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Infrared spectroscopic studies on gramicidin ion-channels: relation to the mechanisms of anesthesia

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Fourier transform infrared spectroscopic studies are reported on gramicidin ion-channels in phospholipid bilayers and the effects on the spectra of the anesthetics and related compounds (methoxyflurane, halothane, chloroform, carbon tetrachloride, *n*-pentane and *n*-decane) have been determined. The addition of anesthetics containing the 'acidic hydrogen' caused unique changes particularly on the amide I bands at 1639 cm^{-1} and 1670 cm^{-1}. The 1639 cm^{-1} band became more intense while the intensity near 1670 cm^{-1} decreased dramatically. These effects were not observed with carbon tetrachloride, *n*-pentane and *n*-decane. The 1670 cm^{-1} band is interpreted as arising from the carbonyls involved in the head-to-head hydrogen-bonded dimerization where the relationship between chains is analogous to that of the antiparallel β-pleated sheet structure and the anesthetics with 'acidic hydrogens' are considered to disrupt the hydrogen-bonded dimerization by competitive hydrogen bonding to the carbonyls at the head-to-head junction. As the dimer-monomer equilibrium is the 'on-off' mechanism for gramicidin ion-channel conductance, the results are considered in terms of the mechanism of action of anesthetics and are taken to suggest, for certain anesthetics, a hydrogen-bonding role to protein ion-channel components.

Introduction

Until recently the most widely accepted theory of general anesthesia has been the lipid-hydrophobic theory. According to it, anesthetic action takes place in the lipid bilayers of the neuronal membrane. It is, indeed, highly probable that, since there exists a good correlation between lipid solubility and anesthetic potency (the Meyer-Overton rule), anesthetics must be taken up in the lipid part of the membrane in order to exert their action. There, according to current theories, they might disorder or inflate the membrane or perturb lipid phase transitions (For reviews see Refs. 1–4).

However, at clinical concentrations certain anesthetics cause little disorder; also the extent of disordering is dependent on the cholesterol content of the membrane. A sound discussion of the problem is found in recent publications by Miller and his co-workers [5–7]. Exceptions to the Meyer-Overton rule have been found earlier [2].

Now, the functioning of the nervous system is conditioned by the permeability of the membrane to ions. This, in turn, depends on the structure of the ion-channel which is formed by proteins crossing the lipid membrane. It is reasonable to suppose that anesthetic action must perturb the ion-channel. This can be thought to happen either by

Abbreviations: H-bond, hydrogen bond; AAPC, 1-alkyl-(C16:0, C18:0)-2-acetoylphosphatidylcholine; lyso-PC, lysophosphatidylcholine (L-α-lysolecithin); CD, circular dichroism.

direct action on the protein or indirectly by altering the structure of the lipid surrounding the protein which contains the ion-channel. Another possibility is to perturb neurotransmitter release at the synapse by changing conditions for neurotransmitter release from the vesicles within which it is contained. The latter possibility has been recently explored by Bangham [8,9], Nichols [10] and Fassoulaki et al. [11] and their co-workers. The two are of course not mutually exclusive.

In the authors' opinion any attempt to understand the mechanisms of anesthesia on the basis of non-polar or hydrophobic interactions only is bound to lead to a one-sided view. The relationship between lipid solubility and anesthetic potency, while it shows that uptake of the anesthetic in the lipid membrane is a necessary precondition for its action, does not inform us about the site of the action. This may be in the lipid, or at the lipid-protein interface, or within the ion-channel itself. Furthermore, there are good reasons to believe that polar interactions play a role in the mechanisms of anesthesia.

It is significant in this respect that the most 'potent' * general anesthetics are halogen containing compounds. The role of fluorine is to make these molecules inert so that they do not readily undergo chemical reactions in the organism while the other halogens promote anesthetic activity. Now, it has been known for some time that in chlorinated (or brominated) solvents the formation of hydrogen bonds is hindered. For example, when studying by infrared spectroscopy the self-association of alcohols (O-H $\cdots$:O-H $\cdots$:O-H $\cdots$) or amines (N-H $\cdots$:N-) or amides (N-H $\cdots$:O = C) first in a saturated hydrocarbon solvent and then in carbon tetrachloride, it immediately appears that the relative intensity of the free (non H-bonded) OH or NH stretching vibrations is much greater and that of the association (by H-bonding) bands is much weaker in CCl_4 than in hydrocarbons [12–14]. While this is not yet a 'polar interaction', much stronger effects are observed when the solvent is a compound containing the so-called 'acidic hydrogen' as is the case with chloroform, halothane, or methoxyflurane [15–19] **. As it happens, these are some of the most potent general anesthetics. On this ground Sandorfy and his co-workers suggested several years ago that the altering of the free/associated equilibrium in H-bonds could be an important factor in the mechanisms of anesthesia [20–24]. Actually, they were proposing a pluralistic theory: 'weak' anesthetics (with no acidic hydrogen) can act in the lipid phase through mainly non-polar van der Waals interactions and thus perturb the ion-channel indirectly by changing the physical state of the surrounding lipid while the strong anesthetics (which do contain an acidic hydrogen) act on the protein and the ion-channel itself. This proposal has been substantiated by a series of infrared spectroscopic studies [20–25], by quantum chemical calculations of the equilibrium constants and Gibbs' free energies for equilibria such as: N-H $\cdots$:O-C + $CHCl_3 \rightleftarrows Cl_3C$-H $\cdots$:O = C + NH [26–28].

This does not imply that the more polar a molecule is the greater its anesthetic potency. Alcohols, for example, are relatively weak anesthetics. If the H-bond forming ability of a molecule is too great it might well be intercepted by water before reaching the proximity of an ion-channel.

This pluralistic theory and the importance of polar interactions (H-bonds are, of course, eminently polar) has not so far been tested in systems approaching biological or clinical conditions.

The present communication reports infrared spectroscopic results on gramicidin A ion-channels. Gramicidin A, a channel forming pentadecapeptide from *Bacillus brevis* of primary structure HCO-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-NH-$(CH_2)_2$OH [29], and analogs and derivatives of this molecule (see Ref. 30 and references therein) when incorporated into planar bilayers exhibit conductance properties that are

* The terms 'potent' and 'weak' are used for convenience and brevity; they are understood to refer to anesthetics with a high and low ED_{50} value, respectively.

** Davies and co-workers [15,16] published extensive correlations between polar interactions and general anesthetic concentrations for halogenated hydrocarbons.

strikingly similar to those of channels from higher organisms. In particular, the more that is learned of higher organism channel phenomenology (see Ref. 31 and references therein) the more it resembles gramicidin A channel transport including issues of magnitude of single-channel currents, ideality of cation selectivity, ion saturation, competition and block [32–34] and single filing of ion and water [35] as well as the occurrence of channel flickering and substrates [31,36]. The vibrational spectra of gramicidin A have been the object of several publications [37–42]. Most of these concentrated on the conformation of gramicidin A in various media. More important for our present purposes are previous infrared studies on gramicidin A-phospholipid systems [43–49].

In the present work gramicidin A is incorporated in the channel state in 1-alkyl(C16 : 0, C18 : 0)-2-acetoylphosphatidylcholine (AAPC) or L-α-lysophosphatidylcholine. The phospholipid AAPC has the advantage that the OH group is replaced by $OCOCH_3$ making it easier to identify the NH bands. Examined here are the interactions of anesthetics with the model ion-channel by infrared spectroscopy in systems where it has been established that the channel structure is that of two gramicidin molecules, each in a $\beta^{6.3}$ helical conformation, associating amino end to amino end (head-to-head) by means of six hydrogen bonds to form a continuous lipid spanning transmembrane channel about 26 Å in length [50].

Materials and Methods

Naturally occurring gramicidin, the known mixture of Phe^{11}- (9%), Tyr^{11}- (19%) and Trp^{11}-gramicidin A (72%), was purchased from ICN Nutritional Biochemicals Corporation, Cleveland, OH, lyophilized from methanol-water and used without further purification. L-α-Lysophosphatidylcholine (lyso-PC) as a lyophilized powder and 1-alkyl(C16 : 0, C18 : 0)-2-acetoylphosphatidyl choline (AAPC) in chloroform solution were purchased from Avanti Polar Lipids, Inc., Birmingham, AL. Purities of the peptide and the lipids were verified by carbon-13 nuclear magnetic resonance. Gramicidin channels were packed into lyso-PC and AAPC following the procedure previously described [30] using either distilled water or ^2H_2O, 99.87% ^2H (Sci-Graphics, Wayne, NJ). The lipid to channel ratio in all samples was 30 : 1 and 0.5 mM NaCl was added to the solutions before incubation. Channel incorporation was verified by observing the circular dichroism spectra of each preparation. In Fig. 1A is given the spectrum of lyso-PC packaged gramicidin in H_2O which is a curve typical of good channel formation [30]. It should be appreciated that in this state the channels are in a lipid bilayer membrane [30] which is a valued model for cell membranes. The dashed curve represents the spectrum observed when gramicidin is associated with the lipid but is not yet in the proper channel conformation.

When incorporating gramicidin into AAPC some differences from the usual lysophosphatidylcholine samples were observed which are interesting to note. As the AAPC was supplied in chloroform solution, the solvent was initially removed by passing argon gas over the lipid in the incubation tube until nearly dry and then placing the tube under high vacuum overnight, after which no trace of solvent was detected by smell. The incorporation procedure was then carried out as usual; however, the circular dichroism spectrum obtained from this preparation (Fig. 1B, dashed curve), did not show complete channel formation even after extensive heating and sonication. A distinct odor of chloroform could be detected in the sample tube after it had been incubated for some time. Subsequent samples of AAPC packaged gramicidin were prepared by first suspending the lipid in water and sonicating until homogeneous and then lyophilizing to remove all solvent before proceeding. A proper CD spectrum could then be easily obtained (Fig. 1B) and no traces of chloroform could be detected in the sample.

All samples were centrifuged to remove any residual unincorporated gramicidin and the supernatants were taken for the infrared spectroscopic studies. A small aliquot of each sample was dried and resuspended in methanol in order to determine the concentration of channels associated with the lipid. Using $\epsilon_{282} = 45\,000$ l/mol per cm, all of the samples were found to contain 5 mM channels ($\pm 5\%$).

50 μl of an aqueous solution of gramicidin + AAPC or gramicidin + lyso-PC were deposited

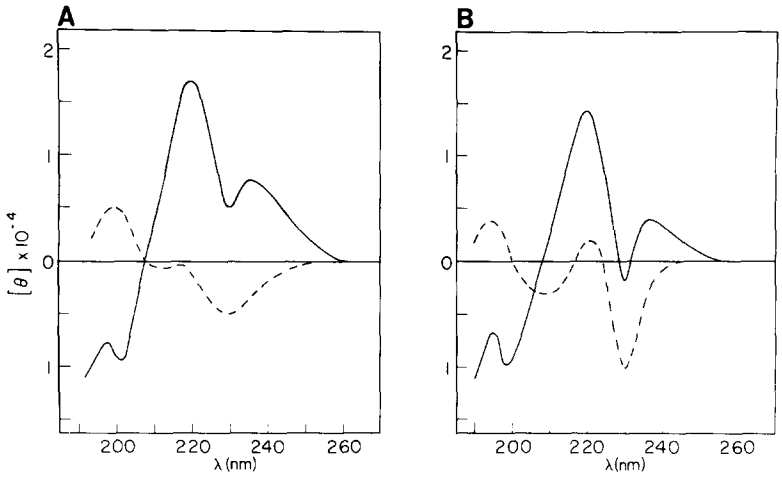

Fig. 1. (A) Circular dichroism spectrum of lysophosphatidylcholine-packaged natural gramicidin A which was used in the infrared studies. A curve such as this indicates that the peptide has achieved the proper channel conformation in the lipid. When gramicidin is associated with the lipid, but has not yet been transformed into the channel conformation, a spectrum represented by the dashed curve is obtained. (B) Circular dichroism spectrum of AAPC-packaged natural gramicidin as in (A). The dashed curve, showing partial incorporation of channels into the lipid, is a spectrum obtained when the sample preparation was attempted with a small amount of chloroform remaining in the lipid.

onto a silver chloride plate using a micropipet. It was let to stand for a day, protected from light, so that excess water could evaporate slowly, leaving a sticky film of hydrated sample. Then for two days it was placed into a desiccator in order to remove as much of the solvent as possible. Even so the sample contained water but less than before, which kept the sample close to biological conditions and, at the same time, made it possible to record the NH-stretching region of the infrared spectrum. When the film was reasonably dry, another AgCl plate was placed on top with a 0.5 mm thick teflon O-ring spacer placed between the two plates in order to help seal the sample and to provide an area for the introduction of anesthetic. The AgCl plates were held firmly together with a screw clamp assembly. After recording the infrared spectrum of the film, the anesthetics and controls were injected with a syringe into the space between the plates through a sealable access port at the bottom of the assembly. The added material was observed to pass evenly over the film without any bubbles and remained in the sample throughout the subsequent infrared scan.

The following anesthetics were applied: [^{2}H]chloroform (Cambridge Isotope Laboratories, 99.8% ^{2}H), halothane (Ayerst), methoxyflurane (Abbott) and enflurane (Ohio Medical Canada). For the sake of comparison carbon tetrachloride (American Chemical), *n*-pentane (Fisher Scientific) and *n*-decane (Philips Petroleum) have also been used. The spectrometer was a Nicolet model 7000 FT-IR instrument with a resolution of 1 cm^{-1}.

Results and Discussion

The NH and CO stretching regions of the spectra will be used to obtain information on the effect of anesthetics on the gramicidin ion-channel. Since the spectra are dependent on the water content of the samples, these were dried sufficiently for the NH bands to become distinguishable. The water bands are still present, however, so that the NH bands are superimposed on a broad water band. The NH stretching bands at 3285 and 3307 cm^{-1}, the carbonyl bands at 1741, 1670 and 1639 cm^{-1} and the NH bending bands at 1552 cm^{-1} will now be considered.

In Fig. 2 the amide NH band at 3285 cm^{-1} is seen to be superimposed on a broader water band. The band at 1741 cm^{-1} correspondings to the ester carbonyl of the lipid. The weak band at 1670 and the stronger band at 1639 cm^{-1} are amide I (mainly carbonyl stretching) bands while the band at 1552 is amide II (mainly NH deformation). With more water (Fig. 3) the complex band at 3285 cm^{-1} becomes broader and the 1670 cm^{-1} band increases in intensity. The latter is probably due to the underlying, increased water bending band.

The effect of methoxyflurane, a powerful anesthetic, is shown in Figs. 4 and 5. The sample for Fig. 4 contained less water and that for Fig. 5 more water. The NH peak moves from 3285 to

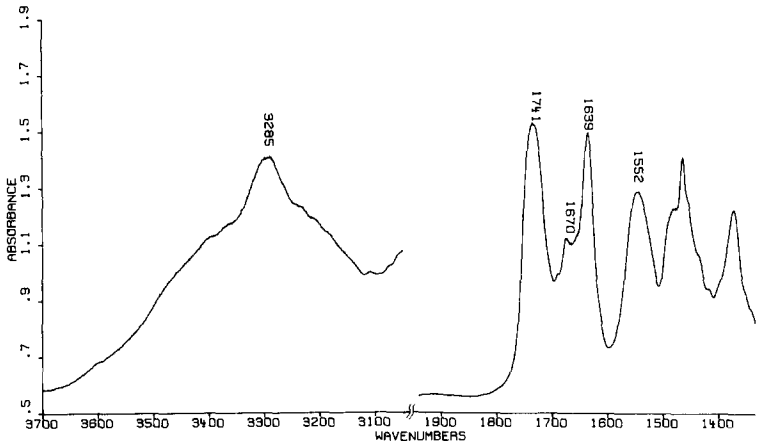

Fig. 2. Parts of the infrared absorption spectrum of a film of AAPC-packaged natural gramicidin.

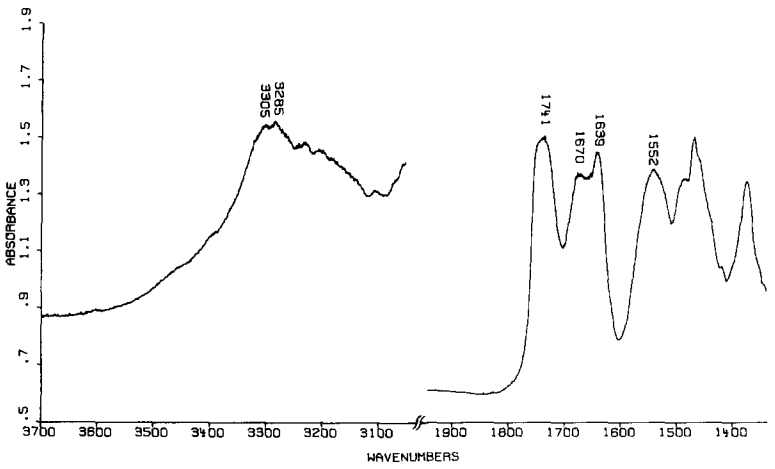

Fig. 3. Same as in Fig. 2 but with more water.

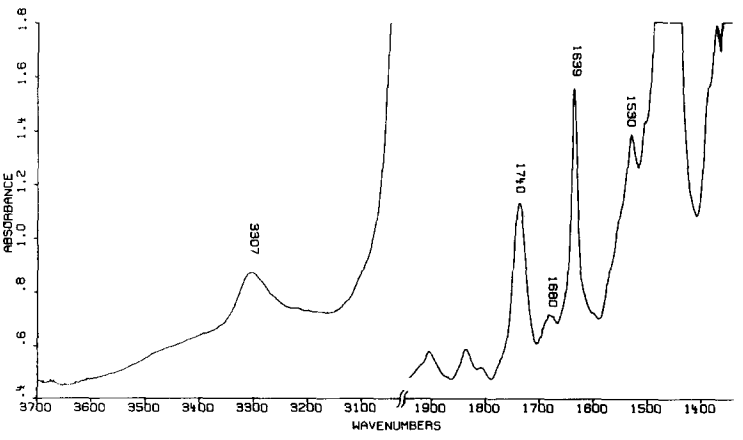

Fig. 4. Parts of the infrared absorption spectrum of a film of AAPC-packaged natural gramicidin in the presence of methoxyflurane.

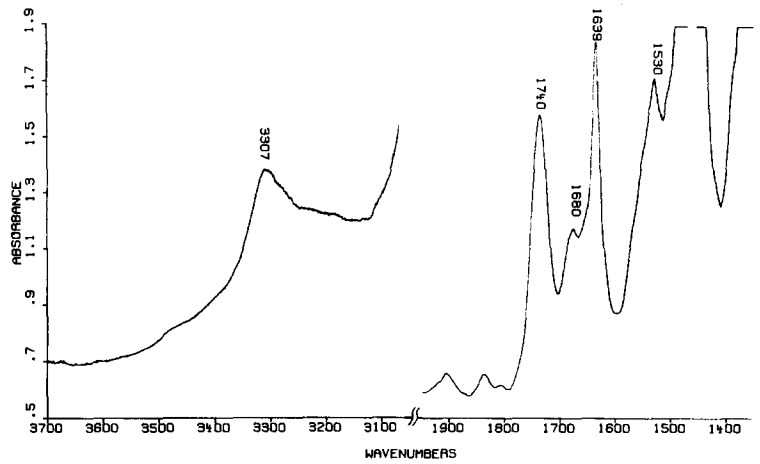

Fig. 5. Same as in Fig. 4 but with more water.

3307 cm^{-1}, indicating less H-bonding of amide NH moieties. The relative intensities of the carbonyl bands undergo significant changes: while the ester band does not seem to be affected, the relative intensities of the two amide I bands are greatly modified, with the 1639 cm^{-1} band gaining intensity the 1670 cm^{-1} band losing intensity and a weak band appearing at 1680 cm^{-1}. No great use can be made of the amide II band, since it is strongly overlapped by an intense band of the anesthetic.

Under these conditions, the assignments of the two amide I bands become essential. A mixture of the phospholipid and the anesthetic does not have a band either at 1670 or at 1639, so these cannot be due to or altered by ester bands. As mentioned above, the broad water deformation band does make a contribution but this cannot account for the much sharper 1670 band. Thus necessarily the 1670 and 1639 cm^{-1} bands are assigned to the amide type carbonyls of gramicidin. Now, according to the known structure of gramicidin A in this system [50] three types of H $\cdots$:O = C bonds are distinguished therein. At the two ends of the channel there are three C = O and three NH groups which are not H-bonded to each other but which would be weakly associated with water. At the head-to-head junction of the two monomers there are six H-bonds with lengths of about 3.20 Å [51]. These are likely to be weak H-bonds, far from linearity. Within each monomer of the channel there are stronger N-H $\cdots$:O = C bonds with N $\cdots$ O distances equal to 2.95 Å [51]. Then it is logical to assign the 1639 cm^{-1} band to the stronger intramonomer H-bonds and the 1670 cm^{-1} band to the weaker H-bonds at the head-to-head junction and, possibly, at the entrance. In fact, of course, the head-to-head junction has an antiparallel arrangement of chains with a hydrogen bonding pattern characteristic of the antiparallel β-pleated sheet which has been repeatedly characterized with a band near 1670 cm^{-1} (Ref. 52 and references therein). Within the monomers of the channel the hydrogen bonding pattern between chains is characteristic of the parallel β-pleated sheet structure which exhibits no band near 1670 cm^{-1} but rather a single amide I band near 1640 cm^{-1} [53].

The perspective, therefore is that the anesthetic can easily perturb the free/associated (i.e. monomer/dimer) equilibrium of the weaker H-bonds; as a consequence, the 1670 cm^{-1} band is greatly weakened. 'Free' (or almost free) NH groups are thus produced which cause the shift of the NH stretching band to 3307 cm^{-1}. In exchange, C-H $\cdots$:O = C type H-bonds are produced with the anesthetic. The proof for this is the presence of an associated C^2H stretching band at 2222 cm^{-1} when C^2HCl_3 is used as the anesthetic (see Fig. 6). Except for the CH, respectively C^2H bands, the spectra are not otherwise influenced by the replacement of H by ^2H. (No other deuterated anesthetics have been available for the study).

According to the foregoing interpretation many of the weaker N-H $\cdots$:O = C bonds are transformed into C-H $\cdots$:O = C. The equilibrium N-

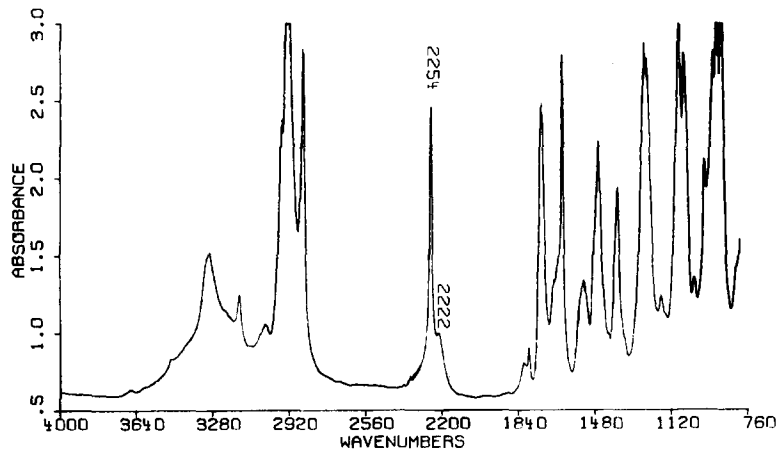

Fig. 6. Infrared absorption spectrum of a film of AAPC-packaged natural gramicidin in the presence of C^2HCl_3.

H · · · :O = C + $CHCl_3$ ⇌ C-H · · · :O = C + NH has been the topic of both theoretical and experimental studies. It could be shown by computing equilibrium constants and Gibbs' free energies [27] and by infrared spectroscopy [20–24] that a high proportion (about 40% at room temperature in the case of the dimer of formamide) of the original H-bonds are replaced by carbonyl/C-H hydrogen bonds even though the latter are weak H-bonds. The carbonyl band corresponding to them might be in near coincidence with the 1639 cm^{-1} band boosting its apparent intensity. Perfect coincidence is not expected, however, and therefore a closer look was taken at the 1639 cm^{-1} band. When an anesthetic is added to the sample, a new shoulder appears at about 1650–1660 cm^{-1} on the high frequency side of the 1639 cm^{-1} band. It is best seen when C^2HCl_3 is used as the anesthetic (Fig. 6). It has also been observed in $CHCl_3$ that the amide II (NH bending) band shifts by about 15 cm^{-1} to lower frequencies. This is in keeping with the high frequency shift of the NH band and the opening and weakening of a part of the H-bonds. methoxyflurane, halothane and chloroform were each used as anesthetics and each gave similar results. For obtaining a check on these results carbon tetrachloride, *n*-pentane and *n*-decane have also been used. CCl_4 and *n*-pentane have only a very slight effect on the relative intensities of the 1670 and 1639 cm^{-1} bands (data not included) whereas *n*-decane has none (Fig. 7). They do not influence the NH stretching and bending bands either. This is just what had been expected in view of the weak anesthetic potency of these compounds.

In a most important contribution Hladky and

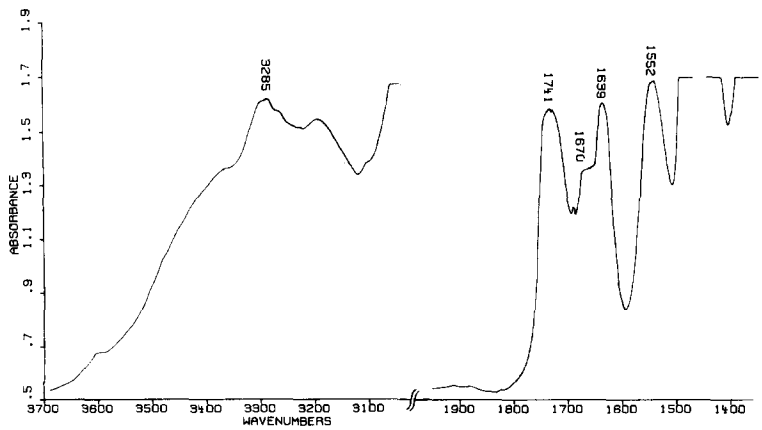

Fig. 7. Part of the infrared absorption spectrum of a film of AAPC-packaged natural gramicidin in the presence of *n*-decane.

Haydon were the first to demonstrate and characterize the channel-forming properties of gramicidin [54,55]. Since that time Haydon and co-workers [56–58] have very effectively utilized this model system for developing an understanding of the molecular mechanisms of anesthesia. They have emphasized the neutral anesthetics, the *n*-alkanols and *n*-alkanes, and have presented evidence that *n*-alkanols and *n*-alkanes act to decrease gramicidin channel conductance by destablizing the conducting dimer through increasing lipid bilayer tension but noting at the same time that alkanols could 'affect the strength of the hydrogen bonding between the two parts of the channel dimer'. [58]. The *n*-pentane and *n*-decane results obtained here are entirely consistent with their findings derived from studies on planar bilayers. What has been contributed specifically by the present infrared studies is evidence that chloroform, halothane and methoxyflurane act directly at the head-to-head junction by competitive hydrogen bonding to destablize the dimer. These results are also consistent with the previous demonstration that physiologically relevant concentrations of halothane (concentrations of 1 mM or less in the bathing medium) decrease channel lifetime [59,60].

Conclusions

The obvious (and minimal) conclusion from these results is that potent anesthetics do interfere with the N-H $\cdots$:O = C hydrogen bonds of the gramicidin A ion-channel. This is clearly demonstrated by the quite significant changes in the intensity ratio of the 1670 and 1639 cm^{-1} amide carbonyl bands. It is furthermore very likely that the H-bonds which are mainly affected are the weaker ones at the head-to-head junction and the amide-water H-bonds at the entrances of the channel.

These results are in line with the previous work of Urry and co-workers [59,60] and more recently by Haydon and co-workers [61] who have shown by single-channel current measurements that physiologically used concentrations of halothane greatly decrease gramicidin channel lifetime by destablizing the conducting dimeric channel, whereas mole fraction quantities (e.g., 0.4 mole fraction of decane in the lipid) are required to have a similar effect [61].

Thus anesthetics have a direct effect on the gramicidin ion-channel which is imbedded within a lipid bilayer model of a cell membrane. The perturbation can involve changes in H-bond strength, specifically the hydrogen bonded association of subunits required for channel formation. Assuming that these results can be extended to ion-channels of higher organisms, they should have an influence on our ideas concerning the mechanisms of anesthesia.

Acknowledgements

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