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Formation of Ion-Translocating Oligomers by Nigericin

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Summary. At pH 4.0, $>10^{-7}$ M nigericin was found capable of conducting net charge transfer across bimolecular lecithin membranes, with a stoichiometry of three uncharged ionophore moieties per cation. At neutral or alkaline pH, nigericin catalyzed the transfer of net charge through dimer forms. In agreement with these results, quantitative analysis of nigericin-potassium complexes formed at pH 4.0 showed a 3 : 1 ratio, and a 2 : 1 ratio at neutral or alkaline pH. A 1 : 1 stoichiometry was observed when the ionophore complex was not transferred from methanol-water to chloroform. Moreover, ^{1}H-NMR spectra of nigericin-cation complexes formed at pH 4.0, displayed clear-cut chemical shift variations different to those observed at neutral or alkaline pH. Thus, it is apparent that acid pH causes a transition from dimeric to trimeric forms of nigericin-cation complexes. The membrane conductance increased up to ten times when negatively charged phosphatidyl glycerol was used, while the conductance decreased in positively charged cetylpyridinium containing membranes at pH 4.0. These results suggest that the nigericin-K^+ oligomeric complex is positively charged. In this respect, pK_a values around 8.0 were obtained for the nigericin carboxylate group in media of different dielectric constant, indicating that this chemical group is undissociated under these conditions. Moreover, the values for the complex formation constants as well as the ΔG values calculated for the dimers and trimers indicated that such ionophore cation oligomeric complexes are thermodynamically stable.

Key Words nigericin · ionic translocators · nigericin oligomeric complexes · carboxylic ionophores · ion transport · pH-dependent transport

Introduction

Nigericin is a monocarboxylic ionophore, whose macrocyclic structure has been found to chlatrate alkali metal cations through the coordinating polar oxygen atoms (O_1, O_2, O_5, O_6, O_7) as reported by X-ray diffraction (Steinrauf et al., 1968). Oxygen O_8 also participates according to ^{1}H-NMR data (Rodios & Anteunis, 1977) (Fig. 1). At concentrations between 10^{-9} and 10^{-7} M, nigericin promotes an electrically silent cation-H^+ exchange through a monomeric 1 : 1 mobile carrier mechanism (Pressman, 1968; Henderson et al., 1969; Ashton & Steinrauf, 1970). At concentrations greater than 10^{-7} M, the ionophore catalyzes the transfer of net charge as a mobile dimer at pH 8 (Markin et al., 1975; Toro et al., 1976).

Other carboxylic ionophores such as X-537A (lasalocid acid) have also been found to complex as monomers and dimers with metal ions (Pressman, 1968; Celis et al., 1974; Degani & Friedman, 1974; Chiang & Paul, 1977; Degani et al., 1981; Bolte et al., 1982; Homan & Eisenberg, 1985). X-537A has also been found to form trimers with lanthanum (Everett et al., 1983). A-23187 (calcimycin) is able to complex divalent cations as a monomer or charge neutral dimer (Case et al., 1974; Wulf & Pohl, 1977; Pfeiffer & Deber, 1979; Tissier et al., 1979; Blau et al., 1984). The property to aggregate into dimers is also shared by etheromycin (Donis et al., 1981) and the calcium ionophore ionomycin (Anteunis & Verhegge, 1981). Other carboxylic antibiotics subjected to monomer-dimer transitions are X-14547A (Bolte et al., 1982) and grisorixin (Sandeaux et al., 1978), while neither the monensins (Estrada-O. et al., 1967*b*) nor dianemycin, HLR-X-206 or Lilly A217 (Lardy et al., 1967) show the ability to aggregate in oligomers or to conduct net charge across membranes at acidic or alkaline pH (Toro et al., *data to be published elsewhere*).

Evidence presented in this paper through membrane conductance measurements, ^{1}H-NMR data

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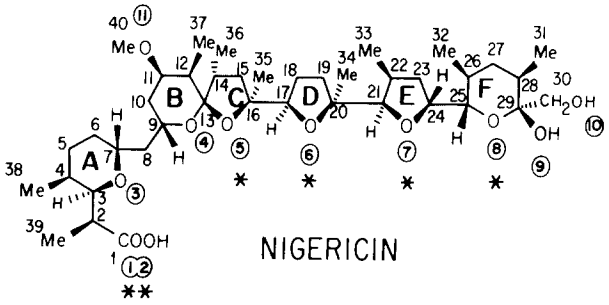

Fig. 1. Chemical formula of nigericin. The numbering system follows that in Rodios and Anteunis (1977). Those atoms marked by an asterisk are the liganding oxygens as mentioned in the text

and constant complex formation measurements, indicate that undissociated nigericin fluctuates from dimer $(N_2H_2K)^+$ to trimer positively charged complexes $(N_3H_3K)^+$, as a function of its concentration and the environmental pH of the aqueous medium from which the complex is extracted to an apolar phase.

Materials and Methods

Lipid Bilayer Experiments

Black lipid membranes (BLM) were obtained and the electric conductance measurements as well as the biionic potential measurements were carried out as described previously (Celis et al., 1974; Toro et al., 1976). The membranes were painted from a solution of 20 mg/ml column chromatography purified lecithin (Singleton et al., 1965), and 15 mg/ml cholesterol in *n*-octane. Where indicated, phosphatidyl glycerol and cetylpyridinium were utilized to confer negative and positive surface charge, respectively, to the membranes.

Nigericin-free acid was a kind gift from Dr. Philip A. Miller (Hoffmann-La Roche Inc., Nutley, New Jersey).

Dioxane and $CDCl_3$ were spectroscopic grade (Merck). All other chemicals were reagent grade. Glass redistilled water was employed for the solutions.

^{1}H-NMR Spectra and Preparation of Ionophore-Cation Complexes

The spectra were taken in $CDCl_3$ in a Varian EM-390, 90 MHz NMR spectrometer. Tetramethylsilane (TMS) was used as internal standard. Spectra of nigericin-free acid was obtained directly from the sample provided by Hoffmann-La Roche. Nigericin potassium salt was prepared from the free acid and an excess of potassium carbonate (100 : 1), added to the free acid previously dissolved in $CHCl_3$. The carbonate was divided in three parts, adding one part at a time to the ionophore solution. After each addition, the suspension was vigorously stirred for 3 min. The carbonate was allowed to sediment and the supernatant was removed. This procedure was repeated five times from the beginning. The suspension was centrifuged and the supernatant filtered. The nigericin potassium salt was recovered by evaporating the solvent. The following experimental procedure was intended to simulate the conditions of complex formation when the ionophore was added to lipid bilayer membrane experiments. Samples of K^+-nigericin salt or nigericin-free acid were dissolved in methanol-water 95% (vol/vol) solution. Potassium chloride (0.1 M) was added to the former salt and pH was adjusted to 4 and to 6–8 with HCl for both samples (free acid and K^+-salt). After 24 hr, the solvent was evaporated under N_2 (g) and water was eliminated by lyophilization. Nigericin-free acid or its K^+-salt, were extracted in $CHCl_3$. The solvent was evaporated under N_2 and the samples dissolved in $CDCl_3$ for ^{1}H-NMR studies. The assignments in the spectra of the signals: Me_{35}, vicinal to O_5; H_{21}, vicinal to O_7 and Me_{40} linked to O_{11}, which were found to show the more significant chemical shifts, were taken from a previous report (Rodios & Anteunis, 1977).

Potentiometric Measurements

Potentiometric measurements were made in a 5-ml glass cell with continuous stirring. Temperature was maintained constant by means of a circulating flux of water. Simultaneous reading of pH and K^+ were made by a pH glass electrode (Beckman 39505) and a cation-selective electrode (Beckman 39047) with a calomel reference electrode (Beckman 41239) connected to a pair of potentiometers (Beckman 4500 and 3550). Their Nernstian response was corroborated before using. Dioxane-water mixtures were utilized to have media with different dielectric constant (ε): 45% (vol/vol), ε = 38.48 and 30% (vol/vol), ε = 51.9 (Harne & Ave, 1967). K_a determinations (Meites & Thomas, 1958) of nigericin were obtained in these conditions. A fresh and standardized solution of tetramethylammonium hydroxide (TMAOH) was employed as a titrant. To standardize the procedure, pK_a of acetic acid was determined in dioxane-H_2O 45% (vol/vol) at 30°C. The obtained value was 6.64 ± 0.02, which is in accordance to that described previously (Harne & Ave, 1967). Formation constants (*Kf*) of the complexes of nigericin-potassium were determined in the solutions described above at different pH.

Quantitative Analysis of K^+ in the Ionophore Metal Complexes

Samples dissolved in 20% methanol-H_2O were analyzed for potassium content in a Corning 400 flame photometer.

Results

Effect of Nigericin and K^+ on Lipid Bilayer Membrane Conductance at pH 4.0

The dependence of the membrane conductance (*Go*) on ionophore concentration and surface charge in the presence of K^+ at pH 4.0, is presented in Fig. 2. It is observed that there is a linear relationship between log *Go vs.* log nigericin concentration with a slope of 3, indicating that an ionophore trimer is the translocator species which catalyzes K^+ movements across the membrane at pH 4.0. In order to prove changes in surface potential of bi-

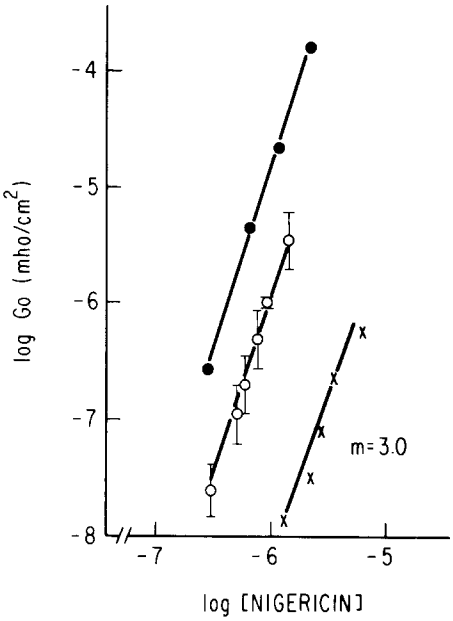

Fig. 2. Dependence of the electrical conductance of BLM on nigericin concentration at pH 4.0. An ethanolic solution of nigericin is added to the inside Teflon® compartment at room temperature with constant stirring. The buffer medium contains 50 mM formic acid and 100 mM KCl adjusted with TEA at pH 4.0. The lipid bilayer composition for ○ lecithin-cholesterol neutral membranes was 20 : 15 mg/ml; for ● lecithin-cholesterol-phosphatidyl glycerol negatively charged films was (9.3 : 10.7 : 5 mg/ml) and for × positively charged bilayers, it was, lecithin-cholesterol (20 : 15 mg/ml) and cetyl pyridinium chloride (1.4×10^{-4} M). The percentage of cholesterol was kept constant in the three cases. Each point represents a minimum of five experimental determinations in different membranes

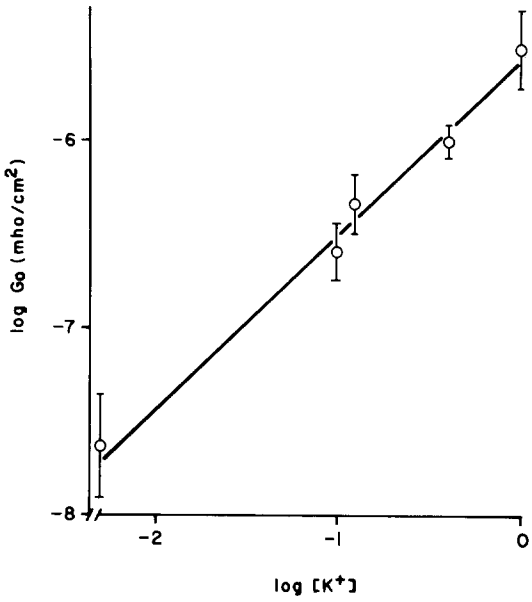

Fig. 3. Dependence of the conductance mediated by nigericin on potassium concentration. The bathing medium contains: 50 mM formic acid and KCl adjusted with TEA at pH 4.0. Nigericin concentration is kept constant (7.3×10^{-7} M). Membranes are formed from a lecithin-cholesterol solution (20 : 15 mg/ml). Bars have the usual meaning. Other conditions are as in Fig. 2

layer membranes, *Go* measurements have been made with positive or negative permeant species (McLaughlin, 1972; Schäfer et al., 1974; Roy et al., 1981). Following this criteria, we used charged membranes to check the sign of the nigericin-cation complex. Accordingly, it is apparent that the trimeric complex is positively charged, since *Go* is higher in negatively charged membranes (phosphatidyl glycerol) than in positively charged surfaces (cetylpyridinium) with respect to the neutral ones. Figure 3 shows that the membrane conductance is linearly dependent on K^+ concentration with a slope of 1, indicating that only one K^+ is involved in the translocating complex at pH 4.0.

Transition of Ionic Selectivity Observed for Nigericin at pH 4.0 in BLM

Biionic potentials measured in BLM in presence of nigericin at pH 4.0 show a different selectivity pattern for monovalent cations, as compared with those obtained at alkaline (8.0) pH.

A transition $Na^+ > Cs^+$ is observed at pH 4.0, with respect to a $Na^+ < Cs^+$ found at pH 8.0, implying a selectivity change for the presumable trimeric complexes (above results) with respect to the dimeric ones (Toro et al., 1976), from ionic sequence V to IV in Eisenman's series (Table 1).

Effect of Nigericin on Lipid Membrane Conductance Measurements at pH 6.0 and 9.4

The relationship between log *Go vs.* log nigericin concentration showed a value of 2.0, when the pH of the medium was adjusted to 6.0 or 9.4 (Fig. 4). This indicates that a dimeric species is the K^+ conducting translocator at near neutral or alkaline pH.

Dependence of pH and Solvent Polarity on the Stoichiometry of Nigericin-K^+ Complexes

Table 2 indicates that a monomeric nigericin-K^+ complex is detected when the complex is not dissolved in methanol-water, nor exposed to 100 mM KCl during 24 hr, and is not extracted to chloroform (this condition is called untreated sample). On the other hand, when the cation-translocator complex

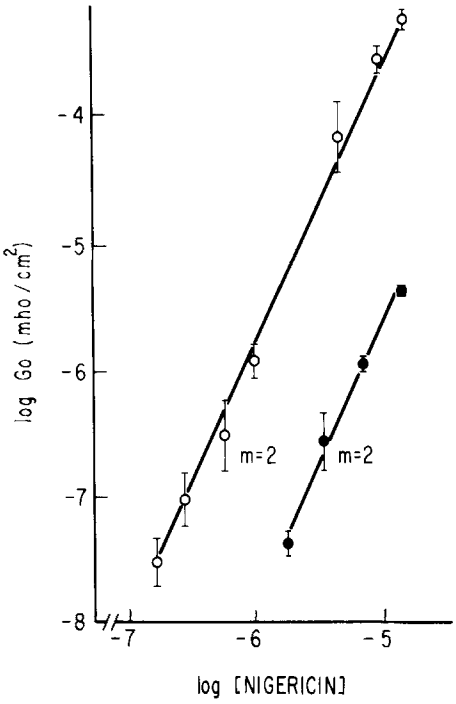

Fig. 4. Dependence of the BLM conductance on nigericin concentration at pH 6.0 and 9.4. The medium contains: ○ 50 mM histidine and 100 mM KCl adjusted at pH 6.0; ● 50 mM glicine and 100 mM KCl adjusted at pH 9.4. Membranes are painted from a solution of lecithin-cholesterol (20:15 mg/ml). Bars indicate standard deviation. Other conditions are the same as in Fig. 2

Table 1. Biionic potentials (E_{AB}) for lipid bilayers in the presence of nigericin, and its ionic selectivity sequence at different pH values[a]

Electrolyte A (in) : B (out)	E_{AB} (mV)	
	pH 4.0	pH 8.0
$K^+ : Rb^+$	−15	−10.87
$K^+ : Na^+$	−30	−12.6
$K^+ : Cs^+$	−28	−16.83
$K^+ : Li^+$	−36	−28.6

pH	Selectivity sequence	Eisenman's series
8.0	$K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$	V
4.0	$K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$	IV

[a] The medium contains: 100 mM metal cation chloride and either 0.05 M formic acid adjusted with TEA at pH 4.0, or 0.5 M Tris adjusted with HCl at pH 8.0. Nigericin concentration is 2.43×10^{-6} M. The selectivity sequences are derived from the results above.

experiences the exposure to different solvents and KCl, stable dimeric complexes are obtained at pH 6 to 8, meanwhile stable trimers are detected at pH 4.

Table 2. Stoichiometry of nigericin-potassium complexes at different pH values[a]

Samples of nigericin-potassium complexes	Nigericin/K^+ ratio
1. Untreated	1.21 ± 0.01
2. Adjusted at pH 6 to 8	2.02 ± 0.02
3. Adjusted at pH 4	3.08 ± 0.17

[a] In the untreated sample the nigericin-potassium complex was not exposed for 24 hr to 0.1 M KCl methanol-water solution, or pH adjustment, nor to $CHCl_3$ extraction, as was the case for samples number 2 and 3. Detail description of the preparation of these samples is indicated in Materials and Methods. Potassium was quantified by flame photometry in triplicate.

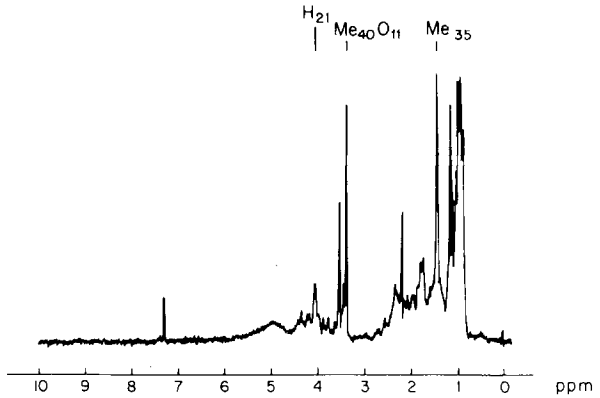

Fig. 5. 90-MHz ^{1}H-NMR spectrum in $CDCl_3$ of nigericin-free acid. TMS was used as internal

Effect of pH on the ^{1}H-NMR Signals of Nigericin-K^+ Complexes

Figure 5 shows the 90 MHz ^{1}H-NMR spectrum of nigericin (free acid) with the assignments of H_{21}, Me_{35} and O-Me_{40}. The proton shift values (in ppm from TMS internal) observed for nigericin-free acid in $CDCl_3$ (Table 3) are almost identical to those reported previously for the sodium salt of this ionophore (Rodios & Anteunis, 1977). Table 3 indicates the chemical shift values for the ^{1}H-NMR spectra of nigericin-free acid compared with its potassium salt, as a function of pH. Firstly it can be seen that there were significant chemical shifts, when nigericin-potassium complexes were formed. Furthermore, broadening of these signals was observed when the complexes were allowed to form at pH 4.

Nigericin pK_a Values and Ionophore-K^+ Formation Constants

Table 4 displays the pK_a values obtained for nigericin in media of different dielectric constant (ε). As

Table 3. Proton shift values (ppm from TMS internal) of 90-MHz ^{1}H-NMR spectra for nigericin-free acid and its potassium complexes in $CDCl_3$

A. Sample	Signals		
	H_{21}	Me_{35}	$O_{11}Me_{40}$
Nigericin-free acid	4.02	1.42	3.35
Nigericin-potassium	4.25	1.6	3.62

B. Sample	H_{21}		Me_{35}		$O_{11}Me_{40}$	
	a)	b)	a)	b)	a)	b)
Nigericin free acid	4.0	3.9	1.42	1.40-s	3.36	3.40
Nigericin-potassium	4.13	vb	1.50	1.45-b	3.51	3.52-b

A. Untreated samples. B. Samples adjusted at different pH values. Nigericin-potassium sample was treated as in Table 2, while nigericin-free acid was subjected to the same procedure as the potassium complex, except for the presence of 0.1 M KCl in the methanol-water solution. b: broad, s: small, vb: very broad. pH values were, for nigericin-free acid sample: a) 5.67 and b) 4.03; and for its potassium complex: a) 7.65 and b) 3.8.

Table 4. Determination of pK values for nigericin in solvents of different dielectric constant[a]

Solvent	ε (25°C)	pK_a
Ethanol	24	8.45[b]
Dioxan-H_2O (45% vol/vol)	38.48	7.76 ± 0.11
Dioxan-H_2O (30% vol/vol)	51.9	6.93 ± 0.13

[a] Nigericin (10^{-3} M) dissolved in dioxan-H_2O 45% (vol/vol) was titrated with 1 M standardized (TMA OH) as indicated in Materials and Methods, and nigericin (10^{-4} M) was titrated in dioxan-H_2O 30% (vol/vol). Two concentrations of nigericin were employed (10^{-3} and 10^{-4} M) according to its solubility in media of different dielectric constant.
[b] Pressman, 1973.

expected, the pK_a becomes smaller with the increase of ε, indicating greater molecular dissociation as hydrophobicity decreases. Moreover, it is noteworthy that the ionophore remains practically undissociated at acid and neutral pH in a predominantly nonpolar environment. Table 5 shows the calculated formation constants (log *Kf*) of the complexes of nigericin with K^+ at different pH and ε values. It is observed that log *Kf* decreases with the increase of pH, irrespective of the influence of the dielectric constant. This suggests that the probability of the ionophore to form chlatrates with K^+ increases as the pH decreases. From Table 5 the values of $\Delta G = -19.89$ kJ/mol ($\varepsilon = 38.48$, pH 4) and $\Delta G = -22.67$ kJ/mol ($\varepsilon = 38.48$, pH 7.76) were calculated, data which corroborates the thermodynamic feasibility of complex formation. These results compare favorably with those previously obtained by Lutz et al. (1971). Also it should be pointed out that no significant changes in pH are observed when the concentration of K^+ is increased during complex formation, indicating that K^+ does not displace H^+ in the latter process.

Discussion

The carboxylic ionophores are a special subclass of metal cation translocators produced by actinomycetes. Several of them have the ability to aggregate in dimers or trimers when wrapping around the metal cation for transport purposes. Such is the case of compound X-14547A (Bolte et al., 1982), grisorixin (Sandeaux et al., 1978), X-537A (Celis et al., 1974) and nigericin (Toro et al., 1976). However, others which otherwise are almost identical to the former group, except for their different cationic selectivity, do not aggregate in oligomeric forms, nor do they have the ability to transfer net charge across black lipid membranes. (Toro et al., *unpublished data*). Such is the case of the monensins A, B and C (Estrada-O. et al., 1967*b*), dianemycin, HLR-X-206 and compound Lilly A-217 (Lardy et al., 1967). Thus the factors which have been shown in the present manuscript to facilitate the aggregation of cation-conducting oligomers of nigericin, are discussed in the following lines.

Dependence of the Aggregation on the Concentration of Ionophore Monomers

Previous information by Pressman et al. (1967), showed that at concentrations below 10^{-7} M, nigericin catalyzes a monomeric 1 : 1 electroneutral transport of alkali metal cations and protons. Increasing the ionophore concentration above 10^{-7} M, caused not only that dimers of nigericin translocate the net charge of K^+ across lipid membranes at neutral pH (Toro et al., 1976), but also the uncoupling of energy conservation by this compound in intact mitochondria (Estrada-O. et al., 1967*a*). This manuscript demonstrates that only at concentrations above 10^{-7} M and at acid pH, nigericin aggregates in trimers to transfer K^+ across the hydrophobic membrane (Fig. 2). It is likely that the amphipatic properties of nigericin contribute to the establishment of a micellial-like process which determines the formation of mobile dimeric and trimeric conducting oligomers. This process is shared with nigericin by compounds X-537A (Celis et al., 1974) and grisorixin (Sandeaux et al., 1978) but not by the

Table 5. Log *Kf* values of the complexes of nigericin with potassium in media of different pH and ε values[a]

ε = 38.48		ε = 51.9	
pH	log *Kf*	pH	log *Kf*
4	3.98 ± 0.025	4	4.15 ± 0.05
7.76	3.49 ± 0.01	6.93	3.51 ± 0.17
11.52	2.93 ± 0.07	9.86	2.51 ± 0.064

[a] Nigericin (10^{-4} M) dissolved in dioxan-H_2O was titrated with KCl. Dioxan-H_2O mixtures were as in Table 3. pH was adjusted with: HCl at pH 4.0; TEA at pH 7.76 and 6.93; and TMAOH at pH 11.52 and 9.86.

monensins, dianemycin and HLR-X-206 (Toro et al., *data to be published elsewhere*).

Dependence of the Self-Assembly Process of Nigericin on pH

The existence of the mobile trimeric species of nigericin complexed with one potassium suggested by the conductance results obtained in BLM at pH 4.0 (Figs. 2 and 3) was corroborated by the stoichiometry of 3 : 1 found for the ionophore-K^+ complex at pH 4.0 (Table 2). Also, in accordance with the second-order dependence for the ionic conductance catalyzed by nigericin in black lipid membranes at neutral pH (Toro et al., 1976 and Fig. 4) a 2 : 1 ionophore-K^+ stoichiometry was obtained at pH 6 to 8 (Table 2). Therefore, it is very likely that protonation of the undissociated moieties of the ionophore contribute to the transition between dimers and trimers, which presumably occurs at the membrane interface.

Dependence of the Aggregation on the Polarity of the Solvent

Only when concentrations of nigericin higher than 10^{-7} M are exposed to acid or neutral pH, and to the membrane or to solvents of different polarity, the ionophore is capable of forming dimers or trimers. When potassium salt of nigericin is not transferred from a polar to a nonpolar solvent, a 1 : 1 stoichiometry for the ionophore-cation complex is obtained (Table 2). Moreover, similar to nigericin, X-ray diffraction studies of X-537A (Chiang & Paul, 1977) have shown that the latter carboxylic ionophore forms dimers in nonpolar media, reverting to a monomeric structure in a polar environment. Furthermore, some intents to obtain the X-ray diffraction pattern for the grisorixin dimers, assumed to exist from the bilayer conductance measurements have failed; only monomers have been observed (Sandeaux et al., 1978). Thus, it appears that the polarity of the solvent has an influence on the structure of the metal ion-carboxylic ionophore complex.

Structural Flexibility Requirements for the Aggregation Process

^{1}H-NMR data shown in Table 3 corroborate important inter-atomic structural changes of the nigericin molecule caused by the cation chlatration and by the effect of pH in such process. This indicates that the carboxylic ionophore possesses an important structural flexibility which can be modulated by the cation, by the pH and by the transfer from a polar to an apolar solvent.

The Electric Charge of the Transferred Cation-Oligomer Complex

Nigericin promotes higher conductance at pH 4.0 in negatively charged membranes, with respect to positively charged or neutral surfaces (Fig. 2). These results lead us to suggest that the trimeric complex of nigericin formed at acid pH has positive charge. This assumption is similar in nature, although in an inverse fashion, to the experiments and conclusions obtained by Mc Laughlin (1972), Shäfer et al. (1974) and Roy et al. (1981) to qualify the sign of the membrane surface charge in lipid bilayers from the *Go* changes mediated by mobile ion complexes with known charge.

It is also possible to suggest that the conducting oligomer of nigericin is protonated. Nigericin pK_a values indeed confirm that protonated molecules are involved in the translocating trimer (Table 4). Under the above-mentioned conditions, the ionophore cannot be dissociated in a hydrophobic medium. The value of K_a represents a Nig-H^+ binding affinity which resides in the 10^7 order. This value is significantly greater than the nigericin-K^+ affinity constant, surrounding the 10^3 to 10^4 range (Table 5), which explains why the cation cannot remove H^+ from the molecule. In fact, the NH-K^+ binding constant is greater than that for the N^-K^+ complex, which is consistent with the finding that coordination increases when the carboxyl terminal group is protonated.

A Model Transport Mechanism for the Nigericin Trimer

The above observations lead us to suggest a cyclic transport mechanism for the nigericin trimer. Due

to their amphipatic properties, it is likely that high concentration of protonated monomers and dimers aggregate in the presence of cation and pH 4.0, above a "critical micelle-like concentration," to form the conducting oligomer, the charged trimer $(N_3H_3K)^+$, assembled at the water-membrane interface, transfers the cation across the membrane down the electrical potential gradient, while the ionophore devoid of cation (NH) which is neutral, diffuses back across the membrane. A similar mechanism implemented with two nigericin molecules has been suggested to occur for nigericin at pH 8 (Toro et al., 1976).

Part of the results of this paper were presented at the American Society of Biological Chemists 71st Annual Meeting and Biophysical Society 24th Annual Meeting in New Orleans. Also, at the 12th International Congress of Biochemistry in Perth, Australia.

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