MODE OF ACTION OF MONAMYCIN. EVIDENCE FOR THE FORMATION OF A COMPLEX BETWEEN THE MONAMYCIN CYCLODEPSIPEPTIDE ANTIBIOTICS AND SOME CATIONS IN SOLUTION.

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SUMMARY

The structural relationships of the monamycins to the ionophorous and the surface active peptide antibiotics are briefly discussed. Micro-electrophoresis, microbiological assay data and gel formation suggest that the monamycins complex with K^+, Rb^+ Cs^+ but not readily with Na^+ or Li^+ ions. Their antibacterial action seems due, however, to their lytic effects on cell membranes rather than to any ion transporting properties.

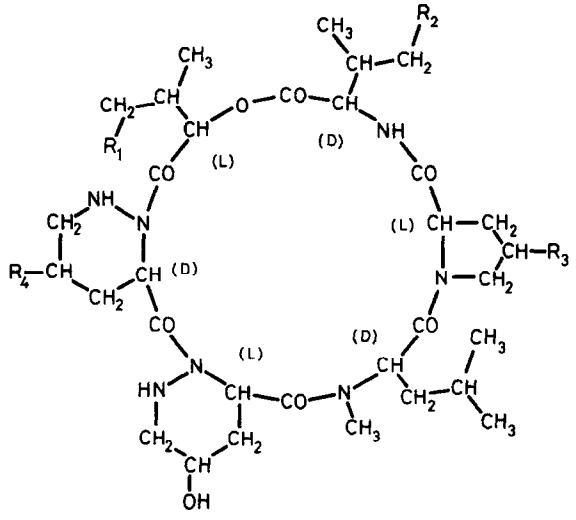
INTRODUCTION

Cation transport by valinomycin, the enniatins and the actins in biological and artificial membrane systems are now well established phenomena (1 - 4). These properties result, in part, from their cyclic structures which enable them to form highly specific complexes with monovalent cations (5 - 8).

The monamycins are a new series of cyclohexadepsipeptide antibiotics (9). All are composed of one hydroxy acid and five amino-acids, the configurations of which alternate LDLDLD around an eighteen membered ring (Figure 1). Mode of action studies provided evidence that they formed complexes with potassium, rubidium and caesium ions. This communication describes this evidence and discusses the possible relationship of the chelate to the antibiotic activity.

MATERIALS AND METHODS

Monamycin, isolated from culture broths of Streptomyces jamaicensis, was purified by differential crystallisation and countercurrent



Monamycin D_1 $R_1 = CH_3, R_2 = H, R_3 = CH_3, R_4 = H$.

(Substitutions $R_1 = H$ or CH_3; $R_2 = H$ or CH_3; $R_3 = H$ or CH_3; $R_4 = H$ or Cl)

Figure 1. Structure of the monamycin cyclodepsipeptide antibiotics.

distribution (Hall, M. J. and Hassall C. H. - to be published). Two products were used in these studies; unchlorinated monamycin D_1 (M.W. = 691) contaminated with smaller amounts of monamycins A - F and the chlorine containing monamycin H_1 (M.W. = 725) contaminated with small amounts of monamycins G and I (see ref. 9). The antibiotics were added as ethanolic solutions and controls using ethanol alone were incorporated into each experiment.

The micro-electrophoresis apparatus, modified from that of Gittens and James (10), was calibrated using human erythrocytes which have a known mobility of 1.31 microns/sec/volt/cm. when suspended in M/15 phosphate buffer at pH 7.35 (11). All measurements were made at 25°C at the upper stationary layer, the position of which had been previously determined (12). The potential gradient across the rectangular observation cell was between 5 and 10 volts/cm. calculated by the method of Moyer(13). Electrode compartments were filled with either 3.5M-KCl or 3.5M-NaCl. Bacterial suspensions were prepared

by centrifuging late log-phase cells of Staphylococcus aureus (NCTC 6571) for 10 mins. at 2800 g, resuspending the pellet in phosphate buffer (ionic strength 0.01, pH 7.0) recentrifuging and finally resuspending to give 0.1 mg dry wt. of cells per ml. Antibiotic was added before the suspension was introduced into the apparatus. Cetyl pyridinium chloride (CPC) was dissolved in the buffer prior to the final resuspension of the cells. Measurements (25 cells timed over 110 microns) were carried out with the current passing first in one direction and then in the other, thus allowing calculation of the average mobility in microns/sec/volt/cm.

The microbiological assay employed a large plate agar diffusion technique using an 8 x 8 quasi-Latin Square layout (14) and S.aureus NCTC 6571 as test organism. The medium (mannitol agar) had the composition (g/l), yeast extract, 2.5; peptone P (oxoid), 10; lactose, 2; d-mannitol, 10; NaCl, 10; agar, 13; tap water to 1 l. Potassium or sodium salts (0 - 5 g/l) were added as required. 200 ml. of this medium (previously sterilised) was melted, cooled to 45°C, inoculated with 1 ml. of a suspension of log phase S.aureus and poured into a level 12" x 12" glass plate. When set the samples (0.075 ml), were applied on 13 mm. antibiotic assay discs (Whatman) and the plate incubated at 37°C for 18 hours before measurement of the inhibition zones.

Gel formation between monamycin and various inorganic salts was detected by mixing equimolar portions of an ethanolic solution of monamycin with aqueous solutions of the salt. The mixture was then extracted twice with ether (5 ml) the extracts combined and monamycin recovery estimated by assaying.

RESULTS

Micro-electrophoresis. Untreated cells of S.aureus had a mobility of 2.1 ± 0.1 microns/sec/volt/cm. In potassium phosphate buffer, a decrease in mobility began at 30μM antibiotic concentration (Fig. 2a.). Charge reversal occurred at 100μM, the mobility finally stabilising at 300 - 350μM in the reverse direction. A very similar curve was obtained for the cationic detergent CPC. Reversal was not observed when a sodium phosphate buffer was used (Fig. 2b) though a slight lowering of mobility occurred at 90μM. Thereafter, mobility decreased only slightly with concentrations up to 700μM. Addition of 0.01M KCl to the sodium phosphate

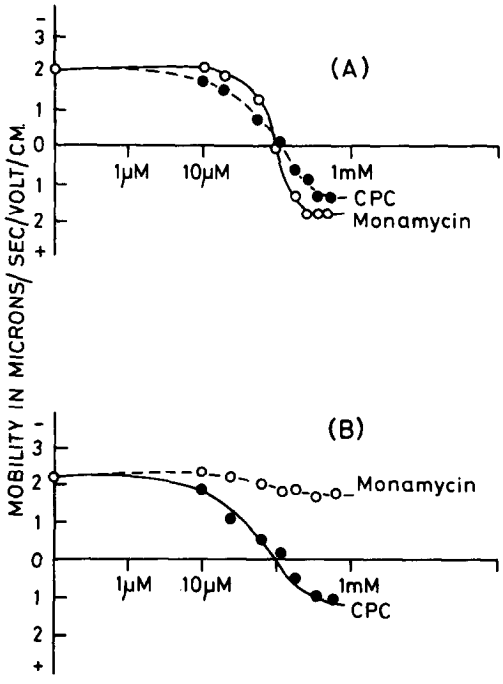


Figure 2. Effect of monamycin (691) on the electrophoretic mobility of S. aureus cells in (a) potassium and (b) sodium phosphate buffers (pH 7.0 at 25°C).

buffer, however, resulted in a reversal of charge similar to that shown in figure 2a. The curve for CPC was not affected by the change in buffers and neither the CPC nor the monamycin curves altered when the pH was lowered to 5.0.

The cell suspensions, initially almost entirely diplococci, were observed to aggregate at a monamycin concentration of 50μM in a potassium phosphate buffer. At 100μM concentration the clumps had attained their maximum size of several hundred cells. The mobility of the aggregates was similar to that of the few remaining isolated diplococci. With sodium phosphate buffer, cells showed little aggregation at any concentration. Slight aggregation was noted with CPC at concentrations above 200μM in both buffers.

Zone development on agar diffusion assay plates. The effects of Li, Na, K, Rb and Cs chlorides on assay zone sizes are shown in Figure 3. Addition of 0 - 5 g/l of LiCl, NaCl or CsCl had no effect on

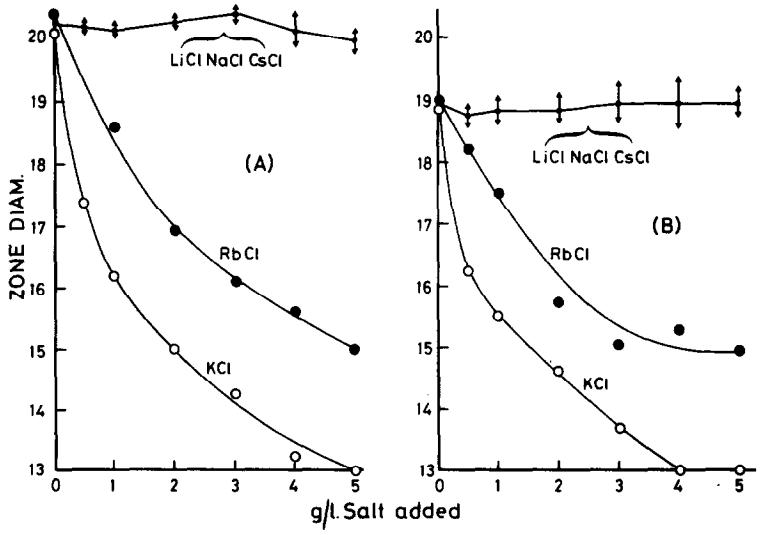


Figure 3. Effect of chlorides of Li, Na, K, Rb and Cs on inhibition zone sizes on microbiological assay plates. (a) monamycin (M.W. 691), (b) chloro-monamycin (M.W. 725)

zone size, while increasing concentrations of KCl (and to a less extent RbCl) progressively reduced the size. Zones were completely eliminated by 5g/l KCl for monamycin D_1 and by 4 g/l KCl for monamycin H_1. RbCl did not totally prevent zone development in either case. Results were similar when K_2HPO_4 or $KHCO_3$ were used in place of KCl.

Gel formation between monamycin and inorganic salts. The mixing of potassium, rubidium or caesium salts with either monamycin resulted in instant gel formation. Sodium salts, $BaCl_2$ and $AgNO_3$ produced a slight floccular precipitate after 10 - 15 minutes. Lithium chloride and all other bivalent salts tested were without effect (Table 1). Extraction of the gels and subsequent assay indicated that 80 - 100% of the antibiotic was bound in solution. Between 10% and 20% was bound in tubes containing a precipitate. In the remainder and in the control tubes 90 - 100% of the monamycin could be recovered.

DISCUSSION

The ionophorus antibiotics fall into two categories, those which induce ion transport and those which abolish or reverse it (15, 16). The former (the enniatins, the actins, valinomycin and the gramicidins) are neutral peptides which lack ionisable groups and possess a regular

alternation of L and D configurations while the latter (nigericin, dianemycin) are monocarboxylic acids. Both groups are lipid soluble and of low molecular weight (500 - 1500). A third category of low molecular weight peptide antibiotics (polymyxins, gramicidin S, bacitracin and tyrocidin) possess surface active properties and cause leakage of cell constituents (including cations) by gross membrane damage. They possess a macrocyclic ring system and basic amino-acid or fatty acid residues such as ornithine, glutamine, asparagine and α-γ-diaminobutyric acid but lack the regular alternation of configurations (17). Their mode of action is similar to the cationic detergents cetyltrimethylammonium bromide (CTAB) and CPC. The positive charge on the basic peptide antibiotics probably results from protonation of their free amino nitrogen atoms. The view that these compounds behave as cationic detergents is supported by the pH dependence of their antibacterial activity, by the observation that some are able to reverse the electrophoretic mobility of bacteria (one of the properties of CTAB and CPC) and by the observed reduction of the surface tension of water by polymyxin and tyrocidin (18 - 21).

The monamycins are somewhat intermediate in structure, in possessing a regular LDLDLD alternation of configurations, a low molecular weight (675 - 739), lipid solubility and an 18 membered ring while also possessing a hydroxyl group and two imino-nitrogen atoms which could, at certain pH values, undergo protonation.

The reversal of electrophoretic mobility by the monamycins in potasssium phosphate buffers and their similarity to CPC in this respect suggested that these antibiotics acted as cationic detergents. This view was supported by the appearance (under electron microscopy) of aggregated bacterial cells (M. J. Hall - to be published). They showed evidence of lysis similar to cells of S.aureus treated with CTAB (22) and Pseudomonas aeruginosa treated with polymyxin (23). However, the failure to reverse mobility in sodium phosphate buffers suggested that the cation played a part in the behaviour of monamycin. The re-establishment of reversal after the addition of KCl confirmed this view and indicated that a positively charged monamycin-potassium complex might be formed. The repression of assay-zone formation by several different salts of potassium suggests a less specific role for the anion. The agglutination of cells was also

Table I. Complex formation between monamycin D_1 and certain inorganic salts and its effect on the subsequent recovery of monamycin by ether extraction. (++ = immediate gel formation, + = floccular precipitate, - = no effect, ppt = slight precipitate).

Salt	Gel Formation	Monamycin recovery (μg/ml)	Ionic radius (Å) of cation	Salt	Gel Formation	Monamycin recovery (μg/ml)	Ionic radius of cation (Å)
K_2HPO_4	++	16	1.33	NaCl	+	84	0.95
KH_2PO_4	++	18	1.33	Na_2HPO_4	+	79	0.95
$KHSO_4$	++	18	1.33	$NaCO_3$	+	79	0.95
KI	++	10	1.33	NaOH	+	87	0.95
KCl	++	8	1.33				
KOH	++	8	1.33	LiCl	-	96	0.60
$KHCO_3$	++	12	1.33				
				$BaCl_2$	ppt	79	1.35
CsCl	++	15	1.69	$AgNO_3$	ppt	68	1.26
				$MgCl_2$	-	93	0.65
RbCl	++	17	1.48	CONTROL*	-	94	-

*The extract of the control should contain 100 μg/ml.
All figures are means of at least 4 determinations.

consistent with the view of the monamycins as cationic agents. It was reported that CTAB caused agglutination of S.aureus at the point of maximum adsorption of the compound, 300 - 400μg/ml (22),and that polymyxin agglutinated cells lacking the surface O antigen (24). Although cyclodepsipeptide molecules have been reported to form aggregates in solution (3), they do not appear to agglutinate bacterial cells.

In the aqueous system employed in the assay a fairly high degree of specificity for K^+ and Rb^+ was suggested. However, the data of Table 1 indicated that some association could occur between monamycin, caesium and possibly sodium in aqueous ethanol. Ivanov et.al. (5) have shown that the conformation of valinomycin in solution varied according to the polarity of the solvent. The differing specificities suggested by the above data for monamycin might also result from different conformations in water and aqueous ethanol. The monamycins and actins have similar specificities for cations, namely $K^+ > Rb^+ = Cs^+ > Na^+ > Li^+$ (16).

The precision relationship of complex formation to antibiosis is not clear. The decreasing size of assay zones shows that either the monamycin-cation complex was insoluble and was failing to reach its site of action or that the complex itself had little or no activity. In one case only (the monamycin H_1/KI complex) was the gel taken to dryness. The resulting powder (analysing for 1 mole of monamycin per mole of $KI.6H_2O$) was insoluble except in dimethylformamide and dimethylsulphoxide, in which it was very slightly soluble (R. B. Morton - private communication). Attempts to assay the complex have, therefore, been unsuccessful.

During an attempt to determine whether the monamycins possessed ion transporting properties, Professor Chappell and his co-workers observed that low concentrations (1 - 5μg/ml) lysed phospho-lipid micelles, bacterial chromatophores and isolated rat liver mitochondria. Dr. P. Mueller found that monamycin lowered the resistance of bimolecular lipid membranes by 100 fold, was not selective between Na^+ and K^+ and was electrically inactive. Thus, despite the apparent complex formation, the data presented suggests a mode of action more akin to the basic peptides than to the ionophorous antibiotics.

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