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RELAXATION STUDIES ON COMPLEX FORMATION OF MACROCYCLIC AND OPEN CHAIN ANTIBIOTICS WITH MONOVALENT CATIONS *

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The stability constants for the 1 : 1 complexes of macrocyclic antibiotics (nonactin, monactin, dinactin and trinactin) with Li^+, Na^+, K^+, Rb^+, Cs^+, NH_4^+ and for the Na^+-complexes with the open chain compounds nigericin and monensin in methanol solution have been determined. The relaxation amplitude method was employed to obtain both the equilibrium constants and the enthalpies of reaction. The kinetics were studied with the help of temperature-jump, electric-field pulse and ultrasonic absorption techniques. Although complex formation of the metal ions with the antibiotics involves multidentate ligand chelation, the formation rates are in general very high, i.e. close to the limits imposed for diffusion controlled processes. The data for the macrotetrolides indicate the existence of conformational transition prior to complexation. A sequential substitution or "redressing" mechanism is proposed which is in accord with the high rates of complex formation. The selectivity patterns, as expressed by the equilibrium constants, are similar to those observed for the transport of metal ions across membranes in presence of the antibiotics. Selectivity results from an optimal balance between the strength of metal ion solvation and the stability of the individual metal complex, which in turn is governed by the conformational flexibility of the antibiotics.

1. Introduction

Macrocyclic carriers as well as some related open chain compounds are capable of increasing markedly the cation permeability of natural and artificial membranes [1–6]. Moreover, those substances usually also exhibit a striking selectivity for the transport of certain cations. The ability of discriminating different ions is characteristic of many biological membranes. Important physiological processes, among which excitation of the nerve membrane represents the most prominent example, are based on such a phenomenon. Therefore, an elucidation of the mechanism of selective binding of cations to antibiotics may provide a clue to the carrier action irrespective of whether these compounds actually fulfill such a function in biological systems.

A reasonable approach to the problem is to study first the static – i.e. equilibrium – interactions of the antibiotics with alkali ions in order to learn more about the relationship between molecular structure and conformation. Secondly, it is very important to investigate the mechanism of complexation. This requires kinetic studies of the binding process. Formation and dissociation of the cation antibiotic-complex are essential steps in carrier function regardless of whether the metal ion transport is mediated by a mobile carrier or some "single file" pore, which is an integral part of the membrane structure. In both cases, ligands must be provided, which are able to compete with the solvent molecules for binding in the inner coordination sphere of the metal ion. Close contact between the solvated cation and the chelating ligand is a fundamental requirement for selectivity. It is this property which accounts for the high thermodynamic stability of the complex and hence for the unique transport properties.

Equilibrium and kinetic measurements on two classes of antibiotics are described in this article. The systems investigated are (1) the macrotetrolides: non-, mon-, din-, and trinacton and (2) the open chain compounds nigericin and monensin. Macrotetrolides

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can be isolated from microorganisms such as actinomycetes. Their chemical composition [7] as well as their spatial structure [8] (e.g. of the K-nonactin complex) are well known. Data about their binding strengths [9] have already been reported. Nigericin [10] and monensin [12,13] can be isolated from *Streptomyces hygroscopicus* and *Streptomyces cinnamonensis* respectively. The crystal structure of the silver complex of nigericin [11] and of several of the monovalent metal cation complexes of monensin [14,15] have been published.

2. Principles of methods

For our investigations we applied three different relaxation methods, permitting a determination of rate and stability constants and enthalpies of reaction as well. Two of the procedures demand for the presence of an indicator [16]. A detailed description of the relaxation amplitude- and substitution titration-method is presented in ref. [17].

2.1. Relaxation amplitude method

The relaxation amplitude, associated with the perturbation of the complex equilibrium, involving an indicator (In^-) and a metal ion (M^+):

$$In^- + M^+ \rightleftharpoons InM \tag{1}$$

is given by

$$\delta E_1 = l(\epsilon_{InM} - \epsilon_{In})\Gamma_{In}\frac{\Delta H}{RT}\frac{\delta T}{T}. \tag{2}$$

δE is the amplitude measured in absorbance units, l the length of the light path, ϵ_{InM} and ϵ_{In} are the extinction coefficients of InM and In^- respectively. The metal ion is assumed not to contribute to optical absorption. K_{In} represents the stability constant for the metal-indicator system (1). The amplitude factor Γ_{In} is defined as:

$$\Gamma_{In} = \frac{c_{In}\,c_M}{c_{In} + c_M + K_{In}^{-1}}. \tag{3}$$

In the presence of the antibiotic "X" a competitive reaction is to be considered:

$$X + M^+ \rightleftharpoons XM^+. \tag{4}$$

The overall relaxation amplitude — both systems being present — then reads:

$$\delta E_2 = l(\epsilon_{InM} - \epsilon_{In})\frac{\Gamma_{In}}{1 - \Gamma_{In}\Gamma_X/c_M^2} \times \left[\frac{\Delta H_{In} - (\Gamma_X/c_M)\Delta H_X}{RT}\right]\frac{\delta T}{T}, \tag{5}$$

where

$$\Gamma_X = \frac{c_X\,c_M}{c_X + c_M + K_X^{-1}} \tag{6}$$

and $K_X = c_{XM}/c_X\,c_M$ is the respective (apparent) stability constant of the carrier complex (4) referring to a defined ionic strength. δT always refers to the same T-jump in both experiments, i.e. in presence and absence of the antibiotic, starting from identical initial temperatures. Under the condition that the concentrations of the unreacted indicator c_{In} - and the metal ion c_{M^+} are exactly the same in *both* solutions and furthermore that $\Gamma_{In}/c_M \ll 1$, the amplitude difference simply reduces to:

$$(\delta E_1 - \delta E_2) = l(\epsilon_{InM} - \epsilon_{In})\frac{\Gamma_{In}\Gamma_X}{c_M}\frac{\Delta H_X}{RT}\frac{\delta T}{T}. \tag{7}$$

The relation $\Gamma_{In}/c_M \ll 1$ expresses the fact that the indicator is present at a concentration $c_{In}^0 \ll c_M^0$ and hence does not exert any buffering effect on the equilibrium, involving the antibiotic X.

For $c_M^0 = c_X^0$, Γ_X can be simplified to the form

$$\Gamma_X/c_M = \frac{1}{2}\left[1 - \frac{1}{\sqrt{1 + 4K_X c_X^0}}\right]. \tag{8}$$

Evaluation of eq. (5) is based on the quantity

$$f = \frac{\Gamma_X \Delta H_X}{c_M RT}, \tag{9}$$

which results directly from the experimental data. Although both ΔH_X *and* K_X are unknown one can work out a suitable iteration procedure starting from using guessed values of ΔH_X.

Combining eqs. (8) and (9) one obtains:

$$\phi = \left(1 - \frac{2f\,RT}{H_X}\right)^{-2} - 1 = 4K_X c_X^0. \qquad (10)$$

By plotting ϕ versus c_X^0 one generates a family of curves. A straight line with zero intercept will appear only for the correct value of ΔH_X.

The procedure is such that the correct ΔH_X can be convergently approached by iteration. The slope of the straight line yields the parameter $4K_X$.

2.2. *Substitution titration method* [17]

In the presence of an indicator with unique optical properties (as discussed above for the relaxation amplitude method), the change in absorbance ΔE at a given wavelength upon formation of InM is:

$$\Delta E = c_{InM}(\epsilon_{InM} - \epsilon_{In}). \qquad (11)$$

Defining

$$\eta = c_{InM}/c_{In}^0 = \Delta E/(E_\infty - E_0), \qquad (12)$$

with $(E_\infty - E_0)$ being $c_{In}^0(\epsilon_{InM} - \epsilon_{In})$ and applying the known equations

$$c_{In}^0 = c_{InM} + c_{In}; \qquad K_{In} = c_{InM}/c_{In}c_M,$$

the metal ion concentration c_M can be expressed as:

$$c_M = \frac{\eta}{1-\eta} K_{In}^{-1}. \qquad (13)$$

Combination with the conservation and mass action relations for the X-system:

$$c_X^0 = c_X + c_{XM}, \qquad c_M^0 = c_M + c_{InM} + c_{XM},$$

$$K_X = c_{XM}/c_X c_M$$

leads to:

$$\frac{\eta}{1-\eta} + K_{In}(c_X^0 - c_M^0 + \eta c_I^0)$$

$$= \frac{K_{In}^2}{K_X}\left(c_M^0 \frac{1-\eta}{\eta} - c_I^0(1-\eta)\right) - \frac{K_{In}}{K_X}. \qquad (14)$$

For $c_X^0 = c_M^0$ and at constant $c_{In}^0 \ll c_M^0$, K_{In}^{-1} (no buffering by the indicator) one can simplify eq.(14) to:

$$\frac{\eta}{1-\eta} = \frac{K_{In}^2}{K_X}\,\frac{1-\eta}{\eta}\,c_X^0 - \frac{K_{In}}{K_X}. \qquad (15)$$

This form suggests a plot of $\eta/(1-\eta)$ versus $[(1-\eta)/\eta]\,c_X^0$ resulting in a straight line, yielding both K_{In} and K_X from intercept and slope.

2.3. *Direct UV spectrophotometric titration*

The third method employed in order to obtain equilibrium parameters is a direct spectrophotometric titration. K_X can be determined [18] from the linear plots of $1/(\bar{\epsilon} - \epsilon_X)$ versus $1/c_M$ in accordance with

$$\frac{1}{\bar{\epsilon} - \epsilon_X} = \frac{1}{\epsilon_{XM} - \epsilon_X}\left[1 + \frac{1}{K_X c_M}\right]. \qquad (16)$$

The average extinction coefficient $\bar{\epsilon}$ is given by $E/l c_X^0$, where E is the absorbance of the reaction mixture. ϵ_X and ϵ_{XM} represent the extinction coefficients of the antibiotic and its complex respectively. c_M is the concentration of the unreacted cation (non-absorbing) as calculated via an iteration procedure.

All kinetic data were obtained from relaxation studies [19], using

1) the temperature jump technique,
2) the sound absorption method.

The recombination of the metal ion with the antibiotic can be directly detected by recording the re-equilibration following the perturbation induced by the T-jump. In the presence of an indicator, the observed relaxation time τ is related to the rate parameters as:

$$\tau^{-1} = k_f\{(1 - \Gamma_{In}/c_M)c_X + c_M\} + k_d, \qquad (17)$$

where k_f is the rate constant for the formation of the metal ion-antibiotic complex, and k_d that for its dissociation. The factor $(1 - \Gamma_{In}/c_M)$ describes the buffering action of the indicator exerted on the X-system. This effect is usually negligible, i.e. the factor is just one. Eq. (17) then reduces to:

$$\tau^{-1} = k_f(c_X + c_M) + k_d. \qquad (18)$$

With $K_X = k_f/k_d$ the explicit form of eq. (18) reads:

$$\tau^{-2} = k_f^2\{(c_M^0 - c_X^0)^2 + 2K_X^{-1}(c_M^0 + c_X^0) + K_X^{-2}\}. \qquad (19)$$

For an evaluation, only the overall concentrations of

the reactants M and X are required to be known. This expression can be further simplified if $c_M^0 = c_X^0$, in order to provide a linear dependence of τ^{-2} on the sum of the weighed-in concentrations: $(c_M^0 + c_X^0)$. The rate constants, k_f and k_d, and hence the equilibrium constant K_X result from the slope $(2k_f^2 K_X^{-1} = 2k_f k_d)$ and intercept k_d^2 respectively.

Sound absorption measurements yield information on rates in the form of a frequency dependence of the "absorption per wavelength": $\mu = \alpha\lambda$ where α stands for the sound absorption coefficient describing the exponential decay $\sim e^{-\alpha x}$ of a sound amplitude with distance x and λ is the wavelength.

$$\mu = A'\omega + \sum_i B_i' \frac{\omega\tau_i}{1+\omega^2\tau_i^2}, \tag{20}$$

ω is the angular frequency $\omega = 2\pi\nu$, τ_i are the time constants of (chemical) relaxation effects, while A' and B_i' are characteristic constants. The term $A'\omega$ comprises all so called classical absorption phenomena (due to finite viscosity, heat conductivity and structural flexibility of the medium). It increases linearly with ω, as long as observations are made at frequencies far below the critical relaxation range ($>10^9$ Hz), characteristic of these "classical" effects [20]. The B_i'-terms describe amplitudes of chemical relaxation occurring in the frequency range of observation. These amplitude factors of which each characterizes an isolated reaction step can be explicitly correlated with the equilibrium parameters of that very step:

$$B_i' = \frac{\pi\Gamma_i}{RT\kappa_s}\left[\Delta V_i - \frac{\alpha_p}{\rho c_p}\Delta H_i\right]^2, \tag{21}$$

where $\Gamma_i = (\Sigma_k n_k^2/c_k)^{-1}$ (n_k being the stoichiometric numbers and c_k the concentrations of reaction partners and products involved in the particular reaction step). ΔV_i and ΔH_i represent the corresponding volume and enthalpy of reaction, κ_s the adiabatic compressibility, α_p the thermal expansion coefficient, ρ the density, and c_p the specific heat under constant pressure, the latter all referring to the solvent. Explicit expressions for Γ_i are given in eqs. (3) and (6).

For the antibiotic systems under study, two chemical reaction steps turned out to be of importance: a fast conformational change of the antibiotic, and the subsequent association with the metal ion:

$$\begin{array}{ccc} & X_1 & \\ & \uparrow\downarrow & \\ k_{21} & & k_{12} \\ & \downarrow\uparrow & \\ X_2 + M^+ & \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} & MX_2^+ \end{array} \tag{22}$$

For such a scheme, the relaxation times — assuming the conformation change to occur fast as compared to metal complex formation — are given by

$$\tau_I^{-1} = k_{12} + k_{21} \tag{23}$$

$$\begin{aligned} \tau_{II}^{-1} &= k_{23}\left\{\frac{c_M}{1+K_{21}} + c_{X_2}\right\} + k_{32} \\ &= \frac{k_{23}}{1+K_{21}}\{c_M + c_{X_1} + c_{X_2} + K_X^{-1}\}, \end{aligned} \tag{24}$$

with $K_{21} = c_{X_1}/c_{X_2} = k_{21}/k_{12}$ and $K_X = c_{MX}/[c_M(c_{X_1} + c_{X_2})]$.

If the overall equilibrium constant K_X is known, one can easily calculate c_M and the sum $(c_{X_1} + c_{X_2})$ using the total concentrations for M and X, and then deduce, from τ^{-1}, a value for k_{32} and $k_{23}' = k_{23}/(1 + K_{21})$. If, in addition, K_{21} can be determined separately (e.g. from the fast relaxation effect) the true value for the rate constant k_{23} is also obtainable.

3. Material and methods

Nonactin, monactin, dinactin, and trinactin were donated by Ciba AG (Basel, Switzerland) *. The structures of the macrotetrolides are shown in fig. 1. The open chain antibiotics nigericin, and monensin (cf. fig. 2) were gifts from Eli Lilly Comp. **. Both substances were isolated as sodium salts. Tetrabutylammonium perchlorate was purchased from G.F. Smith Chemical Co. The chloride salts of all other cations used were "suprapur" grade from E. Merck. Murexide and absolute methanol were Merck products as well. In some cases, the equilibrium quotients had to be determined with the help of an indicator. Murexide (fig. 3) turned out to be very suitable for the indication of sodium ions, for which it forms a relatively stable complex in methanol ($K_{In} = 2.55 \times 10^3\ M^{-1}$)

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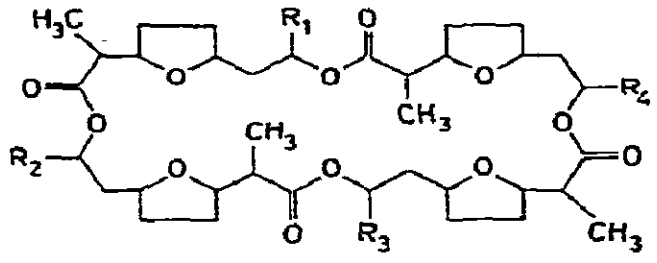
** We should like to thank especially Dr. J.W. Chamberlin.



$R_1 = R_2 = R_3 = R_4 = CH_3$		Nonactin
$R_1 = R_2 = R_3 = CH_3$	$R_4 = C_2H_5$	Monactin
$R_1 = R_3 = CH_3$	$R_2 = R_4 = C_2H_5$	Dinactin
$R_1 = CH_3$	$R_2 = R_3 = R_4 = C_2H_5$	Trinactin
	$R_1 = R_2 = R_3 = R_4 = C_2H_5$	Tetranactin

Fig. 1. Chemical composition and structure of macrotetrolides.

[16], expressed by a pronounced spectral shift. With this indicator it was possible to measure precisely the relaxation amplitudes using the improved version of the temperature-jump relaxation apparatus [21] which has a risetime of $\leqslant 1$ μs and a high differential sensitivity for the relative signal down to 5×10^{-5}. The investigations were carried out at 4900 Å, which refers to the maximum absorbance of the sodium-murexide complex. ΔH_X and K_X were evaluated according to the procedure outlined in 2.1, i.e. by guessing values for ΔH_X and plotting ϕ versus c_X^0 (cf. eq. (10)). A PDP 8 computer was programmed to perform this iteration process *. All the spectra were recorded with

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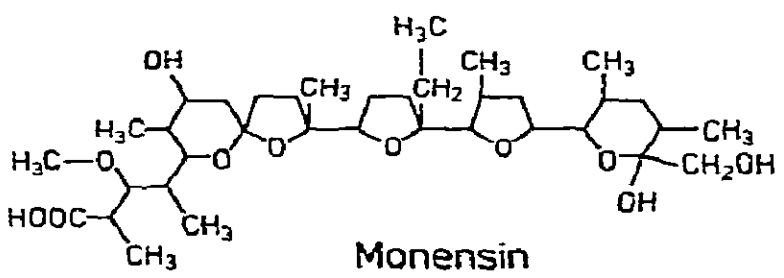
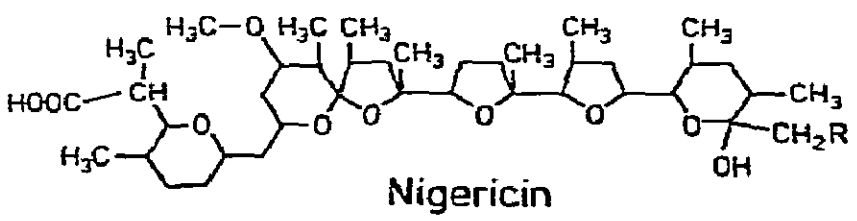
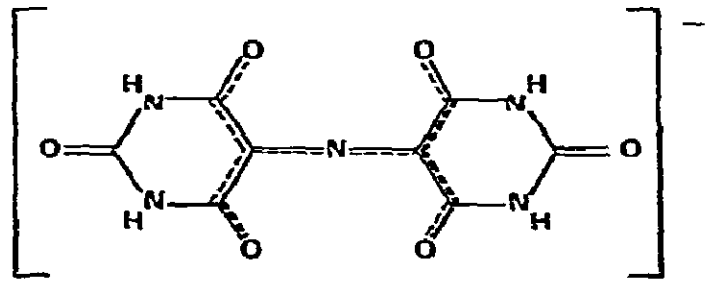
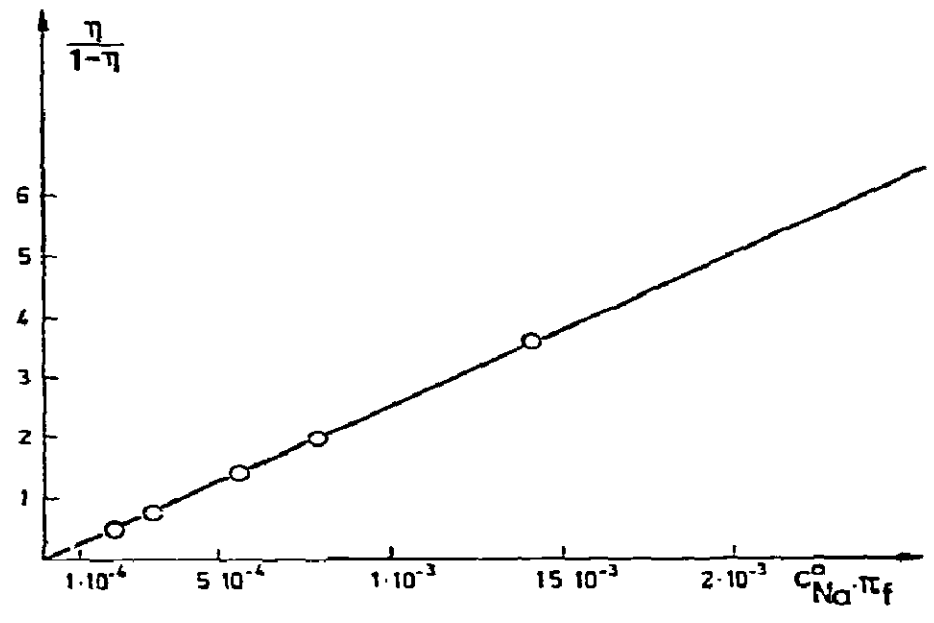




R: OH [Nigericin]
R: CH_2OCH_3 [Grisorixin]

Fig. 2. Chemical composition and structure of open chain antibiotics.





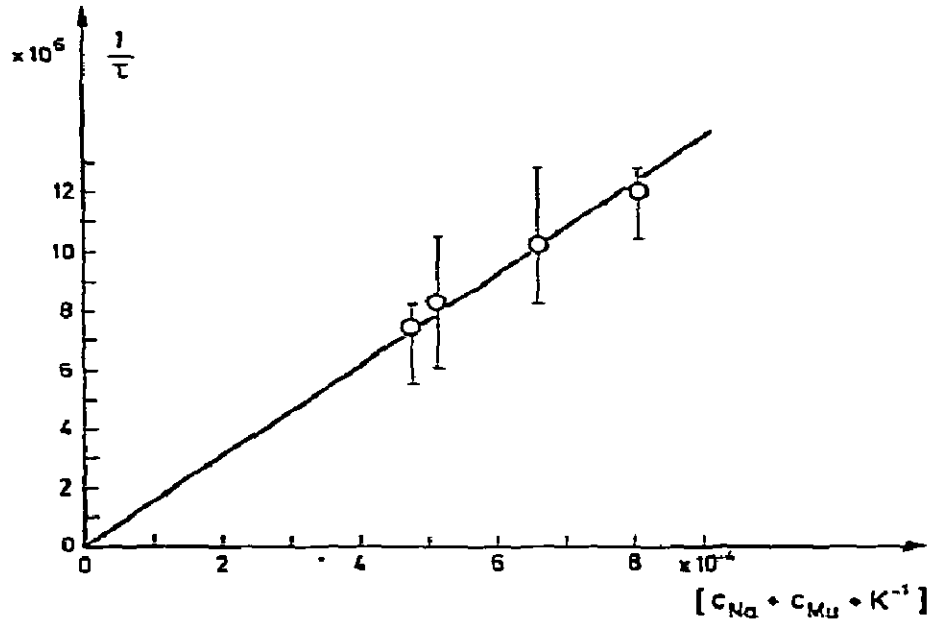


Fig. 3. a) The indicator murexide (anion of purpuric acid). b) Titration curve of Na-murexide: Extinction ratio $\eta/(1-\eta)$ as a function of the weighed-in concentration of Na^+ (corrected by the product of activity coefficients). c) Concentration dependence of the reciprocal relaxation time for complex equilibration between Na^+ and murexide.

the help of a Cary 14 spectrophotometer equipped with thermostated cell holders. A 0–1.0 absorbance unit slide wire was used for the experiments with murexide as indicator. In this case eq. (15) was applied for an evaluation of K_X.

All spectrophotometric, electric field pulse and sound absorption measurements were carried out at 25°C. In the T-jump experiments δT amounted to 6°C yielding a final temperature of 25°C.

Unfortunately, in methanol solutions alkali ions with a size equal to or larger than potassium precipitate as murexide complexes. Spectral changes due to the formation of ammonium-murexide complexes are very small. Therefore the macrotetrolides were directly titrated following the small absorbance changes upon complexation at 2200 Å ($\epsilon \approx 400$) with the help of a 0–0.5 absorbance unit expansion scale slide wire. K_X was determined from the linear plot of $1/(\bar{\epsilon} - \epsilon_X)$ versus $1/c_M$ represented by eq. (16).

Kinetic investigations of complex formation between metal ions with the antibiotics using murexide as an indicator were carried out at 4900 Å. The temperature-jump technique mentioned above, and an electric field pulse method [22] were applied. Some conformational studies of antibiotics by means of the ultrasonic absorption method required the application of two different instruments: a resonance and a pulse technique. They are particularly adapted to this kind of study [23,24] demanding only 0.9 ml of solution (for the resonance method) or 2.3 ml (for the pulse method). The frequencies covered by these instruments range from about 0.4 to 100 MHz.

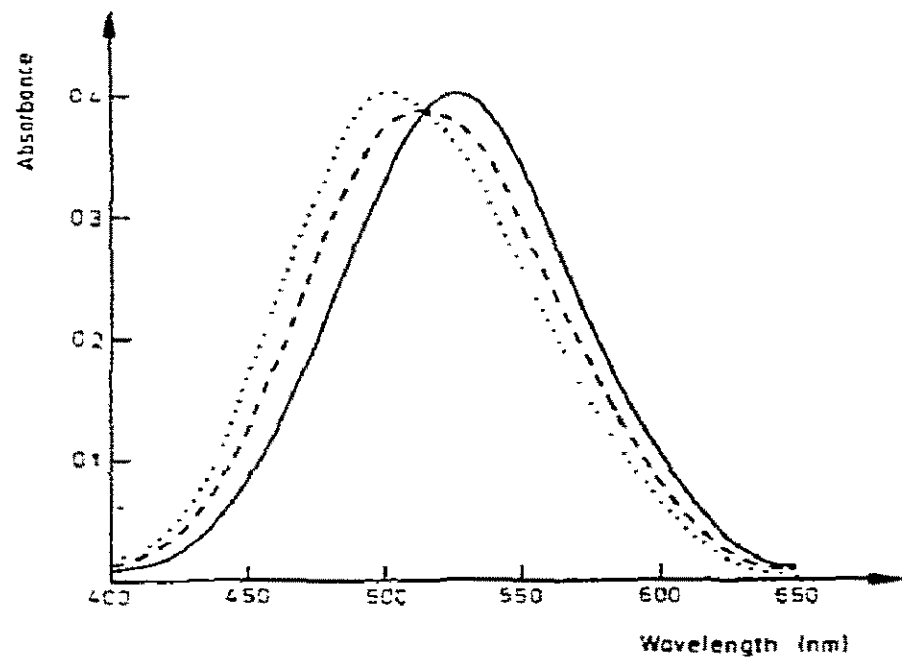


Fig. 4. —— Absorption curve of 3.4×10^{-5} M murexide solution in methanol. – – – Absorption curve of a solution containing – in addition to 3.4×10^{-5} M murexide – 6.0×10^{-4} M nigericin and 6.0×10^{-4} M Na^+. This spectrum is identical with the absorbance of a 3.4×10^{-5} M murexide solution in presence of 1.7×10^{-4} M Na^+. ····· Absorption curve of 3.4×10^{-5} M murexide containing in addition 2.0×10^{-3} M nigericin and 2.0×10^{-3} M Na^+. This curve is identical with the spectrum of a 3.4×10^{-5} M murexide solution in the presence of 3.6×10^{-4} M Na^+.

4. Results

Methanol was the solvent used throughout because the antibiotics studied are only slightly soluble in water. It has been shown that the solvation of a cation in methanol [25] is of a similar strength as in water.

The equilibrium parameters for complex formation between sodium and the various antibiotics were obtained by an indirect titration procedure, involving a detection of the absorbance changes of the sodium-murexide complex at 4900 Å. For the relaxation amplitude method, the spectrum of each murexide containing sample was recorded before carrying out the T-jump experiments (see fig. 4). It was ensured – starting from identical initial murexide concentrations that in each pair of samples, one containing the antibiotic and the other not, the concentrations of uncomplexed murexide and sodium were exactly the same. In fig. 5 the amplitudes observed for the nigericin system are shown as examples. In fig. 6 the ϕ values obtained from eq. (10) are plotted versus c_X^0. In this particular plot the abscissa c_X^0 is multiplied by Π_f (the product of activity coefficients) because a net change of charge results from complexation. The value of the mean activity coefficient of the sodium ions in methanol was calculated from literature data [25]. The amplitude method is so sensitive that a deviation of 0.1 kcal/mol in a total ΔH_X of 2 kcal/mol can easily be detected. In the studies of the neutral macrotetrolides variation of ionic strength using tetrabutyl ammonium perchlorate did not yield any significant change of K_X and ΔH_X. The data obtained by the relaxation amplitude method for all systems treated in this paper are given in table 1.

The method of substitution titration was applied to several of the systems under study. A typical plot of $\eta/(1-\eta)$ versus $c_M^0(1-\eta)/\eta$ for dinactin with Na^+ is shown in fig. 7. The equilibrium parameters determined are in agreement with those obtained by relaxation studies.

As was already mentioned, murexide complexes of

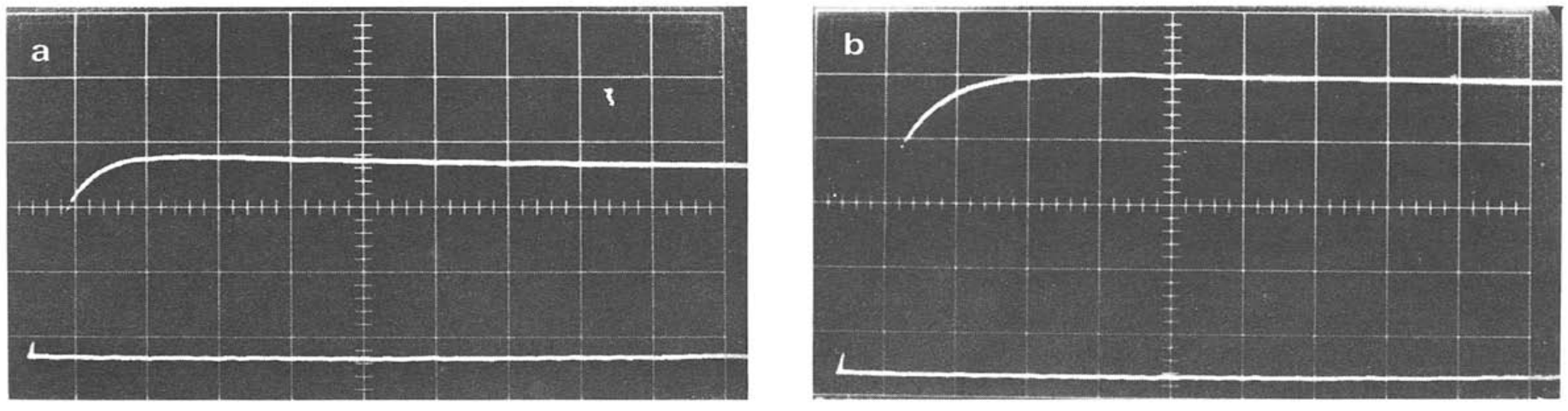


Fig. 5. Oscillograms of T-jump experiments with a Na-murexide (curve a) and a Na-murexide-nigericin system (curve b), respectively. The sensitivity is 0.00068 absorbance units/division and the time scale 10 ms/division, δT is 6.6°. Sample and reference solution refer to one of the absorption curves (dashed line) in fig. 4.

K^+, Rb^+, and Cs^+, precipitate in methanol. The spectral shift of murexide upon complexation with NH_4^+ is not significantly large. Therefore the stability constants of the corresponding macrotetrolide complexes were determined by direct observation of the small absorbance changes at 2200 Å which are due to their association with the cation. The data were plotted as $1/(\bar{\epsilon}-\epsilon_X)$ versus $1/c_M$ and the results are listed in table 2. The values for K_X determined by the three

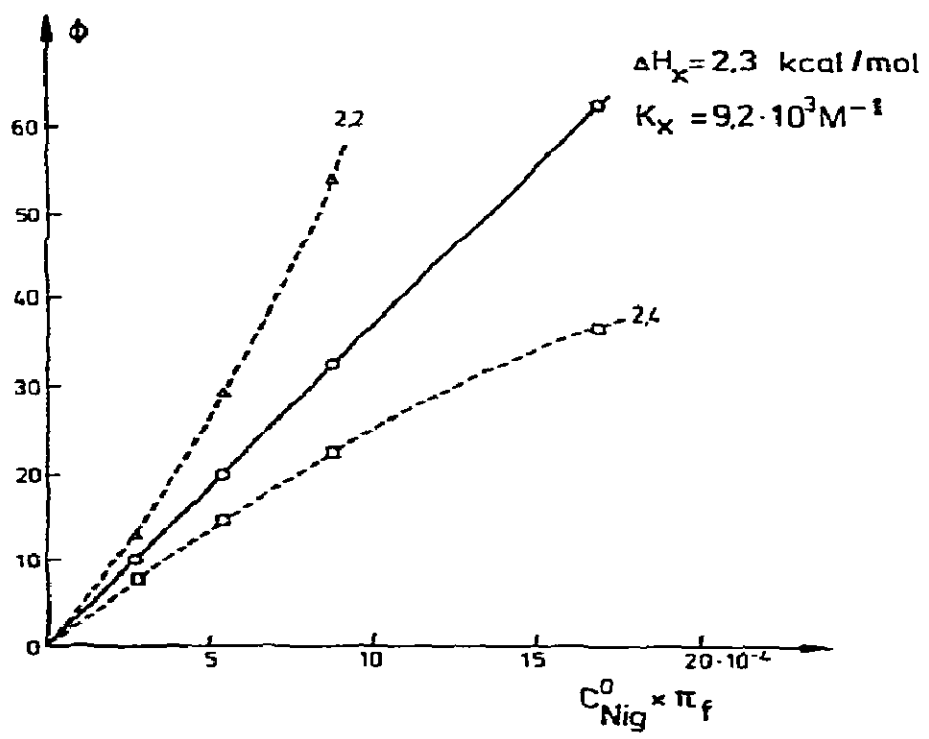


Fig. 6. Evaluation of the reaction enthalpy, ΔH_X and the stability constant, K_X, for Na^+-nigericin complex formation in methanol from relaxation amplitudes. ϕ was calculated according to eq. (10).

Table 1
Equilibrium constants (K_X^0) and reaction enthalpies obtained by temperature jump method for sodium ion in methanol solution at 25°C

	K_X^0 [M^{-1}]	ΔH_X [kcal]
Nonactin	$(1.7 \pm 0.3) \times 10^2$ a)	~-5
Monactin	5×10^2	
	$(5.8 \pm 0.7) \times 10^2$ a)	-6
Dinactin	$(1.1 \pm 0.2) \times 10^3$	
	$(1.0 \pm 0.2) \times 10^3$ a)	-6.6 ± 0.2
Trinactin	$(1.9 \pm 0.2) \times 10^3$	
	$(1.7 \pm 0.2) \times 10^3$ a)	-7.3 ± 0.2
Monensin	$>1 \times 10^6$	–
Nigericin	$(9.2 \pm 0.5) \times 10^3$	+2.3

a) Values obtained by method described in eq. (14).

different titration procedures resemble each other very well, as can be seen from a comparison of the corresponding numbers in table 1 and table 2.

The temperature-jump apparatus was employed for a measurement of the rates of complex formation. In most cases, however, it turned out that the reactions were too fast to be resolved by the standard device. However, by using a 1×10^{-8} farad capacitor for the discharge in the presence of a high ionic strength of the medium (0.1 M tetrabutylammonium perchlorate) a single relaxation time of about 10 μs for the sodium-dinactin and sodium-trinactin system could be observed. The corresponding rate constants are reported in table 3.

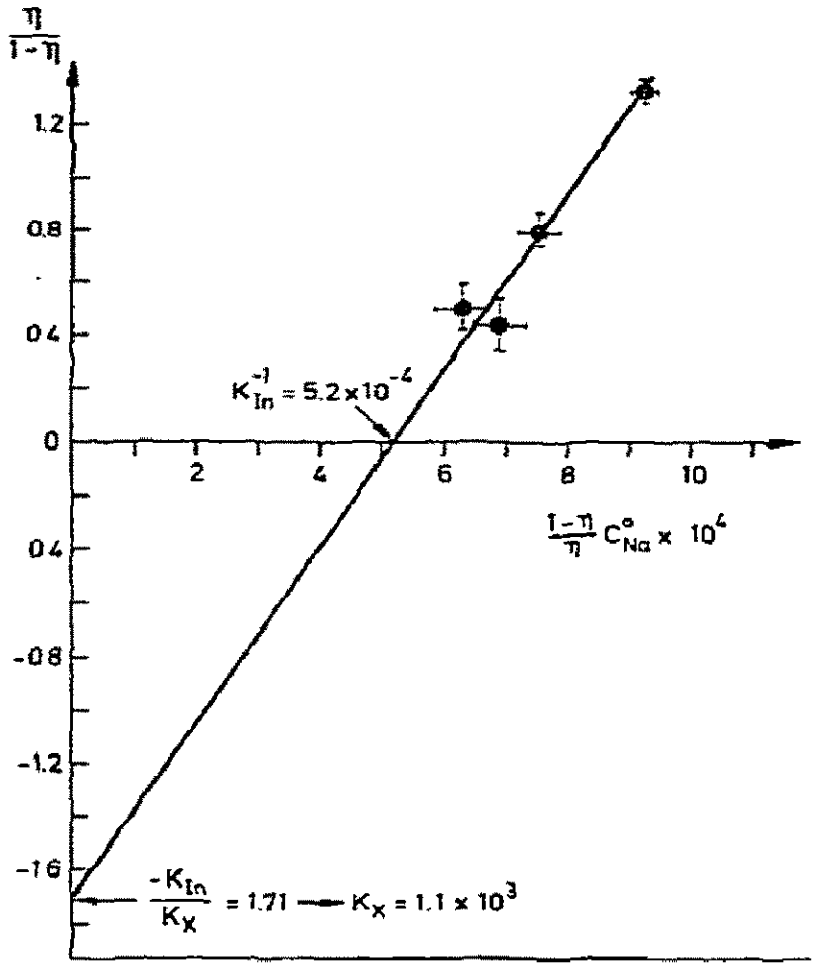


Fig. 7. Spectrophotometric substitution titration according to eq. (15). Sample: sodium-dinactin, indicator: murexide.

The electric field pulse technique was also tried. However, a finite field effect is to be expected only if the reaction under study is associated with a neutralization of charges. This does not hold for the combination of alkali ions with the neutral macrotetrolides, although it is true for their reactions with murexide and nigericin. While the rate constants for formation of alkali ion murexide complexes could be determined from the relaxation of the dissociation field effect, this method was not successful with nigericin where only lower bounds of rates could be deduced. The high rates found for murexide, indeed, suggest that the rate constants for nigericin are above $2 \times 10^{10}\ M^{-1}s^{-1}$, closely resembling the limiting values to be expected for diffusion controlled processes.

Since dinactin is sufficiently (up to 0.05 M) soluble in methanol its sound absorption could also be detected. With the help of both the resonance and pulse techniques it was possible to determine finite absorption values in the frequency range of 4×10^5 to 10^8 Hz. The results are given in a log ($\alpha\lambda$) versus log (ν) plot according to eq. (20).

The measured absorption for a dinactin solution of 0.048 M is represented by curve I in fig. 8. The remaining chemical effect after subtraction of the "classical" term is shown in curve I_a. This additional absorption can be attributed to a relaxation caused by a conformational transition of the dinactin molecule. The frequency term $\omega\tau/(1+\tau^2\omega^2)$ of I_a indicates a single relaxation effect appearing at 2.8×10^7 Hz, which corresponds to a relaxation time of 5.7 ns ($\tau = (2\pi\nu)^{-1}$). This relaxation time is independent of the dinactin concentration, as is demonstrated by curve II or II_a respectively, which refer to a lower concentration of dinactin. As to be expected, the amplitude of the absorption curve decreases with decreasing dinactin concentration. Curve II also indicates that in the presence of Cs^+ a second relaxation effect with an ampli-

Table 2
Equilibrium quotients [a] obtained by spectrophotometric titration in methanol solution at 25°C

		Li^+	Na^+	K^+	Rb^+	Cs^+	NH_4^+
Nonactin	K_X^i, M^{-1}	<2	$(2.3 \pm 0.3) \times 10^2$	$(1.4 \pm 0.2) \times 10^4$	$(1.4 \pm 0.2) \times 10^4$	$(1.5 \pm 0.2) \times 10^3$	$(2.4 \pm 0.4) \times 10^4$
	K_X^i/K_X^K		0.016	1.0	1.0	0.11	1.7
Monactin	K_X^i, M^{-1}	<2	$(4.0 \pm 0.7) \times 10^2$	$(2.4 \pm 0.4) \times 10^4$	$(2.4 \pm 0.3) \times 10^4$	$(2.0 \pm 0.3) \times 10^3$	$(4.8 \pm 0.7) \times 10^4$
	K_X^i/K_X^K		0.017	1.0	1.0	0.08	2.0
Dinactin	K_X^i, M^{-1}	<2	$(1.1 \pm 0.1) \times 10^3$	$(4.3 \pm 0.5) \times 10^4$	$(4.2 \pm 0.5) \times 10^4$	$(4.2 \pm 1.2) \times 10^3$	$(9.1 \pm 0.9) \times 10^4$
	K_X^i/K_X^K		0.025	1.0	0.98	0.1	2.1
	K_X^i, M^{-1}	<2	$(1.7 \pm 0.2) \times 10^3$	$(9.1 \pm 0.9) \times 10^4$	$(7.7 \pm 1.4) \times 10^4$	$(1.0 \pm 0.1) \times 10^4$	$(2.1 \pm 0.3) \times 10^5$
	K_X^i/K_X^K		0.019	1.0	0.85	0.11	2.3

[a] The index i refers to the particular cation, while all ratios are normalized with respect to the equilibrium constant of the potassium complex.

Table 3
Rate constants obtained in methanol solution at 25°C

	$k_f[M^{-1}s^{-1}]$	$k_r[s^{-1}]$	K_{21}	$k_{12}[s^{-1}]$	$k_{21}[s^{-1}]$	$k_{23}[M^{-1}s^{-1}]$	$k_{32}[s^{-1}]$	Method a)
Monactin + Na^+	3×10^8	6×10^5						U.S.
Dinactin + Na^+	5×10^7	4.6×10^4	1.7	6.5×10^7	1.1×10^8	1.3×10^8	4.3×10^4	U.S.
	6.3×10^7	5.8×10^4				1.6×10^8	5.4×10^4	T.J.
Dinactin + Cs^+	3.4×10^8	8.2×10^4	1.7	6.5×10^7	1.1×10^8	7.8×10^8	5.1×10^4	U.S.
Trinactin + Na^+	7.2×10^7	4.2×10^4	1.2			1.6×10^8	4.2×10^4	T.J.
Nigericin + Na^+	$\geq 1 \times 10^{10}$	$\geq 1.1 \times 10^5$						E.F.

a) U.S. = ultrasonic absorption method; T.J. = temperature jump method; E.F. = electric field pulse technique.

tude of $\mu_{max} = 0.22 \times 10^{-3}$ occurs at a frequency of 8.2×10^5 Hz, yielding a relaxation time of approximately 0.19 μs. In this case the relaxation time also shows concentration dependence. When the Cs^+-dinactin solution was diluted to half of its original concentration, the relaxation time increased to 0.36 μs while the amplitude changed slightly to $\mu_{max} = 0.20 \times 10^{-3}$. When Cs^+ is replaced by Na^+, the relaxation time becomes 0.35 μs and the amplitude: 0.26×10^{-3} (referring to a solution containing 0.11 M Na^+ and 0.045 M dinactin). After dilution of this solution to half of its original concentration, a relaxation time of 0.61 μs with $\mu_{max} = 0.24 \times 10^{-3}$ could be evaluated. From these data, values for k_f and k_d were calculated. The magnitude of k_f for dinactin is lower than for dibenzo-30-crown-10. For comparison k_f for the Cs^+-dibenzo-30-crown-10 complex amounts to $8 \times 10^8\ M^{-1}\ s^{-1}$ [27].

If the relaxation time observed at 28 MHz is due to a conformational change of the dinactin itself, and if one of the conformational states is likely to form the metal complex, then the simplified reaction scheme (22) as presented in section 2 may hold. Applying eq. (24) and estimating K_{21} to be 1.7, a k_{23} value for the complex formation of Cs^+ with dinactin is obtained, which is comparable to the rate constant of Cs^+ complexing with dibenzo-30-crown-10. Based on this procedure, all rate constants, i.e. k_{12}, k_{21}, k_{23} and k_{32} could be evaluated. The resulting data are given in table 3.

Furthermore, referring to eq. (21) and using the known thermodynamic data for methanol [28,29] at 25°C, $\alpha_p = 1.1 \times 10^{-3}\ deg^{-1}$, $\rho = 0.79\ g/cm^3$, $c_p = 0.608$ cal/g deg, $\kappa_s = 1.05\ 10^{-4}\ atm^{-1}$, $R = 82.05\ cm^3\ atm\ mol^{-1}\ deg^{-1}$ the value of ΔV can be determined as well. For the sodium-dinactin complex ΔV amounts to $-16.5\ cm^3/mol$.

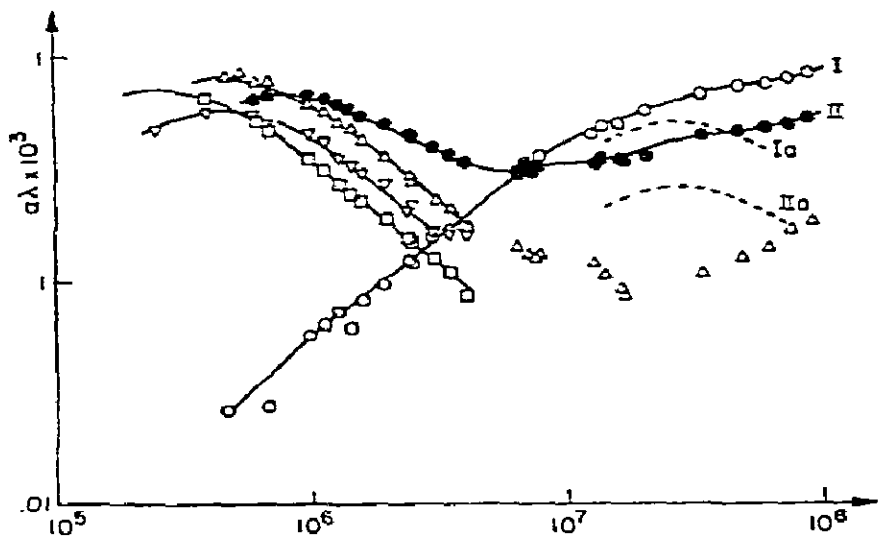


Fig. 8. Log-log plot of absorption per wavelength versus frequency for the dinactin system in methanol at 25°C. ○: dinactin (0.048 M), ●: dinactin (0.048 M) and CsCl (0.031 M). ▽: dinactin (0.024 M) + CsCl (0.0155 M), △: dinactin (0.045 M) + NaCl (0.11 M), ■: dinactin (0.0225 M) + NaCl (0.055 M).

5. Discussion

The equilibrium data in general fit very well a 1 : 1 complex formation scheme. The values are in agreement with those determined by Pioda et al. [9] from vapor phase osmometric measurements of nonactin and monactin. The conclusions are also consistent with the results of the X-ray studies of K^+-nonactin [8], Ag-nigericin [11], and Ag-monensin [15]. The stability constants of the macrotetrolide series show a selectivity pattern which is remarkably similar to that observed for the transport of alkali metal ions across membranes in presence of the macrotetrolides [3],

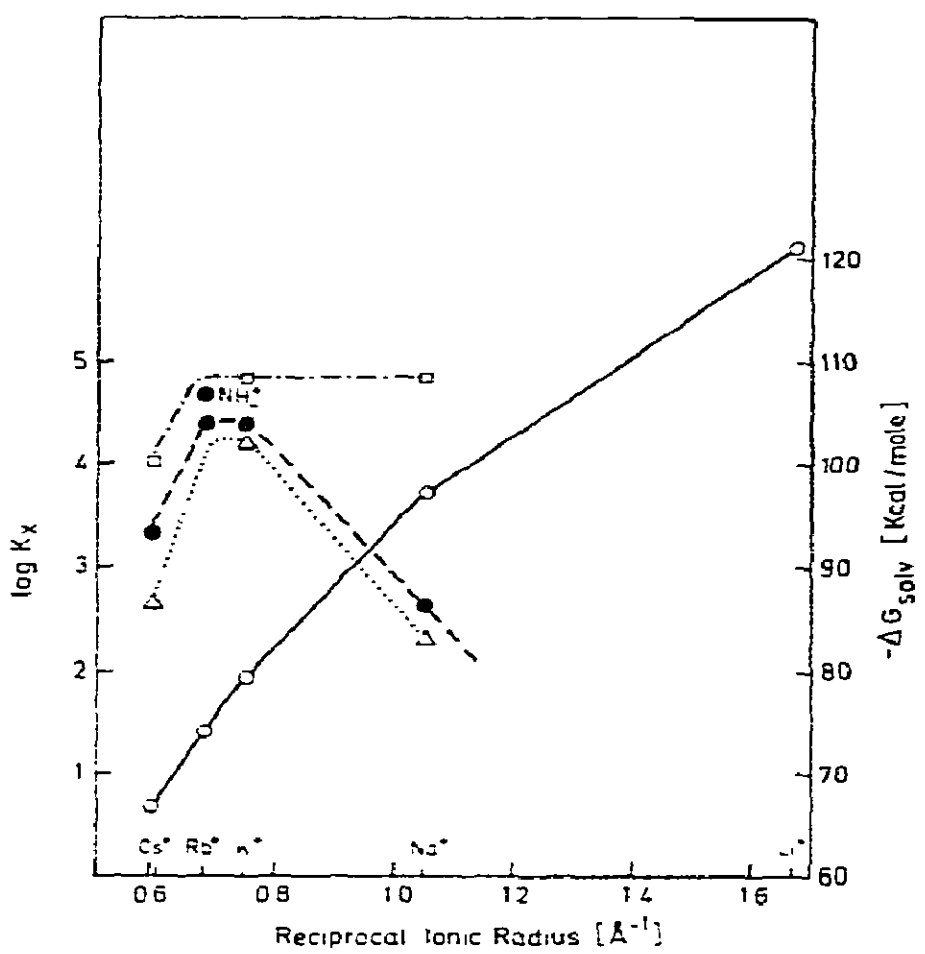


Fig. 9. Correlation between the logarithm of stability constant for complex formation (log K_X), free energy of solvation ($-\Delta G_{solv}$) and reciprocal ionic radius for Li^+, Na^+, K^+, Rb^+, Cs^+ and NH_4^+. The solid line (○) refers to $-\Delta G_{solv}$. The broken lines represent 1) (– – –) log K_X for the cation-monactin systems in methanol (●) (including the value for NH_4^+), 2) (– · –) and 3) (• • •) log K_X for the cation-nonactin system in dry acetone (□) [33] and in acetone-D_2O (Δ), respectively.

which indicates that the selectivity exhibited in the transport is essentially caused by the binding properties of the carrier.

Fig. 9 shows the correlation between the stability constants of the cation-macrotetrolide complexes and the reciprocal crystal radii [30] as well as the hydration energies [31] of the alkali metal ions. Beginning with cesium the stability constants increase with decreasing radii and the concomitantly increasing hydration energies. For potassium, however, the stability reaches a maximum, then followed by a sharp drop for sodium and lithium. This particular behaviour can be rationalized as the superposition of the following effects:

(i) Removal of solvent molecules from the inner coordination sphere of the alkali metal ion: This means "paying" for the free energy of solvation. The formation of complexes with the antibiotics involves the replacement of all solvent molecules from the inner coordination sphere of the metal ion. The fact of complete inner sphere substitution is supported by at least two kinds of evidence. (a) No solvent molecule – coordinated to the metal ion – was found by the X-ray studies of KSCN-nonactin, Ag-nigericin, and Ag-monensin. (b) Proton magnetic resonance spectra of the K^+-nonactin complexes [32] in dry acetone and acetone-D_2O mixtures lead to the same conclusion. The vicinal coupling constants also indicate that only an unhydrated ion is present in the cavity formed by the nonactin molecule. All this evidence is further suported by the finding that Na^+, K^+, and Cs^+ possess [33] nearly equal affinities for nonactin in dry acetone, but the situation changes drastically when a 0.55 mole fraction of D_2O is added. In the latter case, the stability constants were markedly reduced, namely in such a way that the binding of K^+ to nonactin becomes highly favorable compared to that of Na^+ and Cs^+ (see fig. 9). These results demonstrate the important role of the free energy of solvation of the alkali metal ions with respect to their selectivity pattern for a given chelating ligand.

(ii) Desolvation of the antibiotic associated with a supply of energy required to change its conformation from the "free" to the "complexed" state. The data in table 2 show that for a given metal ion there is a clear trend in the complex stability in the order trinactin > dinactin > monactin > nonactin. This trend is caused by the successive substitution of a hydrogen atom by a methyl group at the R position in the homologue series as demonstrated in fig. 1. The most hydrophobic macrotetrolide, trinactin, is likely to possess the lowest solvation energy in a relatively polar solvent like methanol (dielectric constant of 31.5). Binding of a cation makes it less hydrophobic due to a finite net charge.

The disappearance of the relaxation effect attributed to the conformational transition as a consequence of complex formation between the macrotetrolide and the metal ion (as observed by ultrasonic absorption, cf. fig. 8), suggests a strong stabilization of one conformational state due to the binding of the metal ion. The conformational transition was also detected [33] by proton magnetic resonance presuming that the nonactin ring undergoes a sizable conformational change upon incorporation of an alkali metal ion. The

X-ray data by Kilbourn et al. [8] indicate a quite different configuration of uncomplexed nonactin as compared to that of KSCN_ nonactin. The important role of conformational energy with respect to the selectivity for alkali metal ions has also been reported for valinomycin [34].

(iii) Optimal binding of the antibiotic – using its polar groups as ligands – to the metal ion. The size of the cavity relative to that of the metal ion governs the optimal compensation of all energy terms involved. The antibiotics resemble multidentate ligands. The cation is located in the cavity formed by these ligands which in turn envelope the metal ion completely. Steric hindrance and ligand-ligand repulsion limit the conformational flexibility. Therefore it is conceivable that a particular size of the metal ion could achieve an "optimal fit" for a given cavity. The "best fit" condition would provide a closest distance of the metal ion to the hydrophilic groups, but it is ultimately defined by the superposition of the different energy terms. Metal ions smaller than the optimal size of the cavity – supposed they are located in the center – would yield no smaller energy increment for binding as those with a space filling fit. However, they require a quite larger energy for complete desolvation, with the effect that complex stability decreases. For ions larger than the optimal size both increments decrease, but the larger increment for ligand binding prevails, otherwise the complex would not be stable. It depends on the particular structure of the antibiotic, for which metal ion size the compensation of energy terms yields maximum stability constants. For example, the relatively rigid cyclodecapeptide, antamanide [35], forms a considerably more stable complex with Na^+ than with K^+, while the dodecadepsipeptide, valinomycin [36], forms the most stable complex with Rb^+, the Na^+ complex being quite weak. In addition, the stability of the complex is related to the exact local distribution of ligands and their coincidence with the coordination properties of the metal ion. This is demonstrated by the fact that the NH_4^+ ion, which has a radius of 1.43 Å, forms a very stable macrotetrolide complex, while showing only weak interaction with dibenzo-30-crown-10 [27] although both compounds are selective for K^+. In the alkali metal ions the charge is distributed more uniformly than in NH_4^+, where the four hydrogens at which the positive charge is concentrated, form a tetrahedral configuration. The four furan and the four keto oxygens of the macrotetrolides, when coordinated with a cation, assume an approximately cubic octadentate symmetry. With the help of CPK models one can show that the tetrahedral charge distribution of the NH_4^+ fits very well this cubic arrangements of ligands. On the other hand, the oxygen ligands in dibenzo-30-crown-10 cannot provide a similarly favorable configuration for NH_4^+. For $[NH_3(CH_3)]^+$ the complex stability with trinactin [37] reduces by at least a factor of 100. Studies of nuclear magnetic resonance [38] using $^{23}Na^+$ further indicate the importance of the relative position of the metal ion with respect to the coordinated ligands.

In conclusion, the selectivity can be expressed only by a superposition of several free energy terms:

$$-RT \ln K_X = \Delta G_{Bind} - \Delta G_{Hyd}(M) - \Delta G_{Hyd}(X) + \Delta G_{Conf}(X), \quad (25)$$

where ΔG_{Bind}, $\Delta G_{Hyd}(M)$, $\Delta G_{Hyd}(X)$, $\Delta G_{Conf}(X)$ are the free energy increments for metal-ligand bond formation, metal ion solvation, ligand solvation, and ligand conformational change, respectively. Any difference of 1.36 kcal/mol at 25°C is equivalent to a factor of 10 in the stability constants. This picture of alkali ion carrier specificity and dynamics has been developed several years ago [39,40]. It is strongly supported by the experimental data collected so far.

The dynamic studies indicate that dinactin by itself exhibits a very fast conformational transition with a concentration independent relaxation time of 5.7 ns. This suggests that the macrotetrolide molecules are very flexible and exist in at least two configurations. The rate constants of this interconversion were calculated for the simple reaction scheme (cf. expression (22)). The estimated value for K_{21} accounts for such a flexibility.

The high value of k_{23} implies that the substitution mechanism [39,40] must occur stepwise. Since the solvation energies involved are quite large (60–100 kcal/mol) appreciable activation energies were to be expected, if energy balance would not apply to every single step of substitution. In other words: the metal ion cannot strip off all its solvent molecules at once before entering the cavity. On the other hand, the stepwise substitution mechanism – a kind of redressing – provides an immediate compensation of the

energy "paid" for desolvation in a 1 : 1 exchange [39, 40]. It is known that replacement of a *single* solvent molecule from the inner coordination shell of an alkali metal ion can be accomplished within about 10^{-9} s [41,42]. The enormous flexibility of the macrotetrolide molecule favours strongly its fast complexation with the cation. If it were less flexible, as is the case for valinomycin and antamanide, a slower rate of complex formation would consequently result [43–45]. The fact that only a single relaxation time for the whole process is observed, emphasizes that the initial and final states are the predominant ones and that intermediates occur only transiently.

An efficient carrier should be selective, which demands a high K_X-value, for the particular substrate:

$$K_X = \frac{k_{23}}{k_{32}} \left(\frac{k_{12}}{k_{21} + k_{12}} \right).$$

On the other hand, it should also have a high turn-over rate in the transport of ions, which requires k_{32} to be as high as possible, otherwise it would block the unloading of ions. The fact that indeed high k_{23}-values have been found is of significance with respect to optimal performance in selective transport.

The open-chain antibiotics studied contain a carboxylic group which is negatively charged at neutral pH. X-ray diffraction data show that in the Ag-complexes of both monensin [14] and nigericin [11] the carboxylate groups are not directly involved in forming the metal oxygen bonds. Instead they are used in order to stabilize the annular conformation via strong hydrogen bonds with the two hydroxyl groups at the opposite end of the chain. The metal ion then is enclosed in this conformation in a similar fashion as in the case of macrotetrolides. The ether and hydroxyl oxygen atoms point inward coordinating the metal ion whereas the exterior of the complex consists almost completely of the hydrocarbon-sections of the antibiotic. The crystal form [15] of K-monensin is isomorphous with that of the silver complex; also the Na complex, although not isomorphous, is very similar.

The two hydrogen bonds seem to play an important role in alkali metal ion binding. This is especially obvious from studies with grisorixin, an antibiotic isolated [46] from *Streptomyces griseus*. The structure of grisorixin is similar to that of nigericin except for a $-CH_2OCH_3$ group, which replaces the $-CH_2OH$ group at the cyclic ether ring opposite to the carboxylic group. Therefore, it cannot form two hydrogen bonds like nigericin. In studies [47] of grisorixin in the presence of Na^+ and murexide no spectral shifts of the indicator could be detected, indicating that the Na^+-grisorixin complex, if present at all, is at least 500 times less stable than the corresponding nigericin complex.

All evidence obtained by X-ray crystallography clearly shows that the antibiotic molecule is wrapped around the metal ion in a special manner: the polar groups point to the center of the molecule while the non-polar groups are arranged at the surface providing a lipophilic coat, which is essential for the function of these compounds as membrane carriers.

It is believed that coordination principles similar to those which determine the ion selectivity of antibiotics are also valid for the binding of monovalent cations to enzymes. The effectiveness of a given monovalent cation in modifying enzymatic activity, is likely to be governed by a change in conformation of the protein

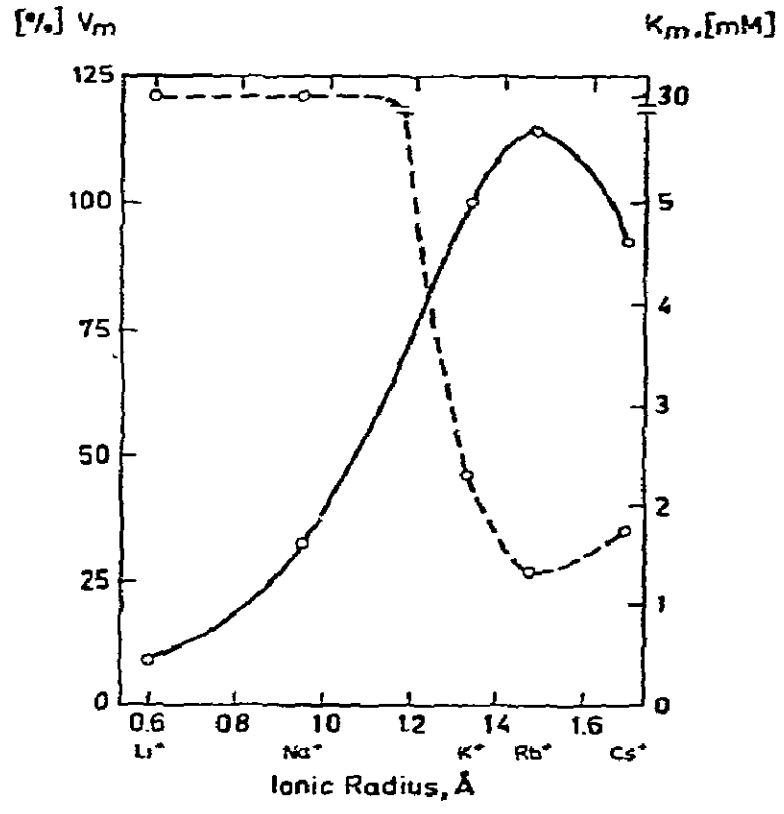


Fig. 10. Correlations between relative enzymatic activity in terms of the maximum velocity V_m, assuming 100% for K^+ (solid line), Michaelis constant K_m (broken line) and ionic radius of alkali metal ions for the system rat liver pyruvate carboxylase. The values were obtained from ref. [48].

induced by the formation of a metal-protein complex. In fig. 10, this behavior is exemplified with rat liver pyruvate carboxylase. The relative maximum velocity (V_m) and the Michaelis constant K_m (i.e. the concentration of a given cation required to obtain half of V_m) are plotted as function of the radii of those alkali metal ions which activate the enzyme [48]. The relative apparent binding affinities decrease according to the series $Rb^+ > Cs^+ > K^+ \gg Na^+ \sim Li^+$, while effects on the maximum velocity follow the order $Rb^+ > K^+ > Cs^+ \gg Na^+ > Li^+$. These two series exhibit certain similarities. Rb^+ enhances the maximum velocity most effectively and also forms the most stable complex with the enzyme. The data suggest that the alkali cations activate the enzyme by forming complexes and triggering off conformational changes. The degree of activation thus is dependent on the specific conformational change induced.

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