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# THE AGGREGATION OF ALAMETHICIN

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SUMMARY

Interfacial tension data and ultracentrifugal studies of solutions of the cyclic polypeptide alamethicin indicate that some form of micellisation takes place in aqueous solvents at very low concentrations. The aggregates increase in size as the concentration increases, at fixed ionic strength, up to about 17 monomers per micelle at 0.8 %. The presence of 6 M urea reduces the size to about 5 monomers per micelle and the presence of ethanol or methanol inhibits micellisation. Increase in ionic strength increases the aggregation number and the aggregation is also increased by lowering the pH. These facts are discussed in light of the "bilayer excitation" properties of this substance.

## INTRODUCTION

The cyclic polypeptide antibiotic alamethicin is one of the very few well-characterised molecules known to impart electrical "excitability" to certain bilayers or "black" films of lipid and protein[1]. The effect is similar to that imparted to bilayers by the anonymous material 'EIM'[2] and such systems are at present the best synthetic model of the nerve membrane, in terms of ion translocation and electrical properties in general[3]. The theory of the action of the cyclic polypeptide is based on reversible molecular aggregation and stems from the strongly non-linear (sixth power) dependence of ion conduction on peptide concentration. This dependence demands an aggregated-monomer 'pore' or 'carrier' which is strongly controlled or 'gated' by the electrical potential existing, or applied, across the bilayer membrane. In order to examine the characteristics of the physicochemical aggregation of alamethicin therefore, the studies described below were performed. Recently the extent of interaction with phospholipid has been investigated[4] and the amino-acid sequence determined[5].

## MATERIALS AND METHODS

Water was triply distilled in borosilicate glass, including an alkaline permanganate stage. Salts were 'specpure' (Johnson, Matthy and Co., England). Chloroform and buffer salts were 'Analar' grade. Ethanol, methanol and urea were 'Aristar' grade (British Drug Houses). *n*-Decane was 'spectroscopically pure', and treated with activated aluminia before use. Alamethicin was quoted as being over 99 % pure;

batch No. 8831-CEM-93.2 and CEM.82, from Upjohn Chemical Co., U.S.A. pH measurements were made with an EIL meter, Model 23A, using a miniature electrode.

*Partial specific volume.*

A solution of alamethicin, dried to constant weight at room temperature was dissolved in ethanol (approx. 6 mg/g) and densities determined in an 8.7-ml stoppered pyknometer equilibrated at 25° ± 0.02°. A minimum of 4 weighings of each solution was averaged and the partial specific volume ($\bar{v}$) calculated from

$$\bar{v} = \frac{W_1}{wW_2W_3} \cdot \rho \cdot (W_2 - W_3 + wW_3)$$

in which $W_1$, $W_2$ and $W_3$ are the weights of water, solvent and solution in the pyknometer, $w$ is the weight of alamethicin dissolved in $W_3$ g of solution and $\rho$ the density of water. For alamethicin in ethanol $\bar{v} = 0.820$.

*Specific refractive increment*

A solution of alamethicin (dried to constant weight) was made up in ethanol by weight and its weight/volume concentration determined. The refractive increment was obtained in a Price–Phoenix differential refractometer in which the increment between solvent and solution, $\Delta n$, is proportional to a measured image displacement $\Delta d$. All measurements were made at 20° using light of 546 nm. Found, $\Delta n/\Delta c = 0.1493$ ml/g.

*Sedimentation*

The sedimentation coefficients and molecular weights were determined using a Spinco electrically driven ultracentrifuge with inclined Schlieren optics, the diagonal bar being replaced by a ¼-phase plate. Temperatures were maintained at 25°. Except for those runs intended for measurement of a diffusion coefficient, maximum permitted speeds were used throughout. Sedimentation velocity measurements were made using a 12-mm synthetic boundary cell as designed by PICKELS *et al.*[6]. Sedimentation coefficients were calculated from a least squares straight line plot of log $r$ against $t$ using the formula:

$$s = \frac{1}{W^2 r} \cdot \frac{dr}{dt} = \frac{1}{W^2} \cdot \frac{\Delta \log x}{\Delta t}$$

A computer programme provided by Mrs. G. Gilbert of Birmingham University and modified for use with a Ferranti Mercury computer was used for all routine calculations.

Molecular weights were determined by the sedimentation and diffusion procedure employing Svedberg's equation and confirmed in several instances using the approach-to-equilibrium method described by ARCHIBALD[7].

Diffusion coefficients were measured in a double-sector, synthetic boundary cell, by using the height–area formula[8] and obtaining the slope of a least squares straight line plot of $(A/H^2_{max})$ against $t$. Diffusion coefficients calculated from low speed (10000 rev./min) and high speed (60000 rev./min) runs were not significantly different.

*Interfacial tensions*

Interfacial tensions were determined by the drop detachment method at 30° using mutually saturated aqueous/*n*-decane phases and a 0.75-mm diameter stainless steel orifice. An Agla syringe was employed to measure the volume detached and the tension determined from Tate's Law. Densities of decane and aqueous phases were determined using a 5-ml pyknometer and the corrections of Hawkins and Brown were applied after accurate measurements of orifice diameter by travelling microscope. The drop image was projected at a magnification of × 50 in order to ascertain the critical detachment volume and to detect 'wetting' of the steel nozzle as described[9].

## RESULTS

In low dielectric solvents such as alcohol the material is quite soluble and the sedimentation results shown in Table I indicate that, in absolute ethanol, alamethicin is essentially in the monomeric form at all concentrations. The data show no concentration dependence. Similar values for $s_{20,w}$ are obtained in ethanol containing 10 % water, 10 % aqueous 0.5 M NaCl or 50 % water.

Diffusion coefficients in absolute alcohol have been obtained by the 'height/area' method using the ultracentrifuge results, enabling molecular weight values to be determined, Table II.

An aggregation of alamethicin at higher concentrations in aqueous solvents

TABLE I

SEDIMENTATION COEFFICIENTS IN ETHANOL

| *Concn.* (%, *w/v*) | $s_{20,w}$ | *S.E.* |
|---|---|---|
| 0.1 | 0.3640 | 0.0060 |
| 0.2 | 0.3797 | 0.0087 |
| 0.3 | 0.3622 | 0.0065 |
| 0.4 | 0.3811 | 0.0063 |
| 0.5 | 0.3495 | 0.0080 |
| 0.6 | 0.3689 | 0.0135 |
| 0.8 | 0.3519 | 0.0048 |
| 1.0 | 0.3426 | 0.0048 |
| | $\bar{s}_{20,w} = 0.363$ | |

TABLE II

MOLECULAR WEIGHT IN ETHANOL

| *Concn.* (%, *w/v*) | *Rev./min* | $D_{20,w}$ ($\times 10^{-6}$) | *Mol. wt.* |
|---|---|---|---|
| 0.66 | 59 780 | 1.95 | 1670 |
| 1.00 | 59 780 | 1.83 | 1780 |
| 1.00 | 15 000 | 1.78 | 1840 |
| Theoretical mol. wt. (ref. 5) | | | 1691 |

was shown by ultracentrifugal studies. Thus in phosphate buffer ($I$ = 0.2) at pH 8.0 a concentration dependent plot in the concentration range 0.5–0.2 %, w/v (by serial dilution) gave the results shown in Table III. A significant 'molecular' weight dependence on concentration remained, Table IV, in the presence of urea.

Clearly urea has a definite, though limited, disaggregation influence and does not render the solution of alamethicin monomeric except at the lowest alamethicin concentration.

The influence of ionic strength and electrostatic interaction probably plays a fundamental role in the bilayer 'excitability' property of alamethicin — a function

TABLE III

SEDIMENTATION COEFFICIENTS FOR ALAMETHICIN IN PHOSPHATE BUFFER, pH 8.0 ($I$ = 0.2)

| *Concn.* (%, *w/v*) | $s_{20,w}$ | *S.E.* | *Mol. wt.* | *Aggregation No.* |
|---|---|---|---|---|
| 0.2 | 1.853 | 0.031 | — | — |
| 0.3 | 1.935 | 0.024 | — | — |
| 0.4 | 2.161 | 0.023 | — | — |
| 0.5 | 2.239 | 0.015 | — | — |
| 0.6 | 2.649 | 0.05 | 27 000 | 16.2 |
| 0.8 | 2.787 | 0.062 | 28 000 | 16.8 |

where $s = 1.56 + 1.39\,c$

TABLE IV

SEDIMENTATION COEFFICIENTS IN 6 M UREA/PHOSPHATE, pH 8.0, $I$ = 0.2

| *Concn.* (%, *w/v*) | $s_{20,w}$ | *S.E.* | *Mol. wt.* | *Aggregation No.* |
|---|---|---|---|---|
| 0.15 | 0.474 | 0.2 | 1500 | 1 |
| 0.30 | 0.787 | 0.056 | — | — |
| 0.40 | 1.036 | 0.041 | — | — |
| 0.60 | 1.305 | 0.035 | 6900 | 4.1 |
| 0.80 | 1.376 | 0.046 | 7900 | 4.7 |

where $s = 0.362 + 1.41\,c$

TABLE V

INFLUENCE OF IONIC STRENGTH ON $s_{20,w}$, AQUEOUS SOLUTION

| $I$ $(PO_4)^{3-}$ | *Concn.* (%, *w/v*) | $s_{20,w}$ |
|---|---|---|
| 0.006 | 0.20 | 0.89 |
| 0.200 | 0.20 | 1.56 |
| 0.200 | 0.05 | 0.78 |
| 0.200 | 0.13 | 1.20* |
| 1.200 | 0.13 | 2.25 |

* By interpolation.

which is controllable by an ion gradient or an imposed transmembrane electrical potential. It is clear from Table V that the aggregation of the polypeptide is dependent on ionic environment, the $s$ value increasing considerably over a 200-fold increase in ionic strength. A primary 'charge effect' on the $s$ value cannot be ruled out, however.

In full aqueous systems the monomer is only present at extreme dilutions where detection by ultracentrifugal methods cannot be applied; however, the amphiphilic character of the molecule enables its surface activity to be measured with reasonable accuracy and the 'critical micellar concentration' determined below which the monomeric form exists.

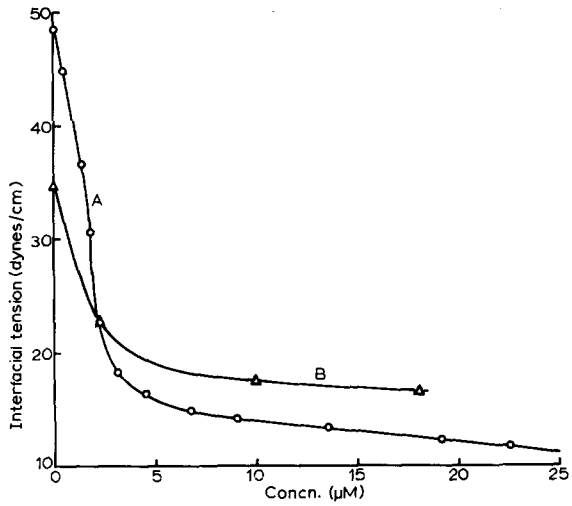


Fig. 1. Interfacial tension ($\gamma_a$) curves of alamethicin solutions *vs.* *n*-decane at 30° and pH 8.0. Aqueous phase: A, 0.05 M KCl; B, 6 M urea.

Previous work has shown that alamethicin is highly surface active[1] and this was quantitatively confirmed in the present study of the decane–water interface. Fig. 1, Curve A, shows the variation in the interfacial tension where the aqueous phase is dilute NaOH at pH 8.0 and 30°. In this phase the critical micellar concentration determined from the curve is 2.4 $\mu$M.

In the presence of 6 M urea the lowering of interfacial tension is much less marked (Curve B, Fig. 1) but the critical micellar concentration is not much altered; however, the 'minimum' tension value is increased by several dynes.

## DISCUSSION

The amino acid composition of alamethicin[1] is predominantly of hydrophobic residues. Thus only about 17 % of the content comprises relatively polar components, compared to about 45 % in most normal proteins[10]. Nevertheless the molecule is relatively soluble in aqueous solvents at high pH and electrometric titration has shown the presence of an acidic group with a pK of 5.5[1]. This was confirmed in the present work by titration in the presence of 50 % ethanol. In this case the $pK_a$ is approx. 5.8, as previously reported[4].

The sedimentation analysis as a whole indicates that the alamethicin is present

as a reasonably homogeneous molecular weight entity, only one peak being detected throughout (Fig. 2). This confirmed the experience of PAYNE *et al.*[5] during chromatographic investigations of the amino-acid sequence although they report a discrepancy with the original analysis[1] of one methylalanine residue, giving an average molecular weight of 1690.5. This compares very favourably with the weight determined here in alcohol at low concentrations. Recent thin-layer chromatography analysis, however, shows that at least two additional components of similar amino acid content are present in significant quantity[11]. The belief that the molecular characteristics, weight and shape, are very similar is supported by the lack of discrimination in the ultracentrifugal analysis. This microheterogeneity is typical of polypeptides synthesised by non-ribosomal routes[12].

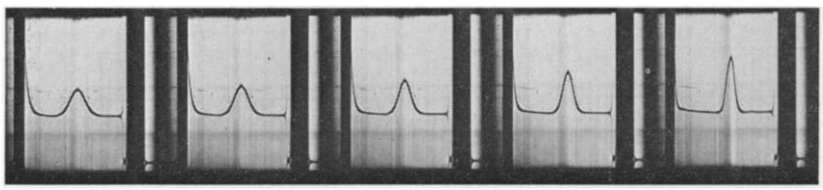

Fig. 2. Analytical ultracentrifuge profile of alamethicin sedimenting in absolute ethanol. 0.6% (w/v) alamethicin at 59780 rev./min; $t = 25°$; interval between frames, 8 min.

The noticeable and steady drop in interfacial tension above the critical micellar concentration must be due to additional alamethicin molecules packing into the interface and causing progressive folding of the ring with removal of portions of it further into the oil phase[4]. This may arise either from some concentration dependence of the monomer-micelle equilibrium 'constant' (*i.e.* if the micelle size or structure varied with concentration) or from an appreciable amphipathic property of the micelle. This latter property is contrary to that found for soap micelles but is consistent with the postulated mechanisms of alamethicin 'pore' action[3]. The adsorbed film pressure, $\pi = \gamma_w - \gamma_a$, = 33 dynes/cm, at the critical micellar concentration and the very low value of the critical concentration reflect the highly amphipathic nature of the monomeric molecule and the strong tendency to aggregate by 'hydrophobic' forces at extreme dilutions. Again this is in accord with its property of effecting multiple ion transport at low concentrations where it is highly aggregated in aqueous substrate and relatively unaffected by 6 M urea.

Micelles in general are also stabilised by mutual binding to metal ions and by reduction in carboxyl electrostatic interaction on increase of the solvent ionic strength, see Table V. Aggregation would thus be facilitated on the high side of an ion gradient and decreased on the low side and so long as such a gradient existed the difference in free energy between the two configurational states of the 'pore' or 'carrier' system could result in a net transfer of ions across the gradient if such ions were bound to the micellar structure. This transfer rate would depend on the magnitude of any potential applied across the system, such as an additional source of energy operating on the 'field receptor' (which in alamethicin is likely to be the glutamine–glutamic acid side chain). Thus the 'ion-gate' might be closed or reversed by a potential difference which moved the alamethicin ion chelate against the ion gradient either bodily or conformationally.

The sedimentation data suggest that although some hydrogen bonding aggregation occurs the inherent aggregation is probably due to entropic forces and this conclusion is supported by model building studies. These indicate that the normal H-bonded parallel or anti-parallel pleated sheet structures are not possible for stacked alamethicin monomers due to the presence of the methylalanine residues. Fig. 3 illustrates this point, showing three stacked molecules arranged in an anti-parallel pleated sheet 'pore' format, where the –N–H–O nearest approach distance is above 3 Å. Such a dynamic mechanism as that postulated by MUELLER AND RUDIN[3] would require a low activation energy which might be provided by rather weak bonding of sub-units, particularly if the dissembly of the 'pore' did not involve a highly cooperative bond-breaking mechanism. This weak association would also tend to give rise to the nonuniqueness of the micelle size, or aggregate number, as reflected in the interfacial tension data and therefore, although higher order aggregates may be somewhat H-bonded, the active micelle is more likely to be associated hydrophobically in a quite small aggregate, such as a dimer. The alamethicin ring can be orientated such that one 'face' is largely hydrophobic and the other hydrophilic

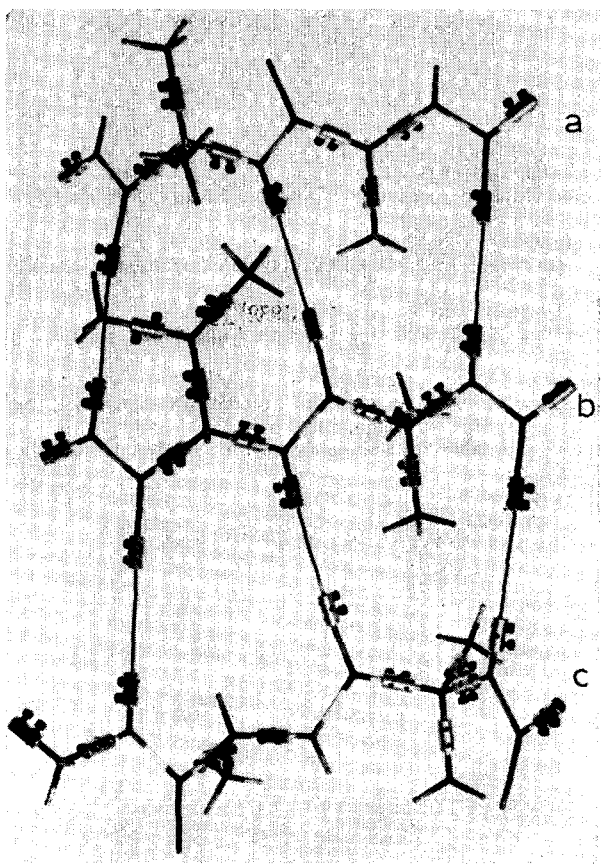


Fig. 3. Portions of three 'stacked' alamethicin rings (a, b, c) arranged in a H-bonded $\beta$-sheet structure. Van der Waals distances between $\alpha$-carbon groups of the methylalanine and of neighbouring residues on adjacent molecules are impossibly small.

giving the possibility of a dimer bonded by hydrophobic (entropic) interaction. Higher order micelles might possibly be formed by limited H-bond formation between 'outer' imide–carboxyl interaction. The H-bonding effect suggested by the influence of urea on the ultracentrifugal characteristics could, on the other hand, be intramolecular. Thus the micellar format could be composed of partly helical alamethicin molecules, a reasonable interpretation of the interfacial tension and optical data (A. I. McMULLEN[11] and A. I. McMULLEN, I. MARLBOROUGH AND P. BAYLEY[13]). Here the stability of the micelle would be affected indirectly by the change in configuration of the individual molecules from a partly helical content, under the influence of urea, to a more random conformation, allowing the dimer or other 'stacked' system to form in more polar environment.

It seems possible, therefore, that the obvious structure of a series of stacked rings within the membrane may not be correct and consideration should be given to the importance of some 'helical molecule aggregate' as postulated above.

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