

Interactions between Metal Cations and the Ionophore Lasalocid. Part 12: ¹Alkaline-earth–Lasalocid 1–2 Complex Salts in Chloroform. Formation and Structure

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Extraction of alkaline-earth cations M^{2+} in water by lasalocid HA in chloroform results in formation of neutral complex salts MA_2 . Corresponding reaction constants are obtained. An increase in the selectivity of lasalocid as a function of cation size is observed. A systematic investigation of the structure in chloroform of the species formed with all the alkaline-earth cations is carried out using ¹H and ¹³C NMR spectroscopy. The two anion ligands consistently play a strongly dissymmetric role but significant structure variations are observed according to the cation involved, coordination sites, respective location of the two ligands, and rate of exchange between them. Data on lasalocid–divalent cation interaction processes in water–membranes, either natural or model, are tentatively related to these findings.

Lasalocid (Fig. 1), a carboxylic polyether of bacterial origin, is known to facilitate transportation across membranes of both monovalent and divalent cations. Many aspects of the interaction of alkaline-earth cations with lasalocid have been investigated: formation of various complexes^{2–7} including kinetic aspects,³ structure of the species formed in solution^{2,8–12} and in the solid state^{13,14} and transportation across model systems.^{4,15,16}

This paper focuses on species formed in weakly protic, weakly polar media. Characterization and formation constants of complexes resulting from the interactions of lasalocid and alkaline-earth cations are jointly attained using cation extraction of the aqueous phase at variable pH. Though many studies of this type have been devoted to such equilibria in homogeneous media, *i.e.* in organic solvents, few have involved these equilibria in heterogeneous liquid systems, *i.e.* in biphasic water–organic systems, which are nevertheless more representative of water–membrane systems.

The selectivity of lasalocid for the various alkaline-earth cations, in terms of both formation constants and cation transport across an organic phase, was expected to be linked to the structure of the complexes formed in these organic phases. Accordingly a systematic ¹³C and ¹H NMR study of the various alkaline-earth lasalocid salts was carried out following on from our previous work on lasalocid acid,¹⁷ anion¹⁷ and alkali-metal salts structures^{17–18} in chloroform. As regards alkaline-earths, previous NMR data focus on the calcium salt.¹¹ Our purpose here was to ascertain how the structure of these species, *i.e.* the location of the cation, its bonding sites and the conformation of the ligand, varied with the cation involved. Literature data on analogous paramagnetic cation (Mn^{2+} and Cu^{2+}) lasalocid salts^{19,20} provided useful additional information for this purpose.

Experimental

Chemicals.—Lasalocid and its tetramethylammonium salt were obtained and purified as previously stated.²¹ Its alkaline-earth salts were prepared by treatment of a chloroform solution of the lasalocid tetramethylammonium salt with an aqueous solution of the appropriate alkaline-earth chloride. The chloroform phase was washed with purified water, and then

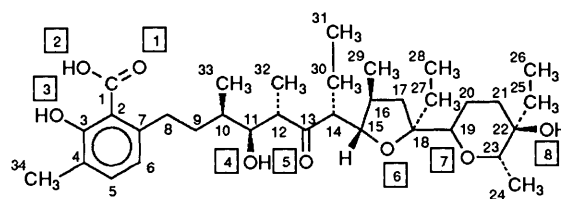


Fig. 1 Lasalocid formula showing carbon and oxygen numbering scheme

decanted and the solvent evaporated. To carry away the last traces of water, the substrate was dissolved in acetone, and then the solvent was evaporated, this operation being repeated twice. The salt thus obtained was left to stand in a PTFE-coated dish for 24 h at 40 °C, in a vacuum oven. Its purity was then checked by metal ion EDTA titration after reextraction.

The solvent used in the extraction experiments was chloroform stabilized with 0.9% by mass of ethanol, a *pro-analysis* product from Merck, Darmstadt, Germany. The deuteriochloroform used in the NMR experiments, of the best quality grade available, was obtained from the CEA, France. Water was distilled three times in a quartz apparatus and then deaerated.

Alkaline-earth chlorides were 'suprapur' grade from Merck Company, except the barium salt which was a 'for analysis' grade from Prolabo, Paris, France. Bis-Tris was from Sigma Company and triethanolamine was carefully distilled from a Fluka 'puriss' sample.

Extraction Experiments.—The procedure was analogous to that previously described for lasalocid–alkali-metal cations²² or monensin–alkaline-earth cations²³ in the same biphasic solvent system.

Buffering of the aqueous solutions was achieved using mixtures of hydrochloric acid and either Bis-Tris [bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane] for pH from 5 to 7.5 or triethanolamine for pH from 6 to 9. Monitoring of the pH of the aqueous phase and assay of the overall metal concentration in the organic phase by flame spectrometry were as previously described.²³

Equilibration of equal volumes of chloroform and water results in changes in both volume and composition of the two

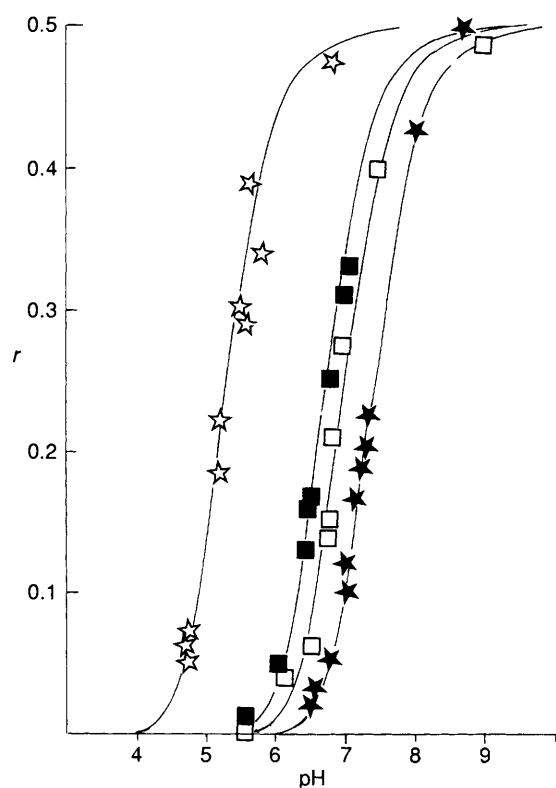


Fig. 2 Extraction of alkaline-earth cations in water ($c_M^o = 10^{-2}$ mol dm $^{-3}$) by lasalocid in chloroform ($c_A^o = 2 \times 10^{-3}$ mol dm $^{-3}$). Extraction ratio r as a function of pH of aqueous solution: \star , Mg $^{2+}$; \square , Ca $^{2+}$; \blacksquare , Sr $^{2+}$; \star , Ba $^{2+}$

Table 1 Formation constants of 2-1 lasalocid complexes of alkaline-earth cations in the water-chloroform solvent system according to reaction (1) (298 K, molar scale of concentration in the two phases) in terms of $\log_{10} K_1$ (estimated accuracy ± 0.2 log unit)

Cation	$\log K_1$
Mg $^{2+}$	-9.7
Ca $^{2+}$	-8.4
Sr $^{2+}$	-8.2
Ba $^{2+}$	-7.5

phases. In percent by mass, starting with chloroform 99.10 stabilized with ethanol 0.90, the composition at equilibrium at 25 °C were found to be: 24 chloroform 99.84, ethanol 0.11, water 0.05 for the organic phase, water 98.05, ethanol 1.14, chloroform 0.81 for the aqueous phase and the initial-to-final volume ratios $\xi = 0.981$ for the aqueous phase and $\bar{\xi} = 1.01$ for the organic one.

NMR Experiments.—Spectra and contour plots corresponding to ^1H - ^1H and ^1H - ^{13}C correlations were obtained as previously 17 using a Bruker MSL 300 instrument.

Results

Formation of Lasalocid-Alkaline-earth Complexes in the Water-Chloroform Solvent System.—Extraction of the metal cation from the aqueous phase by the ionophore in the organic phase was expressed, as previously, $^{22-24}$ by the ratio r defined as the quotient in the organic phase of the overall metal concentration and the overall ionophore concentration; $r = \bar{c}_M/\bar{\xi}c_A^o$ where c and \bar{c} are the molar actual concentrations in the aqueous and organic phases, c^o and \bar{c}^o the corresponding

initial ones, ξ and $\bar{\xi}$ the initial over final volumes of the phases, M and A standing for overall metal and ionophore. The extraction curves, given by ratio r versus pH of the aqueous phase, have the usual S-shaped form with an asymptote $r = 0.5$, as illustrated in Fig. 2. This corresponds to major formation of MA $_2$ species from lasalocid HA and cations M $^{2+}$ according to relation (1), in which the species in the organic phase are



upperlined. If such a reaction predominates, *i.e.*, if formation of complexes other than MA $_2$ can be neglected, pH and $\text{p}K_1$ are related by eqn. (2), in which y_2 is the mean molar activity

$$\text{pH} = \frac{1}{2} \text{p}K_1 - \frac{1}{2} \log \xi \bar{\xi} y_2 - \frac{1}{2} \log \bar{c}_A^o - \frac{1}{2} \log [(c_M^o - r \bar{c}_A^o)(1 - 2r)^2/r] \quad (2)$$

coefficient for 2-1 electrolytes in the aqueous phase, estimated as previously. 23

From eqn. (2) it is clear that provided initial concentration conditions are identical (here $c_M^o = 10^{-2}$ and $\bar{c}_A^o = 2 \times 10^{-3}$ mol dm $^{-3}$), the shape of curve $r = f(\text{pH})$ does not depend on the equilibrium constant K_1 . The actual value of this constant only determines the location of the curve on the pH scale. Using eqn. 2, $\text{p}K_1$ can be calculated for each experimental (r , pH) couple. Full line curves plotted on Fig. 2 were determined using K_1 values thus obtained. This data shows for the four cations that MA $_2$ complex salts are mainly formed and that no appreciable partition of these species between the two phases occurs (otherwise the asymptote would be at $r = y$ with $y < 0.5$). Mean $\text{p}K_1$ values, reported in Table 1, vary as a function of the size of the cation, maximum complexation being for the barium ion.

NMR Spectra of 1-2 Alkaline-earth-Lasalocid Complex Salts in Chloroform.—Both ^1H and ^{13}C (broad band and J -modulated spin echo) NMR spectra are well resolved at ambient temperature for MgA $_2$, SrA $_2$ and BaA $_2$, though some linewidth increases are observed on the proton spectra, mainly for magnesium and to a lesser extent strontium salts. Unambiguous assignment of ^1H and ^{13}C resonance frequencies was then easily derived, as already described, 17 from their ^1H - ^1H (COSY 45) and ^1H - ^{13}C correlation contour plots. This was carried out independently for these three lasalocid salts. New complete data on their ^1H and ^{13}C chemical shifts in chloroform are given in Tables 2 and 3.

The specific behaviour of the calcium salt is noteworthy. On the ^{13}C , ^1H -decoupled spectrum, the signals corresponding to C-13, C-18 and C-30 were strongly enlarged at ordinary temperature as shown in Fig. 3 in which the calcium salt spectrum is compared with the strontium salt spectrum. As shown in Fig. 4, with the exception of 5-H, 6-H and 34-H, ^1H signals were enlarged so that it was not possible to obtain readable ^1H - ^1H and ^1H - ^{13}C contour plots. ^{13}C and ^1H resonance frequencies for CaA $_2$ were therefore assigned by analogy with the other species. In this way, ^{13}C chemical shifts can be reported (also in Table 2) for all the carbons of the molecule, but chemical shifts for only a limit number of protons, those easily accessible from the simple spectra, are reported in Table 3. When the temperature was decreased, further enlargement of the ^{13}C signals occurred, followed by the splitting of most of them, as shown at the bottom of Fig. 3, in the spectra at 233 K.

Discussion

Selectivity of Lasalocid for Alkaline-earth Cations.—The selectivity sequence observed here for the formation of the MA $_2$

Table 2 Observed mean ^{13}C chemical shift for lasalocid anion in lasalocid-alkaline-earth neutral salts in CDCl_3 (room temp., concentration $\sim 0.1 \text{ mol dm}^{-3}$, referred to Me_4Si in CDCl_3 , carbons numbering in Fig. 1)

^{13}C	MgA ₂	CaA ₂	SrA ₂	BaA ₂
1	175.5	176.6	177.1	176.6
2	115.8	116.2	116.9	116.4
3	161.1	161.2	160.9	161.7
4	123.3	123.2	122.8	123.0
5	132.8	132.3	131.8	131.8
6	120.7	120.1	119.8	119.6
7	143.8	144.1	144.4	144.5
8	34.5	33.8	32.7	32.7
9	36.4	37.0	38.0	38.3
10	36.0	34.5	33.7	33.7
11	72.2	72.2	70.8	70.8
12	49.4	50.0	49.1	49.2
13	216.6	217.3	218.5	219.9
14	55.7	55.1	55.9	56.4
15	84.3	85.0	83.6	83.8
16	35.0	34.7	34.4	34.4
17	39.6	38.6	38.1	37.8
18	87.4	87.9	87.1	87.5
19	71.7	70.5	70.1	70.3
20	20.7	20.0	19.7	19.6
21	29.1	29.6	29.2	29.0
22	71.4	72.9	72.4	72.7
23	76.9	76.2	77.1	77.1
24	13.6	13.7	13.5	13.3
25	30.7	30.8	31.2	31.4
26	6.4	6.5	6.9	7.0
27	29.4	29.6	29.4	29.9
28	8.9	9.3	9.1	9.1
29	16.7	16.1	16.2	15.9
30	17.4	18.1	16.2	16.1
31	13.0	12.9	13.4	12.7
32	13.4	13.3	11.9	11.9
33	13.8	12.7	12.6	13.5
34	16.0	16.5	15.9	16.1

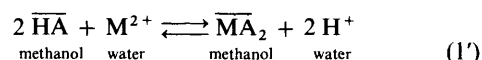
Table 3 Observed mean ^1H chemical shift for lasalocid anion in lasalocid-alkaline-earth neutral salts in CDCl_3 (conditions analogous to data in Table 2)

^1H	MgA ₂	CaA ₂	SrA ₂	BaA ₂
5	6.97	7.02	6.96	6.96
6	6.48	6.50	6.47	6.42
8A	3.55	4.25	4.20	4.22
8B	2.10		2.18	2.05
9A	1.97		1.59	1.58
9B	1.40		1.40	1.48
10	1.77		1.59	1.62
11	4.30	4.42	4.47	4.60
12	2.97	2.85	2.79	2.80
14	2.61		2.56	2.48
15	3.75	3.92	3.85	3.98
16	2.10	2.14	2.18	2.13
17A	1.84		1.77	1.78
17B	1.40	1.25	1.40	1.29
19	3.38	3.45	3.38	3.45
20A	1.63		1.81	1.86
20B	1.40		1.40	1.47
21A	1.40		1.26	1.89
21B				1.54
23	3.66	3.90	4.01	3.94
24	0.95		1.03	1.04
25A	1.06		1.40	1.35
25B				
26	0.72		1.01	0.97
27A	1.66		1.77	1.63
27B	1.40		1.33	1.13
28	0.80	≥ 0.80	0.50	0.56
29	1.04		1.00	1.01
30A	1.94		2.02	2.00
30B	1.40		1.29	1.17
31	0.80		0.83	0.64
32	0.95		0.92	0.96
33	0.97		0.78	0.78
34	2.10	2.21	2.18	2.19

species: $\text{Mg} < \text{Ca} < \text{Sr} < \text{Ba}$ is identical to that previously encountered in methanol.⁶ In order to compare these two sets of data more precisely, analogous reactions in the two solvent systems must be considered. From the results in methanol⁶ for the acid dissociation of the ionophore and the complexation of these cations by the ionophore anion, equilibrium constants and standard Gibbs functions for reaction (3) were calculated.



Hypothetical reaction (1') strictly corresponding to reaction (1)



in the water-chloroform system could then be considered and corresponding data calculated using a thermodynamic cycle. However, safe values for standard Gibbs functions of transfer from water to methanol are unfortunately not yet known for all the alkaline-earth cations.²⁵ Data compared in Fig. 5 are thus standard Gibbs functions for reaction (1) in water-chloroform and for reaction (3) in methanol.

These data for the two solvent systems show analogous trends as a function of the cation radius. This Gibbs function variation is related to both solvation of the cation in water or methanol and differences in solvation of the complex and the free ionophore in methanol or chloroform. In addition, it reflects the variation in the differences between interaction of the cation with the coordinating oxygens of the two ionophore

anion ligands and interaction of the same cation with the oxygens of the solvent, either water or methanol. This means that referring to their state of solvated cations in these protic solvents, barium is more strongly complexed by lasalocid anion than strontium, strontium than calcium and calcium than magnesium. This must be reflected in some structural features.

NMR Data Related to Structure of MA₂ Salts in Chloroform.—NMR data reported here concern only ^{13}C and ^1H chemical shifts. From this and previous data^{11,19-20} in chloroform and from what is known of the barium salt in the solid state,^{13,14} the two anion ligands seem to differ in both conformation and bonding of the cation. Determinations of ^1H - ^1H coupling constants or ^1H - ^1H nuclear Overhauser effects (NOE) were not attempted here since they should reflect a mean situation for the two ligands, which would be rather difficult to interpret.

From the previously mentioned signal linewidth increases observed in the ^{13}C and ^1H spectra, it appears that the exchange of the two anion ligands bound to the cation is more or less rapid according to the cation. The rapidity of this exchange would increase according to the following sequence: $\text{Ca} < \text{Mg} < \text{Sr} < \text{Ba}$. ^{13}C and ^1H chemical shifts reported in Tables 2 and 3 are thus mean values of chemical shifts corresponding to the two exchanging anion ligands. Separation of ^{13}C signals is only achieved for the calcium salt at low temperature but no attempt was made precisely to assign either of the twin resonances to either of the ionophore anions.

Information on coordination sites can be expected from Fig. 6 in which is reported, for all the carbons of the molecule, the variation in the observed chemical shifts from the uncomplexing

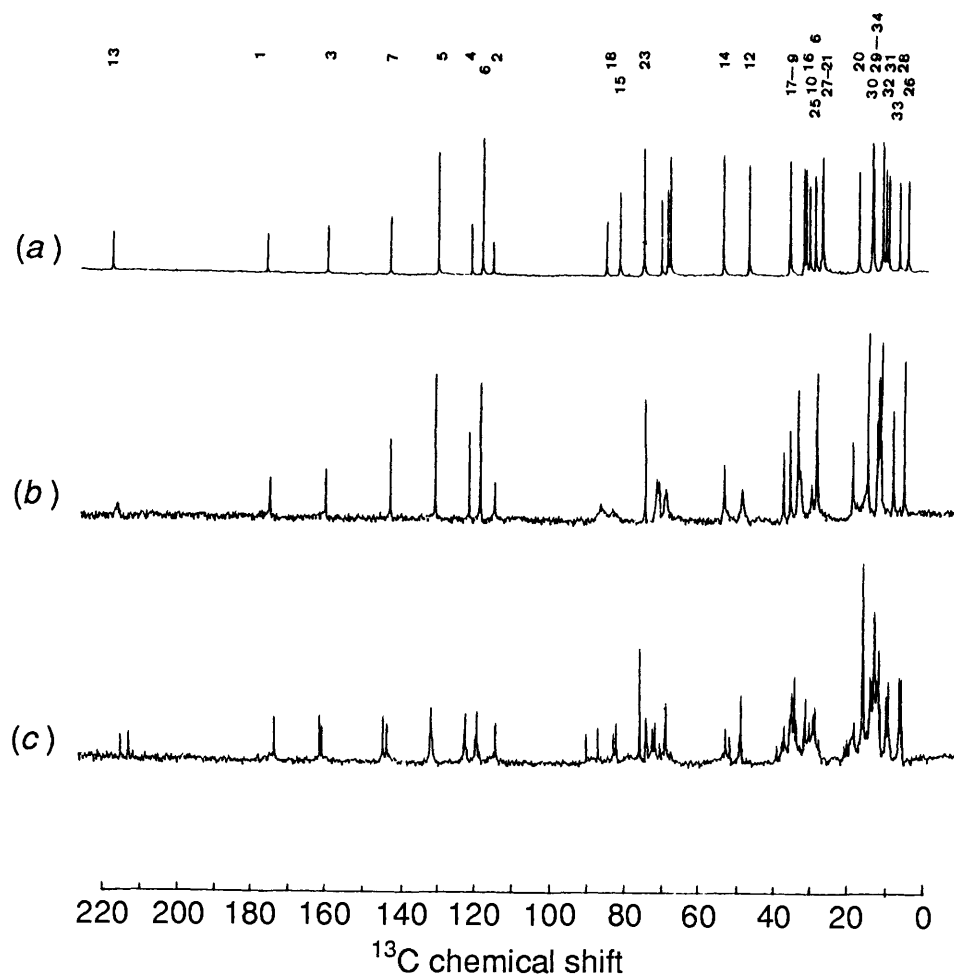


Fig. 3 ^{13}C Spectra of lasalocid alkaline-earth neutral salts: (a) SrA_2 at 296 K; (b) CaA_2 at 297 K; (c) CaA_2 at 233 K (concentration $c \sim 10^{-1} \text{ mol dm}^{-3}$, assignment of carbon signals at top)

lasalocid anion (in its tetraethylammonium salt¹⁷) to the anion involved in the MA_2 complex, both in chloroform at room temp. Displacement of the ^{13}C shifts of the carbons between these two states of the anion can be attributed to both conformational change and electronic effects related to modification or disruption of the internal hydrogen bonds and to coordination of the cation. The latter are particularly sensitive for the carbons adjacent to the oxygens involved in the coordination of the cation and would correspond to a low field displacement of their signals. A complex situation is reflected by Fig. 6. Sensitive differences are observed according to the cation involved. Both the conformation of the carbon backbone and the respective involvement of the various coordination sites are probably affected. Attempts have been made by other authors to use paramagnetic cations to label coordination sites in manganese¹⁹ and copper²⁰ lasalocid complexes. From these and the present data it appears that participation of the various oxygens in the bonding of the cations varies appreciably with the cation concerned. The carboxylic function, probably oxygen O-2 (O-1 being that hydrogen bonded to O-3) is always strongly involved, as is to a lesser extent the terminal hydroxyl oxygen O-8. According to the size of the cation, O-4 and O-5 could exchange their bonding role through some flexion-rotation in the C-10, C-14 area. Inversion between O-6 and O-7 ligation is also possible.

There is no rigorous way to assign the coordination sites to one or the other of the two anion ligands. Concerning the calcium salt, information can be tentatively derived from linewidth increases observed at room temp. and signal splitting

observed at low temperature. It is clear from this data that some of the oxygens have a rather different environment in the two anions ligands; it can then be suggested that oxygens O-4, O-5, O-6 and O-8 of only one of the ligands are involved in the coordination of the calcium ion but that the oxygens of the two carboxylates have a similar role.

Concerning the relative location of the two anions ligands, it helps to consider the solid structures of MA_2 salts; they are known only for two $\text{BaA}_2 \cdot \text{H}_2\text{O}$ crystals.^{13,14} At the molecular level, the structure is essentially the same for these two crystals, a 'head-to-tail' dimeric structure with pseudo-two-fold symmetry. In such a structure the two protons H-28 are just above the two benzene rings as was also shown for some dimeric lasalocid monovalent cation salts.²⁶ In these conditions, consequently to anisotropy related to an aromatic ring current, a strong upfield shift of the H-28 signal occurs.¹⁷ Values obtained here in chloroform for barium and strontium salts (δ 0.56 and 0.50) against δ 0.86 and 0.82 for the free acid and anion, agree well with a head-to-tail location of the two ionophore anion ligands. No such upfield shift of H-28 is observed for the magnesium salt (δ H-28 = 0.80) nor the calcium salt for which no ^1H signal appears before δ 0.8. For these two salts the respective location of the two anion ligands can not thus be stated.

Lasalocid-Alkaline-earth Cation Interactions in Natural and Model Membranes.—Lasalocid is known to monitor divalent cation flux across biological membranes. This was shown, for example in mitochondria²⁷ for Ca^{2+} and Mg^{2+} and in erythrocytes for both these cations²⁸ and Fe^{2+} .²⁹ These and

other divalent cations were also shown to be carried by lasalocid across phospholipid bilayer membranes,^{15,29-32} frequently taken as models of the natural ones. Thus the following question arises: has the data acquired here and elsewhere on the

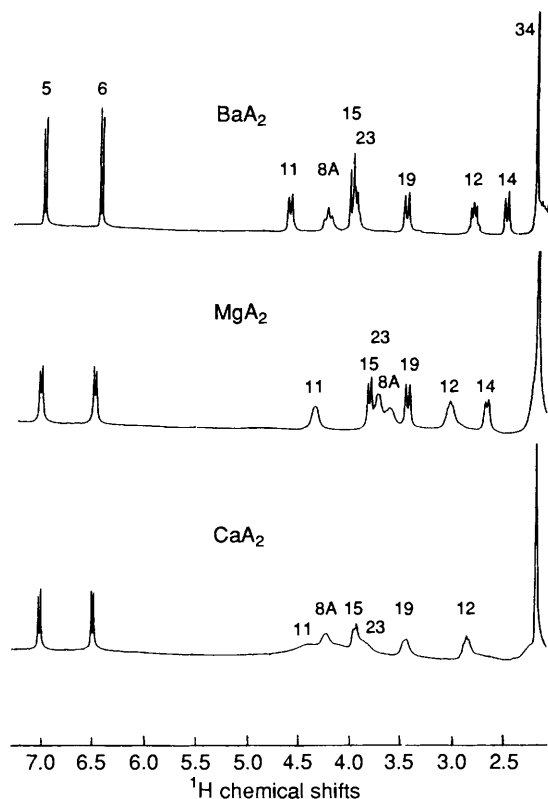


Fig. 4 ^1H spectra in chloroform at room temp. of lasalocid-alkaline-earth neutral salts

formation thermodynamics and the structure of the MA_2 salts in an aprotic apolar solvent such as chloroform any implications for observations made in biological and model systems?

Species migrating in the lipidic phases are assumed to be neutral species. Involvement of mainly the 1-1 complex, MA^+ , as claimed by Blau *et al.*¹⁵ would imply an improbable comigration of an anion soluble in such phases. Thus, it is generally accepted that the species involved in the so-called non-electrogenic transport of the divalent cations M^{2+} by lasalocid HA are their neutral salts MA_2 . This, which corresponds to our findings for cation transport in a water-chloroform-water system,⁴ has been clearly demonstrated in many cases³⁰⁻³³ for bilayer and natural membranes. Our study of the structure of these neutral salts is thus relevant. Owing to its difficulty, no pertinent study of their structure in natural or bilayer membranes has yet been undertaken; their structure in chloroform or other aprotic media is expected to be similar.

With regard to natural and model systems, the available data only concerns determination of cation flux in the presence of lasalocid and is restricted to studies on Ca^{2+} and Mg^{2+} and to cations Mn^{2+} used as paramagnetic probes. No systematic work on the whole series of the alkaline-earth cations has, to our knowledge, been carried out. For magnesium and calcium cations, the higher selectivity of lasalocid for calcium was clearly demonstrated in such translocation experiments.^{30,32} It has also been suggested by Antonenko,³² using a simple translocation model, that $\text{Ca}^{2+}/\text{Mg}^{2+}$ selectivity according to transport is considerably higher than that due to the ratio of the formation constant of the two cations at the interfaces. This would explain large differences in the translocation rate constants of the two salts in the organic phase. Such rate differences are not surprising given the differences suspected here in the structure of CaA_2 and MgA_2 and consequently in the design of their hydrophobic envelopes and in the insulation of their cations and carboxylate groups.

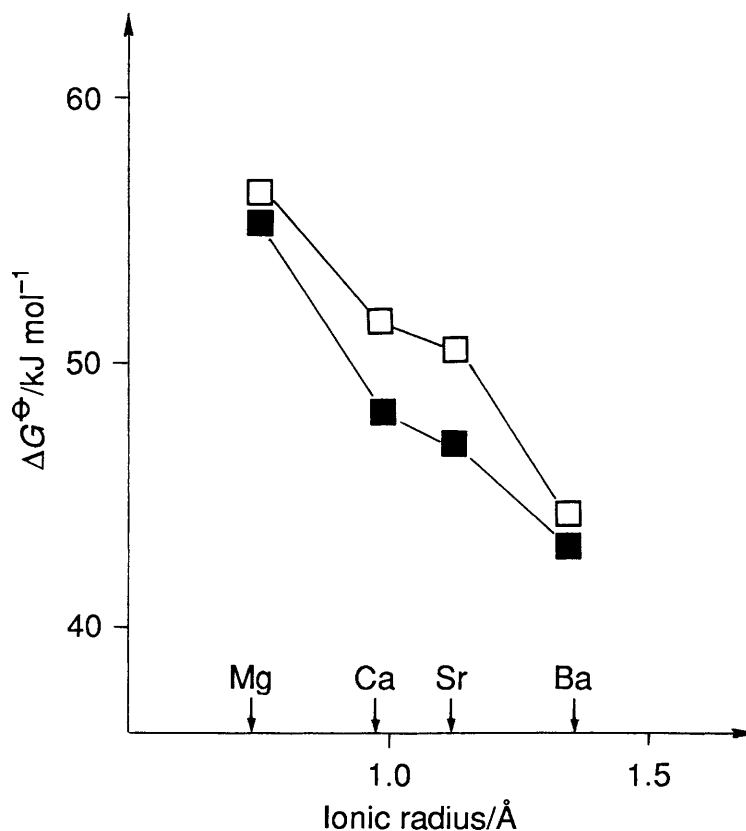


Fig. 5 Standard molar Gibbs functions for formation reactions of lasalocid-alkaline-earth salts in methanol [reaction (1), □] and in the chloroform-water system [reaction (3), ■] as a function of size of cation

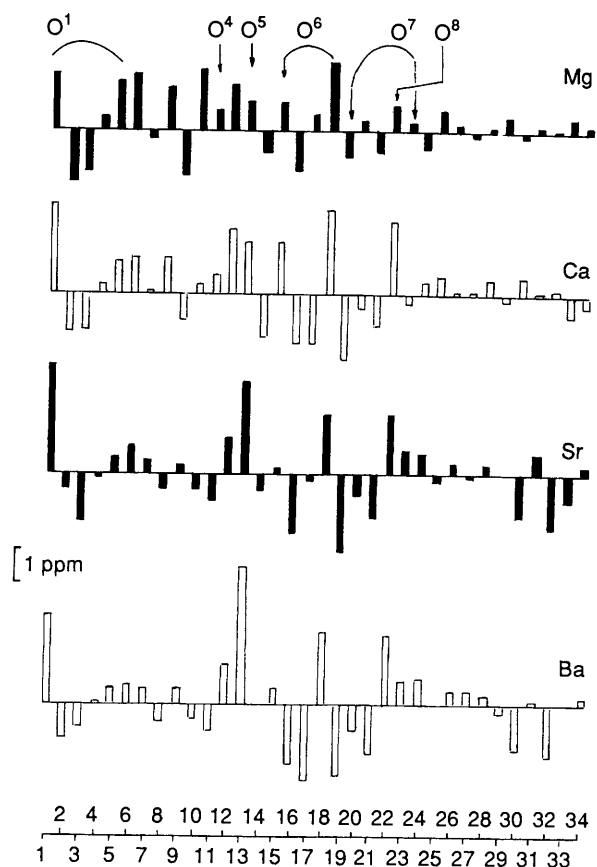


Fig. 6 Variations in ^{13}C chemical shifts in chloroform from uncomplexing lasalocid anion (in the tetraethylammonium salt) to lasalocid anion (engaged in complexation of alkaline-earth cation) using mean values given in Table 1

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