SECOND FEBS–FERDINAND SPRINGER LECTURE*

MEMBRANE ACTIVE COMPLEXONES. CHEMISTRY AND BIOLOGICAL FUNCTION

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One of the fundamental problems of modern biochemistry is the study of the chemical structure and the functioning of biological membranes, which play a dominating part in regulation of the molecular and ionic transport between the cell and its environment. It would be no exaggeration to say that the progress achieved in latter years towards our understanding of phenomena associated with transmembrane ion transport is in one way or another connected with the application of antibiotic ionophores as tools for study of these phenomena. The successful use of such ionophores, forming complexes with alkali (or alkaline earth) metal ions and selectively inducing membrane permeability towards the complexed ion, has in turn stimulated the impetuous development of the chemistry of these unique bio-regulators.

I shall first discuss the 'prime' ionophore, valinomycin, and its numerous analogs, tracing the relation between the physicochemical properties of its various spatial forms and its behavior in the individual stages of the transmembrane ion transport process. Then I shall touch upon some new findings in the series of enniatin ionophores which have convinced us that we have a new type of transmembrane ion carriers, functioning in the form of bi- or trimolecular combinations.

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Valinomycin

The study of valinomycin antibiotics in our laboratory took on a number of directions. On the one hand, the mode of action of valinomycin (fig. 1) and some of its analogs on lipid bilayers was investigated. We consider such bilayers as prototypes of the lipid zones of biological membrane systems. On the other hand, the synthesis of new valinomycin analogs and elucidation of their spectral characteristics was continued, the accumulated data serving as basis for elucidating the conformational states of the free and alkali metal complexed valinomycins. Finally, a parallel study was undertaken of the biological action of the valinomycins on bacteria and mitochondria.

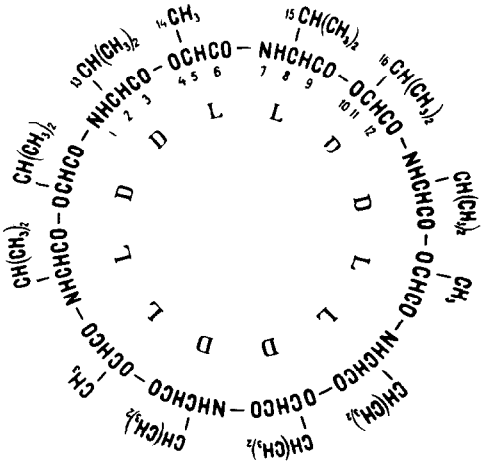


Fig. 1. Valinomycin (I).

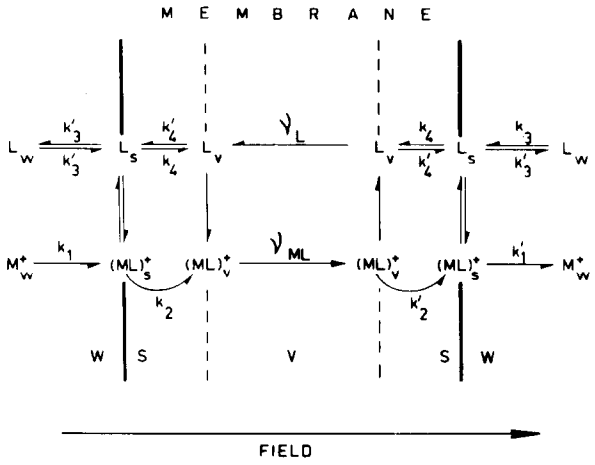
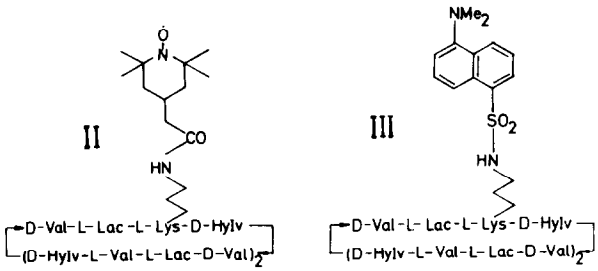


Fig. 2. Ion transport model through phospholipid bilayers in the presence of valinomycin cyclodepsipeptides.

Table 1
Leakage of K^+ from lecithin liposomes in the presence of 10^{-5} M valinomycin and its derivatives II and III



Compound	Leakage of K^+, in % of the initial KNO_3 content, after 5 min at 25°C	% of valinomycin activity
Valinomycin (I)	40.0	100
II	44.0	110
III	32.0	80

The biophysical studies led to a model for the induced ion conductivity shown on fig. 2 [1,2]. First the ion becomes fixed on the membrane surface by heterogeneously complexing with the embedded ionophore molecule. It is then taken up by a free ionophore from within the membrane, the complex cation being further transported by bulk diffusion along a potential gradient. Thus according to the model the membrane resistance can be represented as the sum of 'bulk' and 'boundary' resistances, the former depending upon the concentration and mobilities of the complexes within the membrane and the latter, upon the rates of the heterogeneous complexing reaction, the surface to bulk potassium ion exchange and the intramembranal free cyclodepsipeptide flow. It turned out that Nature has built the molecule of this antibiotic to such perfection that all these stages have very similar rates.

The behavior of valinomycin could also have been described by a similar model, as was done by Stark and Benz [3] in 1971. However, structural peculiarities of the valinomycin analogs (XXVII and XL, see below) caused them considerable sluggishness at one or another of the transmembrane transporting stages which were thereby brought to light and thus included in our model.

We aim to continue our studies on the behavior of valinomycin on artificial membranes, making use of the recently synthesized spin- and fluorescence-labeled derivatives II and III [4], which turned out practically identical to valinomycin in their ability to transport potassium ions across lecithin micelles (table 1).

It follows from our model that for effective ionophore functioning a molecule of the valinomycin group must meet the following requirements; i) ability to form sufficiently stable K^+ complexes; ii) ability to partition between the surface and the internal zone of the membrane, i.e. possession of both lipophilicity and surface active properties; iii) sufficient lipophilicity of the K^+ complex for it to be able to pass through the internal zone of the membrane; iv) sufficiently rapid exchange of the potassium ion between two ionophore molecules.

The 36-membered depsipeptide chain of valinomycin has a wealth of conformational possibilities. In non-polar media it assumes a compact symmetric (C_3) structure (form A on fig. 3a), with the amide CO and NH groups forming an intra molecular hexa hydrogen-bonded 'bracelet'. The ester carbonyls point away from the symmetry axis; all twelve alkyl side chains are situated on the molecular surface. In solvents of medium polarity a flatter structure with three hydrogen bonds, so-called 'propeller', (form B, see fig. 3b) is predominant. Its interior comprises a hydrophobic nucleus of three D-valine isopropyl groups and three lactic acid methyls. This is surround-

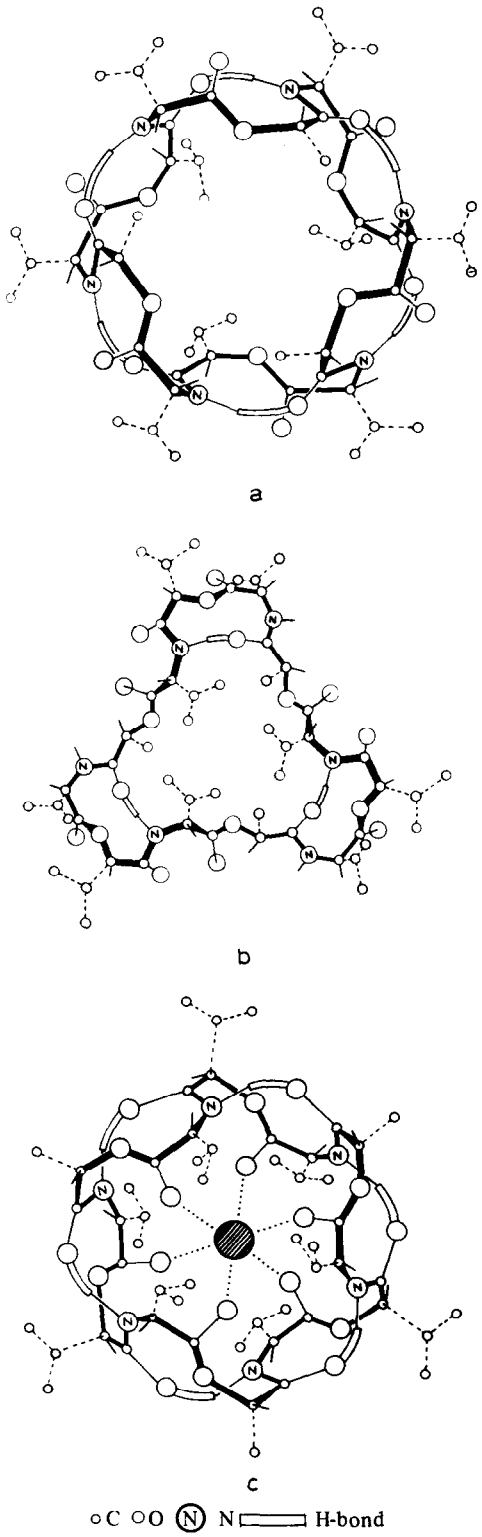


Fig. 3. Conformation of valinomycin in (a) non-polar solvents, (b) solvents of medium polarity and (c) on complexation with K^+.

ed by the depsipeptide chain with the polar amide and ester groups. We consider it very likely that in the internal regions of the membrane, valinomycin assumes conformation A and on its surface, conformation B. In polar media, including water, valinomycin is in the form of an equilibrium mixture of a large number of non-hydrogen bonded energetically similar conformers. Grell et al. [5,6] showed that several more conformers, apparently intermediate between forms A, B and C, can exist in solution.

Interestingly the crystalline state of valinomycin has a completely different conformation (fig. 4), despite recrystallization of the X-ray specimen from isooctane, i.e. from a solution where form A is predominant [7,8]. The structure observed has a pseudo symmetry center. In the crystal, all NH groups form hydrogen bonds, four with amide carbonyls and the remaining two with ester carbonyls. The nonequivalence of the amide and ester carbonyls in crystalline valinomycin is distinctly manifested in both its IR spectrum (fig. 5) and Raman spectrum [9]. For comparison fig. 6 gives the IR spectra of valinomycin in nonpolar solvents, where one can see the absence

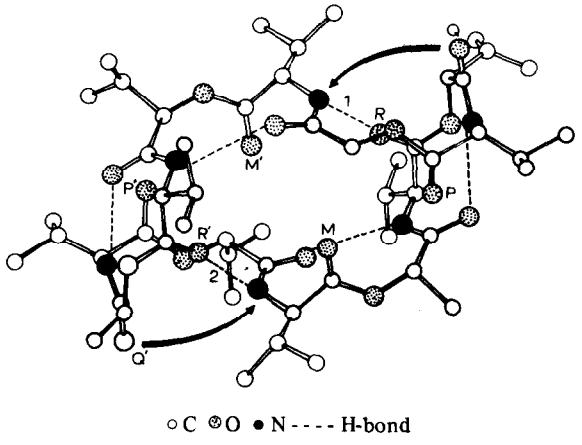


Fig. 4. Conformation of crystalline valinomycin. M and M', P and P', R and R' are ester carbonyl oxygens arranged about a pseudo symmetry center; Q and Q' are non-H-bonded amide oxygens, 1 and 2 – H-bonds of the 5 → 1 type. Arrows mark the direction of Q and Q' displacement after rearrangement of the crystalline conformer into the complexed form.

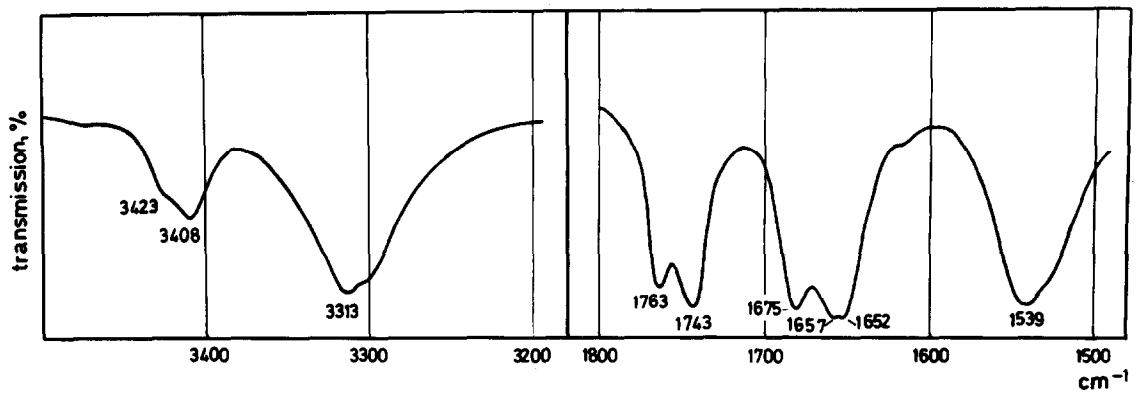


Fig. 5. Infra red spectrum of crystalline valinomycin.

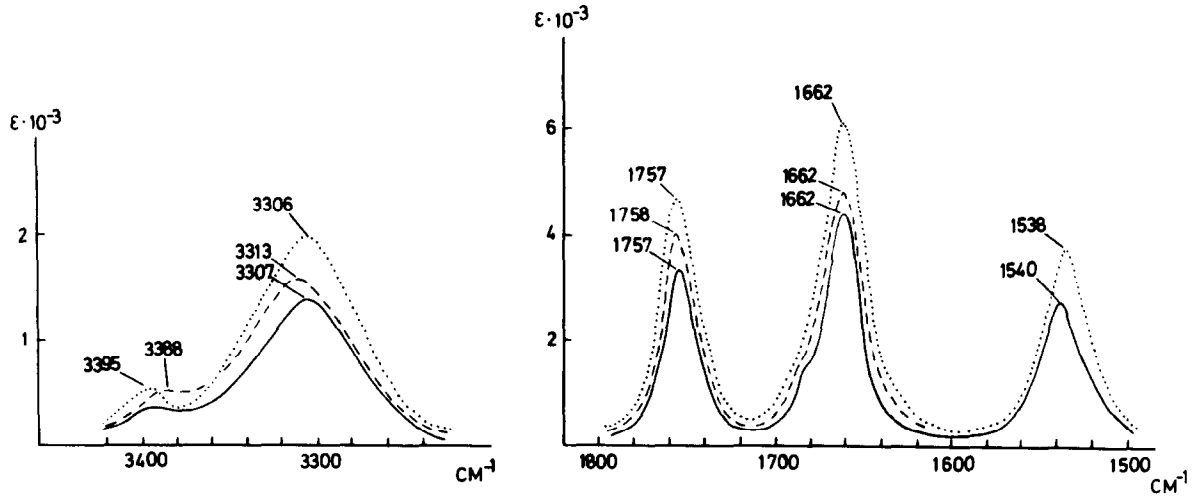


Fig. 6. Infra red spectra of valinomycin in non-polar solvents (—— C_7H_{16}, . . . CCl_4, - - - $CHCl_3$).

of any signs of splitting in the ester and amide carbonyl stretching bands (the lower wavelength shoulder of the amide I band is attributed to the presence of conformers containing less than six intramolecular hydrogen bonds). Valinomycin assumes a rigid conformation in all solvents on complexing with K^+. It has the same hydrogen bond sequence as in form A, but its ester carbonyls are now turned inwards, forming 6 ion–dipole bonds with the centrally situated cation (fig. 3c). The latter is effectively screened from interaction with the solvent and the anion by the depsipeptide skeleton and the pendant side chains. The molecular surface of the complex thus consists mainly of methyl and isopropyl groups, lending it its high hydrophobicity. The same structure was found in the crystalline K^+ complex [10].

The diameter of the complex's cavity (2.7–2.9 Å) corresponds to the size of the potassium and rubidium ions. With cesium ions steric strain arises and the stability constant falls [11].

The observed structural characteristics also clarify the high K/Na complexing selectivity of valinomycin: owing to the rigidity of the bracelet, it is unable to augment the efficiency of the ion–dipole interaction with Na^+ such as to compensate the considerably higher Na^+ than K^+ solvation energy [12].

On the basis of all the above said one can rationalize the complexing capacity of all the known synthetic analogs of valinomycin (tables 2–5).

The K^+ and Cs^+ complexes of compound V com-

Table 2
Valinomycin analogs with altered ring size

No.	Compound	Minimal growth inhibiting concentration (γ/ml)						Stability constants of K^+ complexes (K, 1 × mole^{-1}, C_2H_5OH, 25°C)		
		Staphylococcus aureus 209P	*Staph. aureus* UV-3	*Bacillus mycoides*	*Escherichia coli*	*Mycobacterium phlei*	*Candida albicans*	Na^+	K^+	Cs^+
I	[(D-Val–L-Lac–L-Val–D-HyIv)$_3$] valinomycin	> 50	0.2–0.4	> 50	> 50	0.3	0.2–0.4	20	2×10^6	6.5×10^5
IV	[(D-Val–L-Lac–L-Val–D-HyIv)$_2$] 'octa-valinomycin'	> 100	> 100	> 100	> 100	> 100	> 100	40	10	–
V	[(D-Val–L-Lac–L-Val–D-HyIv)$_4$] 'hexadecavalinomycin'	> 50	> 50	> 50	> 50	> 50	> 50	~ 10	100	500

Table 3
Valinomycin analogs with altered configurations of amino and/or hydroxy acid residues

No.	Compound	Minimal growth inhibiting concentration (γ/ml)						Stability constants of K^+ complexes (K, 1 × mole^{-1}, C_2H_5OH, 25°C)	Free energy of complex formation with K^+ ($-\Delta F=RT\ln K$, kcal × mole^{-1})
		Staphylococcus aureus 209P	*Staph. aureus* UV-3	*Bacillus mycoides*	*Escherichia coli*	*Mycobacterium phlei*	*Candida albicans*		
I	[(D-Val–L-Lac–L-Val–D-HyIv)$_3$] valinomycin	> 50	0.2–0.4	> 50	> 50	0.3	0.2–0.4	2×10^6	8.6
VI	D-Val → L-Val	> 50	2	> 50	> 50	1.5	3–5	11 000	5.5
VII	L-Lac → D-Lac	> 50	> 50	> 50	> 50	> 50	> 50	75 000	6.7
VIII	L-Val → D-Val	> 50	1–2	> 50	> 50	1–2	0.2–0.4	4400	5.0
IX	D-HyIv → L-HyIv	> 50	> 50	> 50	> 50	> 50	> 50	100–150	2.7–2.9
X	3 D-Val → 3 L-Val	> 50	> 50	> 50	> 50	> 50	> 50	< 50	< 2.3
XI	3 L-Val → 3 D-Val	> 50	> 50	> 50	> 50	> 50	> 50	< 50	< 2.3
XII	3 D-HyIv → 3 L-HyIv	> 50	> 50	> 50	> 50	> 50	> 50	< 50	< 2.3
XIII	3 D-Val → 3 L-Val; 3L-Val → 3D-Val	> 25	> 25	> 25	> 25	> 25	> 25	< 50	< 2.3
XIV	[(D-Val–L-HyIv–L-Val–D-HyIv)$_3$] meso-HyIv–valinomycin	> 50	> 50	> 50	> 50	> 50	> 50	3.7×10^5	7.6
XV	D-Val → L-Val	> 25	> 25	> 25	> 25	> 25	> 25	10^6	8.2
XVI	D-Val → L-Val; L-Val → D-Val	> 25	> 25	> 25	> 25	> 25	> 25	10 000	5.5
XVII	2 D-Val → 2 L-Val	> 25	> 25	> 25	> 25	> 25	> 25	11 000	5.5
XVIII	D-HyIv → L-HyIv	> 25	> 25	> 25	> 25	> 25	> 25	1500	4.3
XIX	2 D-HyIv → 2 L-HyIv	> 25	> 25	> 25	> 25	> 25	> 25	< 50	< 2.3
XX	L-HyIv → D-HyIv; D-HyIv → L-HyIv	> 25	> 25	> 25	> 25	> 25	> 25	< 50	< 2.3

Table 4
Valinomycin analogs with replaced ester or amide groupings

No.	Compound	Minimal growth inhibiting concentration (γ/ml)						Stability constants of K^+ complexes (K, 1 × mole^{-1} C_2H_5OH, 25°C)	Free energy of complex formation with K^+ ($-\Delta F=RT\ln K$ kcal × mole^{-1}
		Staphylococcus aureus 209P	*Staph. aureus* UV-3	*Bacillus mycoides*	*Escherichia coli*	*Mycobacterium phlei*	*Candida albicans*		
I	(D-Val–L-Lac–L-Val–D-HyIv)$_3$ valinomycin	> 50	0.2–0.4	> 50	> 50	0.3	0.2–0.4	2 × 10^6	8.6
XXI	D-Val → D-HyIv	> 25	> 25	> 25	> 25	> 25	> 25	42 000	6.3
XXII	L-Lac → L-Ala	4.5	0.1	> 50	> 50	0.2–0.4	4.5	3 × 10^5	7.5
XXIII	2 L-Lac → 2 L-Ala	2–4	2	12	> 50	2	12–18	2.2 × 10^5	7.3
XXIV	L-Val → L-HyIv	> 50	> 50	> 50	> 50	50	> 50	2500	4.7
XXV	L-Val → L-MeVal	> 50	> 50	> 50	> 50	50	> 50	< 50	< 2.3
XXVI	D-HyIv → D-Val	> 50	1	> 50	> 50	1.5	3	5200	5.1
XXVII	3 L-Lac → 3 L-MeAla	> 20	0.4–0.8	> 20	> 20	0.4	> 20	10^7	9.6

Table 5
Valinomycin analogs with altered side chains

No.	Compound	Minimal growth inhibiting concentration (γ/ml)						Stability constants of K^+ complexes (K, 1 × mole^{-1} C_2H_5OH, 25°C)	Free energy of complex formation with K^+ ($-\Delta F=RT\ln K$, kcal × mole^{-1})
		Staphylococcus aureus 209P	*Staph. aureus* UV-3	*Bacillus mycoides*	*Escherichia coli*	*Mycobacterium phlei*	*Candida albicans*		
I	[(D-Val–L-Lac–L-Val–D-HyIv)$_3$] valinomycin	> 50	0.2–0.4	> 50	> 50	0.3	0.2–0.4	2 × 10^6	8.6
XXVIII	D-Val → D-Ala	> 50	0.1–0.2	> 50	> 50	0.4	0.2–0.3	2 × 10^6	8.6
XXIX	2 D-Val → 2 D-Ala	> 50	0.5–0.7	> 50	> 50	0.5–1	1–1.5	88 000	6.8
XXX	3 D-Val → 3 D-Ala	> 50	> 50	> 50	> 50	> 50	> 50	160	3.0
XXXI	L-Val → L-Ala	> 50	0.3–0.5	> 50	> 50	0.2	0.2–0.5	3 × 10^6	8.9
XXXII	2 L-Val → 2 L-Ala	> 50	0.5	> 50	> 50	1	1.5–2	2 × 10^5	7.3
XXXIII	3 L-Val → 3 L-Ala	> 50	1–1.5	> 50	> 50	4.5	4.5	20 000	5.9
XXXIV	D-Val → D-Ala; L-Val → L-Ala	> 50	2	> 50	> 50	1.5	4	10^5	6.9
XXXV	D-Val → D-Leu; L-Val → L-Leu	> 50	2	> 50	> 50	1–2	3–4	1.3 × 10^5	7.0
XXXVI	L-Lac → L-HyIv	> 50	> 50	> 50	> 50	> 50	> 50	4 × 10^5	7.7
XXXVII	3 L-Lac → 3 L-HyIv meso-HyIv–valinomycin	> 50	> 50	> 50	> 50	> 50	> 50	3.7 × 10^5	7.6
XXXVIII	D-HyIv → D-Lac	> 25	0.2	> 25	> 25	0.3	0.2	3 × 10^5	7.5
XXXIX	3 D-HyIv → 3 D-Lac meso-Lac–valinomycin	> 50	> 50	> 50	> 50	> 50	> 50	2.3 × 10^6	8.7
XL	3 L-Lac → 3 L-HyIv; 3 L-Val → 3 L-Ala	> 50	0.2–0.4	> 50	> 50	0.3	0.2–0.4	2.9 × 10^6	8.9

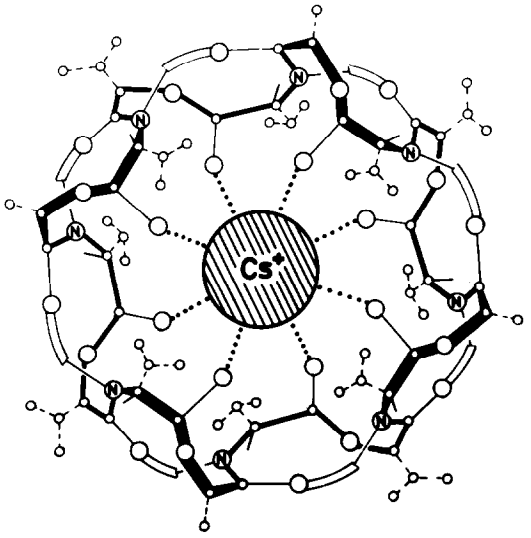


Fig. 7. Conformation of the Cs^+ complex of 'hexadecavalinomycin' (V).

prising four (instead of the valinomycin three) tetradepsipeptide fragments ('hexadecavalinomycin') are in the form of the hydrogen bonded bracelets with the depsipeptide chain folded much like that of the valinomycin complexes (judging from the CD, IR and NMR data) (fig. 7). The internal cavity in the complexing structure of V (diameter 4–5 Å) is too large for effective ion–dipole interaction not only with K^+ but even with Cs^+; although, of course, the latter makes a better fit, explaining the preferential Cs^+ complexation of 'hexadecavalinomycin' in solution (table 2).

Recently we have shown that despite the resultant steric strain 'octa-valinomycin' IV is also capable of complexing Na^+ and K^+ and that, in conformity with the size of the molecule, it manifests preference for Na^+. The K^+ complex of 'octa-valinomycin' is based on the same structural principles as the complex of the naturally occurring antibiotic (fig. 8a) whereas with Na^+, the amide as well as the ester carbonyls take part in the interaction. Under such circumstances further weakening of the hydrogen bonds with their already unfavorable geometry takes place in the Na^+ complex. Fig. 8b shows the conformation in which the cation is interacting only with the amide carbonyls. Most likely the sodium complex is in an equilibrium of structures represented on fig. 8a and 8b together

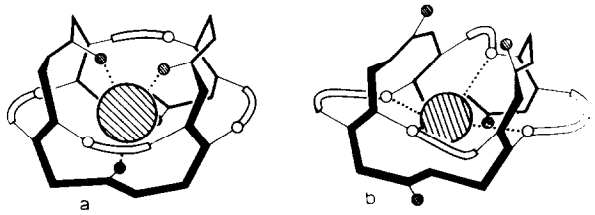


Fig. 8. Conformation of the K^+ complex of 'octa-valinomycin' IV (a) and the hypothetic conformation of its Na^+ complex with all amide groups interacting with the cation (b).

with intermediate conformers.

The diastereomers of valinomycin form less stable complexes and are less potent antibiotics than the parent compound. From an energy standpoint the change in the amino and hydroxy acid configurations in the valinomycin K^+ complex schematically shown in fig. 9 is to a first approximation equivalent to the appearance of new fragments with conformational maps with respect to ϕ 0°, ψ 0°. As one can see from fig. 10 such changes are in all cases accompanied by enhanced non-bonding interaction energies: the energy of the valine and lactic acid fragments is less affected (1.9–4 kcal/mole), than that of the hydroxyisovaleric acid fragments (by 10 kcal/mole) [13]. It thus becomes clear why there is a successive diminution of the stability constants with configurational change of one (compounds VI–IX, XV and XVIII), two (compounds XVI, XVII, XIX and XX) and more (compounds X–XIII) residues, and why the complex is more sensitive to such change in the hydroxy-

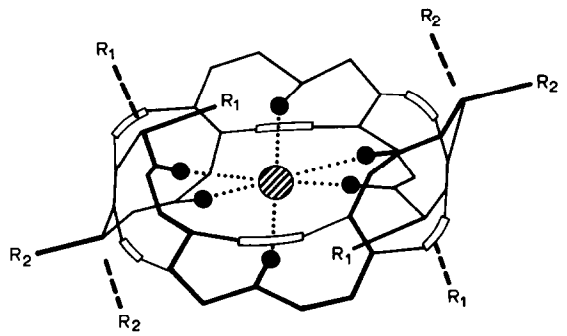


Fig. 9. Schematic representation of the change in configuration of the amino (R_1) and hydroxy acid (R_2) residues in valinomycin·K^+ complex (the dashed lines show the position of the isopropyl groups after the change in configuration).

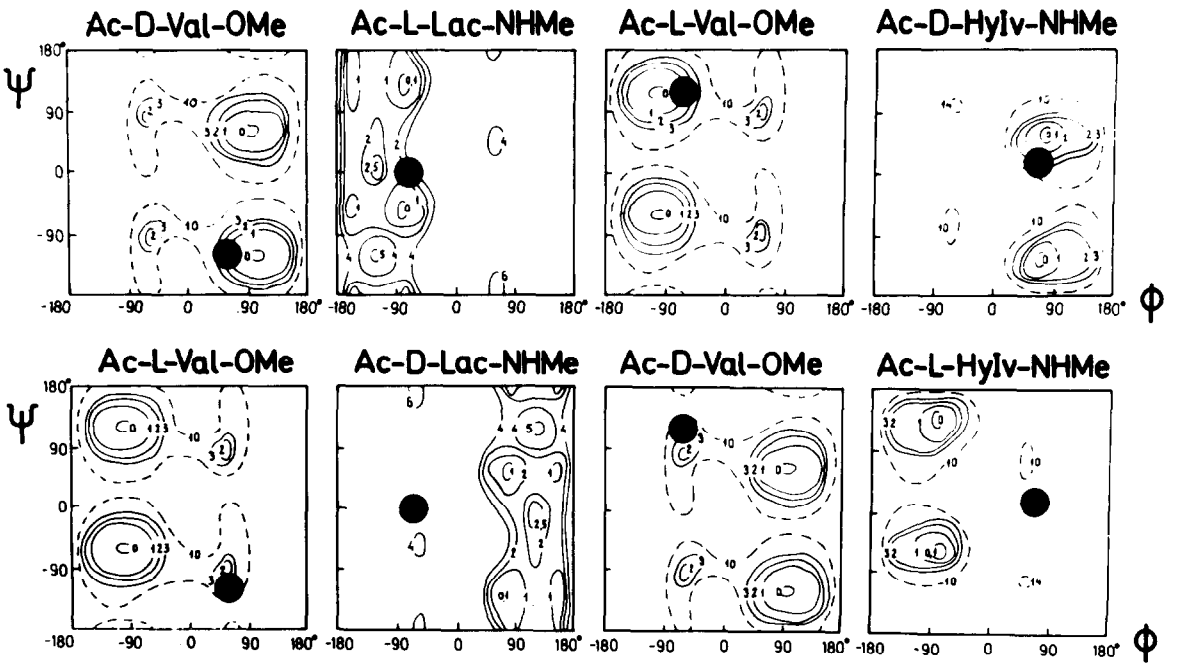


Fig. 10. Conformational maps of the enantiomeric amino and hydroxy fragments of valinomycin. Dark circles – coordinates of the respective fragments of the valinomycin · K^+ complex (upper row) and of its diastereomers (lower row).

isovaleryl moiety (compounds IX, XII and XVIII–XX).

Of considerable interest is the substitution of amide and ester groups in valinomycin by related groupings. On amide for ester substitution a hydrogen bond is lost, giving rise to a ca. 4 kcal · $mole^{-1}$ energy loss (table 4, fig. 11). It is just such an effect (decrease in $-\Delta F$ from 8.6 to 4.7 kcal · $mole^{-1}$) that is observed in compound XXIV. In this case, modification of the antibiotic did not affect the other five H-bonds and the arrangement of carbonyl oxygens about the cation. If, however, the amide group is substituted by an *N*-methyl amide group (fig. 11), not only is one of the hydrogen bonding protons lost, but the formation of neighbouring hydrogen bonds is sterically hindered, and the resultant analog (compound XXV) is even a weaker complexone, if at all (table 4). On the other hand, substitutions of the type L-Lac → L-Ala and D-Hylv → D-Val (compounds XXII, XXIII and XXVI) (table 4, fig. 12) retain the complexing properties; for the hydrogen bonding system is unaffected and the carbonyl groups are free to participate in the ion–dipole interaction. These analogs also show high antibacterial activity.

Analogs with modified aliphatic side chain struc-

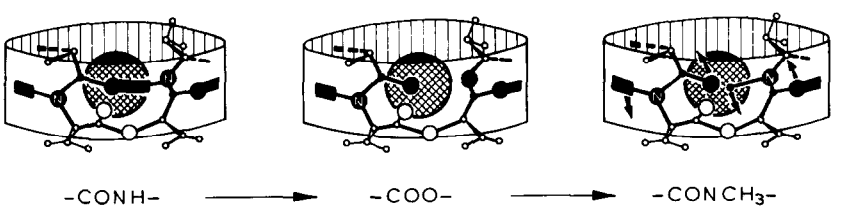


Fig. 11. Schematic representation of the replacement of amide groups by ester or *N*-methyl amide groups in the valinomycin · K^+ complex.

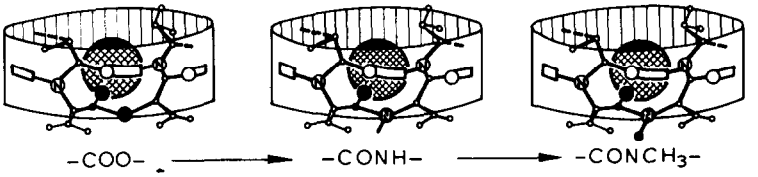


Fig. 12. Schematic representation of the exchange of ester by amide or *N*-methylamide groups in the valinomycin · K^+ complex (the substituted atoms are designated by dark circles).

tures (table 5) are as a rule good complexones. In cases where a decrease in the free energy of complexation is observed (e.g. compounds XXX and XXXIII) it is apparently due to enhanced conformational flexibility of the original depsipeptides rather than steric strain in the complex. In other words, here there is more probably a decrease in free energy on the left hand side of the equilibrium.

$$\text{macrocycle} + K^+ \rightleftarrows (\text{macrocycle} \cdot K)^+ \qquad (1)$$

than an increase on the right hand side.

However, despite the similarity in stability of the complexes of this series of compounds and those of valinomycin in ethanol, they can differ considerably from the latter in other parameters and, therefore, in their behavior in membrane systems. The best elucidated from this aspect is compound XL [1,2,14,15]. In nonpolar solvents and in the complexed state it is conformationally very similar to valinomycin. However in solvents of medium polarity it manifests no signs of forming structure B, to the peculiarities of which we ascribe the high surface activity of valinomycin. In conformity with this analog XL monolayers are less stable than those of valinomycin in the air–water interface and also display a lesser tendency to penetrate the lecithin monolayer.

A distinctive feature of the molecular structure of the K^+–valinomycin complex is the effective screening of the central cation from the solvent, this being partly due to hanging of the valine isopropyl groups over the openings of the 'cylinder' formed by the depsipeptide skeleton. A notably different behavior is exhibited by the K^+·XL complex: as can be seen in fig. 13, in the upper part of the complex it is the

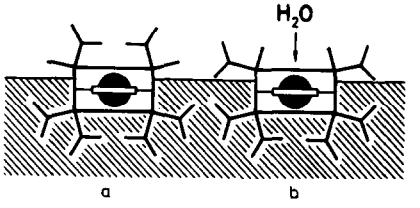


Fig. 13. Schematic drawing of the K^+ complexes of valinomycin (a) and its analog XL (b) on the membrane surface.

alanyl methyls instead of the valyl isopropyl groups which cover the cation, with a correspondingly lower screening effect. As a result, the centrally located ion can now engage in ion–dipole interaction also with the solvent. The energy of this interaction provides for the enhanced stability of the K^+ complex of analog XL, and for a tendency to enlarge the difference in free energies of complexation of valinomycin and analog XL with increasing solvent polarity or on the air–water interface (table 6).

Table 6
Stability constants (K, $1 \times \text{mole}^{-1}$) of the K^+ complexes of valinomycin (I) and its analogs XXVII and XL in different solvents at 25°C.

Compound	EtOH	42 mol% EtOH + 58 mol% H_2O	32 mol% EtOH + 68 mol% H_2O	H_2O (extrapolated values)	Air–water interface
I	2.0×10^6	500	100	0.25	1
XXVII	10^7	1100	3900	100	–
XL	2.6×10^6	1400	100	6	3

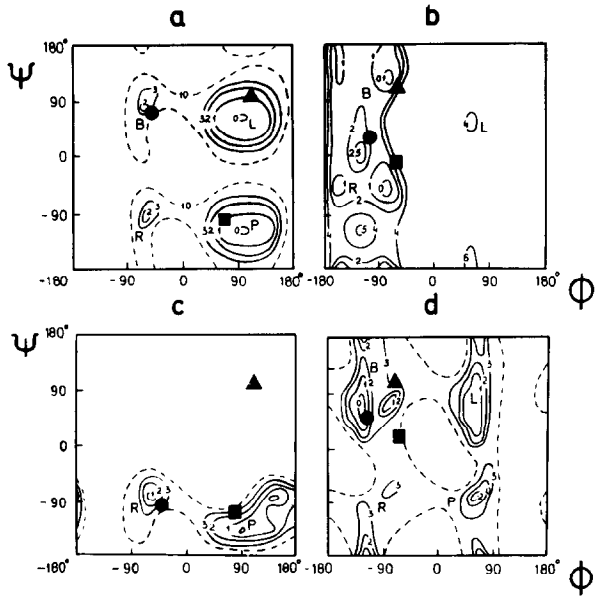


Fig. 14. Conformational maps of Ac–D-Val–OMe (a), Ac–L-Lac–NHMe (b), Ac–D-Ala–NMe_2 (c) and Ac–L-MeAla–NHMe (d). Dark circles indicate coordinates of the corresponding residues in the form A of valinomycin, triangles – in the form B, and squares – in the K^+ complex.

Analog XL behaves differently from valinomycin on bilayers in that under certain conditions the rate of its K^+ ion transport is limited by the flux of free cyclodepsipeptide molecules from within the membrane [1,2].

Thus our structural studies have shed considerable light on the molecular basis of the functioning of valinomycin cyclodepsipeptides in membranes and laid the grounds for the preparation of new, biologically active compounds with predetermined properties.

A striking illustration of the potentialities of this approach serves our results with a new valinomycin analog, compound XXVII in which L-*N*-methylalanine residues are substituted for the L-lactic acid residues. Analysis of the corresponding conformational maps of Ac–D-Ala–$N(CH_3)_2$† and Ac–L-MeAla–NHMe (fig. 14) [17] (instead of the valinomycinic Ac–D-Val–OMe and Ac–L-Lac–NHMe) showed that the ϕ and ψ coordinates of the K^+ complex of valinomycin are close to their allowed regions, i.e. the formation of an analog XXVII complex should not meet with serious steric obstacles. However, compound XXVII has strictly limited non-complexed conformational variability. For instance, the ϕ and ψ parameters of the D-valine residues in the propeller conformation lie in the sterically prohibited region (fig. 14c). From this it follows that the free energies for the right hand side of the system represented by Eq. (1) should not differ in the case of valinomycin and analog XXVII, whereas the free energies for the left hand side are higher for compound XXVII. On this basis we expected it to display a high complexing activity. In fact, as shown by conductiometric measurements the K^+ complex of compound XXVII has a 'record' stability constant, exceeding by at least 1–2 orders of magnitude that of any other cyclodepsipeptide of the valinomycin series (tables 2–6). Apparently the analog obtained by Gisin and Merrifield in which the hydroxy acids are replaced by L- and D-proline residues has similar properties [18].

The steric hindrances to introduction of an *N*-methyl group lead not only to enhanced stability of the K^+ complex, but also to lower rates of the exchange reaction (Eq. 2):

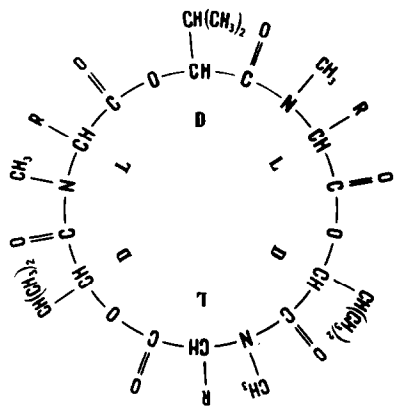
$$\text{macrocycle}^* + \text{macrocycle} \cdot K^+ \rightleftarrows \text{macrocycle}^* \cdot K^+ + \text{macrocycle} \quad (2)$$

As a result, in bilayer experiments, the rate of transition of the ion from the surface to the interior is lowered, making possible detection of this stage but this circumstance also lowers the ionophoric efficiency of analog XXVII. We have here a good illustration of why modification of the valinomycin structure in a way that would have accelerated all stages of the transmembrane ion transport is an exceptionally difficult and still unresolved problem.

Enniatins

Like valinomycin the enniatin antibiotics (fig. 15) are of high structural flexibility. Two basic forms are known for enniatin B (fig. 16), viz., form N,

† The conformational map of a more appropriate model, Ac-D-Val–$N(CH_3)_2$ has not been described in the literature. Experimental data [16] indicate a relatively lower stability for the conformation of this compound with ϕ –60°, ψ –60°; in all the rest, the conformational maps for Ac–D-Val–$N(CH_3)_2$ and Ac–D-Ala–$N(CH_3)_2$ are quite similar.



		R
I	Enniatin A	$-CH(CH_3)C_2H_5$
II	Enniatin B	$-CH(CH_3)_2$
III	Enniatin C	$-CH_2CH(CH_3)_2$
IV	Beauvericin	$-CH_2C_6H_5$

Fig. 15. Antibiotics of the enniatin group.

predominant in non-polar media and form P, found in polar media and in the crystalline state [23].

Both in solution and the crystalline state enniatins form equimolar complexes with alkali metal ions. These have been well elucidated [5,19,20,24–28]. The complex structures (fig. 17) resemble discs of which the cations occupy the centers; the external

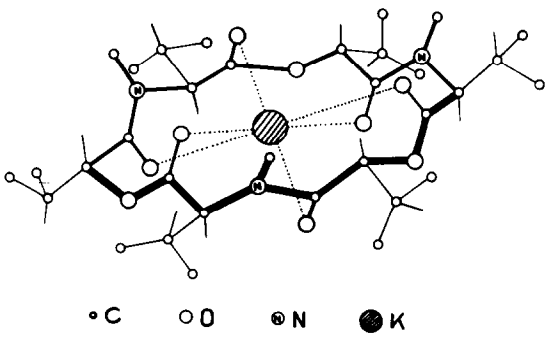
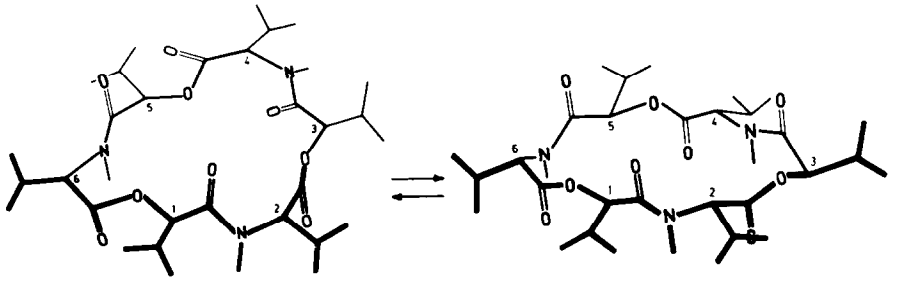


Fig. 17. Conformation of the (enniatin B) · K^+ complex.

boundaries are highly lipophilic. As in the case of the valinomycin complexes, the ion is held in the cavity by ion–dipole interaction with six carbonyl ligands. The conformational lability of the enniatins allows them to 'fit' the cavity to the complexing ion, leading to low complexing selectivity [5,24,25,27].

Alkali ion transport across membranes by the enniatin ionophores has been studied much less extensively than that of valinomycin [24,29–32,36]. It had been taken for granted by analogy with valinomycin that the transport is effected by 1:1 complexes. Our new results show that actually it is more complicated.

Analysis of the NMR spectra of enniatin B (initial concentration c_0) titrated by alkali salts showed that an increase in salt concentration (b_0) and formation



	1	2	3	4	5	6
Φ	89	-118	154	73	-58	-133
Ψ	-109	92	32	-55	-92	140

N

	1	2	3	4	5	6
Φ	74	-103	74	-103	74	-103
Ψ	-136	171	-136	171	-136	171

P

Fig. 16. The principal conformations of enniatin B.

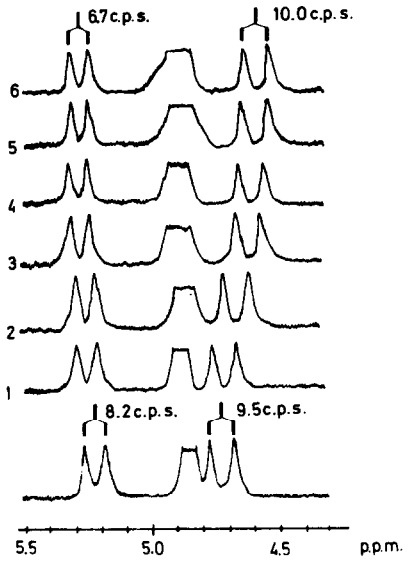


Fig. 18. The C H-NMR signals of enniatin B in CD_3OD ($c_0 = 4.44 \cdot 10^{-2}$ M) for various concentrations of LiCl (b_0). 1) $c_0/b_0 = 0.2$; 2) $c_0/b_0 = 0.66$; 3) $c_0/b_0 = 1.85$; 4) $c_0/b_0 = 3.65$; 5) $c_0/b_0 = 5.40$; 6) $c_0/b_0 = 7.16$.

of complexes are accompanied by changes in the chemical shifts ($\Delta\delta$) and the $^3J_{C^\alpha H - C^\beta H}$ spin–spin coupling constants to a limiting value corresponding to complete complexation (figs. 18–20). As one can see from figs. 19 and 20, the shapes of the curves†† depend upon the nature of the complex cation. In the case of Li^+ smooth $\Delta\delta$ vs. b_0/c_0 curves are obtained the slopes of which are less than for the curves computed for a 1:1 complex with an infinitely high stability constant (fig. 19). In contrast, when enniatin B interacts with K^+ the $\Delta\delta = f(b_0/c_0)$ curves are steeper than the predicted curve in all the solvents studied (fig. 20). A similar phenomenon was observed when enniatin C was titrated with KNCS in CD_3OD. This suggests that for the relatively high macrocycle concentration required in the NMR experiments, the equilibrium involves a significant amount of complexes with a higher than 1:1 macrocycle · M^+ ratio. Since in KNCS titration the experimental $\Delta\delta$ vs. b_0/c_0 plots closely approximate those computed for 2:1 complexes whereas none of them is steeper, we assume that it is the 2:1 complexes which are most probably formed. This is also supported by the non-monotonic change of the proton chemical shifts for enniatin B in CD_3OD and a 1:1 $CDCl_3$–CD_3CN mixture showing an extremum at approximately $b_0/c_0 = 0.5$ (fig. 20b and c). The curves reach a plateau at $b_0/c_0 \approx 3$ (fig. 20), which indicates that, with such excess salt, the 2:1 complex practically completely changes to the 1:1 complex. The different shape of the curves from that on fig. 20 is ascribed to the larger internal shifts between the corresponding proteins in the 2:1 and 1:1 complexes than in $(CD_3)_2SO$. Determination of the stability constant K_2 for the 2:1 complex on the basis of these data leads to values by 1–2 orders of magnitude below K_1 (i.e., ca. 10^2 in ethanol since the K_1 range from 10^3 to 10^4 [24]).

$$CDP + M^+ \rightleftarrows CDP \cdot M^+; K_1 = \frac{[CDP] \cdot [M^+]}{[CDP] \cdot [M^+]}$$

$$CDP + CDP \cdot M^+ \rightleftarrows (CDP)_2 \cdot M^+; K_2 = \frac{[(CDP)_2 \cdot M^+]}{[CDP]\,[CDP \cdot M^+]}$$

Comparison of the enniatin B titration curves in CD_3OD with salts of various metals (figs. 19 and 20) suggests that the stabilities of the 2:1 complexes decrease in the order $K^+ > Cs^+ > Na^+$, no 2:1 complexes being observed for Li^+. It should be noted that the initial slopes of the experimental curves for the CsNCS titration of enniatin B have values between those for the 2:1 and 1:1 complexes, i.e. bear evidence of the presence of 3:2 complexes. The formation of such complexes is in accord with the results of bilayer experiments (see below).

As may be inferred from the similarity of the

†† The presence of a single averaged signal for the free and complexed forms of the macrocyclic compound in the respective regions of the NMR spectra is indicative of a high ion exchange rate:
$CDP \cdot M^+ + CDP^* \rightleftarrows CDP + CDP^* \cdot M^+$ (CDP = cyclodepsipeptide) on the NMR time scale. A different spectral pattern, bearing evidence of slower exchange rates is observed for valinomycin [11,33] and antamanide [34].

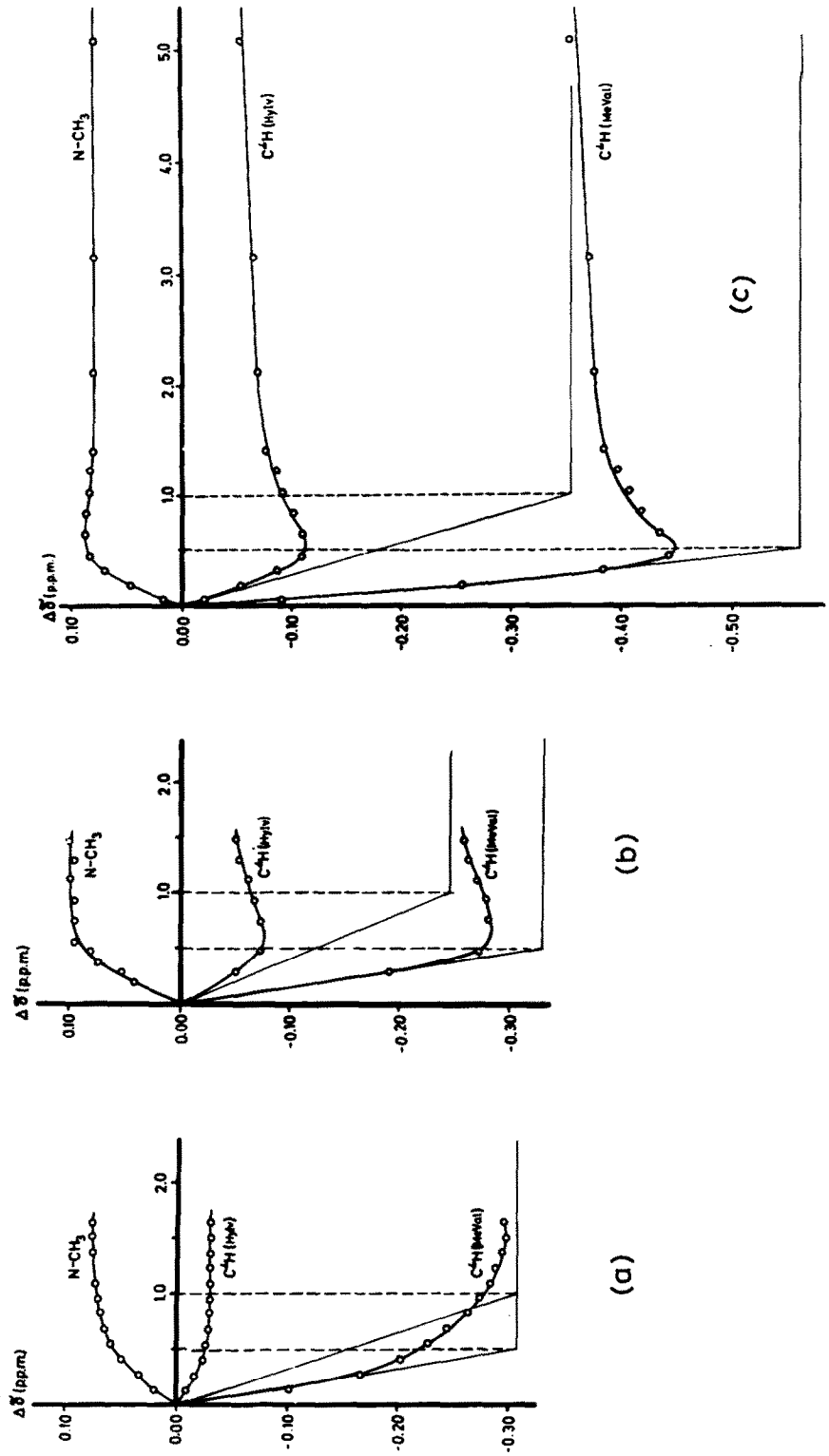


Fig. 19. Enniatin B titration curves in CD_3OD (a) with LiCl, $c_0 = 4.44 \cdot 10^{-2}$ M; (b) with NaNCS, $c_0 = 4.17 \cdot 10^{-2}$ M; (c) with CsNCS, $c_0 = 3.95 \cdot 10^{-2}$ M.

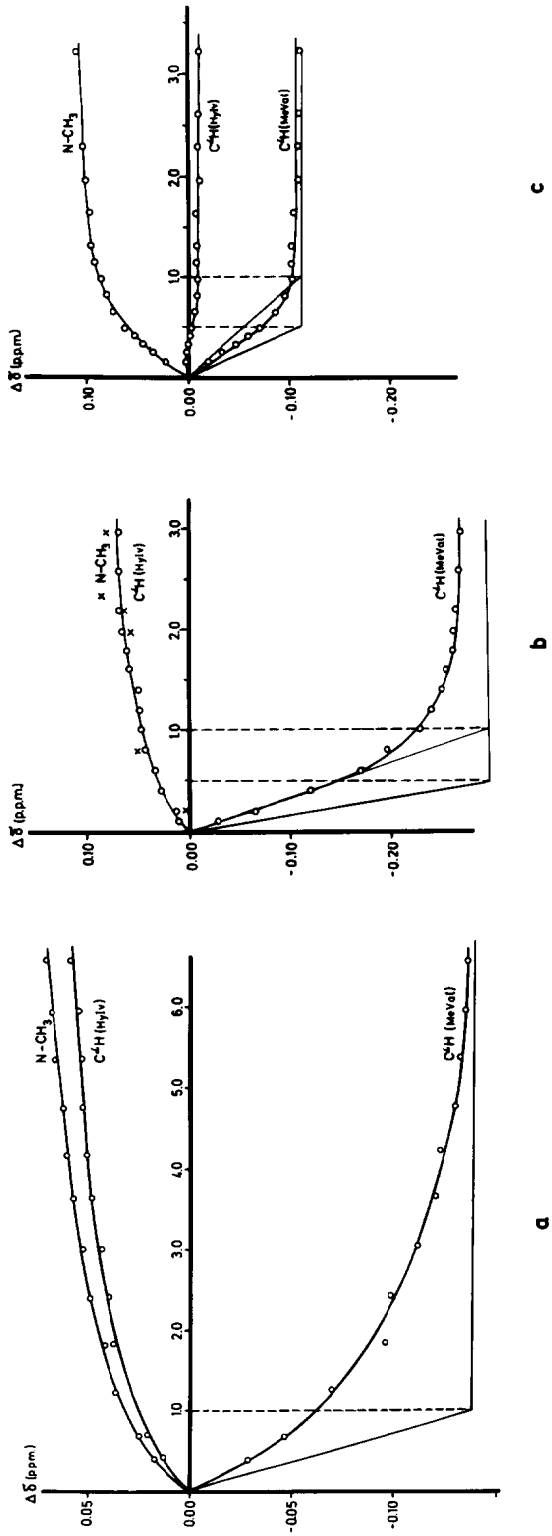


Fig. 20. Enniatin B titration curves with KNCS (a) $(CD_3)_2SO$, $c_o = 7.36 \cdot 10^{-2}$ M; (b) CD_3OD, $c_o = 3.54 \cdot 10^{-2}$ M; (c) CD_3CN: $CDCl_3$ (1:1), $c_o = 4.32 \cdot 10^{-2}$ M.

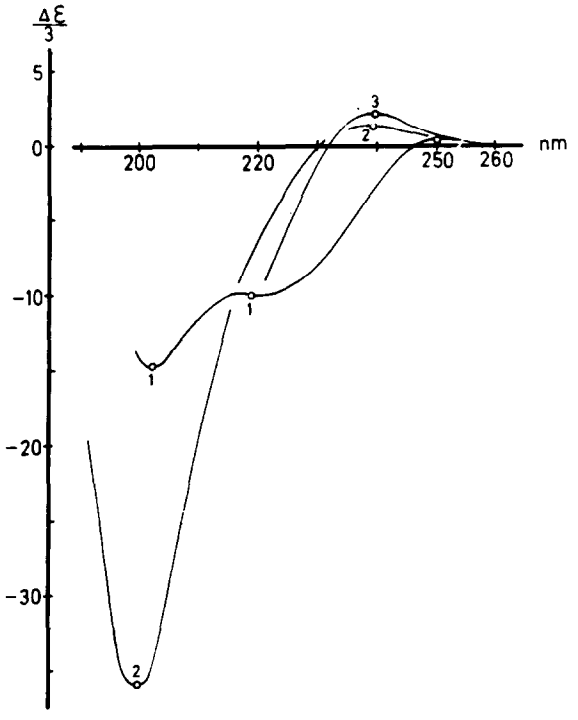


Fig. 21. CD curves in CH_3CN of (1) enniatin B, (2) 1:1 (enniatin B) : K^+ complex, $c_0 = 3.17 \cdot 10^{-4}$ M, $b_0/c_0 = 4.5$, and (3) 2:1 (enniatin B) : K^+ complex, $c_0 = 3.44 \cdot 10^{-2}$ M, $b_0/c_0 = 0.5$.

corresponding CD curves (fig. 21), the cyclodepsipeptide conformation in the 2:1 complexes is similar to that of the well-studied equimolecular complexes.

All the results suggest that the 2:1 complexes are of a sandwich structure with 'amide' (fig. 22a), 'ester' (fig. 22b) and 'mixed' (fig. 22c) types of coordination.

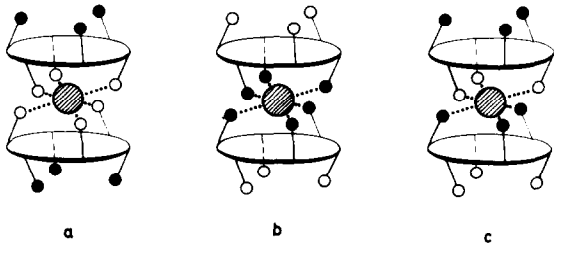


Fig. 22. Possible sandwich complexes for the enniatin antibiotics. •–amide oxygen; ○–ester carbonyl oxygen.

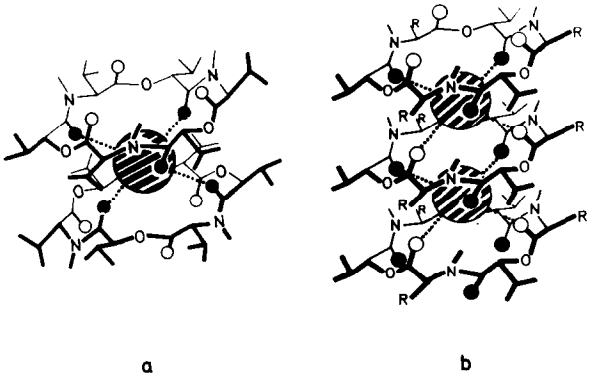


Fig. 23. Possible conformations of the 2:1 (a) and 3:2 (b) complexes.

The last two are the least probable because of spatial hindrance by the *N*-methyl groups. The 'amide' type of sandwich is shown in fig. 23a. In the 3:2 complexes the ligands are, apparently, both amide and ester carbonyl groups (fig. 23b).

Undoubtedly, in the sandwich complexes the cation is more effectively screened from interaction with the solvent and anion than in the equimolecular complexes, thus suggesting the possible participation of the former in the transmembrane ion transport despite their low stability constants. To test this possibility, we studied the effect of enniatin B and beauvericin on the ionic conductivity of bimolecular lipid membranes. The results obtained are shown in fig. 24.

First of all attention is drawn to the second power dependence of potassium conductivity on enniatin B concentration over a wide range of salt concentration (fig. 24a). This suggests that the event of potassium ion transfer across a membrane in fact involves two molecules of enniatin B. However, it was not certain whether the second power is not associated with other features of the transport mechanism. To clarify this matter we synthesized 'bis-enniatin B' – an analogue in which two depsipeptide rings are connected by a sufficiently long and flexible chain, that is, one which has the prerequisites for sandwich formation within a single ionophore molecule (fig. 25). If such an analog would display linear dependence of its concentration on the potassium conductivity of the membrane, the observed second power dependence for enniatin B could then in all probability be attributed to stoichio-

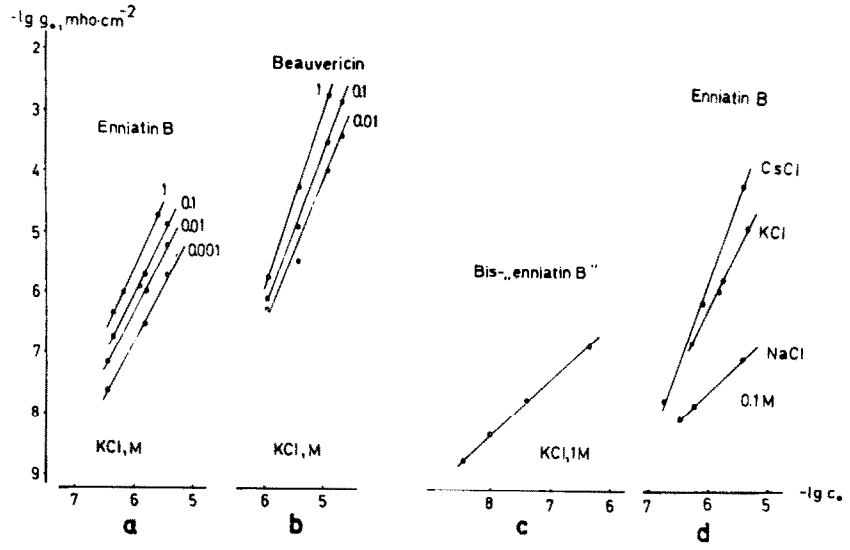


Fig. 24. Bilayer membrane conductivities (g_0) in the presence of enniatins.

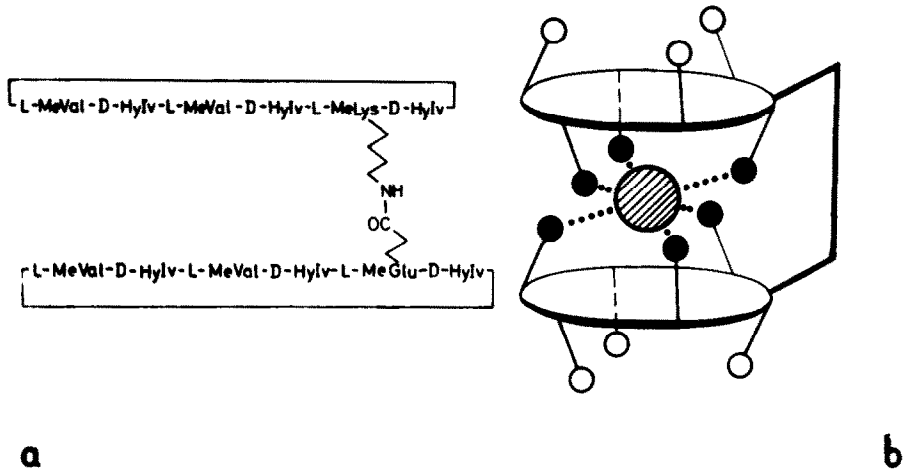


Fig. 25. 'bis-Enniatin B' (a) and the assumed structure of its equimolecular complex (b).

metric factors. Such a linear dependence has indeed been observed (fig. 24c) lending support to the proposal that sandwich complexes are a functional form of enniatin ionophores.

It should also be noted that the power of the c_0-dependence of the enniatin B-induced conductivity is lower for sodium salts and higher for cesium salts than for the potassium salts (fig. 24d). It seems that the main contribution to the Na^+ conductivity is provided by a mechanism involving equimolecular complexes, whereas for Cs^+ three antibiotic molecules are involved in the transport. On the basis of the titration results, it may be assumed that the Cs^+ complexes participating in the transport are of a 3:2 stoichiometry. The high power of the c_0-dependence for the beauvericin-induced conductivity (fig. 24b) also sug-

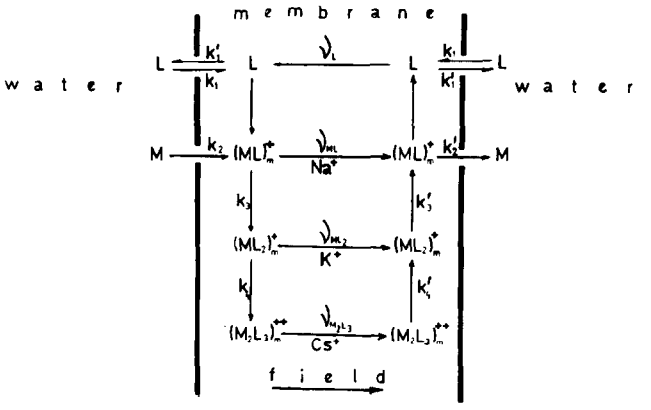


Fig. 26. Ion transport model through phospholipid bilayers in the presence of enniatin cyclodepsipeptides.

gests formation of 'club-sandwiches' by this antibiotic.

Analysis of the data obtained has led to the model of ionic conductivity induced by enniatins shown in fig. 26. It differs markedly from that for the action of valinomycin shown in fig. 2. As with valinomycin the enniatinic model involves heterogeneous binding of the ion by the ionophore molecule situated on the membrane. But then the resultant complex either migrates along the electric potential gradient or binds with free ionophore molecules to form double or triple 'sandwich' complexes capable of moving in the membrane under the influence of the electric field. It follows from this model that the membrane resistance can be represented as the sum of 'boundary' and 'bulk' resistances. The former depends upon the rate of the heterogeneous complexing reaction, the latter upon the concentration and mobilities of the equimolecular double and triple complexes within the membrane.

In this way, the enniatin antibiotics have the unique possibility of selecting from several parallel routes for transmembrane ion transport that which is optimal from the standpoint of ion and antibiotic concentrations and also lipophilicity, mobility and stability of the different complexes.

In the limiting case, when the 'sandwiches' are sufficiently large, one can visualize a channel mechanism of action of the enniatin antibiotics similar to that proposed for the antibiotic gramicidin A. Urry suggested that this linear pentadecapeptide forms a helical dimer (fig. 27a) which spans the membrane from one side to the other. A cation entrapped in the cavity (fig. 27b) can interact with the amide carbonyls twisted somewhat in the direction of the helical axis. In other words the cavity can play the part of a 'pore' in the functioning of gramicidin A in membranes. It is as yet difficult to evaluate the reality of Urry's hypothesis. Veatch and Blout, for instance, propose other structures for the gramicidin A dimers, built according to the double helix type and stabilized by means of numerous interchain H-bonds (private communication). However the idea of an ion conducting channel formed by a comparatively small peptide is very attractive.

The results of the study of peptide ionophores clearly show the advantages of the approach described here to the mechanism of transmembrane ion transport. In a comparatively short time the use of the antibiotic ionophores and their synthetic analogs has yielded valuable information on the functioning of fundamentally different transmembrane ion transport systems from carrier complexones to specific 'pores'. Such variegation notwithstanding, a single principle lies at the bottom of this phenomenon, namely, the ion binds to carbonyl ligands of the peptide (or protein) by ion–dipole interaction, thereby becoming enclosed in a comparatively rigid envelope, which lends the interaction its ion specificity. The lipophilic nature of the periphery of such a complex closing the ion provides the conditions for its ability to function within the membrane. It appears very logical that similar principles should form the basis

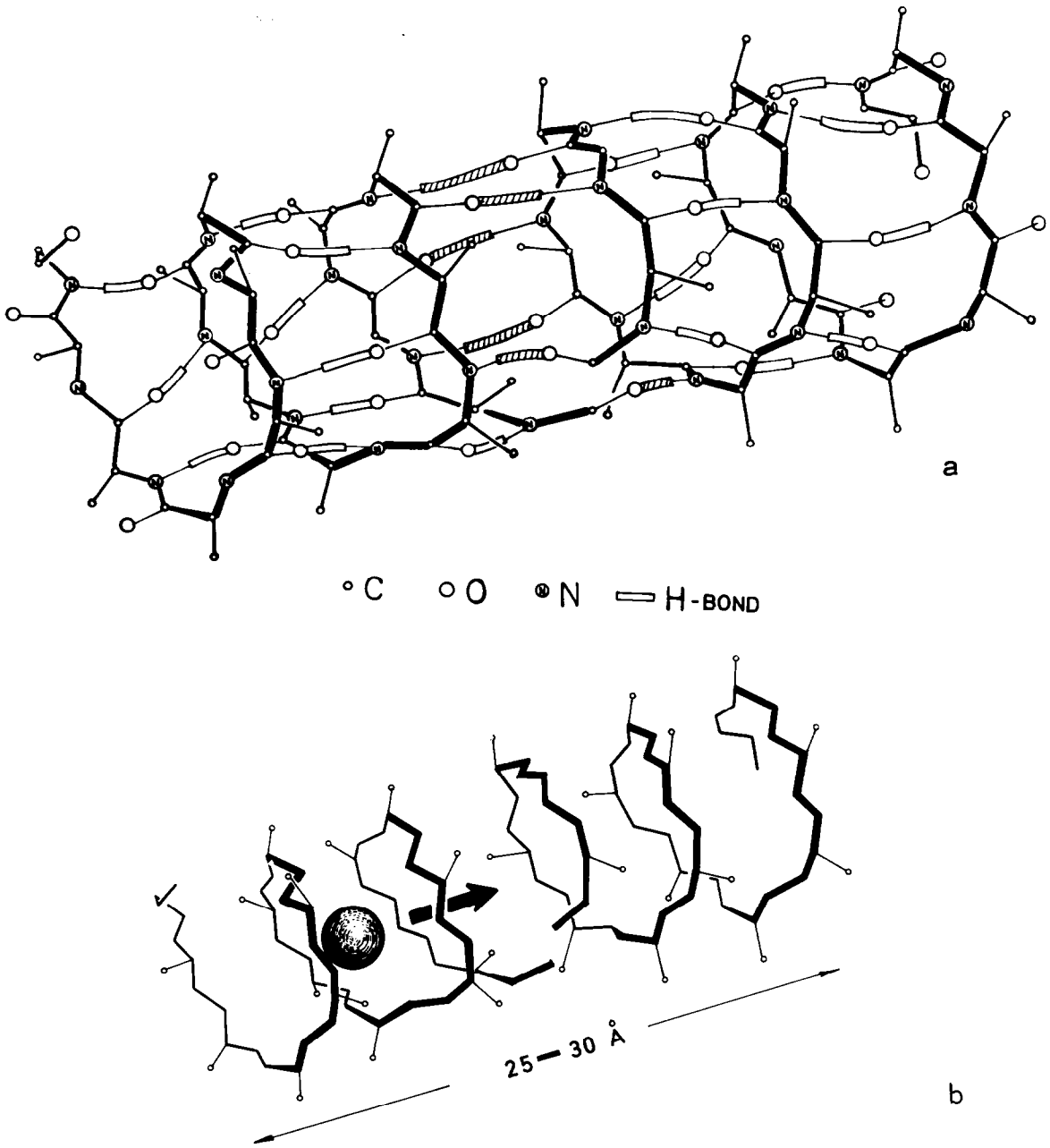


Fig. 27. Schematic representation of the π^6 $(L,D)^-$ helix of gramicidin A (a). In fig. b the cation is shown migrating along the 'pore'.

for ion transport across biological membranes, whether it be the ion-specific components of Na, K-dependent ATP-ase or the ion channels of neural membranes.

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