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SOLUTION AND INTERFACIAL PROPERTIES OF GRAMICIDIN PERTINENT TO ITS EFFECT ON MEMBRANES

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SUMMARY

The behavior of the surface-active material Gramicidin A at the air–water interface and in various solvents was examined.

1. Absorbance at 283 nm as a function of concentration shows that Beer's law is obeyed in the range of 10^{-6} to $5 \cdot 10^{-5}$ M gramicidin in ethanol. Peak fluorescence intensity is linear with gramicidin concentration in ethanol from 10^{-8} to 10^{-6} M.

2. In heptane–water partition experiments, only a very small amount of gramicidin could be detected in either phase but as much as 80 % of the initial gramicidin could be recovered on the experimental vessel's walls. In this regard, the solubility of gramicidin in hexane was found to be less than $1 \cdot 10^{-6}$ M. Thus, gramicidin does not partition as favorably in media of low dielectric constant as do the presumed shuttle carriers, valinomycin and monactin. Solubility in aqueous media is also limited and leads to the growth of aggregates as indicated by increases in light scattering of gramicidin in water as compared to ethanol and by visible turbidity at concentrations above $5 \cdot 10^{-7}$ M.

3. Gramicidin will form monolayers at the air–water interface. A force–area curve of gramicidin shows an initial rise at 350 Å^2, a gentle plateau at 12–16 dynes/cm and 150–180 Å^2 with an imprecise collapse at 18–23 dynes/cm and 130 $\pm$ 10 Å^2. The latter molecular area is in agreement with the cross-sectional area of the helix proposed by D. W. URRY (*Proc. Natl. Acad. Sci. U.S.*, 68 (1971) 672) if the helix is assumed to be perpendicular to the interface. Gramicidin monolayers do not exhibit an elevated surface potential as does valinomycin in the presence of a 1 M KCl subphase.

4. The rate of penetration by gramicidin of a lecithin monolayer and the adsorption of gramicidin at the air–water interface approach proportionality with subphase concentration at concentrations below $1 \cdot 10^{-7}$ M. The rate of adsorption at the air–water interface falls off strikingly with time, while penetration into lecithin monolayers is initially smaller but constant in time. The first-order lecithin monolayer penetration kinetics are consistent with the proposed intramembrane dimerization of gramicidin to form ion-conducting channels.

* This work will constitute part of a thesis to be presented by G. Kemp to the Graduate School of the State University of New York at Buffalo in partial fulfillment of the requirements for the Ph.D. degree.

5. In conclusion, the monolayer data are consistent with the channel mechanism proposed by URRY, and the partition experiments are in contrast to the partition properties of the presumed shuttle carriers.

INTRODUCTION

Gramicidin, isolated from *Bacillus brevis*, consists of a mixture of four related linear pentadecapeptides composed largely of hydrophobic and aromatic residues having formyl and ethanolamine end groups[1,2]. It is known to promote cation permeability changes in both natural and artificial membranes[3–7]. Recently, URRY[8], using the collaborative bilayer transport studies of GOODALL[9], has proposed that gramicidin dimerizes to form helical, ion-conducting transmembrane channels on the basis of peptide conformation studies.

In the course of investigating gramicidin's effect on both natural and model membranes, we have studied its behavior at the air–water interface and in various solvents. We report here the results of these studies and their relevance to the mechanism by which gramicidin increases membrane conductance.

MATERIALS AND METHODS

Gramicidin was obtained from Nutritional Biochemicals, Cleveland, Ohio, and consists of 72 % Gramicidin A, 9 % Gramicidin B, and 19 % Gramicidin C, based upon nuclear magnetic resonance analysis of tryptophan, phenylalanine, and tyrosine content, respectively. (D. W. URRY, personal communication). Valinomycin was obtained from California Biochemical Corporation.

Reagent grade inorganic salts were used throughout the experiments and were roasted at 500° for 4 h to remove traces of organic impurities for the monolayer experiments.

Absorption spectra were measured with a Cary Model 14 Spectrophotometer. Ultraviolet emission spectra were measured with an Aminco-Bowman Spectrophotofluorimeter.

Surface pressure and potential measurements were made using a sliding barrier Langmuir trough constructed of teflon and measuring 30 cm × 5 cm × 2 cm. The measurement of surface pressure and potential have been described previously[10]. All parameters were measured by compression rather than successive addition to a fixed area. Surface adsorption studies were carried out by sweeping the surface clean with the barrier, injecting an ethanolic solution of gramicidin to the stirred subphase next to the stirring bar, and then quickly sweeping the surface to remove any gramicidin that might have been deposited on the surface during the addition process.

The monolayer penetration studies were carried out as follows. The trough was stirred by two magnetic stirrers, one at each end. The lecithin monolayer was formed on the upper end of the trough. An ethanolic concentrate of the gramicidin solution was introduced into the subphase at the lower end of the trough, where the stirring was sufficiently rapid to facilitate thorough mixing.

RESULTS

Gramicidin in alcoholic and aliphatic hydrocarbon solutions

The lower chain alcohols are good solvents for gramicidin. In ethanol, SARGES AND WITKOP[2] have shown that gramicidin exists as a monomer. Ultraviolet absorption and fluorescence spectra of gramicidin in this solvent are typical of tryptophan-containing peptides as shown in Fig. 1a, b. Absorbance at 283 nm as a function of concentration show that Beer's Law is obeyed by $1 \cdot 10^{-6}$ M to $5 \cdot 10^{-5}$ M Gramicidin in ethanol; fluorescence spectra show that the peak fluorescence from $1 \cdot 10^{-8}$ to $1 \cdot 10^{-6}$ M Gramicidin in ethanol is linear with concentration. Thus, convenient concentration assays can be accomplished in ethanol.

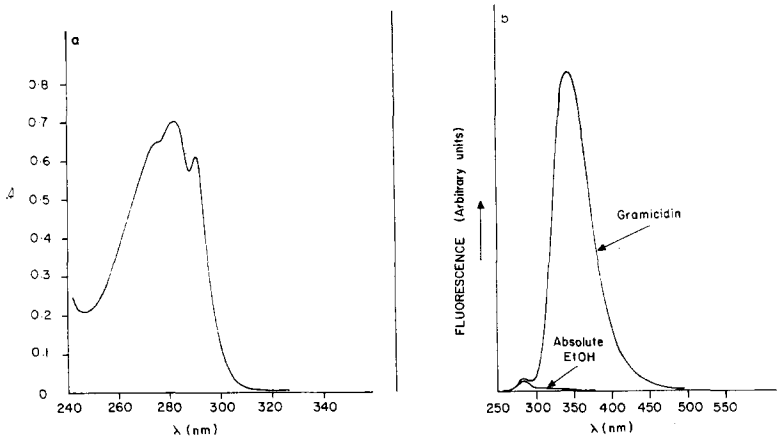

Fig. 1. (a) Ultraviolet absorption spectrum of $2.8 \cdot 10^{-5}$ M gramicidin in ethanol (EtOH). Spectrum recorded with a Cary 14 spectrophotometer. (b) Ultraviolet fluorescence spectrum (uncorrected) of $1 \cdot 10^{-6}$ M gramicidin in ethanol. Spectrum recorded with an Aminco-Bowman spectrophotofluorimeter. Excitation wavelength: 280 nm.

In attempts to determine the partitioning of gramicidin between an aqueous or hydrocarbon phase, a low solubility in either water or heptane was observed. Gramicidin (20 μl of 2.0 mg gramicidin/1.0 ml of ethanol) was introduced into 8.0 ml of either water or an aqueous solution containing 5 mM KCl in separate pyrex erlenmeyer flasks, and an equal volume of heptane was added to each solution. The flasks were shaken overnight but no gramicidin could be detected in either phase using the absorbance assay at 283 nm. The detection limit of gramicidin was $1 \cdot 10^{-6}$ M. These findings suggested that the major portion of the gramicidin was present on the walls of the vessel or at the interface. In confirmation of this suggestion as much as 80% of the unaccounted for gramicidin could be detected upon dissolution of the wall residue in ethanol following decantation of both organic and aqueous phases.

The solubility of gramicidin in aliphatic hydrocarbons was then tested under conditions where the surface area/volume ratio was low, and it was confirmed that gramicidin is quite insoluble in hexane. Additions of sufficient gramicidin in an ethanolic concentrate to make a final concentration of $5 \cdot 10^{-5}$ M and subsequent filtration (0.7 μm pore size) after 2 h leaves no detectable optical density at 283 nm in the

filtrate. Thus, the gramicidin solubility in hexane is less than $1 \cdot 10^{-6}$ M, the detection limit.

While presumed shuttle carriers as nonactin or valinomycin partition favorably in media of low dielectric constant[11–13], gramicidin does not, which suggests that it may act by a different mechanism. In addition, the step conductance experiments of HLADKY AND HAYDON[14], show that gramicidin but not nonactin causes discrete increases in conductance which last longer and are larger than would be expected for a shuttle carrier mechanism in which one or a few ions are transferred in a single trip.

Gramicidin in aqueous solution

The solubility of gramicidin A in aqueous media is also limited[15], presumably due to the abundance of hydrophobic residues. This insolubility leads to a variety of rapid interfacial interactions and self association. We have noted wall residues of gramicidin appearing within minutes in the ethanol washes of quartz, polycarbonate, polyallomer and teflon vessels which had been exposed to aqueous gramicidin solutions for definite times and then aspirated to dryness. A summary of these results appears in Table I. In addition, the growth of aggregates is indicated by changes in the ultraviolet emission spectrum of gramicidin in water as compared to ethanol

TABLE I

ADSORPTION OF GRAMICIDIN TO VARIOUS AQUEOUS–SOLID INTERFACES

At time 0, concentrated gramicidin in ethanol was added to 0.1 M NaCl in the vessel and mixed to give an initial concentration of $1.8 \cdot 10^{-6}$ M. After the desired time, the suspension was quickly aspirated from the vessel until dryness was achieved and an equal volume of ethanol was added back to vessel. After 15 min the ethanol was assayed for gramicidin by fluorimetry. For a control, the same procedure was followed except that the original solution was ethanolic. Control residues averaged 0.6% of the initial gramicidin added. The residue located at the polymer–water interface was assumed to be the difference of the experimental and control values. Apparent surface area to volume ratios were arranged to be approximately equal for each material tested.

Time after gramicidin addition (min)	*Residue of gramicidin left in vessel in % of initial concentration*			
	Quartz	*Polycarbonate**	*Polyallomer**	*Teflon**
1	0.26	3.5	5.4	10.3
5	0.61	4.6	5.7	12.3
60	1.08	6.1	8.3	6.8

* Nominal polymeric composition of the centrifuge tubes used as vessels. Polymer tubes were soaked in ethanol for 36 h prior to experiment to remove any contaminants which would interfere with fluorescence assay.

(Table II) and by visible turbidity at concentrations above about $5 \cdot 10^{-7}$ M. Upon the addition of a few microliters of concentrated gramicidin in ethanol to 0.1 M KCl solution to give a final concentration of $6.2 \cdot 10^{-7}$ M, the scattered intensity at 280 nm (excitation at 280 nm) undergoes an increase of about a factor of 2 relative to the ethanolic value essentially within the time of mixing ($<$ 10 sec). This 2-fold increase in scattered intensity suggests that the initial aggregates formed are dimers assuming they scatter according to Rayleigh's law. In addition, the fluorescent emission at 350 nm is diminished by a factor of about 4. This decrease can presumably be accounted for by increased quenching of the tryptophan residue emission in switching from

TABLE II

CHANGES IN GRAMICIDIN EMISSION SPECTRUM UPON THE ADDITION OF GRAMICIDIN IN AN ETHANOLIC CONCENTRATE TO 0.1 M KCl

Uncorrected spectra recorded with the Aminco Bowman spectrophotofluorimeter at desired times after addition of concentrated gramicidin in 10 μl of absolute ethanol to 2.0 ml of 0.1 M KCl solution to make a final concentration of $6.2 \cdot 10^{-7}$ M gramicidin. The addition was followed by vigorous mechanical stirring.

Time after adding concentrated gramicidin	*Fluorescent intensity at emission maximum** (arbitrary units)*	*Scattered intensity at exciting wavelength, 280 nm (arbitrary units)*
0*	78.0	7
10 sec	19.0	13.0
1 min	15.4	12.5
25 min	14.5	12.0

* The control value at time zero is given by the addition of gramicidin to absolute ethanol.
** Pure solvent scattering and emission has been subtracted.

ethanolic to aqueous solvents[16] and possibly, by additional changes in extinction and quantum yield due to aqueous aggregation. When similar ethanolic additions are made to give final gramicidin concentrations above 10^{-6} M, visible aggregation occurs immediately. We have observed aggregation in twice distilled water, 0.1 M salt solutions, or 1 M ammonium hydroxide. After extended times the aggregates grow and settle out of the suspension. It should be noted that most permeability experiments reported are carried out with a concentration of 10^{-6} M gramicidin or less.

Surface properties of gramicidin

Gramicidin is a surface active material and will form monolayers at the air-water interface. A force area plot of gramicidin is shown in Fig. 2. An initial rise in pressure occurs at an area per molecule of 350 Å^2, and collapse probably occurs at

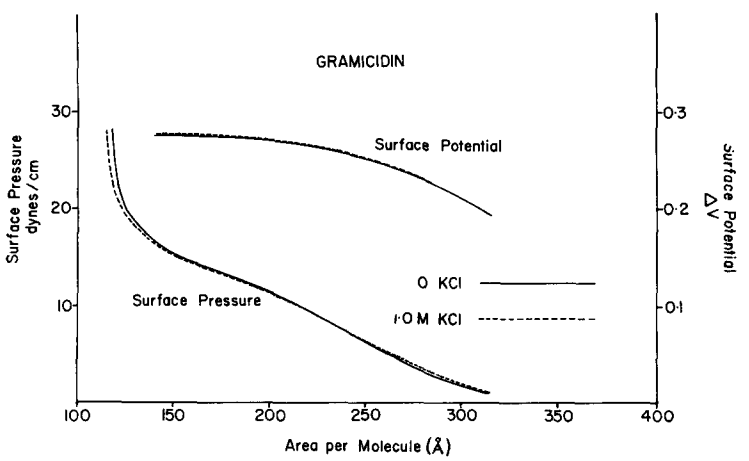

Fig. 2. Surface pressure–area and surface potential–area curves of gramicidin monolayers in the presence and absence of 1.0 M KCl, obtained by compression of gramicidin on a Langmuir trough.

15–18 dynes/cm. The compression is reversible. Unlike most surface active materials, the collapse pressure of the gramicidin film is not easily evaluated. This is due to a slow rate of collapse, and the shape of the force area curve at pressures greater than 20 dynes/cm is therefore dependent upon the rate of compression. A close packed gramicidin film near its collapse pressure has a molecular area of 145 ± 10 Å^2. This is quite consistent with the cross-sectional area of a hybrid between the 4.3_{14} and 4.4_{16} helices proposed as the secondary structure for gramicidin by URRY[8] if the helices are assumed to be perpendicular to the interface. This orientation will allow the molecule to anchor itself to the interface by dipole interactions between the exposed terminal acyl oxygens and the aqueous subphase. It is interesting to note that gramicidin in the presence of high concentrations of KCl does not show the elevated surface potential reported by COLACICCO *et al.*[17] for the shuttle-carrier valinomycin, and as seen in Fig. 3 where 1.0 M KCl was observed to increase the potential to 1.1 V in compressed valinomycin films.

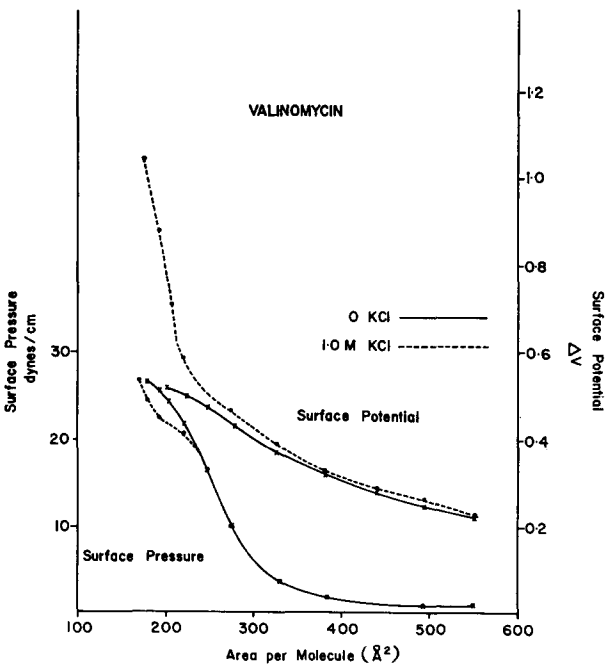

Fig. 3. Surface pressure–area and surface potential–area curves of valinomycin monolayers in the presence and absence of 1.0 M KCl, obtained by addition of consecutive amounts of valinomycin on a Langmuir trough.

In order to better understand the adsorption of gramicidin to membranes, the kinetics of the adsorption at the air–water interface and the penetration into phospholipid monolayers in which it is miscible[18] has been studied. The kinetics of gramicidin adsorption at the air-water interface have been measured in the range of $5 \cdot 10^{-7}$ M to $5 \cdot 10^{-8}$ M, as is shown in Fig. 4. The uptake rate is not linear with time; however, the amount taken up in a given period is nearly directly proportional to the subphase concentration at the lower concentrations used.

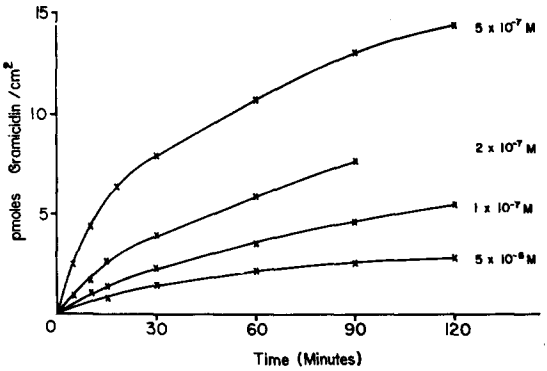

Fig. 4. Rate of gramicidin absorption at the air–water interface. Gramicidin at the designated concentration was introduced into the aqueous subphase and the amount of gramicidin appearing on the surface with time was estimated from a measurement of the area following compression to a designated pressure (2 dynes/cm).

In Fig. 5, the uptake of gramicidin at the air–water interface is compared with the penetration of an egg lecithin monolayer at a pressure of 2 dynes/cm. Again, the penetration rate is approximately proportional to bulk phase concentration. However, in striking contrast to the rate at which gramicidin is adsorbed at the air–water interface, which decreases with time, the rate of penetration of lecithin monolayers is constant with time*. Indeed, if sufficient time is allowed to pass, adsorption at the air–water interface occurs at a rate lower than penetration of the lecithin monolayer at 2 dynes/cm. This result will be discussed later. It is considered that the initial rate of gramicidin adsorption at the air–water interface provides an upper limit

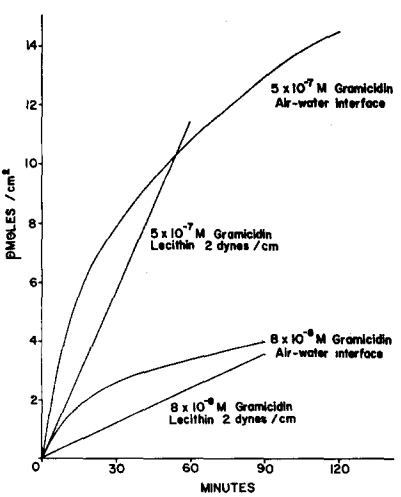

Fig. 5. Rate of gramicidin penetration of lecithin, 2 dynes/cm. The amount of gramicidin appearing at the surface was determined by maintaining constant pressure and measuring the expansion of film area. Mixed gramicidin–lecithin films yield additive areas at 2 dynes/cm.

* In preliminary studies of different phospholipid monolayer compositions at varying film pressures, the rate of penetration has been found to be constant with time, dependent upon phospholipid compositions and to decrease with increasing film pressure.

to the rate at which gramicidin can penetrate a membrane. The presence of a phospholipid film at the interface further hinders the rate of penetration.

In the penetration experiments, it is interesting to note that the rate of gramicidin penetration is very low, indeed much lower than the rate at which cytochrome *c*[19] and other proteins[20] (also D. PAPAHADJOPOULOS AND H. KIMELBERG, personal communication) penetrate the air–water interface. In these experiments it was observed that less than 3 % of the added gramicidin appeared at the interface after 2 h. Although this value depends upon the surface/volume ratio of the vessel used, it is apparent that removal of gramicidin from the subphase by film formation would not substantially decrease the bulk gramicidin concentration during bilayer conductance studies. If these rates are comparable with the rates at which gramicidin penetrates bilayer membranes during conductance measurements, it is apparent that only a very small fraction of gramicidin added to the aqueous phase actually penetrates the membrane to cause the observed conductance changes*.

The presumed shuttle carriers, valinomycin or monactin, establish an equilibrium distribution between the membrane-torus and the bathing solution within 15 min or less when added to the aqueous phase[13]. In contrast to this, the gramicidin-induced conductance of bilayer membranes is still increasing with time one hour after addition[9] (and J. CAPELLA and C. WENNER unpublished results) as would be expected from the time course of gramicidin penetration of phospholipid monolayers.

DISCUSSION

The picture which emerges from the aqueous solubility data is that when an ethanolic gramicidin solution is diluted by an aqueous medium, the ethanolic solvation is lost during the mixing time. This leaves monomeric gramicidin in aqueous solution: a highly unstable species. As a consequence, gramicidin will adsorb to interfaces in the system, and self-associate. The adsorptive processes and probably the formation of small polymers are rapid, being substantially completed on the order of minutes or less, while the large aggregate growth occurs on a longer time scale.

Since penetration proceeds at a constant rate for at least an hour, even at $5 \cdot 10^{-7}$ M initial phase gramicidin concentration, it appears that dimers and perhaps larger aggregates can penetrate these films. On the other hand, there is a decreasing rate of adsorption with time at the air–water interface. To explain these data, it is suggested that only gramicidin monomers and low polymers can be adsorbed to the air–water interface. Since gramicidin is miscible in phospholipid monolayers, it is proposed that phospholipid monolayers are good interfacial solvents for gramicidin in any aggregation state while the dissolution of larger aggregates at the air–water interface is less favorable.

It has been proposed[8,9,14] that the second-order membrane conductance kinetics observed by TOSTESON[7] and GOODALL[9] arise from formation and dissociation of gramicidin dimers within the bilayer membrane. Such a model requires that the penetration of the membrane be first-order with respect to bulk phase gramicidin concentration as we have observed in phospholipid monolayers.

* This may be in marked contrast to valinomycin. If the volume of the membrane-forming solution present is 1 μl and the volume of the cell 10 ml, then approx. 70 % of the added valinomycin would be adsorbed as calculated from the partition coefficient reported by STARK AND BENZ[13].

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REFERENCES

1 L. K. RAMACHANDRAN, *Biochemistry*, 2 (1963) 1138.
2 R. SARGES AND B. WITKOP, *J. Am. Chem. Soc.*, 87 (1965) 2011.
3 B. C. PRESSMAN, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 1076.
4 C. WENNER AND J. HACKNEY, *Biochemistry*, 8 (1967) 930.
5 D. A. HAYDON, *J. Am. Oil Chem. Soc.*, 45 (1968) 230.
6 P. J. F. HENDERSON, J. D. MCGIVAN AND J. B. CHAPPELL, *Biochem. J.*, 111 (1969) 521.
7 D. TOSTESON, T. E. ANDREOLI, M. TIEFFENBERG AND P. COOK, *J. Gen. Physiol.*, 51 (1969) 373.
8 D. W. URRY, *Proc. Natl. Acad. Sci. U.S.*, 68 (1971) 672.
9 M. GOODALL, *Biochim. Biophys. Acta*, 219 (1970) 28.
10 D. PAPAHADJOPOULOS, *Biochim. Biophys. Acta*, 168 (1968) 240.
11 B. C. PRESSMAN, *Fed. Proc.*, 27 (1968) 1283.
12 G. SZABO, G. EISENMAN AND S. M. CIANI, in F. SNELL, *Coral Gables Conference on Physical Principles of Biological Membranes*, Gordon and Breach Publishers, New York, 1970, p. 81.
13 G. STARK AND R. BENZ, *J. Membrane Biol.*, 5 (1971) 133.
14 S. HLADKY AND D. HAYDON, *Nature*, 225 (1970) 451.
15 E. LIBERMAN AND V. TOPALY, *Biochim. Biophys. Acta*, 163 (1968) 125.
16 R. COWGILL, *Biochim. Biophys. Acta*, 133 (1967) 6.
17 G. COLACICCO, E. E. GORDON AND G. BERCHENKO, *Biophys. J.*, 8 (1968) A-22 (Abstracts).
18 G. KEMP, T. DOUGHERTY, K. JACOBSON AND C. E. WENNER, *Biophys. J.*, 11 (1971) 311a.
19 P. J. QUINN AND R. M. C. DAWSON, *Biochem. J.*, 115 (1969) 65.
20 G. COLACICCO, *J. Colloid Sci.*, 29 (1969) 345.