservative guess can be made by calculating the free energy of interaction between water and a single Al-O bond moment. To make this calculation we used Eqn. 2

$$E = -2\mu \cdot \mu_2 / 4\pi r^3 \varepsilon_0 \qquad (2)$$

where E is the bond energy in kJ $\cdot$ mol^{-1}, μ_1 is the dipole moment of water ($6 \cdot 10^{-30}$ C $\cdot$ m), r is the distance between the molecular dipoles (about $2.1 \cdot 10^{-10}$ m for the distance between the water dipole and mid-length of the Al-O bond), and ε_0 is the permittivity of a vacuum ($8.8 \cdot 10^{-12}$ C$^2 \cdot$ m$^2 \cdot$ J^{-1}) [21]. The term μ_2 generally refers to the dipole moment of the second compound, in this case the Al^{3+}-oxyanion complex. However, the direction of each of the three Al-O bonds is unknown, which prevents summation of the bond moment vectors to yield the molecular dipole moment. To overcome this we solved Eqn. 2 for the interaction between a single Al-O bond moment and a single water molecule. To calculate the bond moment we used Eqn. 3

$$\mu_2 = qr' \qquad (3)$$

where q is the equal and opposite charge on the atoms of the bond and r' is the distance between the charges [21]. Assuming equal distribution of the +3 charge of Al between the three oxyanions, the net charge of each is zero. However, using Pauling's electronegativity values for Al and O, we calculate a partial ionic character to the bond of 0.4; that is, 0.4 electron equivalents of charge on the Al per Al-O bond. Given ionic radii of Al^{3+} and O^{-1} equal to 67.5 pM and 170 pM respectively, r' becomes 237 pm. Plugging these values into Eqn. 3 yields a bond moment, μ_2, of $1.5 \cdot 10^{-29}$ C $\cdot$ m. Finally, solving Eqn. 2 for the interaction between a single water and a single Al-O bond moment, we find $E = 1.8 \cdot 10^{-19}$ J per dipole-dipole bond or approximately 25 kcal $\cdot$ mol^{-1} of dipole bonds. This value is small relative to the ion-dipole bond between water and Al^{3+} (about 146 kcal $\cdot$ mol^{-1}) but it is significantly larger than Pauling's calculation of 7.6 kcal $\cdot$ mol^{-1} for a single hydrogen bond between water and a carboxyl group [22]. Thus, the neutral aluminum-citrate complex would be expected to have a higher activation energy for crossing the water/lipid interface than does citric acid. Our permeability data support this. This result may also explain some earlier, unpublished octanol-water partition data from our lab. In those experiments the solvent-to-water ratio was set to allow detection of neutral aluminum-citrate transfer between phases assuming the same partition coefficient as citric acid, i.e. 0.02 [23]. We were never able to detect loss of aluminum citrate from the aqueous phase, which now can be explained by the large bond energy between the dipoles of water and the bound metal.

If the permeability of neutral aluminum citrate is as low as our data indicate, how can we explain the increase in tissue Al associated with dietary supplements of citrate? One possibility is that passive diffusion across biological membranes is significantly different from diffusion across liposomes. Permeability of hydrophilic solutes is typically greater in biological membranes than in liposomes [24]. However, Aubert and Motais [25] were unable to detect erythrocyte permeation by citric acid, in support of the liposome model.

Another plausible explanation for increased absorption of Al in the presence of citrate is a carrier-mediated process. Bergsma and Konings [26] demonstrated that energy-dependent citrate uptake in *Bacillis subtilis* requires formation of complexes with divalent cations. In that mechanism, a protonmotive force drives symport of the monovalent anionic complex with a proton. This mechanism is consistent with transport of the anionic aluminum citrate complex because in both cases the charged site is an unbound, ionized carboxyl group. Another possible carrier-mediated mechanism is Al-citrate competition with iron uptake. For instance, Hussein et al. [27] have demonstrated induction of a citrate-dependent iron transport mechanism in *Escherichia coli*. In this process the ferric-dicitrate complex binds to the inner or outer bacterial membrane and the iron is removed, then transported by a membrane bound receptor protein. Al can also form a dicitrate complex [2,4] that could complete for these receptor sites. One problem with this scenario is the unusually high

specificity of Fe carriers for Fe(III). For example Cline et al. [28] showed that in the presence of 10 μM Al^{3+} and 1 nM Fe^{3+} at pH 4.0, the siderophore desferrioxanine-B is quantitatively bound to Fe^{3+}.

In summary, we have shown that lipid bilayer permeation by neutral aluminum citrate is slow. Comparisons with α-hydroxy carboxylic acids and trimethylcitrate suggest that the rate of permeation is limited by hydrogen bonding between water and the citrate ligand, and by dipole-dipole bonds between water and the bound Al. We conclude that observed increases in tissue Al associated with high levels of Al and citrate, in the intestine of rats and man, cannot be explained by neutral aluminum-citrate permeation of lipid bilayers.

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References221

1 Haug, A. (1984) CRC Crit. Rev. Plant Sci. 1, 345–373.
2 Martin, R.B. (1986) J. Inorg. Biochem. 28, 181–187.
3 Slanina, P., Frech, W., Ekstrom, L., Loof, L., Slorach, S. and Cedergren, A. (1986) Clin. Chem. 32, 539–541.
4 Slanina, P., Falkeborn, Y., Frech, W. and Cedergren, A. (1984) Fd. Chem. Toxic. 22, 391–397.
5 Gutknecht, J. and Walter, A. (1981) Biochim. Biophys. Acta 644, 153–156.
6 Parsegian, A. (1969) Nature 221, 844–846.
7 Poznansky, M., Tong, S., White, P.C., Milgram, J.M. and Solomon, A.K. (1976) J. Gen. Physiol. 67, 45–66.
8 Stein, W.D. (1967) The Movement of Molecules Across Cell Membranes, p. 76, Academic Press, New York.
9 Cohen, B.E. (1975) J. Membr. Biol. 20, 205–234.
10 Barchfeld, G.L. and Deamer, D.W. (1988) Biochim. Biophys. Acta 944, 40–48.
11 Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Biochim. Biophys. Acta 858, 161–168.
12 Jackson, M.L. (1958) Soil Chemical Analysis, pp. 151–153, Prentice-Hall/Englewood Cliffs, NJ.
13 Kano, K. and Fendler, J.H. (1978) Biochim. Biophys. Acta 509, 289–299.
14 Bertsch, P.M., Thomas, G.W. and Barnhisel, R.I. (1986) Soil. Sci. Soc. Am. J. 50, 825–830.
15 Chowhan, Z.T., Yotsuyanagi, T. and Higuchi, W.I. (1972) Biochim. Biophys. Acta 266, 320–332.
16 Nater, E.A., Burau, R.G. and Akeson, M. (1989) Anal. Chim. Acta, (in press).
17 Deamer, D.W. and Nichols, J.W. (1983) Proc. Natl. Acad. Sci. USA 80, 165–168.
18 Cohen, B.E. and Bangham, A.D. (1972) Nature 236, 173–174.
19 Jackson, G.E. (1982) S. Afr. J. Cehm. 35, 89–92.
20 Tikhonov, V.N. (1973) Analytical Chemistry of Aluminum, p. 14, Wiley, New York.
21 Huheey, J.E. (1983) Inorganic Chemistry, 3rd Edn. p. 161 and p. 265, Harper & Row, New York.
22 Pauling, L. (1960) The Nature of the Chemical Bond, 3rd Edn., pp. 477–478, Cornell U. Press, Ithaca, NY.
23 Leo, A., Hansch, C. and Elkins, D. (1971) Chem. Rev. 71, 525–616.
24 Deamer, D.W. and Bramhall, J. (1986) Chem. Phys. Lipids 40, 167–188.
25 Aubert, L. and Motais, R. (1975) J. Physiol. 246, 159–179.
26 Bergsma, J. and Konings, W.N. (1983) Eur. J. Biochem. 134, 151–156.
27 Hussein, S., Hantke, K. and Braun, V. (1981) Eur. J. Biochem. 117, 431–437.
28 Cline, G.R., Powell, P.E., Szaniszlo, P.J. and Reid, C.P.P. (1983) Soil Sci. 136, 145–157.
29 Sha'afi, R.I., Gary-Bobo, C.M. and Solomon, A.K. (1971) J. Gen. Physiol. 58, 238–258.