Interaction of Quaternary Ammonium Bactericides with Biological Materials II: Insoluble Monolayer Studies

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Abstract □ The behavior of a series of bactericidal alkylbenzyldimethylammonium chlorides in the presence of various materials spread at the air/water interface was examined, with a view to elucidating the manner these compounds initiate bacteriolysis in Gram-positive and Gram-negative organisms. A monolayer of the protein gliadin was chosen to represent the Gram-positive bacterial wall, while a gliadin–cephalin film was used to simulate the Gram-negative bacterial wall. Interactions between these monolayers and the various alkylbenzyldimethylammonium chloride homologs, alone and in the presence of protamine, were followed by monitoring surface pressure, surface area, and surface potential as a function of time. Recompression studies were also undertaken. The results suggest that with Gram-positive organisms the bactericide first becomes associated with the protein in the cell wall; subsequent penetration leads to disruption of the cell membrane. In the Gram-negative wall, the phospholipid present affords the organism a degree of protection from the bactericide. This effect is removed in the presence of protamine.

Keyphrases □ Quaternary ammonium bactericides—interaction with gliadin and gliadin–cephalin monolayers, presence and absence of protamine □ Gliadin and gliadin–cephalin monolayers—interaction with quaternary ammonium bactericides, presence and absence of protamine □ Cephalin–gliadin and gliadin monolayers—interaction of quaternary ammonium bactericides, presence and absence of protamine □ Protamine—effect on interaction of quaternary ammonium bactericides with gliadin and gliadin–cephalin monolayers □ Bactericidal activity—quaternary ammonium compounds

In another paper (1), the literature concerning the action of the quaternary ammonium bactericides and the methods used to elucidate their mechanisms of action were reviewed. The presence of an interaction between the quaternary ammonium bactericides of the alkylbenzyldimethylammonium chloride series and the phospholipid cephalin also was established *via* a conductometric analysis of aqueous solutions of these materials. This latter experiment was carried out to establish that the bactericide and the phospholipid indigenous to the Gram-negative bacterial cell wall are able to interact with each other.

The present paper concerns itself with the interactions occurring between the bactericide and insoluble monolayers spread at the air/water interface, designed as models for the Gram-positive and Gram-negative bacterial cell walls. A film of the insoluble protein gliadin was used to model the Gram-positive cell wall, while a protein–phospholipid film of gliadin and cephalin was used to mimic the Gram-negative cell wall. The bactericide under study was injected into the aqueous subphase beneath the insoluble monolayer and its interaction was monitored as the time-dependent change in surface pressure or area and surface potential. In addition, an effort was made to elucidate the role played by protamine in altering the susceptibility of Gram-negative organisms to the quaternary ammonium bactericides. These results were then interpreted in view of the bacteriological activity of this series of compounds on Gram-positive and Gram-negative organisms.

MATERIALS AND METHODS

Alkylbenzyldimethylammonium Chloride—The source, purity, and characteristics of the alkylbenzyldimethylammonium chloride series were described previously (1).

Gliadin—The molecular weight of gliadin[1], a protein derived from wheat, was taken to be 27,000, as determined from monolayer properties of the water-insoluble protein (2). Gliadin is reported to be soluble in 70% ethanol (3). However, the protein, as purchased, in 70% ethanol contained insoluble (proteinaceous) material. Therefore, a purification and quantification procedure was used, which involved filtration of the solution containing insoluble material followed by a gravimetric analysis for the total protein in solution (4).

Cephalin—Synthetic L-α-(β,γ-dipalmitoyl)phosphatidylethanolamine[2] was used as received. The cephalin was dissolved in a mixture of chloroform and ethanol (90:10) for the insoluble monolayer studies.

Protamine—Protamine sulfate[3] was used as received. It contained 67–70% arginine and 24 ± 1.5% nitrogen. A molecular weight of 7000 (5) was used to calculate the molar strength of the aqueous solutions used.

Gliadin–Cephalin Solution—A solution in chloroform–ethanol–water (67:24:9) was used in the formation of a mixed protein–phospholipid monolayer.

Film Balance Studies—The apparatus, a Wilhelmy-type film balance, and the calibration procedure used were described in detail elsewhere (6). The time for equilibration of the film with the barriers set for maximum area was 20 min. The rate of compression was 2.54 cm. min.$^{-1}$.

Penetration Studies—Since both the gliadin and gliadin–cephalin films showed the phenomenon of surface pressure relaxation (4), the compressed films were allowed to relax to their equilibrium pressure prior to the start of any penetration studies. This was accomplished by compressing the film to a preselected area and then allowing the film to reach equilibrium. The area to which the film was compressed was such that the equilibrium film pressure, $F_{eq.}$, at that area was greater than that pressure generated by the alkylbenzyldimethylammonium chloride itself at the air/water interface for the subphase concentration employed in the study. Following attainment of the equilibrium surface pressure, between 0.5 and 5.0 ml. of the subphase was removed from behind the barriers and replaced by an equal volume of solution containing the desired amount of alkylbenzyldimethylammonium chloride. The transfer of the solution was accomplished by means of a glass syringe fitted with a 7.62-cm. (3-in.), 18-gauge hypodermic needle. Homogeneity of the subphase was ensured by gently drawing the subphase into the syringe and discharging it several times under the surface. Penetration into the monolayer was followed as a function of the change in surface pressure or surface area and the change of surface potential with time, the initial time being that point when the alkylbenzyldimethylammonium chloride was introduced into the subphase. Monolayer penetration was monitored until an equilibrium condition was reached or 90 min. had elapsed, whichever was the sooner. At that time, the penetrated monolayer was compressed to its collapsed area.

[1] Nutritional Biochemical Corp., Cleveland, Ohio.
[2] California Corporation for Biochemical Research.
[3] Salmine, Nutritional Biochemical Corp., Cleveland, Ohio.

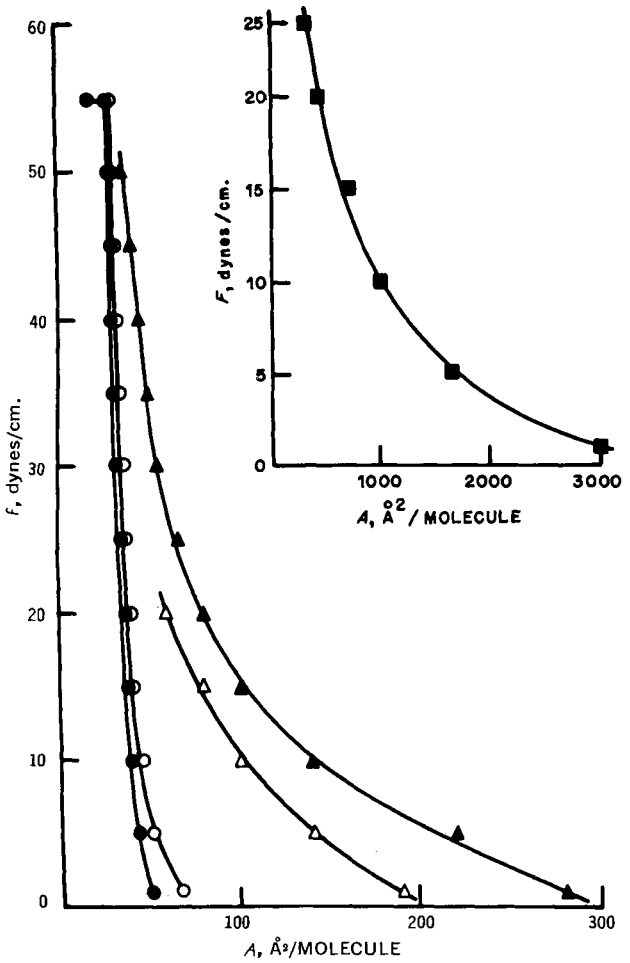

Figure 1—*Surface pressure-area relationships for gliadin* (■), *gliadin–cephalin mixed film* (▲), *gliadin–cephalin derived film* (△), *cephalin* (●), *and cephalin on protamine subphase* (○).

For the studies of monolayer penetration by alkylbenzyldimethylammonium chloride at constant surface pressure, the surface area was increased manually to maintain a constant surface pressure. The distance of separation of the barriers, and, hence, the area per molecule were determined as a function of time.

Surface Potential Measurements—Surface potential measurements were determined by the ionizing electrode method described by Gaines (7). The ionizing electrode consisted of a static eliminator[4] containing 500 μc. of ^{210}Po impregnated in foil. A wire lead was soldered onto the stainless steel back of the unit. The whole electrode was then fastened to an insulated clamp and supported 5 mm. above the water surface in the trough and between the two barriers. The reference electrode was a standard KCl pH electrode[5] immersed in the subphase behind the stationary barrier. The two electrodes were connected by shielded cables to an electrometer[6]. The surface potential, ΔV, was taken as the difference of potential in millivolts between the clean water surface and the film-covered surface. It was not possible to measure the surface potential during the compression phases of the studies due to electrical interference from the electric motor driving the movable barrier.

RESULTS

Definition of Terms—For the purposes of this work, *association* of the penetrant with the insoluble film is defined as the irreversible entry of alkylbenzyldimethylammonium chloride into the film in such a way that it interacts with the film and is not subsequently expelled by compression of the penetrated film. *Penetration* is the general entrance of the alkylbenzyldimethylammonium chloride into the insoluble film and may or may not include association of the penetrant with the film. If the material is not associated, then it can be expelled by compression of the film. Association and penetration are accompanied by a change in both the surface pressure and surface potential.

Anchorage of the alkylbenzyldimethylammonium chloride onto the insoluble film signifies interaction and attachment of the alkylbenzyldimethylammonium chloride in the subphase to the film and involves the attraction between oppositely charged sites on the polar head groups in the subphase. Since this interaction does not occur in the interface but rather immediately below it, it is accompanied by a change in the surface potential with little or no effect on the surface pressure. *Adsorption* onto the film is similar to anchorage but is a more passive, nonspecific process which includes the entrance of the alkyl chain into the interfacial area. Surfactant molecules will be adsorbed at the interface even in the absence of a film. The effect of adsorption on the surface pressure and surface potential is variable.

Table I—Comparison of ΔF and $\Delta(\Delta V)$ for Alkylbenzyldimethylammonium Chloride Homologs 90 min. after Penetration Was Initiated

Homolog	—824 Å²/Molecule— ΔF[a], dynes/cm.	$\Delta(\Delta V)$, mv.	4000 Å²/ Molecule, ΔF[b], dynes/cm.	Surface Tension Lowering Produced by 1.6×10^{-5} *M* Alkylbenzyldimethylammonium Chloride, dynes/cm.
12	1.4	+74	1.0	0.2
13	2.0	+93	1.2	0.3
14	3.4	+112	2.3	0.7
15	4.8	+147	3.7	1.2
16	5.5	+157	7.6	—

[a] Surface pressure of film prior to penetration = 6.6 dynes/cm. [b] Surface pressure of film prior to penetration = 0.8 dyne/cm.

***F–A* Characteristics of Insoluble Films**—*Gliadin*—The surface pressure-area per molecule (*F–A*) isotherm for gliadin is shown in Fig. 1. The surface potential of the spread film was found to be +110 mv. at the maximum area (4000 Å²/molecule) and +328 mv. at the minimum area (324 Å²/molecule). The rate of compression was 503 Å²/molecule/min.

Cephalin—The *F–A* isotherm for cephalin is shown in Fig. 1. The film had a well-defined collapse point at a molecular area of 28.1 Å² and a film pressure of 54 dynes/cm. The extrapolated area per molecule was 39.6 Å²/molecule. The film had a surface potential of +255 mv. at the maximum area (156 Å²/molecule) and +390 mv. at the minimum area. The compression rate was 21.0 Å²/molecule/min.

Gliadin–Cephalin Monolayers—The solution used to produce the mixed monolayer contained a molar ratio of 16:1 cephalin to glia-

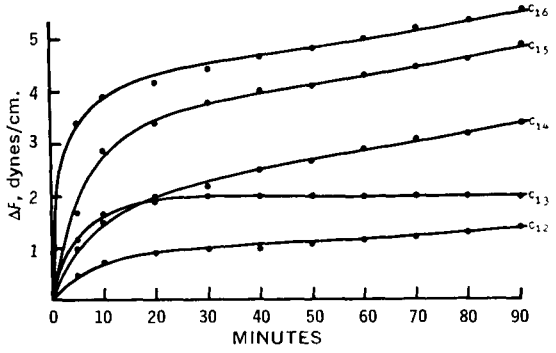

Figure 2—*Increase in surface pressure with time for gliadin monolayers penetrated by alkylbenzyldimethylammonium chloride (subphase concentration = 1.6×10^{-5} M) at constant surface area.*

[4] Staticmaster, Will Scientific, catalog No. 24887.
[5] Beckman Instruments, Inc., No. 31970.
[6] Keithley Instruments, model 610B.

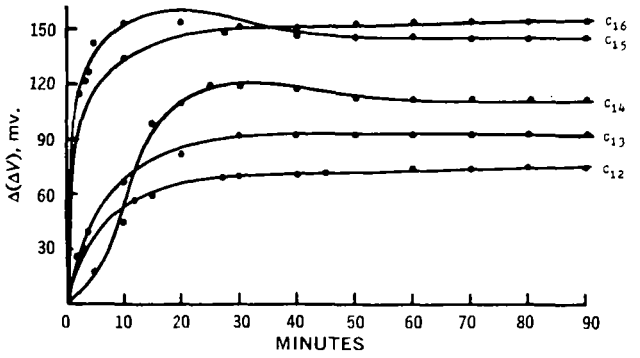

Figure 3—*Increase in surface potential with time for gliadin monolayers penetrated by alkylbenzyldimethylammonium chloride (subphase concentration = 1.6 × 10⁻⁵ M) at constant surface area. Initial surface potential = 264 ± 10 mv.*

din. This ratio is approximately equal to the ratio of 50:20 % w/w for protein to phospholipid commonly found in Gram-negative bacteria (8). The *F-A* isotherm for the mixed gliadin-cephalin film is shown in Fig. 1. The compressed film collapsed at a surface pressure of 50.2 dynes/cm. and an area of 34.9 Å²/molecule and gave an extrapolated area per molecule of 43.5 Å². The surface potential of the spread film was +257 mv. at the maximum area (297 Å²/molecule) and +366 mv. at the minimum area. The compression rate was 39.5 Å²/molecule/min.

Effect of Protamine—To determine if protamine would adsorb onto or penetrate into a spread film, protamine sulfate was injected into the subphase beneath one of the previously mentioned films, and the surface pressure and surface potential were monitored. When the subphase was 3.4 × 10⁻⁶ *M* with respect to the protamine, no effect on the surface pressure-area properties of the gliadin or gliadin-cephalin films was observed. A slight expansion was produced in the cephalin film (Fig. 1). With this same concentration of protamine, the surface potential remained constant for the gliadin-cephalin films but increased 38 mv. for the cephalin film.

Penetration Studies—The surface pressure or area increase and the change in surface potential were used to monitor the penetration of the C_{12}-C_{16} alkylbenzyldimethylammonium chloride molecules into the monolayer systems described previously. For the gliadin films, the penetration was followed as: (*a*) the change in the surface pressure, the surface area being held constant, and (*b*) the change in surface area, the surface pressure being held constant.

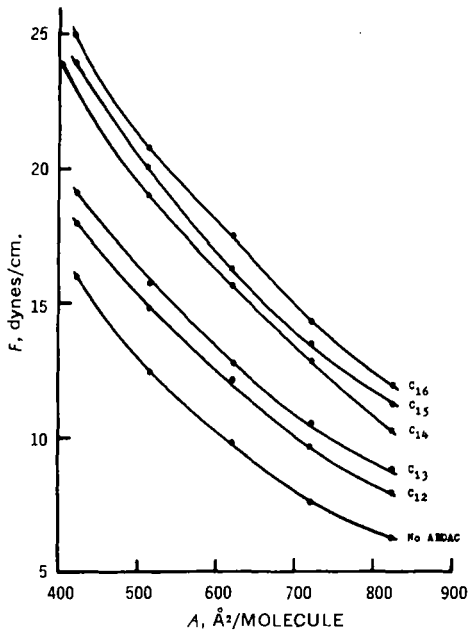

Figure 4—*Recompression of gliadin films previously penetrated at a constant area of 824 Å²/molecule. Subphase concentration of alkylbenzyldimethylammonium chloride = 1.6 × 10⁻⁵ M.*

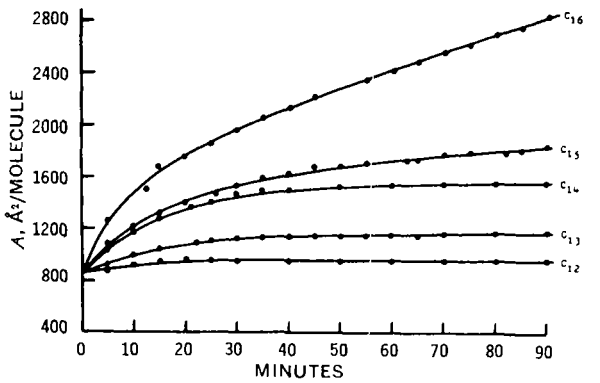

Figure 5—*Increase in surface area with time for gliadin monolayers penetrated by alkylbenzyldimethylammonium chloride (subphase concentration = 1.6 × 10⁻⁵ M) at constant surface pressure of 6.6 dynes/cm.*

For the gliadin-cephalin mixed film, only Method *a* was employed.

Gliadin Monolayers: Penetration at Constant Area—Gliadin films penetrated at constant area showed an initial rapid increase in film pressure and surface potential, followed by a more gradual increase or a leveling off. The penetration was followed for at least 90 min., at which time there was either no change in the surface pressure and surface potential or a constant gradual increase that, with the higher chain homologs, continued for over 4 hr. without any apparent plateau being reached. Therefore, the value at the end of 90 min. was taken so that a comparison of the results could be shown (Table I). Plots of ΔF and $\Delta(\Delta V)$ *versus* time are shown in Figs. 2 and 3, respectively. Linear relationships were observed between ΔF and alkyl chain length ($r = 0.99$) and $\Delta(\Delta V)$ and alkyl chain length ($r = 0.99$).

Studies were also carried out using differing initial areas and surface pressure; the concentration of alkylbenzyldimethylammonium chloride in the subphase was also varied. It was found that changes of the order of ±50 Å²/molecule in the initial area and ±0.5 dyne/cm. in the initial film pressure did not significantly alter the results shown in Table I. To see a more marked effect, gliadin films occupying an area of 4000 Å²/molecule and having a film pressure of 0.75 dyne/cm. were penetrated by a concentration of

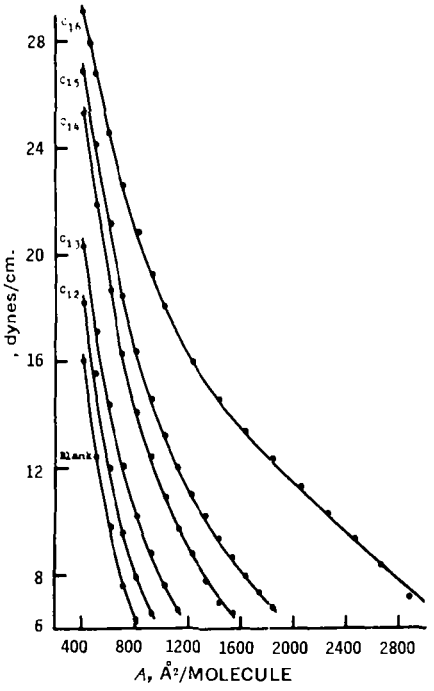

Figure 6—*Recompression of gliadin monolayers previously penetrated at a constant surface pressure of 6.6 dynes/cm. Subphase concentration of alkylbenzyldimethylammonium chloride = 1.6 × 10⁻⁵ M.*

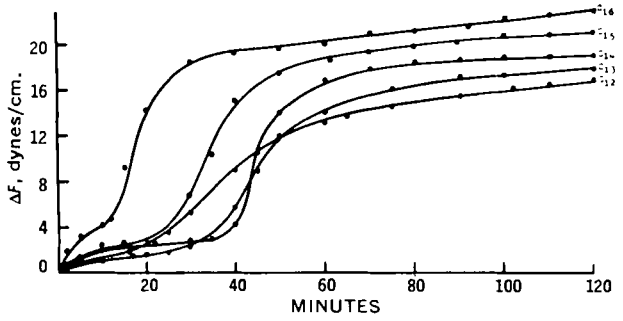

Figure 7—*Change in surface pressure with time for gliadin–cephalin monolayers penetrated by alkylbenzyldimethylammonium chloride (subphase concentration = 1.6 × 10⁻⁵ M) at a constant area of 139 Å²/molecule.*

1.6×10^{-5} *M* drug in the subphase. Only ΔF was monitored in this study; the results are shown in Table I. As the concentration of the drug in the subphase increased, the observed change in surface pressure also increased. Following penetration at constant area, the films were further compressed to their collapse area in order to differentiate between adsorbed and associated alkylbenzyldimethylammonium chloride molecules. Typical results are shown in Fig. 4.

Gliadin Monolayers: Penetration at Constant Pressure—The penetration of gliadin films was also studied as a function of changing surface area, the surface pressure being held constant. Matalon (9) showed that additional quantitative information on the molecular association between a film and penetrating material can be obtained by this technique. The change in area, as a function of time, due to penetration of gliadin by alkylbenzyldimethylammonium chloride homologs is shown in Fig. 5. Following expansion at constant film pressure, the films were recompressed to their minimum area so as to expel any solute adsorbed by the film. The *F–A* isotherms resulting from this recompression are shown in Fig. 6.

Experiments were also performed to determine the effect of the film pressure and drug concentration in the subphase on the magnitude of the penetration. The equilibrium film pressure prior to penetration was varied from 4.8 to 7.8 dynes/cm., and the concentration was 1.92×10^{-5} *M* alkylbenzyldimethylammonium chloride in the subphase. The results of these experiments indicated that there was no difference in the value of the extrapolated area per molecule, A_e, within the range of values studied.

Gliadin–Cephalin Mixed Films—The changes in surface pressure and surface potential were also used to monitor the penetration of the mixed gliadin–cephalin films at constant area. These experiments were carried out with the C_{12}–C_{16} alkylbenzyldimethylammonium chloride homologs. The penetration of the mixed monolayer showed an initial rapid increase in surface pressure, followed by a plateau (Fig. 7). The plateau pressure (approximately 2–4 dynes/cm. above the initial equilibrium film pressure) was normally reached within 10 min. following the introduction of the alkyl-

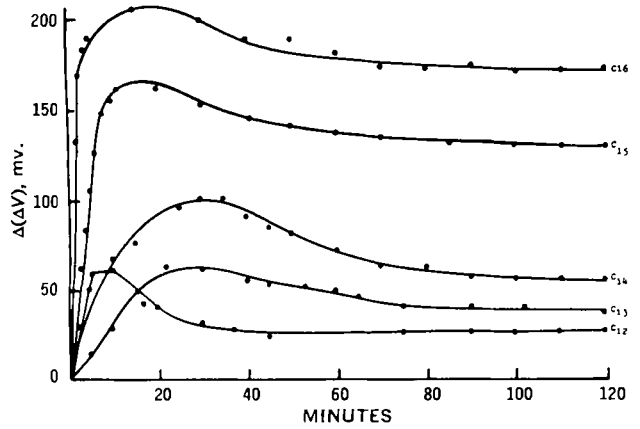

Figure 8—*Change in surface potential with time for gliadin–cephalin monolayers penetrated by alkylbenzylammonium chloride (subphase concentration = 1.6 × 10⁻⁵ M) at a constant area of 139 Å²/molecule.*

Table II—Penetration of Cephalin–Gliadin Films[a] by Alkylbenzyldimethylammonium Chloride Homologs

Homolog[b]	ΔF[c,d], dynes/cm.	$\Delta(\Delta V)$[c], mv.
12	17.0	+37
13	18.0	+27
14	19.7	+57
15	21.3	+135
16	23.2	+174

[a] Area = 139 Å²/molecule. [b] Subphase concentration = 1.6×10^{-5} *M*. [c] After 120 min. [d] Initial equilibrium film pressure = 8.3 ± 0.2 dynes/cm.

benzyldimethylammonium chloride homolog into the subphase. After a finite period of time, the surface pressure again began to increase rapidly. This second phase of the surface pressure increase was then followed by either a slow increase or a leveling off of the *F–t* plot.

The surface potential also increased rapidly initially and reached a plateau level at approximately the same time as the surface pressure (Fig. 8). However, as the second phase of surface pressure occurred (Fig. 7), the surface potential began to decrease. The results are summarized in Table II. Linear relationships were also observed between ΔF and alkyl chain length ($r = 0.99$) and $\Delta(\Delta V)$ and alkyl chain length ($r = 0.91$). Compression curves of the previously penetrated mixed monolayers are shown in Fig. 9.

It was not possible to study the penetration into the mixed film at constant pressure because the film could not be expanded rapidly enough without: (*a*) causing disturbances in the surface, and/or (*b*) maintaining a constant film pressure during the second phase penetration. However, the attempted experiments indicated that the same type of process occurred as with penetration of the mixed film at constant area, *i.e.*, an initial increase and plateau followed by a rapid increase in the surface area.

Penetration of Alkylbenzyldimethylammonium Chloride into Gliadin Films in the Presence of Protamine—The procedure was the same as that used previously, and the penetration was followed as a function of the change in surface pressure and surface potential. Following the introduction of the C_{14} alkylbenzyldimethylammonium chloride homolog into the subphase (1.6×10^{-5} *M* concentration), there was an immediate and rapid increase in the surface pressure to a plateau level which was reached within 10 min. This increase was paralleled by a similar increase and plateau in the

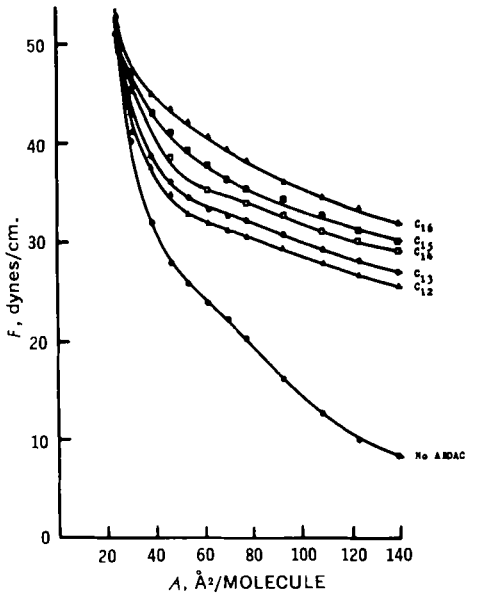

Figure 9—*Compression of gliadin–cephalin monolayers previously penetrated at a constant area of 139 Å²/molecule. Subphase concentration of alkylbenzyldimethylammonium chloride = 1.6 × 10⁻⁵ M.*

surface potential. A plot of the change in surface pressure and potential as a function of time is shown in Fig. 10*a*.

Penetration of Alkylbenzyldimethylammonium Chloride into Gliadin Cephalin Films in the Presence of Protamine—The procedure and conditions for this study were those used for the preceding study. The changes in surface pressure and potential *versus* time are shown in Fig. 10*b*.

DISCUSSION

Cutler *et al.* (10) reported that peak bactericidal activity for the alkylbenzyldimethylammonium chloride homologs occurs at an alkyl chain length of 14 carbon atoms when tested against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Salmonella typhosa* and *Pseudomonas aeruginosa*) organisms. To gain insight into the mechanism of action of the alkylbenzyldimethylammonium chloride homologs against Gram-positive and Gram-negative organisms, various *in vitro* experiments were performed with the aim of correlating some of the physical properties with the observed bactericidal activity of this series of compounds.

The initial rapid increase in surface pressure shown in Fig. 2, which occurs within 10–15 min., is attributed to the penetration of alkylbenzyldimethylammonium chloride into, and its association with, the gliadin monolayer. The surface potential (Fig. 3) also shows a rapid increase during this time period. However, the rate of increase of the surface potential decreases and then levels off within the first 10–15 min., whereas the surface pressure continues to increase but more gradually. The $\Delta(\Delta V)$ data indicate that the distribution of alkylbenzyldimethylammonium chloride between the interfacial region and the subphase is essentially complete within 15 min. of its introduction into the subphase. However, the subsequent penetration process is slower and continues, although at a slower rate, beyond the first 15 min.

Following anchorage to the gliadin monolayer, it can be postulated that the alkylbenzyldimethylammonium chloride molecule undergoes a reorientation, followed by penetration of the hydrocarbon tail and benzyl ring into the monolayer. This reorientation is necessary because the initial step, prior to actual penetration, presumably involves an interaction between the polar head groups of the gliadin and alkylbenzyldimethylammonium chloride. Following this reorientation, the hydrophobic portion of the alkylbenzyldimethylammonium chloride molecule then associates with the hydrophobic amino acid side chains.

The compression to minimum area of a film previously penetrated at constant area can provide further information on the association complex formed between the materials. That the compressed penetrated films shown in Fig. 4 attained a higher film pressure at the minimum area indicates that the penetrant is not expelled from the interfacial area but is held there by a strong association with the gliadin.

The data presented in Table I indicate that the results observed were not due to simple passive adsorption of the surface-active material at the interface. Thus, the surface pressures generated by the presence of 1.6×10^{-5} *M* alkylbenzyldimethylammonium chloride confirm the observation that the affinity of alkylbenzyldimethylammonium chloride for the insoluble film is greater than the tendency toward simple passive adsorption. Consequently, given sufficient area, alkylbenzyldimethylammonium chloride molecules will penetrate the insoluble film until all the sites where association can take place are filled.

It was shown (9) that data on the formation of the association complex can also be gained from penetration studies conducted at a constant surface pressure and monitored as the change in surface area with time. The curves generated by this technique (Fig. 5) are similar in profile to those for the association between cholesterol and sodium cetyl sulfate and between cholesterol and saponin (9). Recompression (Fig. 6) again shows the presence of a strong association between gliadin and alkylbenzyldimethylammonium chloride.

The expansion curves in Fig. 5 are the result of two simultaneous processes which can be described as follows (9): (*a*) interaction of the solute with the film-forming molecules, the stoichiometry of which is indicated by the value of the area, extrapolated to zero time, and (*b*) solution of the injected solute in the surface structure as demonstrated by the linear expansion.

Extrapolation of the linear gradual increase to zero time will yield the area occupied by the stoichiometric complex formed between the two molecules (9). The values of A_e, the area reached by extrapolation of the linear portion of the corrected curve to zero time, are given in Table III. From a knowledge of the area occupied by the gliadin molecules and the cross-sectional area of the alkylbenzyldimethylammonium chloride molecule (11), the stoichiometry of the complex can be determined. The molecular ratio of alkylbenzyldimethylammonium chloride to gliadin is also presented in Table III.

Table III—Data Derived from Extrapolation of Linear Region of Gliadin Films Penetrated at Constant Pressure

Homolog	A_e, Å²/Molecule	Area Due to Alkylbenzyldimethylammonium Chloride, Å²	Ratio of Alkylbenzyldimethylammonium Chloride to Gliadin
12	970	146	1.7
13	1090	266	3.1
14	1480	656	7.7
15	1520	696	8.2
16	1640	816	9.6

The penetration of alkylbenzyldimethylammonium chloride into gliadin–cephalin mixed films was followed as a function of surface pressure and surface potential *versus* time. Plots of these functions are shown in Figs. 7 and 8. Penetration into the gliadin–cephalin films is characterized by an initial increase in surface pressure followed by a plateau. This plateau is reached within 10 min. of the introduction of the alkylbenzyldimethylammonium chloride into the subphase and has a duration of up to 40 min., depending on the homolog. During this same time interval, the surface potential rises rapidly to a peak and then starts a gradual decline. Comparison of Figs. 7 and 8 during this time period with Figs. 2 and 3 (gliadin monolayer) indicates that this rise in surface pressure and surface potential is due to the penetration of alkylbenzyldimethylammonium chloride and its association with the gliadin component of the mixed monolayer.

After this plateau region, a second, more drastic, process takes place. During this phase, the surface pressure increases rapidly and then assumes an equilibrium value. At the same time, the surface potential decreases slightly. This surface pressure increase is attributed to penetration of the alkylbenzyldimethylammonium chloride into, and interaction with, the cephalin component of the monolayer, since this second phase process does not occur with the gliadin monolayer. No explanation is advanced at this time for the small, but real, decrease in surface potential.

Figure 9 shows the compression isotherms of the gliadin–cephalin monolayers previously penetrated at constant area by the alkylbenzyldimethylammonium chloride homologs. As seen from this figure, the penetrated films collapse at the same area and pressure as the nonpenetrated film. This indicates that during the course of compression, the alkylbenzyldimethylammonium chloride is progressively forced out of the monolayer, which then behaves identically with the two-component gliadin cephalin film. Previous work (1) demonstrated a bulk phase ionic interaction between cephalin and alkylbenzyldimethylammonium chloride. In addition, it can be shown that the *F–A* isotherm for the mixed gliadin–cephalin film is slightly more expanded than expected on the basis of the isotherm derived from the isotherms for the two single-component systems. This latter phenomenon suggests only a minor interaction between gliadin and cephalin, since a more condensed film would be expected if a strong interaction existed. On this basis, a rationalization of the behavior of alkylbenzyldimethylammonium chloride, in the presence of the gliadin–cephalin mixed monolayer can be proposed. Thus, the initial stage involves penetration of alkylbenzyldimethylammonium chloride into and association with (through London forces) the gliadin component of the mixed monolayer. Subsequently, the alkylbenzyldimethylammonium chloride penetrates and interacts with the cephalin molecules in the mixed monolayer; the interaction in this case is ionic in nature. Upon compression of the penetrated monolayer, the alkylbenzyldimethylammonium chloride that is associated with the cephalin is expelled from the monolayer but remains anchored in the top layer of the subphase such that the polar head of the alkylbenzyldimethylammonium chloride is adjacent to the negatively charged site on the

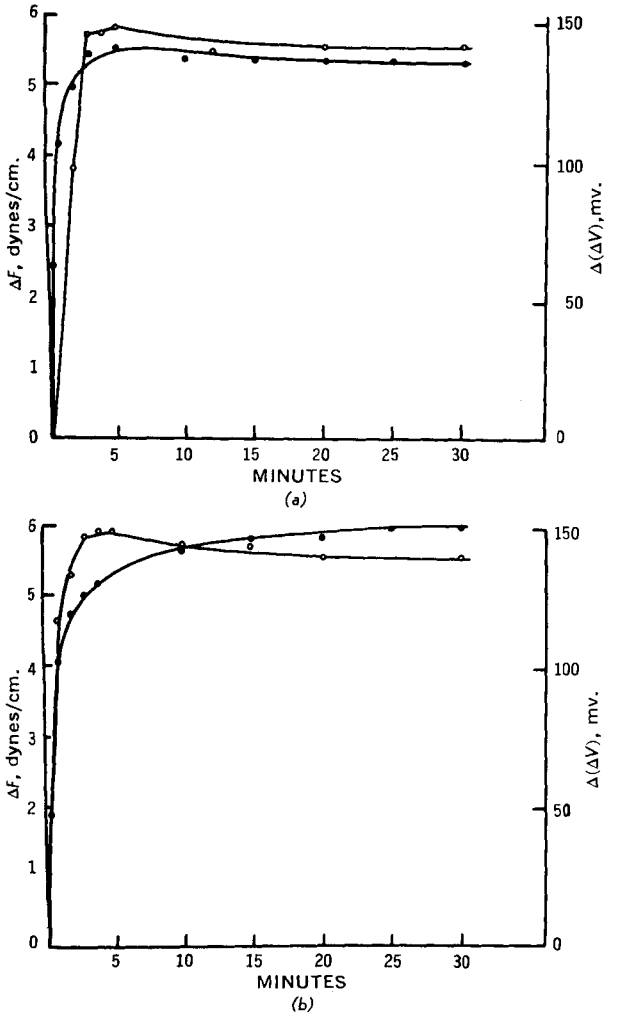

Figure 10—*Penetration of C_{14} alkylbenzyldimethylammonium chloride into:* (a) *gliadin film in the presence of protamine at a constant area of 824 Å²/molecule, and* (b) *gliadin–cephalin film at a constant area of 139 Å²/molecule in the presence of protamine. Key:* ●, ΔF; *and* ○, Δ(ΔV).

cephalin. It is possible that at the relatively high surface pressures obtained at maximum compression, the gliadin may also be expelled from the monolayer. Unfortunately, the present data do not permit an unequivocal statement on this possibility. Were this to happen, it would presumably also occur in the compression of the simple (*i.e.*, unpenetrated) mixed gliadin–cephalin monolayer.

The ΔF and $\Delta(\Delta V)$ *versus* time plots for the penetration of alkylbenzyldimethylammonium chloride into gliadin (Fig. 10*a*) and gliadin–cephalin (Fig. 10*b*) monolayers in the presence of protamine indicate that the same penetration process is occurring in both cases. The gliadin content in the two films was essentially the same, the pure monolayer of protamine containing 9.82×10^{14} molecules and the mixed monolayer containing 7.70×10^{14} molecules of gliadin. That the penetration of the two monolayers was nearly identical indicates that the presence of protamine screened the cephalin in the monolayer to the point that it was unable to interact with the alkylbenzyldimethylammonium chloride. Thus, the C_{14} alkylbenzyldimethylammonium chloride homolog interacted only with the gliadin component of the film; since there was approximately an equal amount of gliadin in each monolayer, the surface pressure and surface potential increase was similar.

Protamine is a highly basic protein having an isoelectric point greater than 12 (12). This is due to the high percentage (approximately 70%) of the basic amino acid arginine. It was shown (13) that protamine forms an insoluble precipitate with cephalin. However, as mentioned previously and as would be expected, the cephalin remains in the interface. Certainly, the magnitude of the time-dependent rise in ΔF resembles that with gliadin on water (Fig. 2) rather than that with gliadin–cephalin on water (Fig. 7). The results obtained here imply that, as might be expected, the cephalin remains at the interface. It seems likely that the protamine attaches itself by charge neutralization to the negatively charged site on the cephalin molecule. In the absence of protamine this would be the site for attachment of the penetrating alkylbenzyldimethylammonium chloride ion. With the effective elimination (by neutralization) of this charged site from the interface, the alkylbenzyldimethylammonium chloride ion must depend upon the gliadin for its ability to anchor onto and penetrate into the film.

CONCLUSIONS

In view of the experimental results and ensuing discussion derived from the model bacterial cell wall, a general theory can be put forward on the mechanism of action of the homologous series of alkylbenzyldimethylammonium chloride bactericides. It must be realized, however, that the studies discussed here are concerned with only the first event occurring in the sequence of events leading to cell lysis (14), namely, adsorption onto and penetration of the surface of the porous bacterial cell wall. The model does not concern itself with transport through the cell wall and the underlying cell membrane, although it does allow extrapolation of the results obtained to these situations.

The observation that peak activity of the alkylbenzyldimethylammonium chloride series resides with the C_{14} homolog is probably related to more than one physical property of the series. It is unlikely that the C_{14} homolog would possess maximum activity in a series due to its particular intrinsic structure, especially since its action has been theorized to be nonspecific. Rather, it appears that this homolog owes its activity to a combination of effects which determine the relative ease with which the homolog can get to the active site and then exert its action. Thus, the hydrocarbon chain of the molecule is needed so that the material will be adsorbed at the bacterial interface in sufficient quantities. This is related to the fact that the longer the hydrocarbon chain, the greater the tendency to be adsorbed at an interface. Also, it is certain that the onium head group is needed for bactericidal activity. This onium head group also lends aqueous solubility to the molecule; thus, its function should not be overshadowed by the hydrocarbon chain. As such, the magnitude of the bactericidal activity of a particular homolog would be dependent on a balance between two physical properties, the aqueous solubility and the relative surface activity, one of which decreases with increasing chain length and the other of which increases.

A gliadin monolayer was used as the model system for Gram-positive bacteria whose cell walls are principally protein. The penetration studies conducted indicate that the alkylbenzyldimethylammonium chloride molecule was able to penetrate into and become bound to the protein molecules in the insoluble monolayer. Extrapolation of these results to the bacterial cell wall suggests that the alkylbenzyldimethylammonium chloride ion exerts its effect through the development of London forces with the lipophilic side chains of amino acid residues of the protein. As a result of this "solution" within the cell wall, the bactericide is then able to penetrate through the cell wall and disrupt the vital cell membrane. This disruption would then lead to the irreversible permeability change and gross leakage from the cells, which is the first step in bacterial cell death (14).

The observation that Gram-negative organisms are more resistant than Gram-positive organisms to the action of ionic detergents has been ascribed to the difference in lipid content of the cell walls, the Gram-negative membrane having much greater amounts of phospholipid (15). Therefore, a gliadin–cephalin mixed monolayer was used as the model for the Gram-negative cell wall.

As noted previously, large quantities of the alkylbenzyldimethylammonium chloride homologs were able to penetrate into the gliadin–cephalin monolayers in a two-step process. However, following penetration, the alkylbenzyldimethylammonium chloride did not appear to be associated with the film, being expelled from the mixed monolayer on compression. However, it is likely that, because of the ionic interaction between alkylbenzyldimethylammonium chloride and cephalin, the former remains anchored in the subphase immediately below the cephalin. The phospholipid thus serves to protect the cell membrane by reacting ionically with the alkylbenzyldimethylammonium chloride. Only after all ionic sites on the phospholipid have reacted with the alkylbenzyldimethylammonium chloride can the bactericide penetrate through the cell wall and initiate

the increase in cell permeability that precedes death. This would account for the fact that higher concentrations of alkylbenzyldimethylammonium chloride are needed to produce the same bactericidal effect in Gram-negative organisms than in Gram-positive organisms.

It was previously observed (16) that the prior addition of small amounts of cephalin to the bulk phase made Gram-positive bacteria more resistant to the action of quaternary ammonium compounds. There are two possible explanations to account for this observation, one or both of which may be operative. One possible explanation is that the phospholipid is adsorbed onto the cell wall and remains there, possibly partially penetrated or merely attached by ionic interactions. When the cationic bactericide is added to the solution and adsorbed onto the cell, it may interact ionically with the cephalin in much the same way as is proposed for the simulated Gram-negative cell wall. In this way, the Gram-positive organisms would be protected. Another possible explanation is that the cephalin in solution interacts with the alkylbenzyldimethylammonium chloride in solution, thereby producing an inactive interaction product. As a result, the effective concentration of the bactericide would be decreased. The conductometric studies (1) confirmed that cephalin and alkylbenzyldimethylammonium chloride reacted with each other in solution, probably through ion-pair formation to form a large complex. Thus, this explanation is also feasible.

It was also observed (17) that addition of protamine to the bulk phase causes Gram-negative bacteria to become more susceptible to the action of quaternary ammonium bactericides. Monolayer studies indicated that protamine is able to interact with the cephalin in the film. This interaction between cephalin and protamine could then nullify the ionic interaction of the phospholipid with the alkylbenzyldimethylammonium chloride. The model may then be considered as having been changed from that representing a Gram-negative bacterial cell wall to that simulating a Gram-positive cell wall. The bactericide would then be free to interact with the protein and freely penetrate the cell wall and cell membrane.

Thus, as a result of studies on a simulated bacterial cell wall, it is felt that the alkylbenzyldimethylammonium chloride series of quaternary ammonium bactericides exert their action by development of London forces with the protein component of the cell wall. This association then leads to penetration of the wall and alteration of the integrity of the cell membrane, with a resulting change in its permeability. This change in permeability allows low molecular weight metabolites to leak out of the cell, followed by complete lysis of the cell by the action of its own autolytic enzymes.

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