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Raman Spectroscopic Study of the Valinomycin–KSCN Complex

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This paper reports the first Raman spectroscopic study of the potassium complex of the cation-specific antibiotic valinomycin. Complete Raman spectra (140 to 3600 cm^{-1}) of crystalline valinomycin–KSCN and its CCl_4, $CHCl_3$ and C_2H_5OH solutions are presented and used to probe the structure of the complex in these environments. In all cases a single, narrow peak is observed in the *ester* C=O stretch region (1750 to 1775 cm^{-1}) which contrasts strongly with the broad bands observed in solutions of uncomplexed valinomycin. This is consistent with the presence of a single conformation in which all six ester C=O groups co-ordinate an enclosed potassium ion. We find that although the ester C=O stretch frequencies of the complex are similar in the solid state and in non-polar solution (~1770 cm^{-1}) they are considerably different in the presence of polar solvents (~1756 cm^{-1}); this may indicate that the complexed potassium ion is still free to interact with nearby solvent ions (and possibly its counterion) through gaps in the hydrophobic "shield" provided by the hydrocarbon residues of valinomycin. In contrast the *amide* C=O frequencies of the complex (~1650 cm^{-1}) are solvent-independent. These groups are apparently strongly hydrogen-bonded to provide a rather rigid, compact framework for the complex conformation.

1. Introduction

The macrocyclic depsipeptide valinomycin ($C_{54}N_6O_{18}H_{90}$) is a membrane-active antibiotic with remarkably selective ion-binding properties. Its ability to facilitate potassium ion transport across mitochondrial membranes (Pressman, 1965) and egg lecithin bilayers (Shemyakin *et al.*, 1969) suggests that a study of its complexation mechanisms may help elucidate selective ionic transport in other systems.

The valinomycin molecule consists of the sequence (L-valine, D-α-hydroxyisovaleric acid, D-valine, L-lactic acid) repeated three times (Shemyakin *et al.*, 1963). The structure of uncomplexed valinomycin crystals (monoclinic, space group $P2_1$) grown from warm *n*-octane has been determined by X-ray diffraction (Duax *et al.*, 1972). Unique to this conformation is the presence of intramolecularly hydrogen-bonded ester C=O groups (Fig. 1). Although recent Raman spectroscopic data indicate that this structure is maintained in valinomycin recrystallized from several other solvents (CCl_4, $CHCl_3$, $CH_3(CH_2)_2Cl$, CH_3CN), a different solid-state conformation is found to exist in valinomycin freshly recrystallized from *p*-dioxane or *o*-dichlorobenzene (Rothschild *et al.*, 1973; Asher, Rothschild, Anastassakis & Stanley, manuscript in preparation).

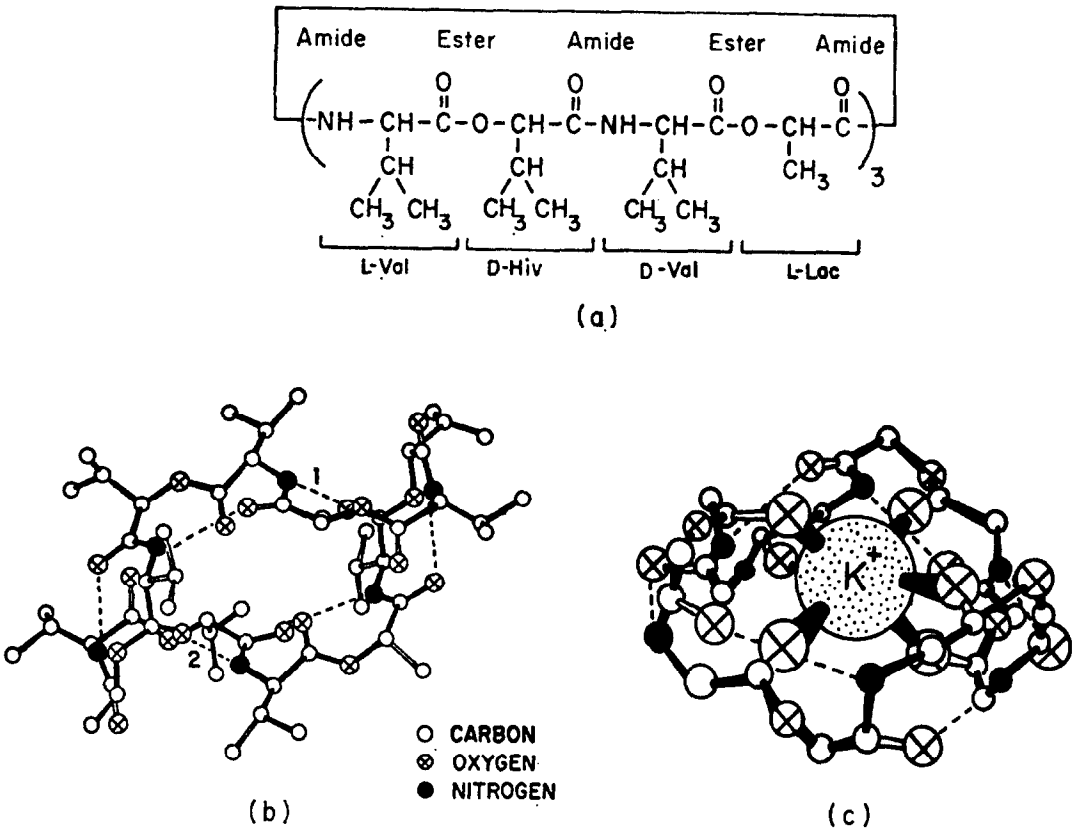

FIG. 1. (a) Primary sequence of valinomycin showing alternating amide and ester linkages. Hiv, α-hydroxyisovaleric acid; Lac, lactic acid.

(b) Structure of uncomplexed valinomycin crystallized from *n*-octane; from Duax *et al.* (1972). 1 and 2 indicate ester C=O . . . HN hydrogen bonding.

(c) Structure of the valinomycin–K^+ complex; from Pinkerton *et al.* (1969), in which hydrocarbon sidegroups have been omitted. All six amide C=O groups are H-bonded, and all six ester C=O groups co-ordinate the enclosed cation.

A variety of different valinomycin conformations exists in solution; their structure and relative concentrations at equilibrium depend on the polarity and hydrogen-bonding ability of the solvent. Nuclear magnetic resonance (Haynes *et al.*, 1969; Shemyakin *et al.*, 1969; Patel, 1973; Patel & Tonelli, 1973; Grell & Funck, 1973), infrared absorption spectroscopy (Shemyakin *et al.*, 1969; Grell & Funck, 1973) and more recently, laser Raman spectroscopy (Rothschild, Asher, Anastassakis & Stanley, manuscript in preparation) have been used to investigate the details of these conformations. In the last case this mixture of conformations is responsible for the appearance of broad bands in the amide (=1650 cm^{-1}) and ester (=1750 cm^{-1}) C=O stretch region.

The structure of the valinomycin–potassium ion complex was determined by Pinkerton *et al.* (1969) using X-ray crystallographic methods. The crystals were grown from a 1:1 mixture of chloroform and *m*-xylene which was shaken with an aqueous solution of potassium aurichloride ($KAuCl_4$). Such crystals are yellow, triclinic (space group P_1) with one molecule of complex per unit cell. Complex

formation depended critically on the counterion used. The potassium ion was found to be held in the internal cavity of the valinomycin molecule by co-ordination to all six ester C=O groups (Fig. 1(c)); the K^+---O distance was found to be ~2·7 Å. The valinomycin backbone forms three complete sine waves as it circles around the enclosed ion; this framework is held comparatively rigid by intramolecular H-bonds formed by all six amide C=O groups (H. . . O distances ~1·9 Å). The X-ray structure of the crystalline complex suggests that valinomycin carries potassium ions across the hydrophobic region of membranes by surrounding them in a "cage" whose outer surface (containing 12 aliphatic residues) is considerably less hydrophilic than the bare ion.

This conformation of the complex has been inferred to exist in chloroform and methanol solutions by nuclear magnetic resonance (Haynes *et al.*, 1969; Shemyakin *et al.*, 1969; Ohnishi & Urry, 1970; Patel, 1973; Patel & Tonelli, 1973) and infrared absorption (Shemyakin *et al.*, 1969; Grell & Funck, 1973) techniques. For example, the infrared spectrum of valinomycin in chloroform displays amide and ester C=O stretch frequencies at 1661 cm^{-1} and 1755 cm^{-1}, respectively; these shift to 1657 cm^{-1} and 1739 cm^{-1}, on adding n-$C_{11}H_{23}OSO_3K$ (Shemyakin *et al.*, 1969). This downward shift and the concomitant narrowing of the ester C=O peak is taken as evidence for the uniform bonding of all six ester C=O groups to an enclosed potassium ion.

This paper reports the first Raman spectroscopic study of the valinomycin–potassium complex. Unlike the references previously cited, it includes observations of the complex in the solid state and in non-polar (CCl_4) solution. The former provides a more direct comparison with the X-ray crystallographic structure of Pinkerton *et al.* (1969); the latter allows one to observe the effects of solvent polarity on the conformation of the complex.

2. Materials and Methods

Uncomplexed valinomycin was obtained commercially from Calbiochem (San Diego, Calif.) and used without further purification. Valinomycin–potassium complex was prepared by adding excesses of uncomplexed valinomycin and KSCN (or KCl) to ethanol and extracting the supernatant. Additional powdered valinomycin–KSCN complex was obtained from Professor V. T. Ivanov and his colleagues at the Shemyakin Institute for the Chemistry of Natural Products, Moscow, prepared as in Ivanov *et al.* (1973). No spectral differences were observed between samples prepared by these two methods.

Raman spectra were taken of the powdered complex and its solutions in CCl_4, $CHCl_3$, and C_2H_5OH using a SPEX Ramalog 4 system in conjunction with a 4 W argon ion laser (Spectra-Physics Model 124). This system will be described in detail elsewhere (Asher, Rothschild, Anastassakis & Stanley, manuscript in preparation). All samples were held in 1-mm inner diameter glass capillary tubes mounted perpendicular to the scattering plane. The effects of sample fluorescence were minimized by using the violet 4579 Å argon line and moderately slow scanning speeds (3 to 12 cm^{-1}/min). Spectral resolutions of 3 to 5 cm^{-1} were sufficient for our purposes. Optical rotatory dispersion spectra of the valinomycin–KSCN complex in ethanol solution were taken with a Carey model 60 spectropolarimeter.

3. Results

Our results are displayed in Figures 2 and 4 and Tables 1 and 2 (the mode assignments are based on Asher *et al.*, manuscript in preparation). In section (a) to (c) below we will confine our attention to the 1600 to 1800 cm^{-1} region, although complex forma-

tion-induced changes are observed throughout the valinomycin spectrum (section (d)). The stretch vibrations of the amide (~1650 cm^{-1}) and ester (~1750 cm^{-1}) C=O groups have been shown to be particularly sensitive to valinomycin conformation in previous Raman spectroscopic studies (Rothschild *et al.*, 1973).

The Raman spectrum of uncomplexed valinomycin powder recrystallized from *n*-octane (Fig. 2(a)), CCl_4 or $CHCl_3$ contains four distinct peaks in the 1600 to 1800 cm^{-1} region, indicating the presence of both "free" and intramolecularly hydrogen-bonded amide C=O groups (1675 cm^{-1} and 1649 cm^{-1}, respectively) and both

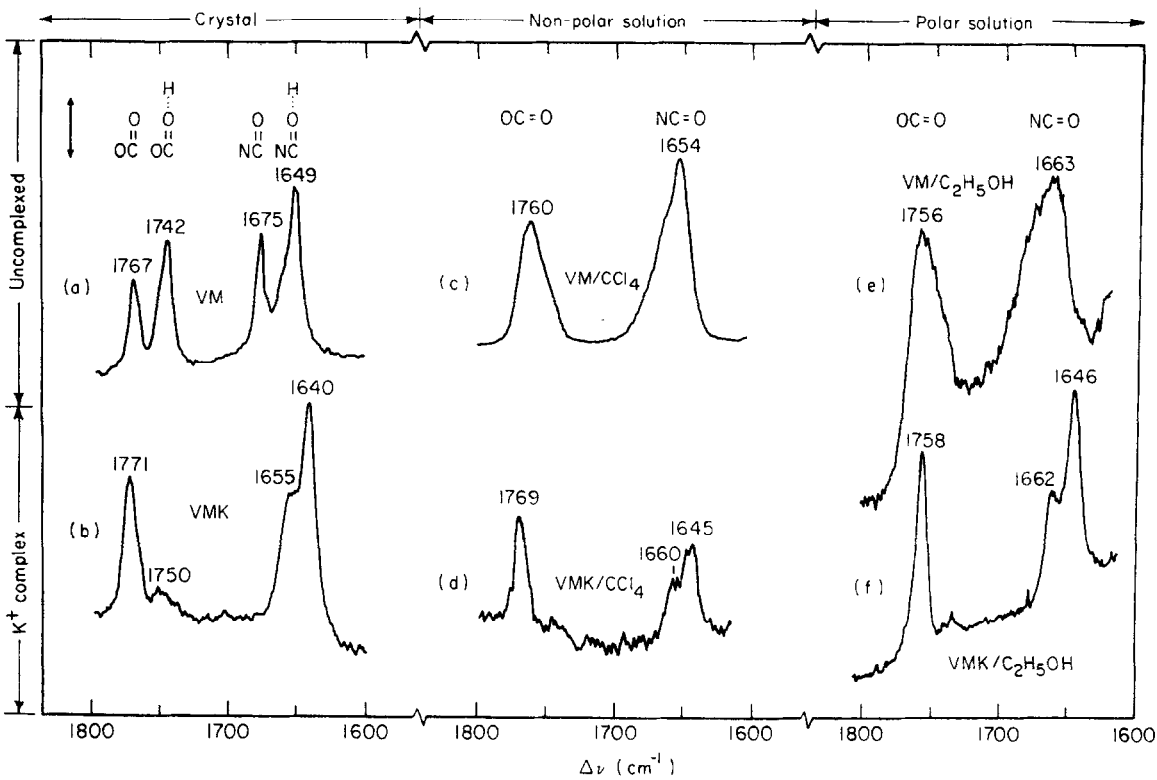

FIG. 2. Raman spectra of the C=O stretch vibrations (1600 to 1800 cm^{-1}) of (a) crystalline uncomplexed valinomycin (VM); (b) crystalline valinomycin–KSCN complex (VMK); (c) uncomplexed valinomycin in CCl_4 solution; (d) valinomycin–KSCN complex in CCl_4 solution; (e) uncomplexed valinomycin in C_2H_5OH solution; (f) valinomycin–KSCN complex in C_2H_5OH solution. Spectral resolution: (a) and (c) 3 cm^{-1}; (b) 4 cm^{-1}; (d) to (f) 5 cm^{-1}. Laser excitation: (a), (b), (e) and (f) 4579 Å; (d) 4727 Å; (c) 4880 Å. Scanning speed: (b) 6 cm^{-1}/min; (c) 12 cm^{-1}/min; (d), (e) and (f) 30 cm^{-1}/min; (a) 60 cm^{-1}/min. The vertical arrow corresponds to: (b) and (d) 100 cts/s; (a), (c), (e) and (f) 300 cts/s. Incident power levels: (a), (c), (e) and (f) 100 mW; (b) 90 mW; (d) 60 mW.

"free" and intramolecularly H-bonded ester C=O groups (1767 cm^{-1} and 1742 cm^{-1}, respectively). This interpretation is consistent with the X-ray structure described by Duax *et al.* (1972) (Fig. 1(b)). We have recently shown (Rothschild *et al.*, 1973; Asher *et al.*, manuscript in preparation) that Raman spectra of uncomplexed valinomycin freshly recrystallized from *o*-dichlorobenzene or *p*-dioxane

contain only the 1767 cm^{-1} ester C=O frequency, which implies a structure having no H-bonded ester C=O groups. This second conformation seems to resemble the predominant form of valinomycin in polar solvents as proposed by Ivanov *et al.* (1969) and Patel & Tonelli (1973). This should make an X-ray determination of its structure more biologically relevant than that of valinomycin crystallized from *n*-octane.

(a) *Solid state*

The Raman spectrum (1600 to 1800 cm^{-1}) of powdered valinomycin–KSCN complex (Fig. 2(b)) is strikingly different from that of uncomplexed valinomycin (Fig. 2(a)). The amide C=O stretch vibrations appear as a close doublet at 1640 and 1655 cm^{-1}. The latter peak is similar in frequency to the 1657 cm^{-1} shoulder observed in uncomplexed valinomycin powder, and the 1654 cm^{-1} band observed in CCl_4 and $CHCl_3$ solutions (Table 1), but the 1640 cm^{-1} frequency is 9 cm^{-1} lower than the lowest amide C=O stretch vibration of uncomplexed valinomycin.

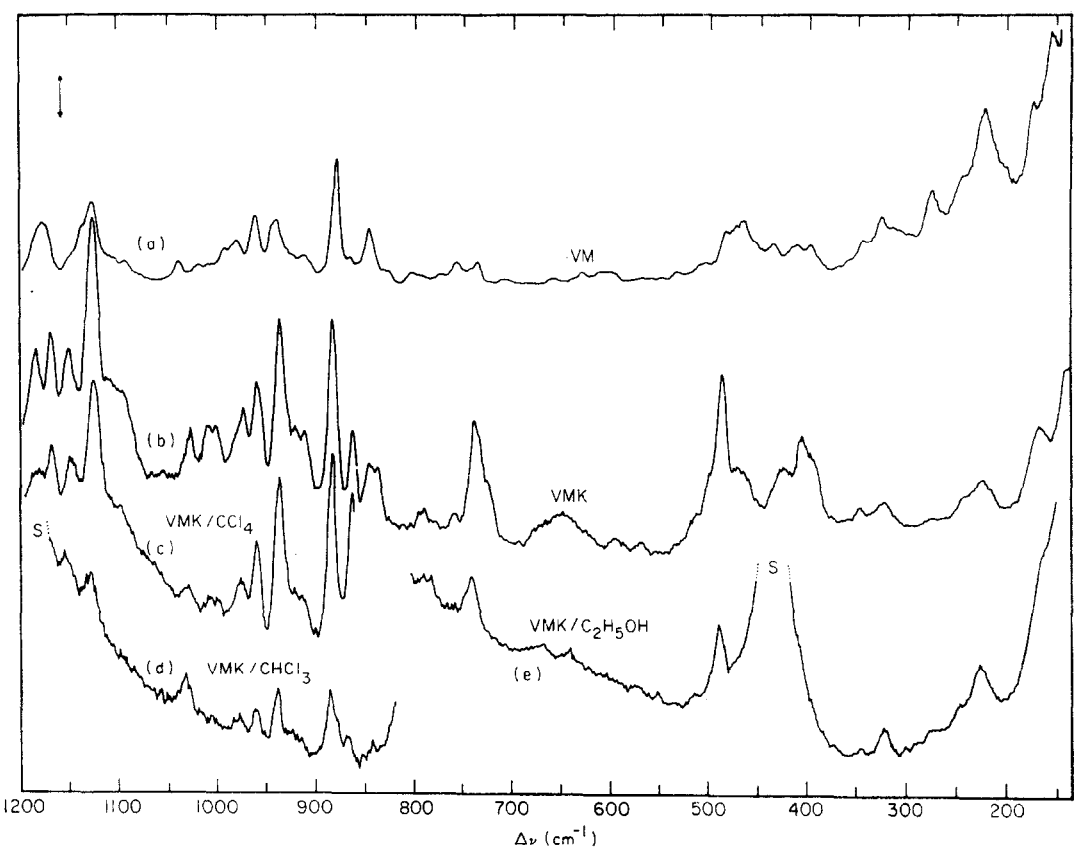

FIG. 3. Raman spectra (150 to 1200 cm^{-1}) of (a) crystalline uncomplexed valinomycin–KSCN complex (VMK); and solutions of the valinomycin–KSCN complex in (c) CCl_4, (d) $CHCl_3$ and (e) C_2H_5OH. Spectral resolution: 5 cm^{-1} (except (a) 3 cm^{-1}). Laser excitation: 4579 Å (except (c) 4727 Å). Scanning speed: (a) 60 cm^{-1}/min; (b), (d) and (e) 30 cm^{-1}/min; (c) 6 cm^{-1}/min. The vertical arrow corresponds to: 300 cts/s (except (c) 100 cts/s; and (b) 150 to 380 cm^{-1} 1000 cts/s). Incident power levels (a) 100 mW; (b) to (d) 60 mW; (e) 50 mW. S denotes solvent peaks. VM, valinomycin.

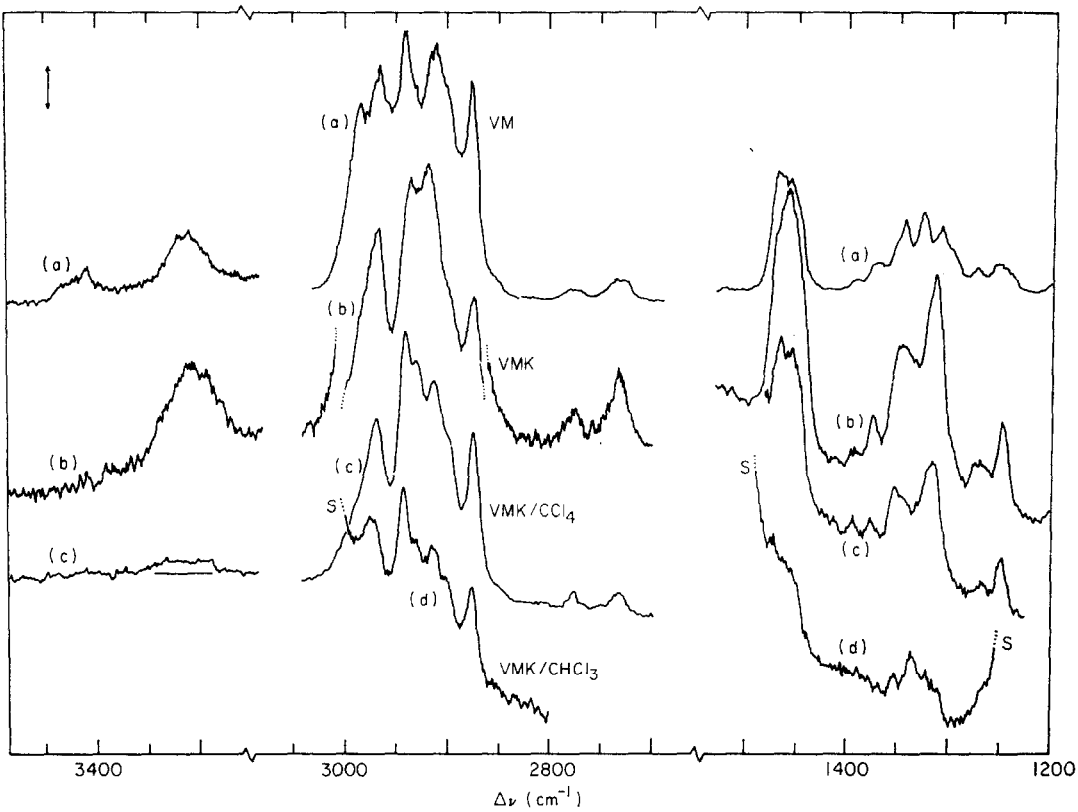

FIG. 4. Raman spectra (1200 to 1400 cm^{-1}, 2700 to 3100 cm^{-1}, 3250 to 3480 cm^{-1}) of (a) crystalline uncomplexed valinomycin (VM); (b) crystalline valinomycin–KSCN complex (VMK); and solutions of the valinomycin–KSCN complex in (c) CCl_4, (d) $CHCl_3$. Conditions as in Fig. 3, except in (b) where: the scanning speed is 6 cm^{-1}/min (1200 to 1500 cm^{-1} and 2700 to 2960 cm^{-1}), 12 cm^{-1}/min (2860 to 3000 cm^{-1}), 30 cm^{-1} (3250 to 3480 cm^{-1}); the spectral resolution is 5 cm^{-1}, except 2 cm^{-1} (2700 to 2860 cm^{-1}); the incident power is 90 mW, except 60 mW (3250 to 3480 cm^{-1}), and the vertical line represents 300 cts/min, except 100 cts/s (2700 to 2860 cm^{-1}). S denotes solvent peaks.

The ester C=O stretch vibration of powdered valinomycin–KSCN appears as a prominent single peak at 1771 cm^{-1}, which is slightly above the free ester C=O frequency of uncomplexed valinomycin (1767 cm^{-1}). There is a small peak near 1750 cm^{-1} which may represent a minority conformation (a similar small peak appears as a shoulder at 1747 cm^{-1} in uncomplexed valinomycin).

(b) *Non-polar solvents*

Both valinomycin and its KSCN complex dissolve readily in CCl_4. The amide and ester C=O vibrations of uncomplexed valinomycin in CCl_4 solution appear as asymmetric broad (~25 cm^{-1}) bands in solution (Fig. 2(c)). This indicates that these C=O groups now exist in a variety of different local environments with varying degrees of exposure to (and interactions with) the solvent (Rothschild *et al.*, manuscript in preparation). In fact independent evidence exists to show that a mixture of several conformations is present (Shemyakin *et al.*, 1969; Patel & Tonelli, 1973).

TABLE 1

C=O stretch frequencies (cm^{-1}) of valinomycin and its K^+ complex

(a) Uncomplexed valinomycin

Raman: Powder		Raman: Solution			Infrared: Solution		
Octane[a]	Dioxane[b]	CCl_4	$CHCl_3$	C_2H_5OH	$CHCl_3$[c]	CH_3OH[d]	Assignment
1649	1650						Amide
(1657)sh		1654B	1655B				C=O
	1663	(1665)sh		1663B	1661		Stretch
1675						1676	
1742							Ester
(1747)sh							C=O
	(1757)sh			1756B	1755	1752	Stretch
		1760B	1760B				
1767	1767						

(b) Valinomycin–KSCN

Methanol[e]	Octane[f]	CCl_4	$CHCl_3$	C_2H_5OH	$CHCl_3$[c]	CH_3OH[d]	Assignment
1640							Amide
	1644	1645	1646B	1646			C=O
1655					1657	1658	Stretch
	(~1660)	1660sh	1664	1662sh			
		(1741)			1739		Ester
(1750)						1745	C=O
			1755				Stretch
				1758			
1771		1769					
	1775						

(c) Complex formation shifts [(b) — (a)]

Powder[g]	Octane[f,h]	CCl_4	$CHCl_3$	C_2H_5OH	$CHCl_3$	CH_3OH	Assignment
−9	−5	−9	−9	−17	−4	−18	Amide
−20	−15	−(5)					C=O
							Stretch
							Ester
+4	+8	+9	−5	+2	−16	−7	C=O
							Stretch

[a] Valinomycin powder crystallized from warm *n*-octane (Calbiochem).
[b] Valinomycin powder recrystallized from *p*-dioxane.
[c] From Ivanov *et al.* (1969) and Shemyakin *et al.* (1969).
[d] From Grell & Funck (1973).
[e] Grown from methanol (or ethanol) solution.
[f] Valinomycin–KSCN powder (e) at the bottom of a capillary tube filled with *n*-octane (valinomycin is not readily dissolved in octane at room temperature).
[g] Powder (e) — powder (a).
[h] Powder (f) — powder (a).

Abbreviations used: sh, shoulder; B, broad; ( ) frequency uncertain.

TABLE 2

Raman spectra (140 to 3600 cm^{-1}) of valinomycin and its KSCN complex

VM[a]	VMK[b]	VM/CCl_4[c]	VMK/CCl_4[d]	VMK/$CHCl_3$[e]	VMK/C_2H_5OH[f]	Assignments
		(130)sh				
(145)	(143)					
158	169*	(160)		165		
(201)						
223	224			(220)sh	225	Skeletal deformation region
246	244				242sh	
274	274				(275)sh	
(298–318)sh						
326	323			322	322	
346	348				347	
398	(397)sh	(392)B				
412	407*	412		428sh	(410)sh	
435	426B*			428		C—CH_3 rock (in plane)
(454)						
466	sh					
474	(470)sh					
484	488			490	491	
(509)	(502)sh					
	(518)sh				(516)sh	
531		530sh				
	(570)				574	
597	(597)	(595)sh			598	Amide VI
610						
631	(627)	635B			(640)B	
	651B*					
		(677)			(670)	
	(728)sh					
736	740				745	Amide V
757	761				(760)	

	794*				792	
804						
821B	839*			840		
847	848	843		849		CH_3 rock
867	864	866	865	865		
880	885*	879	886	886		
912	913	915	(915)	913		C—CH_3 stretch
	923*		(924)	(923)		
940	939	938	939	940	940	CH_3 rock (symmetric)
945						
962	961	960	961	960	962	
981	976	987	(978)	(978)	979	
993	1003*					
1020	1011*	(1010)	(1010)	(1008)		
1039	1029*	1038	(1032)	1030E		C—$(CH_3)_2$ stretch (symmetric)
1096	1098	1096		(1105)sh		
1127	1128	1128	1129	1128		C—$(CH_3)_2$ stretch (antisymmetric)
1140sh		1142				
(1150)	1152		1151	1152	1152sh	CH_3 rock (antisymmetric)
	1170*		1171	1170	1170	
1178		1178				
	1186*		1186		1193	COC vibration
1252	1247*	1247	1247			Amide III
	(1265)sh		(1266)	(1265)sh		
1271	1272	1268				CH vibrations
1307	1313*	1313	1315B	(1312)	1313	
1325		(1329)sh		1332E		
1344	1346	(1334)sh	(1343)sh			
		1351	1352B	1350	1353	CH vibrations
1372	1373	1369	(1376)	(1375)		CH_3 bend (symmetric)
(1391)	1393	1391	(1393)	(1393)		
		1450	1448	1449E		
1454	1457B	1455		1457		CH_3 bend (antisymmetric)
(1461)		1463	1463	1462		
1467						

TABLE 2—*continued*

VM[a]	VMK[b]	VM/CCl_4[c]	VMK/CCl_4[d]	VMK/$CHCl_3$[e]	VMK/C_2H_5OH[f]	Assignments
1640–1770			See Table 1			C=O stretch
	[2060[g]]					
2723						
2731	2730	2730	2731			
2774	2775	2773	2777			
2875	2873	2875	2875			C—$(CH_3)_2$ vibrations
			2896sh			
2913	2918*	2913	2913	2913		
		(2930)sh	2929			
2938	2934	2940	2941	2943		CH_3 stretch (symmetric)
2966	2969	2969	2969	(2975)E		
2984						
3312	3306B	(3306)B	(3290–3354)			NH stretch (bonded)
3406						
(3425)						

[a] Uncomplexed valinomycin powder grown from warm *n*-octane. From Asher *et al.*, manuscript in preparation.
[b] Valinomycin–KSCN complex grown from methanol or ethanol.
[c] Uncomplexed valinomycin (a) in CCl_4 solution. From Rothschild *et al.*, manuscript in preparation.
[d] Valinomycin–KSCN complex (b) in CCl_4 solution
[e] Valinomycin–KSCN complex (b) in chloroform solution.
[f] Valinomycin–KSCN complex (b) in ethanol solution.
[g] The SCN^- peaks appearing in 2000 to 2150 cm^{-1} region are all small for this sample, except for this exceptionally intense SCN^- vibration which appears as tall as the 1455 cm^{-1} peak of valinomycin. Lower frequency modes of KSCN occur near 509, 515, 775, 985, 999, 1490 cm^{-1} in the solid state, and 471, 748, 945 cm^{-1} in aqueous solution; none seem to be observed here.

Abbreviations used: VM, valinomycin; B, broad; sh, shoulder; sl, slant; ( ) frequency uncertain; * frequency shifted ≥ 5 cm^{-1} from the corresponding uncomplexed value, or appearing only in the complex; E, possibly extraneous.

Raman spectra of CCl_4 solutions of the valinomycin–KSCN complex (Fig. 2(d)) show a dramatic narrowing of the ester C=O peak which has about the same width ($\sim$10 cm^{-1}) and frequency (1769 cm^{-1}) as in the solid state. This narrowing, an expected result of complex formation, is not observable in solid-state samples (Fig. 2(b)) since the C=O peaks of uncomplexed valinomycin powder are already quite narrow, i.e. the sample is already "locked" into a single, well-characterized conformation. The frequencies of the amide C=O bands (1645 cm^{-1}, 1660 cm^{-1}) fall between those observed in the crystalline complex (1640 cm^{-1}, 1655 cm^{-1}) and those observed in CCl_4 solutions of uncomplexed valinomycin (1654 cm^{-1}, 1665 cm^{-1}).

(c) *Polar solvents*

Complex formation-induced narrowing of the ester C=O band of valinomycin is also observed in the polar solvents $CHCl_3$ and C_2H_5OH (Fig. 2(f)). In $CHCl_3$ (Table 1) the ester C=O stretch frequency of the complex (1755 cm^{-1}) is $\sim$5 cm^{-1} below that of uncomplexed valinomycin in the same solvent (1760 cm^{-1}). Although the Raman spectra of uncomplexed valinomycin in CCl_4 and $CHCl_3$ solutions appear almost identical in this region, the ester C=O stretch frequency of the complex is $\sim$14 cm^{-1} lower in $CHCl_3$.

The ester C=O frequencies of both complexed and uncomplexed valinomycin in ethanol are near 1757 cm^{-1}, and both are below the corresponding peaks in ClC_4 solutions of valinomycin resulted in spectra similar to those shown in Figure 2(f) solvent-independent.

Several additional procedures were carried out to verify the existence of the valinomycin–K^+ complex in C_2H_5OH. Samples obtained by two different methods (see Materials and Methods) were found to yield identical Raman spectra. Optical rotatory dispersion measurements confirmed the presence of a single maximum near 220 nm, characteristic of the complex conformation (Shemyakin *et al.*, 1969), and the absence of the "polar" (200 nm) and "non-polar" (240 nm) conformations observed in C_2H_5OH solutions of uncomplexed valinomycin. The addition of KCl to C_2H_5OH solutions of valinomycin resulted in spectra similar to those shown in Figure 2(f) (valinomycin–KSCN complex), whereas the addition of NaCl produced no changes from those in Figure 2(e) (uncomplexed valinomycin in C_2H_5OH).

(d) *Raman spectrum outside the 1600 to 1800 cm^{-1} region*

Comparisons of the complete Raman spectra (140 to 3600 cm^{-1}) of valinomycin and its KSCN complex (Figs 3 and 4; Table 2) reveal several differences in addition to those occurring in the 1600 to 1800 cm^{-1} region. Several peaks (488, 864, 939, 1152 cm^{-1}) are greatly increased in relative intensity on complex formation. Some peaks appear only in the complex (651, 794, 923 cm^{-1}); others (1325, 2984 cm^{-1}) occur only in the uncomplexed form. The 1178 cm^{-1} single peak of uncomplexed valinomycin (COC stretch) splits into a 1170, 1186 cm^{-1} doublet in the complex; and the 1252 cm^{-1} amide III vibrations shift to 1247 cm^{-1}. Other complex formation-induced frequency shifts involve the 407, 839, 885 and 1003 cm^{-1} vibrations.

It is significant that major changes occur in the 1300 to 1350 cm^{-1} and 2900 to 2950 cm^{-1} spectral regions upon complex formation. The peaks of these regions represent, primarily, bending and stretching modes involving the hydrocarbon side groups of valinomycin. There are further changes in the frequency and relative

intensity of these modes on dissolving the valinomycin–KSCN complex in CCl_4 or $CHCl_3$. Frequencies in the 1000 to 1050 cm^{-1} region (valyl symmetric stretch) also shift on complex formation; although the 1127 cm^{-1} vibration (valyl antisymmetric stretch) does not.

In the solid state, the amide III vibration near 1250 cm^{-1} increases somewhat in relative intensity upon complexation. There may be additional amide III contributions to the CH band near 1313 cm^{-1}.

4. Discussion

Since the biological significance of valinomycin hinges upon its ability to shield cations (K^+, Rb^+, Cs^+) from the non-polar interior of lipid membranes, an investigation of the valinomycin–K^+ complex in non-polar solvents is particularly important. The Raman spectra of valinomycin–KSCN in the solid state and in CCl_4 solution (Figs 2 to 4) are found to be similar except for changes in the amide C=O stretch (1600 to 1700 cm^{-1}) and valyl stretch (2850 to 3000 cm^{-1}) regions. The former may reflect the breaking of weak intermolecular hydrogen bonds (or the weakening of ionic bonds to the counterion) in solution. The latter may reflect the steric effects of the close packing of the complex (and its counterion) in the solid state. Thus the X-ray structure of the crystalline complex (Pinkerton *et al.*, 1969) would appear to be relevant to non-polar solutions as well. In contrast, Raman spectra of the complex in polar solvents display appreciable frequency shifts in the ester C=O stretch region (1740 to 1780 cm^{-1}) which apparently reflect solvent interactions in the vicinity of the cation co-ordinated by these groups.

In the following sections we discuss our results in the amide (section (a)) and ester (section (b)) C=O stretch regions and in the remainder of the Raman spectrum (section (c)). The significance of the observed solvent-dependence of the ester C=O stretch frequency is elaborated upon in section (d). We also compare our results with those obtained by other methods.

(a) *The amide C=O region (1600 to 1700 cm^{-1})*

The amide C=O stretch region of the valinomycin–KSCN complex displays close doublets (Fig. 2(b), (d) and (f)) with frequencies below those of uncomplexed valinomycin (1649, 1675 cm^{-1}). The absence of the 1675 cm^{-1} peak indicates that now *all* amide C=O groups are H-bonded. The down-shift in the lower peak may represent an increase in the strength of the intramolecular H-bonding of some amide C=O groups beyond that of the "strong" H-bonds of uncomplexed valinomycin. The small-angle X-ray diffraction measurements of Krigbaum *et al.* (1972) show that the radius of gyration of valinomycin molecules in C_2H_5OH solutions drops from ~5·0 Å to ~3·9 Å on adding KSCN. However, X-ray diffraction measurements of the N. . . O distances of complexed and uncomplexed valinomycin crystals vary only between ~2·8 and 3·0 Å in both cases (Pinkerton *et al.*, 1969; Duax personal communication). Our attribution of the complex formation-induced downshift in the former amide C=O stretch frequencies to increased hydrogen bonding is supported by proton nuclear magnetic resonance studies of the NH groups of valinomycin. The valine temperature coefficient shifts from $\sim 75 \times 10^{-4}$ p.p.m./deg. C in dioxane/water solution (in which valinomycin lacks strong hydrogen bonds) to $\sim 30 \times 10^{-4}$ p.p.m./deg. C in dioxane/octane solution (in which all NH groups are strongly hydrogen

bonded; Patel & Tonelli 1973). Significantly, these coefficients are still lower $\sim 19 \times 10^{-4}$ p.p.m./deg. C in the valinomycin–KSCN complex in CH_3OH (Ohnishi & Urry 1970).

The appearance of a close, but well resolved, amide C=O doublet in the Raman spectrum of the complex (Fig. 2), suggests that hydrogen bonds to these groups may be of two discrete, slightly different strengths. This conclusion is supported by recent ^{13}C nuclear magnetic resonance spectra of valinomycin in CD_3OD (Patel, 1973), which show two amide C=O resonances with chemical shifts of 20·64 and 21·76 p.p.m. On complex formation with KSCN these shift, respectively, 1·3 and 0·9 p.p.m. further downfield. (The other two resonances of this region shift 3·6 and 5·1 p.p.m. downfield on complex formation, and were therefore assigned to ester C=O groups.) The observed shifts led Bystrov *et al.* (1972) to suggest that the amide C=O groups may also be interacting directly (albeit weakly) with the K^+ ion.

We find the amide C=O frequencies of the valinomycin–KSCN complex to be virtually independent of solvent polarity (Fig. 2(d) and (f); Table 1). This may be attributed to the strong hydrogen-bonding of these groups, and their shielding by the hydrocarbon side-groups of valinomycin. The appearance of this region in Raman spectra of uncomplexed valinomycin (Fig. 2(c) and (e)) is solvent-dependent due to shifts in the equilibrium concentrations of several, simultaneously-present conformers (Ivanov *et al.*, 1969; Patel & Tonelli, 1973). The amide C=O frequencies of the valinomycin–KSCN complex are ~ 5 cm^{-1} lower in the solid state (Fig. 2(b)) than in solution. This may reflect additional bonding of these groups to adjacent valinomycin molecules or SCN^- anions in the crystal lattice.

(b) *The ester C=O region (1700 to 1800 cm^{-1})*

Raman spectra of the valinomycin–KSCN complex in both the solid state and in *non-polar* (CCl_4) solution display a single, narrow, intense ester C=O peak near 1770 cm^{-1}. This suggests: (i) that all six ester C=O groups are similarly bonded; (ii) that these groups are "locked" into position and shielded from the non-polar solvent (which explains the dramatic narrowing of this peak, compared to that of uncomplexed valinomycin, in CCl_4); (iii) that the structure of this (presumably inner) portion of the complex is the same in both the solid state and in non-polar solution. These observations are consistent with the X-ray crystallographic structure of the valinomycin–$KAuCl_4$ complex (Fig. 1(c)) determined by Pinkerton *et al.* (1969) in which all six ester C=O groups co-ordinate the enclosed cation. No parallel studies (nuclear magnetic resonance, infrared absorption) of valinomycin complexes in the solid state, or in simple (non-hydrogen-bonding) non-polar solutions, are available.

It is not surprising that this frequency (1771 cm^{-1} in solid state, 1769 cm^{-1} in CCl_4) is higher than both the "free" (1767 cm^{-1}) and hydrogen-bonded (1742 cm^{-1}) ester C=O stretch frequencies of crystalline uncomplexed valinomycin (or the 1760 cm^{-1} frequency in CCl_4 solution). The co-ordination of the K^+ ion modifies the electron density distribution in the vicinity of the C=O bond, shifting it further toward the oxygen atom; this changes the effective carbon–oxygen force constant (the second spatial derivative of the local potential energy along the bond). Thus the relative effects of hydrogen-bonding and K^+ co-ordination on the C=O stretch frequency cannot be predicted *a priori*, except by combining a normal mode analysis (opposing C=O groups are coupled *via* the cation) with a local solution of the Schrödinger

equation. Such effects are usually investigated empirically, e.g. by comparing a series of model compounds. For example, it was found that hydrogen bonding consistently lowered the frequency of the amide C=O stretch vibration (Richards & Thompson, 1947; Koenig, 1972). This is an additional reason for attributing the narrowing of ester C=O peaks *unaccompanied* by a frequency shift (Fig. 2(e) and (f)) to some process other than hydrogen bonding.

Narrowing of the ester C=O stretch band in the moderately polar solvents, $CHCl_3$ and C_2H_5OH (Fig. 2(f)), verifies the existence of valinomycin–KSCN complex formation in these solvents as well; however, the ester C=O frequency of the complex (1755 cm^{-1} in $CHCl_3$, 1758 cm^{-1} in C_2H_5OH) is $\sim$12 cm^{-1} lower than its value in CCl_4 solution (Fig. 2(d)). In contrast, the corresponding spectra of uncomplexed valinomycin in CCl_4 and $CHCl_3$ solutions are similar (Table 1); both display broad bands with peaks near 1760 cm^{-1}. This sensitivity of the ester C=O frequency to the solvent is striking since:

(i) the X-ray crystallographic structure of the complex (Fig. 1(c)) has all six ester C=O groups substantially shielded by surrounding parts of the molecule (Pinkerton *et al.*, 1969);

(ii) in any case, the ester C=O groups should be less available in the complex (in which they co-ordinate the K^+ ion) than in the rather open structures of uncomplexed valinomycin solution (in which the ester C=O groups are "free"; Shemyakin *et al.* (1969); Patel & Tonelli (1973));

(iii) the *amide* C=O frequencies of the complex are virtually identical in CCl_4, $CHCl_3$ and C_2H_5OH (Fig. 2(d) and (f); Table 1).

It is interesting to compare these Raman results with infrared absorption measurements of the valinomycin–$KC_{11}H_{23}OSO_3$ complex in $CHCl_3$ (Shemyakin *et al.*, 1969) and the valinomycin–KCl complex in CH_3OH (Grell & Funck, 1973). The infrared and Raman C=O frequencies differ numerically in all cases (Table 1); this is not surprising since Raman-active vibrations are often infrared-inactive (or *vice versa*) depending on the symmetry and selection rules which characterize the vibration. Complex formation in $CHCl_3$ shifts the infrared stretch frequencies of both the amide and ester C=O groups downward (4 cm^{-1}, 16 cm^{-1}, respectively); the corresponding Raman displacements are also downward but different in magnitude (9 cm^{-1}, 5 cm^{-1}, respectively). Complex formation in CH_3OH (or C_2H_5OH) shifts both the infrared and Raman amide C=O frequencies markedly downward ($\sim$17 cm^{-1}); but the corresponding ester C=O frequencies are shifted 7 cm^{-1} downward in the infrared, and 2 cm^{-1} upward in the Raman.

Despite differences in the Raman and infrared frequencies (arising from differences in modes observed and perhaps the choice of counterions as well), these infrared measurements support our conclusions that:

(i) the amide C=O frequencies of the complex are about the same in $CHCl_3$ and C_2H_5OH (or CH_3OH);

(ii) the ester C=O frequency of the complex is significantly lower in $CHCl_3$ than in C_2H_5OH (or CH_3OH);

(iii) the downward shift of the amide C=O frequency on complex formation is largest in C_2H_5OH (CH_3OH), while that of the ester C=O frequency is largest in $CHCl_3$.

Parallel infrared studies of valinomycin complexes in non-polar solvents like CCl_4 might be helpful in verifying some of our other conclusions.

Although valinomycin and its KSCN complex are not readily soluble in octane at room temperature, Raman spectra of valinomycin–KSCN powder at the bottom of a capillary tube filled with *n*-octane yield a still higher ester C=O stretch frequency (1775 cm^{-1}) that is ~6 cm^{-1} above its value in CCl_4, and ~20 cm^{-1} above its value in $CHCl_3$ (Table 1). Again the amide C=O stretch frequencies are basically unchanged.

(c) *Raman spectrum outside the 1600 to 1800 cm^{-1} region*

Observations in other regions of the Raman spectrum of valinomycin (Table 2, Figs 3 and 4) support the existence of widespread conformational changes in the valinomycin backbone on complex formation (Fig. 1(b) and (c)). In particular, the splitting of the 1178 cm^{-1} peak into a sharp doublet (1170, 1186 cm^{-1}) reflects changes in the COC stretch mode induced by the reorientation of the neighboring ester C=O groups in the complex. The latter groups also adjoin the valyl residues of the L and D-valine subunits, which may account for the major changes which occur in the 1300 to 1350 cm^{-1} and 2900 to 2950 cm^{-1} regions on complex formation.

These observations are supported by studies using other techniques. Ivanov *et al.* (1971) report shifts in the 1184 cm^{-1} COC infrared absorption frequency of uncomplexed valinomycin in CCl_4/CH_3CN (2 : 1 v/v) solution from 1194 to 1197 cm^{-1} on the addition of K^+, Rb^+, Cs^+ or even Na^+. Widespread changes in the valinomycin backbone on KSCN complex formation are observed in proton nuclear magnetic resonance spectra in $CDCl_3$ (Haynes *et al.*, 1969) and CD_3OD and CH_3CN (Ivanov *et al.*, 1969). ^{13}C nuclear magnetic resonance studies of valinomycin–KSCN complex formation in $CDCl_3/CD_3OD$ (1:1 v/v) (Bystrov *et al.*, 1972) and CH_3OH (Patel, 1973) indicate ~3 p.p.m. shifts for the $C_{(\alpha)}$ carbons of the valine subunits, but ~1 p.p.m. shifts for the $C_{(\alpha)}$ carbons of lactic and hydroxyisovaleric acid (which are further from the K^+-binding ester C=O groups). Similarly ~1·3 p.p.m. shifts are observed for the $C_{(\beta)}$ carbons of valine but not for hydroxyisovaleric acid.

Finally, Ivanov *et al.* (1973) find far infrared evidence for a direct $K^+ \ldots O$ stretching mode at 171 cm^{-1} in the valinomycin–K^+ complex. Although we observe a mode of similar frequency (169 cm^{-1}) in Raman spectra of the complex (Fig. 3), this may represent a frequency shift in the 158 cm^{-1} mode of uncomplexed valinomycin rather than the appearance of a new stretching mode.

(d) *Further discussion of results in the ester C=O region*

The sensitivity of the ester C=O stretch frequency of the valinomycin–K^+ complex to the polarity of the solvent is of great biological interest. It indicates that the enclosed ion and its surrounding ester C=O groups still interact with nearby solvent molecules, suggesting that this interaction may be involved in the release mechanism for K^+ ions carried to the water interface on the far side of a lipid bilayer; it indicates further that the X-ray structure described by Pinkerton *et al.* (1969) may not reflect in detail the inner structure of the complex in polar solution.

The valinomycin ↔ valinomycin–KSCN exchange rate, as measured in proton nuclear magnetic resonance experiments, is also solvent dependent, e.g. it is $<0{\cdot}2\ s^{-1}$ in pure $CDCl_3$, but $\sim 2{\cdot}0\ s^{-1}$ in $CH_3OH/CDCl_3$ (4 : 1) (Haynes *et al.*, 1969); a K^+ turnover rate of $\sim 200\ s^{-1}$ has been reported in mitochondrial preparations (Pressman *et al.*, 1967).

The role of the counterion in complex formation must also be considered. Pinkerton *et al.* (1969) found complex formation with K^+ to be anion-dependent, with picrate$^-$ and $AuCl_4^-$ giving the best results; their X-ray crystallographic measurements show that the $AuCl_4^-$ anion rests in a nearly spherical cavity formed by adjacent valinomycin–K^+ complex molecules. They conclude that valinomycin selectively transports K^+/picrate$^-$ *pairs* across a $CHCl_3$ barrier from the fact that adding KCl to one side of the barrier effectively increases the migration of both K^+ and picrate$^-$ (but not Cl^-) ions. The ability of valinomycin to solubilize K^+ in decane is also anion-dependent (Tosteson, 1972). Picrate analogs are superior to even such lipid-soluble anions as hexanoate; the trinitrocresolate anion was found to be the most potent (although it dramatically reduces the *selectivity* of valinomycin as well). The SCN^- anion has been found to be closely associated with the co-ordinated Rb^+ ion in X-ray crystallographic studies of the RbSCN–dibenzo-18-crown-6 polyether complex (Bright & Truter, 1970). The SCN^- ion is inserted from above the ring in this ion-pair complex. A similar role is played by the highly polar solvent CH_3CN in the Li^+ antamanide complex (Karle *et al.*, 1973; Karle, 1974*a*,*b*).

These results suggest that the negatively charged $AuCl_4^-$ ion may remain associated with the positively charged valinomycin–K^+ complex even in $CHCl_3$ solution. If the same is true—perhaps in a lesser degree—of the valinomycin–KSCN complex: (i) the observed shift of the ester C=O peak in polar solvents may reflect the increased dissociation of the anion in polar solvents; (ii) the ester C=O groups might be particularly affected since they co-ordinate the positively charged cation, which could interact strongly with a nearby negative ion; (iii) the amide C=O groups (which circle the ring equatorially) would be relatively unaffected by changes in the anion proximity, especially since the density of peripheral hydrocarbon groups is particularly high in that region. It should be noted, however, that recent ^{13}C-nuclear magnetic resonance studies suggest that valinomycin forms no stable association with ClO_4^- counterions in CD_3OD solution (Fedarko, 1973).

Finally, none of our data directly contradicts the (unlikely) possibility that a totally different form of complex exists in $CHCl_3$ and C_2H_5OH solution in which the *amide* C=O groups are solvent-protected and the *ester* C=O groups are solvent-exposed. Similarly, the assignment of the most KSCN-sensitive ^{13}C nuclear magnetic resonance carbonyl resonances of valinomycin in CD_3OD to ester C=O groups was based on the *a priori* assumption that those groups co-ordinate the cation (Patel, 1973).

The situation in other hydrogen-bonding solvents is even more complex. Patel & Tonelli (1973) observe a new, weakly H-bonded, complex conformation in dimethylformamide; recently an unusual mixture of valinomycin conformations has been observed in Raman spectra of dioxane solutions in contact with a KCl-saturated deuterium oxide phase (Rothschild *et al.*, manuscript in preparation). These results indicate that a true understanding of the complex formation and transport mechanisms of valinomycin still requires considerable further research using all available techniques. In particular, polarization studies and investigations of anion effects are under way.

5. Conclusion

We have obtained the first complete Raman spectra of the valinomycin–K^+ complex in the solid state, and in non-polar (CCl_4) and polar ($CHCl_3$, C_2H_5OH) solvents. Major spectral changes, such as the narrowing and frequency shifting of the amide and ester C=O stretch peaks, differentiate complexed from uncomplexed valinomycin. The ester C=O stretch frequency is extremely solvent-sensitive; it is highest in non-polar solvents. In contrast the amide C=O stretch frequencies, while lower than in uncomplexed valinomycin, are solvent-independent.

These results suggest that the complex consists of a tight, rigid carbon framework within which the ester C=O groups and K^+ ion are only partially shielded from external solvent. Gaps in the outer covering of hydrocarbon side-chains could allow a closer approach of counterions or polar solvent molecules to the center of the complex than previously suspected. This hypothesis might be investigated further using valinomycin analogs in which the hydroxyisovaleric acid subunits are replaced with lactic acid or *vice versa* (such analogs have already been synthesized (Shemyakin *et al.*, 1969)). Solvent interactions with the complexed cation may be a biologically significant part of the ion-release mechanism of such ion-carrying molecules.

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REFERENCES

Bright, D. & Truter, M. R. (1970). *Nature (London)*, **225**, 176–177.

Bystrov, V. F., Ivanov, V. T., Koz'min, S. A., Mikhaleva, I. I., Khalilnlina, K. K., Ovchinnikov, Yu. A., Fedin, E. I. & Petrovskii, P. V. (1972). *FEBS Letters*, **21**, 34–38.

Duax, W. L., Hauptman, H., Weeks, C. M. & Norton, D. A. (1972). *Science*, **176**, 911–914.

Fedarko, M. C. (1973). *J. Magnetic Resonance*, **12**, 30–35.

Grell, E. & Funck, T. (1973). *J. Supramol. Struct.* **1**, 307–335.

Haynes, D. H., Kowalsky, A. & Pressman, B. C. (1969). *J. Biol. Chem.* **244**, 502–505.

Ivanov, V. T., Laine, I. A., Abdulaev, N. D., Senyavina, L. B., Popov, E. M., Ovchinnikov, Yu. A. & Shemyakin, M. M. (1969). *Biochem. Biophys. Res. Commun.* **34**, 803–811.

Ivanov, V. T., Laine, I. A., Abdullaev, N. D., Pletnev, V. Z., Linkind, G. M., Arkhinova, C. F., Senyavina, L. B., Mescheryakova, E. N., Ponov, E. M., Bistrov, V. F. & Ovchinnikov, Yu. A. (1971). *Khimia Prirodnikh Soedinenii*, **3**, 221–246.

Ivanov, V. T., Kogan, G. A., Tulchinsky, V. M., Miroshnikov, A. V., Mikhalyova, I. I., Evstratov, A. V., Zenkin, A. A., Kostetsky, P. V., Ovchinnikov, Yu. A. & Lokshin, B. V. (1973). *FEBS Letters*, **30**, 199–204.

Karle, I. L. (1974*a*). *Biochemistry*, **13**, 2155–2162.

Karle, I. L. (1974*b*). *J. Am. Chem. Soc.* in the press.

Karle, I. L., Karle, J., Wieland, T., Burgermeister, W., Faulstich, H. & Witkop, B. (1973). *Proc. Nat. Acad. Sci., U.S.A.* **70**, 1836–1840.

Koenig, J. L. (1972). *J. Polymer Sci.* **D60**, 59–177.

Krigbaum, W. R., Kuegler, F. R. & Oelschlaeger, H. (1972). *Biochemistry*, **11**, 4548–4551.

Ohnishi, M. & Urry, D. W. (1970). *Science*, **168**, 1091–1092.

Patel, D. J. & Tonelli, A. E. (1973). *Biochemistry*, **12**, 486–496.

Patel, D. J. (1973). *Biochemistry*, **12**, 496–501.
Pinkerton, M., Steinrauf, L. K. & Dawkins, P. (1969). *Biochem. Biophys. Res. Commun.* **35**, 512–515.
Pressman, B. C. (1965). *Proc. Nat. Acad. Sci., U.S.A.* **53**, 1076–1083.
Pressman, B. C., Harris, E. J., Jagger, W. S. & Johnson, J. H. (1967). *Proc. Nat. Acad. Sci., U.S.A.* **58**, 1949–1956.
Richards, R. & Thompson, H. (1947). *J. Chem. Soc.* 1248–1260.
Rothschild, K. J., Asher, I. M., Anastassakis, E. & Stanley, H. E. (1973). *Science*, **182**, 384–386.
Rothschild, K. J. & Stanley, H. E. (1974). *Science*, **185**, 616–618.
Shemyakin, M. M., Aldanova, N. A., Vinogradova, E. I. & Feigina, M. Y. (1963). *Tetrahedron Lett.* 1921–1925.
Shemyakin, M. M., Ovchinnikov, Yu. A., Ivanov, V. T., Antonov, V. K., Vinogradova, E. I., Shkrob, A. M., Malenkov, G. G., Evstratov, A. V., Laine, I. A., Melnik, E. I. & Ryabova, I. D. (1969). *J. Membrane Biology*, **1**, 402–430.
Tosteson, D. C. (1972). In *Perspectives in Membrane Biophysics* (Agin, D. P., ed.), pp. 129–145, Gordon and Breach Scientific Publications, New York.

Note added in proof: Recently, the technique of laser Raman spectroscopy has been used to provide information germane to the conformations of gramicidin A (Rothschild & Stanley, 1974), nonactin (Asher, Phillies & Stanley, manuscript in preparation), and other membrane-active antibiotics.