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SELECTIVE ION BINDING AND MEMBRANE ACTIVITY OF SYNTHETIC CYCLOPEPTIDES

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Summary

Four cyclic peptides related to the membrane-active complexones PV, *cyclo*-(L-Pro-L-Val-D-Pro-D-Val)$_3$, and valinomycin were synthesized: (1) *cyclo*-(L-Pro-L-Ala-D-Pro-D-Val)$_3$ or PVPA, (2) *cyclo*-(L-Ala-L-Val-D-Pro-D-Val)$_3$ or PVAV, (3) *cyclo*-(L-Pro-L-Val-D-Pro-D-Val)$_2$-L-Pro-D-Val or PV-10, (4) *cyclo*-(L-Pro-L-Val-D-Pro-D-Val)$_2$ or PV-8. In a two-phase extraction assay the affinity of PV and PVPA for alkali picrates was about three orders or magnitude greater than that of valinomycin. It was about equal to valinomycin for PVAV and much lower for PV-10 and PV-8. PV, PVPA and PVAV showed a selectivity sequence similar to that of valinomycin, namely $K^+ \sim Rb^+ > Cs^+ > Na^+ > Li^+$. In the series PV, PV-10, PV-8 the preference for K^+ over Na^+ was 700, 5 and <1, respectively. Thus, it was possible to reverse the selectivity of PV for K^+ over Na^+ by reducing the ring size from 12 to 8 amino acid residues.

In sheep red cell lipid bilayer membranes PVPA increased the membrane conductance significantly in the presence of either KCl or NaCl but it was less potent than PV. PV-10, PV-8 and PVAV on the other hand were ineffective in this assay. The inactivity of PVAV as a potassium carrier in membranes was in contrast to its high affinity for potassium picrate in two-phase assays. Such a behaviour may be observed of a compound that has too low an aqueous cation binding constant to use the solution-complexation mechanism of PV (Davis et al. (1976) Biochemistry 15, 768—774 and Pinkerton et al. (1969) Biochem. Biophys. Res. Commun. 35, 512—518) and too slow binding and release kinetics to use the interfacial-complexation mechanism of valinomycin.

Abbreviations: PV, *cyclo*-(L-Pro-L-Val-D-Pro-D-Val)$_3$; PVPA, *cyclo*-(L-Pro-L-Ala-D-Pro-D-Val)$_3$; PVAV, *cyclo*-(L-Ala-L-Val-D-Pro-D-Val)$_3$; PV-10, *cyclo*-(L-Pro-L-Val-D-Pro-D-Val)$_2$-L-Pro-D-Val; PV-8, *cyclo*-(L-Pro-L-Val-D-Pro-D-Val)$_2$.

Introduction

Carrier-mediated transport of cations through lipid membranes can be effected by hydrophobic antibiotics like the actins, enniatins and valinomycin [1]. Among these the depsipeptide valinomycin is the most potent agent in inducing potassium selective ion permeability in natural [2—4] and in artificial [5—7] membranes. These effects as well as the antibiotic activity [8] are a consequence of the strong ion binding properties of valinomycin in non-polar media [9].

In order to gain more information on how the primary structure of an ion carrier molecule translates into selective ion binding and efficient translocation of the complex through a lipid membrane the synthesis of a peptide analog of valinomycin was undertaken [10]. Consisting only of proline and valine residues, it was designated PV (Fig. 1). This compound was the first synthetic peptide with ion carrier activity on lipid bilayer membrane [14]. It resembled valinomycin in its capability to form hydrophobic 1 : 1 complexes with alkali ions. However, there were also many properties in which the two compounds diverged markedly. Thus, PV complexed not only with the large alkali ions (K^+, Rb^+, Cs^+) as valinomycin does, but also with Na^+ and Li^+. The affinity for the cations as determined by two-phase extraction experiments, was in general much greater than for valinomycin [11]. X-ray crystallographic analysis of a PV · rubidium picrate complex (Steinrauf, L.K., (1977) personal communication) and NMR spectroscopy revealed that the cation complexes of PV have S_6 symmetry [12] and are essentially isostructural with the K^+ complex of valinomycin [13]. It was estimated that their dissociation rate constants were several orders of magnitude slower than of the corresponding valinomycin complexes. In the absence of cations PV exists in two conformational states that interconvert slowly enough to allow their separate observation by NMR

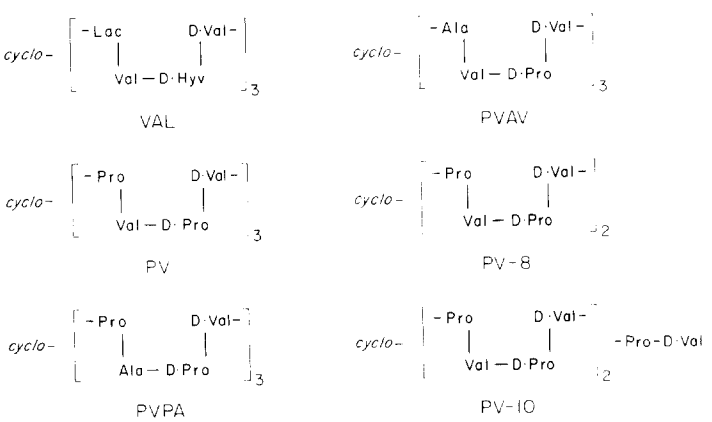


Fig. 1. Primary structures of synthetic cyclopeptides compared with valinomycin (VAL). In each case, one repeating segment representing a β-turn is shown. Note that PV is related to valinomycin by the substitution of D- and L-proline for D-α-hydroxy-isovalerate (D-Hyv) and L-lactate (Lac), respectively. These changes preserve the sequential pattern of D and L residues. They also restrict intramolecular hydrogen bonding to the valine amide protons since proline, like the hydroxy acids, cannot act as a H bond donor.

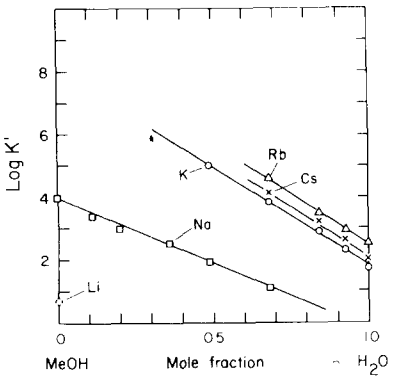


Fig. 2. Apparent stability constants (K', l/mol) of PV with alkali ions in methanol/water mixtures determined spectrophotometrically at 25°C by a method described previously [35,36]. The corresponding values for valinomycin in methanol are: $Li^+ < 5$, Na^+ 4.7, K^+ $8 \cdot 10^4$, Rb^+ $1.8 \cdot 10^5$, Cs^+ $2.6 \cdot 10^4$ [35].

spectroscopy [12]. These slow molecular movements, not observed with valinomycin, are probably due to the reduced flexibility of the backbone induced by the proline rings.

The effects of PV on the ionic permeability of lipid bilayer membranes were different from valinomycin both in qualitative and in quantitative terms. Thus, steady-state measurements showed a PV-induced increase in membrane conductance in K^+ as well as in Na^+ medium [14]. While this conductance was proportional to the aqueous PV concentration, it was four orders of magnitude smaller than with valinomycin. When PV was added only to one side of a bilayer membrane separating otherwise identical solutions of an alkali salt a large zero-current potential developed. This observations together with experiments indicating complexation of PV in water (Fig. 2) and diffusion polarization across lipid bilayers suggested an ion transport mechanism different from that of valinomycin. Indeed, more detailed studies involving electrical relaxation techniques [15,16] showed that most of the PV-induced effects in lipid bilayers are consistent with a carrier mechanism in which binding of the cation occurs in the aqueous phase whereupon the complex crosses the unstirred layers and the membrane just like a large hydrophobic ion. This is, of course, in contrast to the more efficient valinomycin mechanism where complexation takes place at the water-membrane interface followed by a short traverse of the complex through the membrane interior before the ion is released at the opposite interface.

Thus, replacing the hydroxy acid residues in valinomycin by proline residues had resulted in a membrane-active complexone that acts by a different and not previously observed ion transport mechanism. In order to explore this novel mechanism further, four cyclopeptides related to PV (Fig. 1) were designed. *cyclo*-(L-Pro-L-Ala-D-Pro-D-Val)$_3$, PVPA, was chosen to give information on the role of the side chains in ion binding and ion transport. It was expected to answer the question whether asymmetry would impart higher membrane activity on a complexone since PV (low activity) is symmetric while valinomycin (high activity) is asymmetric with regard to the distribution of side chains in the complex. PVAV, *cyclo*-(L-Pro-L-Val-D-Ala-D-Val)$_3$, containing only three

proline residues instead of six as in PV could, on the one hand, show less cation affinity and a different selectivity than PV. On the other hand, it would answer the question whether more than six hydrogen bond donors (amino acid residues with an N-H group) would induce conformational changes and prevent ion binding and transport. The peptides PV-10, *cyclo*-(L-Pro-L-Val-D-Pro-D-Val)$_2$-L-Pro-D-Val, and PV-8, *cyclo*-(L-Pro-L-Val-D-Pro-D-Val)$_2$, were expected to display a different ionic selectivity than PV due to their smaller ring size. This paper describes the synthesis of these peptides and some of their properties.

Materials and Methods

(A) Synthesis of peptides

(1) tert-Butyloxycarbonyl-L-Val-D-Pro-D-Val-L-Pro)$_2$-resin. *tert*-Butyloxycarbonyl-L-Pro-resin was prepared from *tert*-butyloxycarbonyl-L-Pro (Fox Chemical Co., 1556 Industrial St., Los Angeles, Calif.), $CsHCO_3$ (ROC/RIC, Belleville, N.J., 07109) and chloromethylated polystyrene-co-1%-divinylbenzene resin [17] as described previously [18]. A sample of this resin, 3.5 g (3 mmol), was placed into a 100 ml reaction vessel [19] and was treated with suitable agitation [20] according to the following schedule: (a) 100 ml CH_2Cl_2, 3 × 2 min; (b) 60 ml 50% F_3CCOOH in CH_2Cl_2 (v/v), 1 × 2 min, 1 × 25 min; (c) 100 ml CH_2Cl_2, 3 × 1 min; (d) 60 ml 5% diisopropylethylamine in CH_2Cl_2 (v/v), 5 × 2 min; (e) 100 ml CH_2Cl_2, 5 × 1 min; (f) 4.5 ml 1 M dicyalohexylcarbodiimide in CH_2Cl_2, 20 s, followed by 0.98 g (4.5 mmol) *tert*-butyloxycarbonyl-D-Val [21] in 10 ml CH_2Cl_2, 90 min; (g) 100 ml CH_2Cl_2, 3 × 2 min; (h) 100 ml dimethylformamide, 3 × 2 min; (i) 100 ml CH_2Cl_2, 3 × 2 min; (k) repeat f—i. Because of the known susceptibility of dipeptide-resin esters to base [19,22,23] and carboxylic acid [19] catalyzed diketopiperazine formation, for the synthesis of the *tert*-butyloxycarbonyl-tripeptide-resin the above procedure was modified as follows: Step d was reduced to 3 × 2 min and in step f *tert*-butyloxycarbonyl-D-Pro. [17] (0.97 g, 4.5 mmol) solution in 10 ml CH_2Cl_2 was added in several batches over a period of a few min. The remainder of the sequence was built up in the manner described for the *tert*-butyloxycarbonyl-dipeptide-resin using the appropriate *tert*-butyloxycarbonyl-amino acids followed by absolute ethanol, 3 × 2 min, and drying under a stream of air to constant weight. The yield of *tert*-butyloxycarbonyl-octapeptide-resin was 4.1 g containing 1.7 mmol of peptide with an amino acid composition of Pro : Val = 3.6 : 4.3 (calc. 4 : 4). Determination of the free amino groups after coupling at the tetra-, hexa- and octapeptide stage with picric acid [24] gave 4.0, 2.8 and 1.0 μmol/g indicating 99.2, 99.4 and 99.7% coupling yield, respectively. As these values fall within the range of resin blank values (2—4 μM/g) it seems fair to assume that coupling actually went to completion.

(2) H-(L-Val-D-Pro-D-Val-L-Pro)$_2$-OH. *tert*-Butyloxycarbonyl-(L-Val-D-Pro-D-Val-L-Pro)$_2$-resin, 2.0 g (0.82 mmol) was placed into a 100 ml reaction vessel equipped with a tightly sealing Teflon stopcock and Teflon-lined screw cap. Because of the corrosive nature of the HBr and F_3CCOOH vapors it was absolutely necessary to perform the cleavage in a well-ventilated hood. The procedure was as follows: (a) 60 ml CH_2Cl_2, 1 × 10 min; (b) 60 ml F_3CCOOH/CH_2Cl_2 (1 : 1, v/v), 1 × 10 min; (c) 50 ml F_3CCOOH, 2 × $\frac{1}{2}$ min. At this point

(d) a fresh batch of 30 ml F_3CCOOH was added and (e) was overlayered with 30 ml 30% HBr in acetic acid (Eastman Kodak Co., Rochester, N.Y., 14650) and the vessel was sealed tightly. Addition of the HBr solution had to be performed very slowly and carefully in order to prevent premature mixing of the F_3CCOOH and HBr/acetic acid layers. Such mixing, even to a small extent, can cause the HBr gas to boil out as it has a lower solubility in F_3CCOOH than in acetic acid. The tightly sealed vessel was then (f) agitated for 10 min. The cleaved peptide was (g) removed from the resin by opening the stopcock first and collecting the liquid which was expelled at a high rate because of the positive pressure within the vessel. The cleavage mixture was combined with a second fraction obtained by repeating steps d—g and washing the resin with 3 × 20 ml F_3CCOOH. Evaporation in vacuo at 45° C followed by lyophilization from glacial acetic acid gave 950 mg of crude peptide hydrobromide. This material was dissolved in a small volume of methanol, neutralized (to moist pH indicator paper) with pyridine and chromatographed on a 4 × 210 cm column of Sephadex LH-20 in methanol. Since this step did not give a pure product, chromatography was repeated on a 2 × 160 cm column of Bio-Beads SX-1 (Bio-Rad Laboratories, Richmond, Calif. 94804) in CH_2Cl_2/glacial acetic acid (9 . 1, v/v). The peptide emerged as a single peak and was lyophilized from water. It was pure by thin-layer chromatography (silica gel G, *n*-butanol/acetic acid/pyridine/H_2O, 15 : 10 : 3 : 2, v/v; R_f = 0.67). Yield 325 mg, 49% for the cleavage step. Amino acid analysis after hydrolysis with propionic acid/conc. HCl (1 : 1, v/v) at 135° C [25] gave a ratio of Pro : Val = 4.03 : 3.96 (calc. 4 : 4).

(3) cyclo-(L-Pro-L-Val-D-Pro-D-Val)$_2$, PV-8. H-(L-Val-D-Pro-D-Val-L-Pro)$_2$-OH, 650 mg (0.8 mmol) was activated with 440 mg (1.6 mmol) Woodward's Reagent K (Aldrich Chemical Co., Milwaukee, Wisc. 53233) in 80 ml dimethylformamide at 0° C for 3 h according to Blaha and Rudinger [26]. After dilution with 720 ml CH_2Cl_2 and addition of 1.14 ml (8 mmol) triethylamine the peptide was allowed to cyclize for 2 weeks at room temperature. The volatile components were removed in vacuo and the residue was taken up in 60 ml methanol/water (8 : 2, v/v). Mixed bed ion-exchange resin (AG 501-X8 (D), Bio-Rad) was then added in small batches until no more ninhydrin-positive material was detectable in the supernatant by thin-layer chromatography. The resin was filtered off and the filtrate evaporated to dryness and lyophilized from *tert*-butanol. The crude product (336 mg) was further purified by chromatography on a 2.5 × 180 cm Bio-Beads SX-1 column in CH_2Cl_2 from where the peptide eluted as a single, narrow peak. Yield of pure peptide, 178 mg, 28% for the cyclization step. Molecular weight was 784 by isobutane chemical ionization mass spectrometry, and 808 by amino acid analysis (calc. 784), amino acid ratio, Pro : Val = 3.94 : 4.06 (calc. 4 : 4). Analysis by thin-layer chromatography silica gel G plates showed one single, ninhydrin-negative spot which stained blue with the iodine/*o*-tolidine reaction [27] in the following solvent systems: (a) *n*-butanol/acetic acid/pyridine/water (15 : 10 : 3 : 2, v/v), R_F = 0.71; (b) *n*-butanol/acetic acid/water (4 : 1 : 1, v/v), R_F = 0.61; (c) propanol/water (1 : 1, v/v), R_F = 0.72. The product was verified by NMR purity checks (cf. Table III).

(4) cyclo-(L-Pro-LVal-D-Pro-D-Val)$_2$-L-Pro-D-Val, PV-10. The *tert*-butyloxy-

carbonyl-D-Val-L-Pro-(L-Val-D-Pro-D-Val-L-Pro)$_2$-resin was prepared as described for the octapeptide-resin above (section 2) with the exception that an automated peptide synthesizer (Beckman 990) was used. A 200 mg sample of the resin was cleaved with 30% HBr in acetic acid F_3CCOOH (1 : 1, v/v) for 15 min according to the procedure described above (section 3) and the crude peptide was purified by Sephadex LH-20 chromatography in 10^{-3} M HCl to give 64 mg of open chain peptide that was pure by thin-layer chromatography. 49 mg (0.049 mmol) was cyclized for 11 days with Woodward's Reagent K in the manner described above (section 4), purified by dry column silica gel chromatography [28] using *n*-butanol/acetic acid/pyridine/water (15 : 10 : 3 : 2, v/v) and by exhaustive deionization with mixed bed ion exchange resin in 75% methanol. The peptide (11 mg, 23% yield for the cyclization step) was pure by thin-layer chromatography: (a) *n*-butanol/acetic acid/pyridine/water (15 : 10 : 3 : 2, v/v), $R_F = 0.73$; (b) *n*-butanol/acetic acid/water (4 : 1 : 1, v/v), $R_F = 0.45$; (c) *n*-butanol, $R_F = 0.00$—0.10. Amino acid analysis, Pro : Val = 5.05 : 5.00 (calc. 5 : 5). Molecular weight by isobutane chemical ionization mass spectrometry, 980 (calc. 980). The product was verified by NMR purity checks.

(5) cyclo-(L-Pro-L-Ala-D-Pro-D-Val)$_3$, PVPA. This compound was synthesized using the same techniques as for PV-8. The product used in the experiments described here had the following characteristics: Amino acid analysis, Pro : Ala : Val = 6.3 : 3.0 : 2.9 (calc. 6 : 3 : 3); $[\alpha]_D^{27} = +17.5°$ (*c* 0.2, absolute ethanol); molecular weight by isobutane chemical ionization mass spectrometry 1092 (calc. 1092); melting point 295.5°C; thin-layer chromatography (no ninhydrin-positive material present): (a) *n*-butanol, $R_F = 0.0$; (b) *n*-butanol/acetic acid/pyridine/water (15 : 10 : 3 : 2, v/v), two spots (A) $R_F = 0.77$, (B) $R_F = 0.57$; (c) *n*-butanol/acetic acid/water (4 : 1 : 1, v/v), two spots, (A) $R_F = 0.19$, (B) $R_F = 0.32$; relative intensities of (A) : (B) were approx. 4 : 1. The product was verified by NMR purity checks (cf. Table III).

(6) tert-Butyloxycarbonyl-L-[^{14}C]Val-D-Ala-D-Val-L-Pro-(L-Val-D-Ala-D-Val-L-Pro)$_2$-resin. Starting with 8 g *tert*-butyloxycarbonyl-Pro-resin (7 mmol) the peptide chain was built up in the manner described for PV-8. The *tert*-butyloxycarbonyl-D-Ala needed was synthesized according to Schnabel [29] from D-alanine (Fox Chemical Co.) and *tert*-butyloxycarbonyl-azide (Aldrich): Yield, 85%; melting point, 84—86°C; $[\alpha]_D^{25} = 27.5°$, lit. [29] for L-isomer, —25.2°C (*c* 1, glacial acetic acid); elemental analysis found, C. 50.74%; H, 7.96%; N, 7.26% (calc. C, 50.78%; H. 7.99%; N, 7.40%). The last residue, L-valine was attached by reacting the deprotected and neutralized undecapeptide-resin first with 0.15 equivalents of *tert*-butyloxycarbonyl-L-[^{14}C]Val (Schwarz Bioresearch, Orangeburg, N.Y. 10962) and dicyclohexylcarbodiimide for 30 min followed by 2.5 equivalents of *tert*-butyloxycarbonyl-L-Val and dicyclohexylcarbodiimide for 90 min. In this way, about 50% of the available radioactivity was incorporated into the peptide chain. Monitoring the synthesis by hydrolysis and amino acid analysis of samples of the resin after each deprotection step (I—XII) gave the following Pro : Val : Ala ratios (calculated values in parentheses): I, 1 : 0 : 0 (1 : 0 : 0); II, 1.0 : 0.91 : 0 (1 : 1 : 0); III, 1.0 : 0.97 : 0.97 (1 : 1 : 1); IV, 1.0 : 1.93 : 0.97 (1 : 2 : 1); V, 2.0 : 1.79 : 0.9 (2 : 2 : 1); VI, 2.0 : 2.94 : 0.98 (2 : 3 : 1); VII, 2.0 : 2.84 : 2.16 (2 : 3 : 2); VIII, 2.0 : 3.90 : 1.96 (2 : 4 : 2); IX, 3.0 : 4.01 : 1.99 (3 : 4 : 2); X, 3.0 :

TABLE I

CLEAVAGE OF PVAV-RESIN

Fraction	Crude weight (mg)	Peptide content ** (%)	Total peptide (μmol)	Specific radioactivity *** (cpm/μmol)	Amino acid ratios Pro : Val : Ala
Cleavage I	4200 *	88	3090	28 000	3.2 : 6.0 : 3.0
Cleavage II	110 *	52	50	21 000	3.4 : 6.0 : 2.6
Cleavage III	101 *	31	30	26 000	2.7 : 6.0 : 3.2
Remaining resin	7900	5.5	430	27 000 †	2.8 : 6.0 : 2.8

* Lyophilized from glacial acetic acid.

** By amino acid analysis after hydrolysis of the crude product.

*** Determined on a Beckman LS-355 scintillation counter using Aquasol (New England Nuclear, Boston, Mass. 02118) as scintillation liquid.

† After hydrolysis. Counting the resin directly gave a value of 26 400 cpm/μmol peptide.

4.64 : 1.86 (3 : 5 : 2); XI, 3.0 : 5.25 : 3.16 (3 : 5 : 3); XII, 3.0 : 6.35 : 3.20 (3 : 6 : 3). Weight of dry *tert*-butyloxycarbonyl-dodecapeptide-resin, 11.1 g.

(7) H-L-[^{14}C] Val-D-Ala-L-Pro-(L-Val-D-Ala-D-Val-L-Pro)$_2$-OH. 10 g of the deprotected (50% F_3CCOOH, 1 × 2, 1 × 25 min) dodecapeptide-resin (3.57 mmol by amino acid analysis) was treated with 50 ml F_3CCOOH + 50 ml 30% HBr in acetic acid for 10 min as described above (section 3) and washed with 50 ml F_3CCOOH for 1 min. Cleavage mixture and F_3CCOOH-wash were combined, evaporated and the residue was lyophilized from glacial acetic acid. Repeating this procedure twice gave three cleavage fractions (I, II, III). An analysis of these fractions and of the remaining resin is given in Table I.

These results show that most of the peptide (86%) was cleaved and washed out during the first 10 min cleavage period leaving a fraction of peptide presumably physically trapped within the resin) that is washed out only slowly. Note the uniformity of the labelling and of the amino acid ratio in the different fractions.

The cleaved peptide was purified on a Sephadex LH-20 column in methanol as described above (section 3) to give a total of 3.1 g of pure compound. Yield, 44% based on the amount of *tert*-butyloxycarbonyl-Pro-resin at the beginning of the synthesis. Amino acid ratios, Pro : Val : Ala = 2.9 : 6.1 : 3.0 (calc. 3 : 6 : 3). Thin-layer chromatography on silica gel G plates: one ninhydrin-positive spot, *n*-butanol/acetic acid/pyridine/water (15 : 10 : 3 : 2, v/v), R_F = 0.72; *n*-propanol/water (7 : 3, v/v), R_F = 0.44.

(8) cyclo-L-Pro-L-[^{14}C] Val-D-Ala-D-Val-(L-Val-D-Ala-D-Val-L-Pro)$_2$, PVAV. Method a: Similar to Wieland and Birr [30] 110 mg (0.1 mol) H-L-[^{14}C]-Val-D-Ala-D-Val-L-Pro-(L-Val-D-Ala-D-Val-L-Pro)$_2$-OH and 46 mg (0.4 mmol) *N*-hydroxysuccinimide in 20 ml dimethylformamide + 30 ml CH_2Cl_2 was neutralized with diisopropylethylamine (55 μl). Dicyclohexylcarbodiimide, 42 mg (0.2 mmol) was added and the cyclization was allowed to proceed at room temperature for 144 h. Samples (50 μl) were taken periodically, put on silica gel G plates (2.5 × 10 cm) and developed with isopropanol. After drying the gel was scratched from the plates in strips as indicated in Fig. 3 (fractions 1—6) and counted in Aquasol. While this monitoring of the cyclization reaction indicated an over 50% yield of cyclopeptide the isolated yield (by silica gel dry

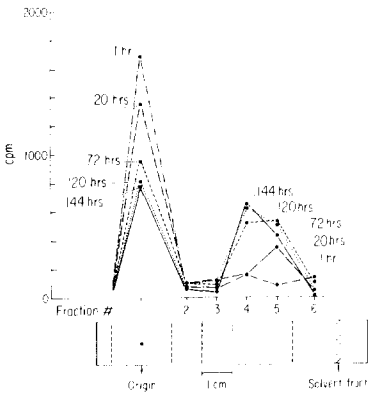


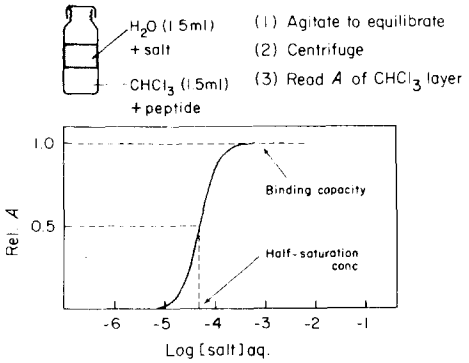
Fig. 3. Monitoring of the cyclization reaction of a radioactive peptide by thin-layer chromatography. The peak in fraction 1 (decreasing with time) is open-chain, that in fractions 4—5 (increasing with time) represents cyclized peptide. The example shown is for the cyclization of PVAV with dicyclohexylcarbodiimide/*N*-hydroxysuccinimide.

column chromatography [28] with isopropanol) was 32 mg (29%). Method b: As described for PV-8, 1.1 g (1 mmol) of the open chain peptide was cyclized with Woodward's Reagent K. Monitoring as described for method a indicated no further reaction after 3 days. The peptide was then purified by silica gel dry column chromatography [28] using *n*-butanol/acetic acid/pyridine/water (15 : 10 : 3 : 3, v/v) to give 480 mg (44% for the cyclization step) of cyclopeptide. Overall yield bases on the starting material, *tert*-butyloxycarbonyl-L-Pro-resin was 19.5%. Per mmol of product 53 mmol each of *tert*-butyloxycarbonyl-L-Val, *tert*-butyloxycarbonyl-D-Ala and *tert*-butyloxycarbonyl-D-Val and 42 mmol of *tert*-butyloxycarbonyl-L-Pro were consumed. Amino acid analysis, Pro : Ala : Val = 2.9 : 2.9 : 6.2 (calc. 3 : 3 : 6). Molecular weight by isobutane chemical ionization mass spectrometry, 1098 (calc. 1098). The product was verified by NMR purity checks (cf. Table III).

(B) Two-phase extraction experiments

(1) Picrates. Picrates were prepared by neutralizing picric acid in aqueous solution with the appropriate cation bicarbonate or hydroxide followed by evaporation to dryness. The salts were recrystallized from ethanol or ethanol/water mixtures. In this way, crystalline picrates of Li^+ ($\cdot 1\ H_2O$), Na^+ ($\cdot 1\ H_2O$), K^+, Rb^+, Cs^+, Tl^+ and NH_4^+ were obtained. They all gave satisfactory elemental analyses.

(2) Extraction experiments. Two-phase extraction experiments were performed similar to Eisenman et al. [31], Haynes and Pressman [34] and Lev (Lev, A.A., Leningrad, personal communication). 1.5 ml of a $3 \cdot 10^{-5}$—$5 \cdot 10^{-5}$ M solution of cyclopeptide in chloroform (Mallinckrodt AR, containing 0.75% ethanol) was equilibrated with 1.5 ml of picrate salt in deionized water by vortexing for 30 s or by agitating on a 180° C wrist action shaker [20] for 30—60 min in a vial sealed with a Teflon-lined screw cap. The vial was centrifuged for 2 min and the picrate uptake into the chloroform layer was measured spectrophotometrically ($\epsilon_{371} = 18\,000$). The dependence of picrate



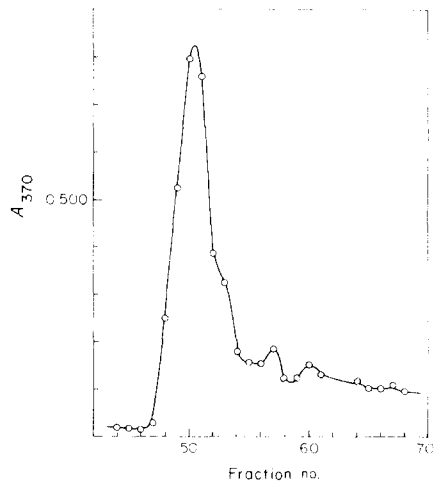


Fig. 4. Two-phase salt extraction assay to determine the affinity of lipophilic complexones for cations and the stoichiometry of the complexes formed.

Fig. 5. Example of the monitoring of the effluent from a column using the assay described in Fig. 4. The sample was a crude, deionized PV preparation (45 mg) that was chromatographed on a 300 ml Sephadex LH-20 column in chloroform/isopropanol (1 : 1, v/v) 50 µl of each 3 ml fraction were combined with 2.0 ml chloroform and 2.0 ml of 1% potassium picrate in water, agitated and the A_{370nm} of the chloroform layer was determined.

uptake on the log of the salt concentration was plotted (after correction for the, at most concentrations, negligible picrate uptake in the absence of peptide) to give a titration curve for each peptide and each salt used. This assay (Fig. 4) gives the stoichiometry of the complex formed (ratio of maximal picrate uptake to peptide concentration) and a measure of the two-phase binding constant, K_{50}. K_{50} is the aqueous salt concentration that half-saturates the peptide in the organic phase. The assay covers the range from $K_{50} \cong 10^{-7}$ M (limit imposed by the extincition coefficient of the anion) to $K_{50} \cong 10^{-2}$ M, depending on the aqueous solubility of the salt used.

This assay was also adapted to monitor the effluent from columns in the purification of ion carriers. A typical examples is shown in Fig. 5. It is a particularly useful assay because most of the ion carriers do not give a ninhydrin reaction and have no significant ultraviolet absorption and therefore cannot be detected by these standard methods of peptide chemistry.

(C) NMR studies

Proton nuclear magnetic resonance (NMR) spectra were recorded in the Fourier-transform mode using a Varian HR-220 spectrometer equipped with a Nicolet 1080 accessory. All samples were prepared by dissolving approx 1—2 mg of the peptide in approx. 0.5 ml of the appropriate deuterated solvent that had been stored over molecular sieves. A drop (approx. 20 µl) of tetramethylsilane was added as a reference and the solutions were filtered through a small plug of glas wool directly into the sample tubes. For variable-temperature experiments, the probe temperature was measured after equilibration using standard, calibrated ethylene glycol or methanol samples.

(D) Membrane studies

The membranes were formed with sheep red blood cell lipids [32] in *n*-decane (≈15—25 mg/ml) using a brush technique. The measurements were carried out with a four-electrode voltage clamp apparatus [33] at room temperature (≈23° C). The voltage was supplied by a ramp generator with stepwise variable voltage change rate (0.1—500 mV/s). The sweep could be interrupted at any time and the voltage clamped at any desired value. Membrane potentials (V_m) were measured with a Keithley 602 electrometer and were recorded on a strip-chart recorder or on an X-Y plotter. Current voltage (*I-V*) curves were obtained directly on the X-Y plotter.

The salts used were reagent grade. *n*-Decane was from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.

Results and Discussion

(A) Cation binding in a chloroform/water two-phase system

Fig. 6 shows the uptake of picrate by cyclopeptides in chloroform when exposed to an aqueous phase containing varying concentrations of potassium picrate. Like PV, PVPA clearly shows saturation at a peptide to picrate ratio of 1 : 1. The two peptides appear to have the same affinity for K^+ as judged by the aqueous salt concentration required to induced 50% picrate uptake (half-saturation concentration, K_{50}), namely, $K_{50} \leqslant 2 \cdot 10^{-7}$ M. However, such a high affinity is at the practical limit of this assay because at all salt concentrations smaller than the peptide concentration essentially all of the picrate added is taken up into the $CHCl_3$ phase. This means that if there were a difference in binding affinity between PV and PVPA it could not be resolved by this assay. Nevertheless, it can be stated that these two peptides have at least a 1000-fold higher affinity for potassium picrate than does valinomycin ($K_{50} = 2.8 \cdot 10^{-4}$ M). The reasons for this, as discussed previously for PV [12], include (a) the higher electron-donating properties of the groups coordinating to the cation (amide vs. ester carbonyl groups) and (b) the reduced flexibility imposed by

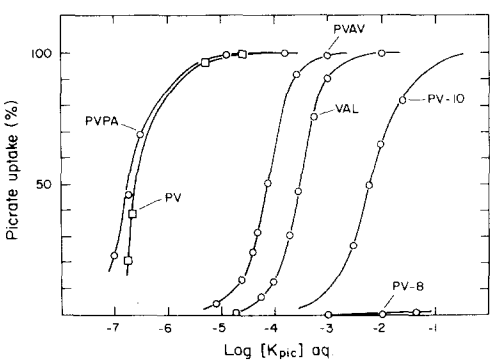


Fig. 6. Two-phase ($CHCl_3/H_2O$) titration of cyclopeptides with potassium picrates. The curves to the left (PVPA, PV) indicate high affinity for potassium picrate, those to the right low affinity (PV-10) or no potassium binding at all (PV-8). The peptide concentration in the chloroform phase was $3 \cdot 10^{-5}$—$5 \cdot 10^{-5}$ M and corresponds to 100% in each case.

TABLE II

HALF-SATURATION CONCENTRATION (K_{50}) OF $3 \cdot 10^{-5}$—$5 \cdot 10^{-5}$ M PEPTIDE IN CHLOROFORM EQUILIBRATED WITH AQUEOUS SOLUTIONS OF PICRATE SALTS

Aqueous salt concentration (mol/l) that converts 50% of the peptide in the organic phase into the cation complex. Determined from titration curves as shown in Figs. 6 and 7.

Ion	Valino-mycin	PV	PVPA	PVAV	PV-10	PV-8
Li^+	—	$2 \cdot 10^{-4}$	$3.7 \cdot 10^{-5}$	$2.5 \cdot 10^{-3}$	$3 \cdot 10^{-2}$	$1.2 \cdot 10^{-1}$
Na^+	$\approx 10^{-1}$	$1.4 \cdot 10^{-4}$	$8 \cdot 10^{-5}$	$2.5 \cdot 10^{-3}$	$3 \cdot 10^{-3}$	$1.6 \cdot 10^{-1}$
K^+	$2.8 \cdot 10^{-4}$	$\approx 2 \cdot 10^{-7}$	$\approx 2 \cdot 10^{-7}$	$8 \cdot 10^{-5}$	$6.3 \cdot 10^{-3}$	>1
Rb^+	$2.2 \cdot 10^{-4}$	$6 \cdot 10^{-7}$	$4.5 \cdot 10^{-7}$	$4 \cdot 10^{-5}$	$7 \cdot 10^{-3}$	>1
Cs^+	$5 \cdot 10^{-4}$	$5 \cdot 10^{-7}$	$7 \cdot 10^{-7}$	$9 \cdot 10^{-5}$	$2 \cdot 10^{-2}$	>1
NH_4^+	$2.8 \cdot 10^{-3}$	$8 \cdot 10^{-7}$	$1 \cdot 10^{-6}$	$2 \cdot 10^{-4}$	—	>1
Tl^+	$1.8 \cdot 10^{-3}$	$1 \cdot 10^{-6}$	—	$7 \cdot 10^{-5}$	—	—

the proline rings which results in a destabilization of the uncomplexed form. PVPA differs from PV only in three amino acid side-chains (methyl vs. isopropyl groups) located on the surface of the molecule. Such a change should not influence greatly the conformation or electronic parameters of the complex. Not surprisingly therefore, the affinity of PVPA and PV are similar not only for potassium but also for all the other cations tested in this assay (Table II).

Quite in contrast, a marked decrease in the binding strength is observed when the nucleophilicity of the carbonyl groups coordinating to the cation is reduced. In PVAV, this was achieved by substituting three alanine residues for three proline residues. Alanine in a peptide bond provides an unsubstituted secondary amide which makes a smaller inductive contribution to the electron density on the carbonyl oxygen than the N-alkylated tertiary amide of proline. As a consequence, PVAV ($K_{50K} = 8 \cdot 10^{-5}$ M) has a two orders of magnitude smaller affinity for potassium picrate than do PV or PVPA. Conversely, comparing PVAV with valinomycin, the substitutions of L-Alanine and D-Proline for L-Lactate and D-α-hydroxy-isovalerate, respectively, apparently have opposing effects so that the affinity of PVAV for cations is similar to valinomycin. This result demonstrates that the availability of nine potential hydrogen bond donors (versus six in valinomycin, PV and PVPA) does not lead to conformation changes which prevent ion binding.

PV-10 formally has only five carbonyl groups available for coordination to the K^+. Accordingly, it binds the K^+ with considerably less strength ($K_{50K} = 6 \cdot 10^{-3}$ M) than PV or valinomycin. This effect is even more pronounced in PV-8 which could provide only four carbonyl groups for coordination. This analogue shows no potassium binding at all ($K_{50} <\sim 1$ M) in this assay.

The affinities of the cyclopeptides for other cations are listed in Table II. In the cases where $K_{50} <\approx 10^{-3}$ M, the saturation level clearly indicates a 1 : 1 stoichiometry of the complexes. It is very likely that this obtains in the other cases as well even if complete saturation was not reached due to the limited aqueous solubility of some of the picrates (e.g. potassium picrate/PV-10, Fig. 4).

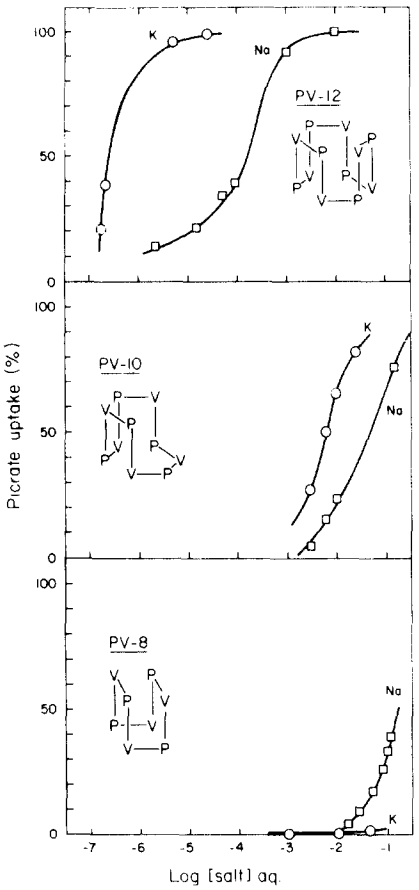


Fig. 7. Selectivity of PV, PV-10 and PV-8 for potassium and sodium picrates in water/chloroform. Note the crossover of the curves going from potassium-selective PV-10 to sodium-selective PV-8.

The ionic selectivity sequence in this assay is similar for all three cyclododecapeptides and parallels that of valinomycin. Thus, PV, PVPA and PVAV have the following selectivity sequence: $K^+ \sim Rb^+ > Cs^+ > Na^+ > Li^+$. The preference of these compounds for potassium over sodium (K_{50Na}/K_{50K}) in this assay is pronounced: valinomycin, >360; Pv, ~700; PVPA, ~400; PVAV, 30.

Fig. 7 shows the K^+/Na^+ selectivity of PV, PV-10 and PV-8. In this series the ring size was successively reduced by two amino acid residues. PV (12 residues) has a 700-fold preference for K^+ over Na^+. This number decreases to about 5 for PV-10 with ten residues. Going to the even smaller PV-8 the curves cross indicating a K^+/Na^+ preference of less than 1. Thus, it has been possible to reverse the selectivity of PV for K^+ over Na^+ by reducing the size of the cavity available for the cation. As discussed earlier there is, however, a strong decrease in the absolute affinity of these peptides for cations. This is presumably due to the reduction of the number of ligand atoms (6, 5, 4, for PV, PV-10, PV-8, respectively).

(B) NMR studies

Proton NMR studies were carried out on both the free and cation-complexed forms of the cyclic peptides described above. A detailed analysis of these studies will be reported elsewhere. The results summarized here include a description of the overall conformation and symmetry properties of the molecules and evidence for complex formation.

As with PV [12] ^{1}H NMR spectra show that in [^{2}H]chloroform, free PVPA exists as a mixture of kinetically stable conformers. About one-third of the PVPA molecules have C_3 symmetry while the remaining two-thirds are distributed about equally into two similar but distinct asymmetric conformers. As judged by variable temperature studies the amide protons of the C_3 symmetric conformer are intramolecularly H bonded. The reaction of these samples with potassium or ammonium picrate give complexes having NMR spectra which indicate the presence of a single species with C_3 symmetry and which are unique for each cation. As judged by the changes in the vicinal coupling constants between NH-C_αH pairs there is a conformation change in the peptide backbone. Based on similar results for valinomycin [42] and PV [12] it is concluded that the conformation of the PVPA complexes (as well as the symmetric form of free PVPA) is of the "A_2" type [43]. This means that the peptide backbone is folded into alternating 1-4 β-turns, giving the molecule a bracelet-like three-dimensional structure.

Both, the free and cation-complexed forms of the PVAV have properties that are similar to those of PV and PVPA except for two notable exceptions. First, only the C_3 symmetric conformer of free PVAV is found to any appreciable extent (>90%) in low-polarity solvents such as chloroform. Secondly, the alkali metal complexes of PVAV, while freely soluble in methylene chloride, are only sparingly soluble (approx. 10^{-4} M) in chloroform although the NMR spectra in the two solvents show that the compounds have the same conformation. Valinomycin complexes show similar solubility differences in going from hexane to chloroform and this has been related to the type of ion-pairing between the positively charged complex and its counter ion [42]. A similar phenomenon may account for the observations with PVAV.

PV-8 is a compact molecule with S_4 symmetry. Its structure is made quite rigid by intramolecular hydrogen bonds that form the alternating 1-4 β-turns so typical of the peptides discussed here. The spectra are virtually temperature independent and do not change upon going from chloroform to acetone. PV-8 forms 1 : 1 complexes with lithium and sodium picrates. This process is accompanied by a distinct change (from 8.5 to 6.8 Hz) in the NH-C_αH vicinal coupling constant (Table III).

(C) Ion carrier activity in lipid bilayers

Fig. 8 shows the dependence of the zero-current, zero-voltage conductance (G_0) of sheep red cell lipid bilayers on the concentration of peptide present in both sides of 1.0 M KCl solutions. PV and PVPA show significant carrier activity as indicated by the increase in G_0 with increased peptide concentration. In both cases the slope of this dependence is close to one which is the expected value for a 1 : 1 carrier · cation complex. PVAV on the other hand has only a small effect on the conductance of sheep red cell lipid bilayers. This is in agree-

TABLE III

CHEMICAL SHIFTS, σ [a], AND COUPLING CONSTANTS, $J_{N,C}$ [b], FOR AMIDE PROTONS OF PEPTIDES AND POTASSIUM · PEPTIDE COMPLEXES [c]

		PVPA		PVAV			PV-8
		D-Val	L-Ala	D-Val	L-Val	L-Ala	D-Val, L-Val
Uncomplexed form	σ	7.97 [d]	7.66 [d]	7.13 [e]	7.90 [e]	6.86 [e]	7.10; 7.11 [f]; 7.12 [g]
	J	7.7	8.5	8.7	5.0	7.8	8.5; 8.5 [f,g]
K^+ complex	σ	8.13; 7.99 [h]	7.88; 7.68 [h]	8.01 [i]	8.38 [i]	6.78 [i]	6.89 [j]; 7.34 [k]
	J	5.2; 5.2 [h]	2.7; — [h]	5.3	4.5	9.0	6.8 [j,k]

[a] In ppm (±0.01) from tetramethylsilane.
[b] In Hz (±0.1).
[c] Except where noted spectra were taken at 25°C and a concentration of approx. 10^{-3} M in [^{2}H]-chloroform.
[d] Temperature coefficient (ppm/degree C) are: D-Val, $5 \cdot 10^{-3}$; L-Ala, $1 \cdot 10^{-3}$.
[e] Temperature coefficient (ppm/degree C) are: D-Val, $1 \cdot 10^{-3}$; L-Val, $1 \cdot 10^{-2}$; L-Ala, $3 \cdot 10^{-3}$.
[f] At −21°C.
[g] In deuteroacetone.
[h] NH_4^+ complex.
[i] Concentration approx. 10^{-4} M.
[j] Li^+ complex in deuterochloroform/deuteroacetone (4 : 1, v/v).
[k] Na^+ complex in deuterochloroform/deuteroacetone (4 : 1, v/v).

ment with results obtained with dioleoyl phosphatidylcholine membranes where it was shown that 10^{-4} M PVAV did not increase the membrane conductance in the presence of 1 M KCl (Gisin, B.F. and Benz, R., (1975) unpublished) indicating an ion carrier potency of PVAV 10^7 times lower than that of valinomycin.

Results obtained using sodium as the cation are shown in Fig. 9. As is the case for potassium, there is essentially no ion carrier activity found for PVAV while PV and PVPA are active. However, 10—100 times larger peptide concentrations are required to induced conductances equal to those observed with potassium. With sodium as well as with potassium, PV is a more potent ion carrier than PVPA.

The purpose of the synthesis of PVPA was to test the possibility that the asymmetry of valinomycin is a significant reason for ith high membrane activity. If that were true, the reduced activity of PV could be explained by the symmetrical distribution of side chains namely, three valine side chains and three proline rings each at the top and the bottom of the molecule (Fig. 1), making it a meso form. PVPA, on the other hand, with three proline rings and three valine side chains at the top and three alanine side chains at the bottom of the molecule is no longer a *meso* form and resemble valinomycin more closely than PV in the asymmetry of its topology. Figs. 8 and 9 show that introducing this change does not lead to a compound with improved ion carrier activity in lipid bilayer membranes. When PVPA is placed only on one side of a membrane separating otherwise identical salt solutions (1 M KCl, 10^{-3} KH_2PO_4, pH 7.4) and appreciable potential difference develops: 16 MV at 10^{-6} M, 34 mV at 10^{-5} M and 34 mV at 10^{-4} M PVPA, the side containing the peptide being negative. This is similar to what was observed with PV [14].

PV-10 and PV-8 have little influence on the membrane conductance in the presence of either K^+ or Na^+ although their capability to bind these ions (see

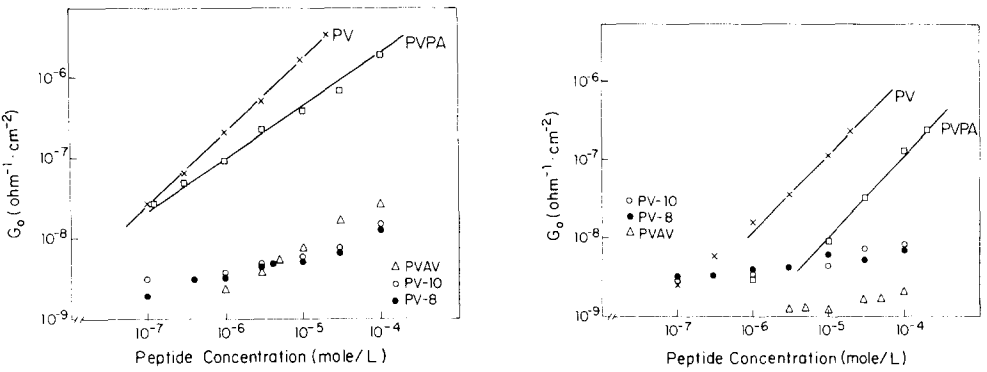


Fig. 8. The effect of cyclopeptide on the zero-current, zero-voltage conductance (G_0) of red cell lipid bilayer membranes in the presence of K^+ (log/log plot). The aqueous solutions separated by the membrane were symmetrical with regard to salt (1 M KCl, 10^{-3} M KH_2PO_4, pH 7.4) and peptide concentration.

Fig. 9. These experiments were performed like those shown in Fig. 8 except that sodium was substituted for potassium (log/log plot).

K^+-PV-10, Na^+-PV-10 and Na^+-PV-8, Figs. 6 and7) has been demonstrated. In this respect they resemble the "crown ethers" which are very much less potent than valinomycin as ion carriers in membrane [37] but which do bind cations in two-phase extraction experiments [38]. It is possible that PV-10 and PV-8 have an aqueous conformation that is too hydrophilic to allow their partitioning into the water-membrane interface.

PVAV shows two-phase cation affinities (e.g. $K_{50K} = 8 \cdot 10^{-5}$ for K^+, Table II) that are about the same as for valinomycin ($K_{50K} = 2.8 \cdot 10^{-4}$). In order to explain the lack of membrane activity of this complexone mechanistic considerations may have to be taken into account. It has been shown that the steady-state conductance of membranes exposed to PV occurs largely by a solution-complexation mechanism [14—16] while valinomycin uses the more efficient interfacial-complexation mechanism [40]. The key features of the latter are high association and dissociation rates of the complex in the membrane-water inface. If the aqueous reactions are slow it is necessary for the cation affinities to be high for appreciable conductance by solution-complexation to occur. Apparently, the affinity of PVAV for cations is too low, in the case of potassium picrate it is 400 times smaller than for PV (Table II), to allow conductance by this mechanism. NMR experiments [12] have shown that the dissociation rate constant of PV presumably due to the high proline content, is several orders of magnitude smaller than that of valinomycin. With three proline residues in its sequence the PVAV molecule may be similarly rigid and possess low rates of cation binding and dissociation. This constraint may preclude the use of the valinomycin mechanism (interfacial complexation). Thus, it appears that PVAV is ineffective because it has access to neither of the two currently known mechanisms of carrier-mediated ion transport through membranes. Its cation binding constants and its rate of reaction with cations in water are too low for a solution-complexation mechanism and its ion binding kinetics are too slow for an interfacial-complexation mechanism.

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References

1 Ovchinnikov, Yu.A., Ivanov, V.T. and Shkrob, A.M. (1974) Membrane-Active Complexones, Elsevier, Amsterdam
2 Murray, W.C. and Begg, R.W. (1959) Arch. Biochem. Biophys. 84, 546—548
3 Moore, C. and Pressman, B.C. (1964) Biochem. Biophys. Res. Commun. 15, 562—567
4 Tosteson, D.C., Cook, P., Andreoli, T. and Tieffenberg, M. (1967) J. Gen. Physiol. 50, 2513—2525
5 Lev, A.A. and Buzhinski, E.P. (1967) Tsitologia 9, 102—106
6 Mueller, P. and Rudin, D.O. (1967) Biochem. Biophys. Res. Commun. 26, 398—404
7 Andreoli, T.E., Tieffenberg, M. and Tosteson, D.C. (1967) J. Gen. Physiol. 50, 2527—2545
8 Harold, F.M. and Baarda, J.R. (1967) J. Bacteriol. 94, 53—60
9 Shemyakin, M.M., Ovchinnikov, Yu.A., Ivanov, V.T., Antonov, V.K., Shkrob, A.M., Mikhaleva, I.I., Evstratov, A.V. and Malenkov, G.G. (1967) Biochem. Biophys. Res. Commun. 29, 834—841
10 Gisin, B.F. and Merrifield, R.B. (1972) J. Am. Chem. Soc. 94, 6165—6170
11 Gisin, B.F. and Davis, D.G. (1973) Biophys. J. 13, 2889, Abstr.
12 Davis, D.G., Gisin, B.F. and Tosteson, D.C. (1976) Biochemistry 15, 786—774
13 Pinkerton, M., Steinrauf, L.K. and Dawkins, P. (1969) Biochem. Biophys. Res. Commun. 35, 512—518
14 Ting-Beall, H.P., Tosteson, M.T., Gisin, B.F. and Tosteson, D.C. (1974) J. Gen. Physiol. 63, 492—508
15 Benz, R., Gisin, B.F., Ting-Beall, H.P., Tosteson, D.C. and Läuger, P. (1976) Biochim. Biophys. Acta 455, 655—684
16 Andersen, O.S., Lev, A.A., Fuchs, M., Ting-Beall, P. and Tosteson, D.C. (1977) Biophys. J. 17, 211a, Abstr.
17 Gisin, B.F. and Merrifield, R.B. (1972) J. Am. Chem. Soc. 94, 6165—6170
18 Gisin, B.F. (1973) Helv. Chim. Acta 56, 1476—1482
19 Gisin, B.F. and Merrifield, R.B. (1972) J. Am. Chem. Soc. 94, 3102—3106
20 Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149—2154
21 Gisin, B.F., Merrifield, R.B. and Tosteson, D.C. (1969) J. Am. Chem. Soc. 91, 2691—2695
22 Rothe, M. and Mazanek, J. (1972) Angew. Chem. 84, 290—291
23 Khosla, M.C., Smeby, R.R. and Bumpus, F.M. (1972) in Chemistry and Biology of Peptides, Proceedings of the Third American Peptide Symposium (Meienhofer, J., ed.), pp. 277—230, Ann Arbor Science Publishers, Inc., Ann Arbor, Mich.
24 Gisin, B.F. (1972) Anal. Chim. Acta 58, 248—249
25 Scotchler, J., Lozier, R. and Robinson, A.B. (1970) J. Org. Chem. 35, 3151—3152
26 Blaha, K. and Rudinger, J. (1965) Collect. Czech. Chem. Commun. 30, 3325—3332
27 Pataki, G. (1966) Dünnschichtchromatographie in der Aminosäure und Peptidchemie, Walter de Gruyter and Co., Berlin
28 Loey, B. and Goodman, M.M. (1970) Prog. Sept. Pur. 3, 73—95
29 Schnabel, E. (1967) Justus Liebig's Ann. Chem. 702, 188—196
30 Wieland, Th. and Birr, Ch. (1972) Justus Liebig's Ann. Chem. 757, 136—146
31 Eisenman, G., Ciani, S. and Szabo, G. (1969) J. Membrane Biol. 1, 294—345
32 Andreoli, T.E., Bangham, J.A. and Tosteson, D.C. (1967) J. Gen. Physiol. 50, 1729—1749
33 Andreoli, T.E. and Troutman, S.L. (1971) J. Gen. Physiol. 57, 464—478
34 Haynes, D.H. and Pressman, B.C. (1974) J. Membrane Biol. 18, 1—21
35 Grell, E., Funck, Th. and Eggers, F. (1972) in Molecular Mechanisms of Antibiotic Action on Protein Biosynthesis and Membranes (Munoz, E., Garcia-Ferrandiz, F. and Vasquez, D., ed.), pp. 646—685, Elsevier, Amsterdam
36 Funck, Th., Eggers, F. and Grell, E. (1972) Chimia 26, 637—641

37 Tosteson, D.C. (1968) Fed. Proc. 27, 1269—1277
38 Pedersen, C.J. (1967) J. Am. Chem. Soc. 89, 2495—2496
39 Stark, G., Ketterer, B., Benz, R. and Läuger, P. (1971) Biophys. J. 11, 981—994
40 Stark, G. and Benz, R. (1970) J. Membrane Biol. 5, 133—153
41 IUPAC-IUB Commision on Biochemical Nomenclature (1971) J. Biol. Chem. 247, 977—983
42 Davis, D.G. and Tosteson, D.C. (1975) Biochemistry 14, 3962—3969
43 Ivanov, V.T., Laine, I.A., Abdulaev, N.D., Senyavina, L.B., Popov, E.M., Ovchinnikov, Yu.A. and Shemyakin, M.M. (1969) Biochem. Biophys. Res. Commun. 34, 803—811