Lytic Agents, Cell Permeability, and Monolayer Penetrability

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ABSTRACT Cell lysis induced by lytic agents is the terminal phase of a series of events leading to membrane disorganization and breadkdown with the release of cellular macromolecules. Permeability changes following exposure to lytic systems may range from selective effects on ion fluxes to gross membrane damage and cell leakage. Lysis can be conceived as an interfacial phenomenon, and the action of surface-active agents on erythrocytes has provided a model in which to investigate relationships between hemolysis and chemical structure, ionic charge, surface tension lowering, and ability to penetrate monolayers of membrane lipid components. Evidence suggests that lysis follows the attainment of surface pressures exceeding a "critical collapse" level and could involve membrane cholesterol or phospholipid. Similarities of chemical composition of membranes from various cell types could account for lytic responses observed on interaction with surface-active agents. Cell membranes usually contain about 20–30% lipid and 50–75% protein. One or two major phospholipids are present in all cell membranes, but sterols are not detectable in bacterial membranes other than those of the *Mycoplasma* group. The rigid cell wall in bacteria has an important bearing on their response to treatment with lytic agents. Removal of the wall renders the protoplast membrane sensitive to rapid lysis with surfactants. Isolated membranes of erythrocytes and bacteria are rapidly dissociated by surface-active agents. Products of dissociation of bacterial membranes have uniform behavior in the ultracentrifuge (sedimentation coefficients 2–3S). Dissociation of membrane proteins from lipids and the isolation and characterization of these proteins will provide a basis for investigating the specificity of interaction of lytic agents with biomembranes.

The lysis or clearing of cell suspensions by lytic agents and lytic systems is the terminal phase of what has long been regarded as an interfacial phenomenon and has been the subject of intensive investigations for many decades. Much valuable information about the nature of cell surfaces had been deduced from these studies, at a time when the cellular membranes and walls were not amenable to the techniques of isolation now commonly employed. With the tremendous growth of our knowledge of the chemistry and structural organization of cell surfaces during the past 10 years, we are in a position to

understand more fully some of the interactions between lytic agents and specific components of biological membranes. Indeed, the basis of the selective biological action of the polyene antibiotics on yeasts and fungi, and not upon bacteria, can be related to the possession of sterols in the membranes of the sensitive microorganisms (Lampen, 1966).

Cell lysis, as determined by the loss of turbidity or optical density of a cell suspension, is the gross manifestation of events which have led to cellular disorganization, disruption, and autolysis. It may be very dramatic, as in the lysis of red blood cells by surface-active agents, or it may be a relatively slow process in which the cell's autolytic enzymes contribute to the dissolution of cellular structures following an initial interaction between surfactant and cell membrane. The latter series of events is believed to occur in bacteria exposed to long-chain, quaternary ammonium compounds (Salton, 1951). It is evident that by the time visible lysis is detectable, the alteration of cell permeability is quite "nonselective," and the cells exhibit marked leakiness resulting from irreversible damage to the osmotic barrier. Thus, under conditions of lysis, the leakage of both high and low molecular weight substances into the suspending fluid may be observed. Surface-active agents such as sodium dodecyl sulfate (SDS) are capable of dissociating cell membranes into "soluble" products (Razin et al., 1965; Salton and Netschey, 1965) and can therefore promote rapid lysis and cell disintegration, with release of macromolecular structures such as ribosomes and DNA. On the other hand, some lytic agents or potentially lytic compounds and systems (e. g. antibody and complement) can induce an early, selective release of intracellular substances. Polyene antibiotics cause an efflux of K^+ from treated yeast cells, thus exhibiting an induction of a permeability change which precedes the subsequent derangement of cellular metabolism (Lampen, 1966). In the more complex antibody-complement "lytic" system, investigated in the studies of Green and Goldberg (Green et al., 1959; Green and Goldberg, 1960), selective changes rather than complete permeability breakdown in animal cells were observed. It is of considerable interest that in the antibody-complement system the loss of macromolecules could be prevented by high protein concentrations, with only slight reduction in the rate of loss of amino acids and nucleotides, and unaltered efflux of K^+ and influx of Na^+ (Green et al., 1959).

It is likely that in studies of the lytic action of surface-active agents on cells, the amounts of the agent used have been those capable of giving a gross biological response, such as lysis or cell death, rather than lower concentrations, which may show some threshold effects on permeability properties. Apart from the instances mentioned above, there are very few studies of reversible permeability changes which could be related to the investigations

of the physicochemical aspects of the interaction of lytic agents with cell membranes.

PHYSICAL CHEMISTRY OF HEMOLYSIS

The red blood cell has provided one of the most widely used "model" systems for investigating some of the physicochemical parameters that are likely to be involved in hemolysis. The mechanism of lysis of erythrocytes by amphipathic molecules has been considered by a number of investigators as an interfacial phenomenon, involving the interaction of surface-active compounds with the surface membrane lipids (Pethica, 1958). Attempts have thus been made to correlate hemolytic activity with surface tension properties, ability to penetrate lipid monolayers, etc. (Pethica and Schulman, 1953; Schulman et al., 1955).

It has been known for some time that for homologous series of lytic compounds, there are some general relationships between hemolytic activity and alkyl chain length (Ponder, 1948). Further studies of this aspect of hemolysis have been made more recently by Hooghwinkel et al. (1965), using a series of alkyltrimethylammonium bromides (hexyl to octadecyl). The relationship between hemolytic activity and alkyl chain length of the cationic compounds established that the effective concentration is minimal for the C_{16} compound. Thus both cationic surface-active substances and anionic alkyl sulfonates (Ponder, 1948) show optimal hemolytic effectiveness with the C_{16} homologue. Such relationships do strongly suggest an interaction between the agents and membrane lipids, although, as emphasized by Ross and Silverstein (1954), the possible role of interactions with protein should not be neglected.

Kondo and Tomizawa (1966) have investigated hemolysis by shorter alkyl chain length (C_4 to C_{12}) anionic and cationic surface-active agents and have found a marked difference between the lytic power of the two classes of compounds. The C_4 and C_6 members of the anionic series had little hemolytic action. These authors concluded that the action of the lower members of the cationic agents was related to the release of phospholipid from the erythrocyte membrane.

It has been known for quite some time that the hemolytic activity of long-chain, ionic, surface-active compounds could be related to their penetration into monolayers of cholesterol (Schulman and Rideal, 1939). The possible importance of monolayer penetrability in understanding the mechanism of hemolysis was later emphasized by Pethica and Schulman (1953). They considered the possibility that the erythrocyte membrane surface complex could be regarded as possessing a critical collapse pressure. Thus, hemolysis could be achieved by simple "detergency," where concentrations of lysin giving surface pressures above 34 dynes/cm would result in irreversible

collapse of the membrane complex. The second mechanism of hemolysis suggested involved the attainment of the critical pressure (above 34 dynes/cm) by reaction of lysin and the erythrocyte membrane cholesterol. The latter behavior was shown by long-chain ionic lysins and saponin. The relationship between hemolytic activity and cholesterol penetration as shown by Pethica and Schulman (1953) is illustrated in Fig. 1.

The possibility that hemolysis was related to the red cell phospholipids

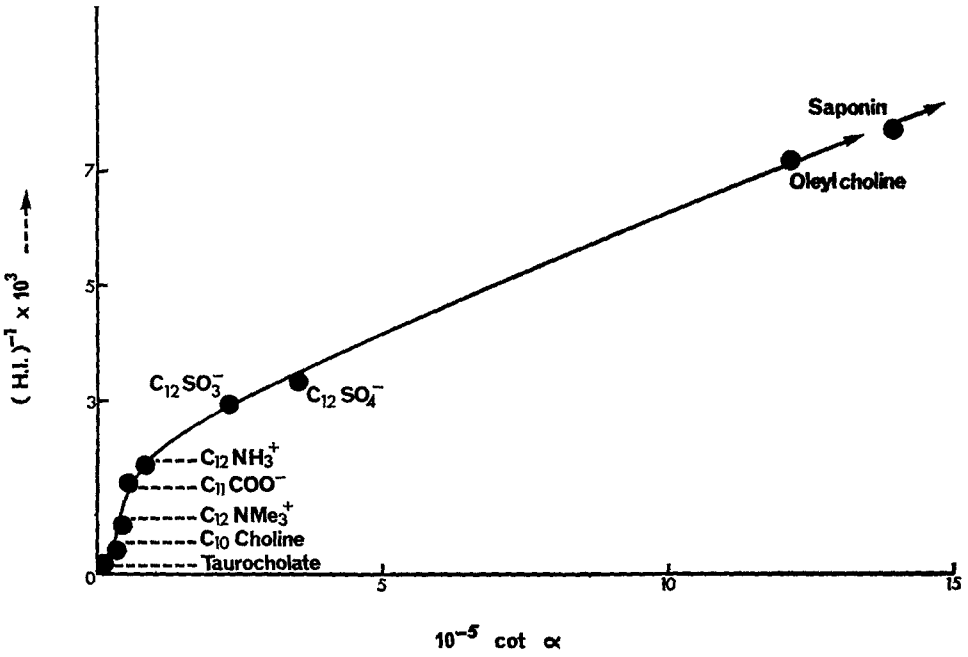

FIGURE 1. The relationship between hemolytic activity $[(H.\ I.)^{-1}]$ and cholesterol monolayer penetration (cot α) for a range of ionic surface-active agents and saponin as presented by the data of Pethica and Schulman (1953) and Pethica and Anderson (1953).

rather than the cholesterol was further considered by Pethica and Anderson (1953). They investigated the penetration of dodecylamine and dodecyl sulfate into lecithin monolayers (Fig. 2) and found that the efficiency of penetration was in the reverse order of their hemolytic activities. This evidence again suggested the possibility that cholesterol was the primary site of action of the hemolysin.

For nonionic lytic agents, Pethica and Schulman (1953) found a correlation between maximum hemolytic activity and the minimum surface tension for the copolymer heptyl alcohol–11 ethylene oxide, as shown in Fig. 3. They thus suggested that in some circumstances the surface tension lowering

may be the more important property, and in others, reaction with cholesterol the dominant factor.

Although the investigations of monolayer penetration have defined some of the physicochemical parameters likely to be encountered in hemolysis,

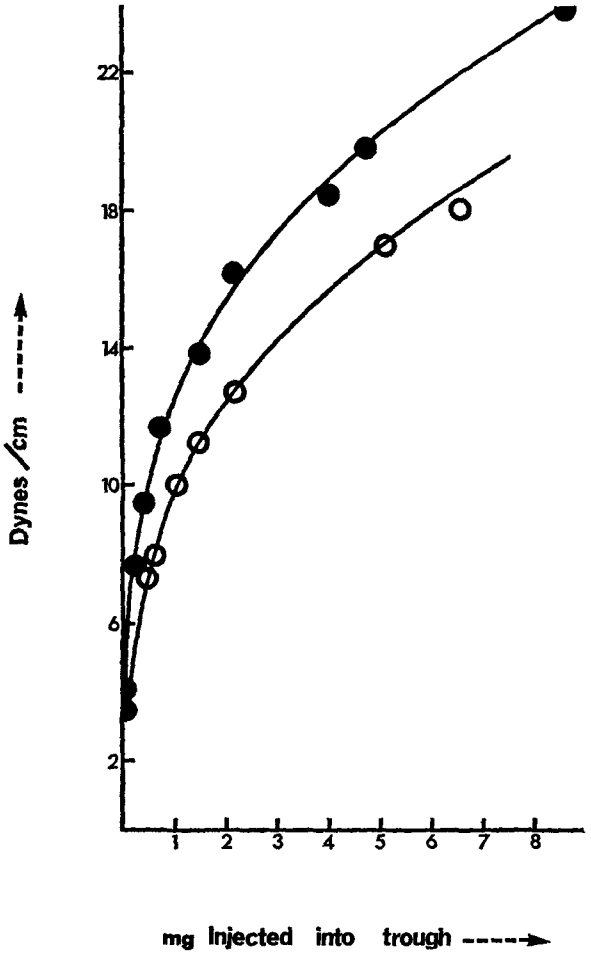

FIGURE 2. The penetration of dodecylamine HCl (●) and sodium dodecyl sulfate (○) into lecithin monolayers spread on isotonic saline at pH 7. The trough volume was 1.2 liters. Data from Pethica and Anderson (1953).

we still do not know enough about the molecular architecture of a functional biomembrane to be certain of the precise nature of the primary sites of reaction between agent and membrane component during cell lysis. The recent excellent work by Shah and Schulman (1967) has established the properties of mixed cholesterol-lecithin monolayers and suggested that the possession of the sterol would increase the flexibility of the membrane. To what extent

this type of monolayer model would increase or decrease the accessibility of cholesterol in the erythrocyte membrane cannot be said at the present time.

Nor can the protein components of cell membranes be neglected in considering their interaction with surface-active agents. Indeed, Ross and Silverstein (1954) have emphasized the possible importance of electrostatic interaction

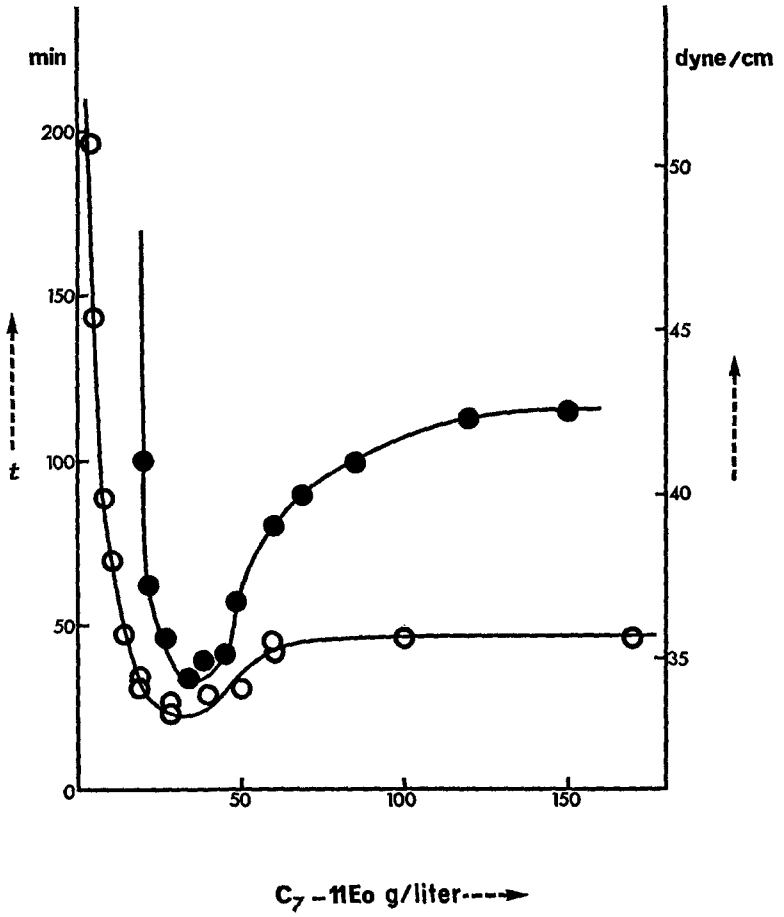

FIGURE 3. The relationship between hemolysis (●) and surface tension (○) lowering for the copolymer heptyl alcohol–11 ethylene oxide (C_7–11Eo) at pH 7. Data from Pethica and Schulman (1953).

of long-chain ions with the protein portion of red cell membranes. Many of the interactions can be investigated more directly with the isolated membrane structures. We have shown that the powerful hemolytic agent SDS rapidly dissociates the membranes isolated from erythrocytes by the method of Dodge et al. (1963). The typical rapid loss of turbidity on treatment of the erythrocyte membranes is illustrated in Fig. 4, such an effect being com-

patible with a nonenzymatic, physicochemical dissociation of the membrane structures. As anticipated from hemolytic studies with homologous series of alkyl sulfates, the longer chain length members (C_{12} and C_{14}) are more effective in dissociating the isolated red blood cell membranes than the C_{10} homologue, as illustrated in Fig. 5. Data obtained with the isolated membrane structures are thus in good accord with results of hemolysis experiments performed on whole erythrocytes.

Until more is known about the nature, arrangement, and association of

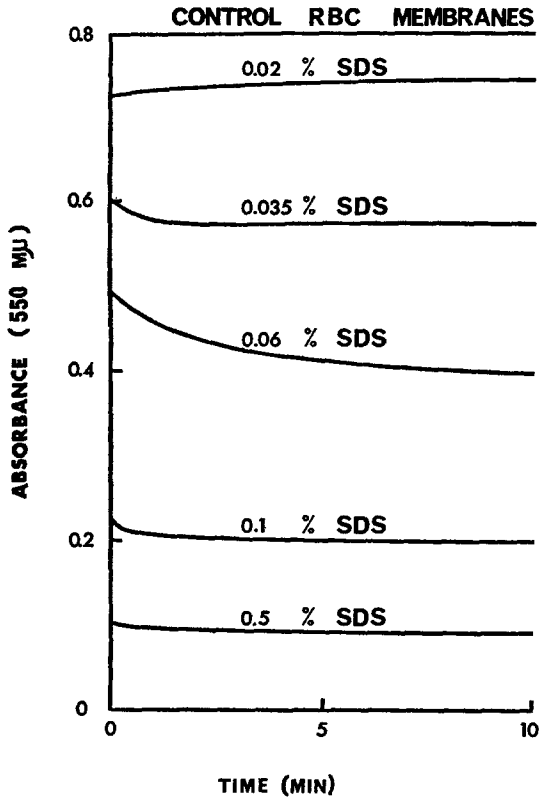

FIGURE 4. Dissociation of isolated red blood cell membranes by sodium dodecyl sulfate (SDS) as determined by loss of absorbance at 550 mμ. Erythrocyte membranes were all adjusted to an absorbance of 0.8 prior to the addition of SDS. Unpublished data of Salton.

membrane proteins with phospholipids and sterols, we cannot set down the sequence of events of the interaction of lytic agents with the membrane components and indicate uniquivocally whether or not one class of membrane component offers a primary target. The type of study performed by Kondo and Tomizawa (1966) on the release of lipid during hemolysis could be extended to the simpler system of the isolated membrane. In this way the relative rates of release of the principal membrane constituents, including phospholipids, sterols, and proteins, could be investigated at levels of surface-active agent below those causing complete dissociation of the structures.

Although the proposed mechanism of lysis from the work on the penetration of monolayers (Pethica and Schulman, 1953) is compelling, there is increasing evidence that the integrity of cell membranes may involve protein-protein interactions through the hydrophobic residues as well as lipid-lipid and lipid-protein associations, and that these factors should also be considered in lytic phenomena.

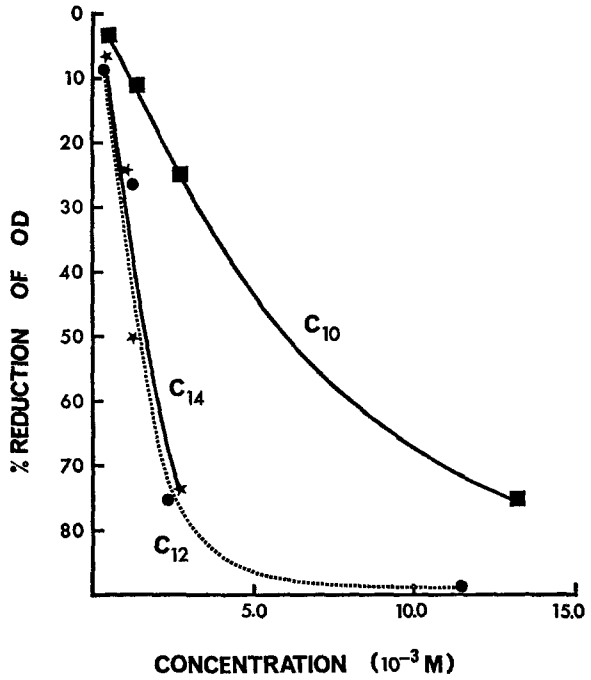

FIGURE 5. Dissociation of isolated red blood cell membranes by long-chain alcohol sulfates (C_{10}, C_{12}, and C_{14}). The extent of dissociation was determined after treated suspension reached a minimum absorbance at 550 mμ, and is expressed as percentage reduction in optical density of the untreated erythrocyte suspension. Unpublished data of Salton.

THE CHEMICAL CONSTITUTION OF CELL MEMBRANES AND THEIR RESPONSE TO LYTIC AGENTS

From the knowledge of the "lipoprotein" nature of the erythrocyte membrane and its response to surface-active agents, it could be inferred that cells showing a similar lytic behavior would possess a surface membrane of the same broad characteristics. Indeed, mammalian cells possess surface membranes of the triple-track type, often referred to as the "unit membrane" (Robertson, 1959). Cells bounded by a single surface membrane appear to be generally sensitive to the lytic action of surface-active agents. We have found

that alcohol sulfates rapidly lyse washed cell suspensions of chick fibroblasts, as shown in Table I. This lytic response is in marked contrast to what we observe when intact bacterial cells possessing a rigid outer wall are exposed to such agents. The lysis of the chick fibroblast cells would thus suggest the presence of an organized lipid-protein surface structure which can be dissociated readily by the long-chain sulfates.

Investigations of the chemical composition of cell membranes isolated from various sources, including animal cells, fungi, and bacteria, have established a great deal of similarity in over-all composition. Biomembranes generally contain about 20–30% lipid, 50–75% protein, and relatively small amounts of polysaccharide. Although proteins constitute the major components of

TABLE I

LYSIS OF CHICK FIBROBLAST CELLS BY LONG-CHAIN ALCOHOL SULFATES*

Chain length, alcohol sulfate	Concentration	Lysis (10 min)
	mM	%
C_{14}	2	50
	1	32
	0.3	12
C_{12}	2.3	64
	1.2	47
	0.4	12
C_{10}	2.6	53
	1.3	32
	0.4	17

* M. R. J. Salton. Unpublished data.

membranes, it has only been in the past few years that investigations have begun to define their properties. Little will be said about the proteins in this discussion except to indicate that they have been described as "structural," "core," "catalytic," and "hydrophobic," and that on dissociation from the lipid they can be resolved into complex mixtures by polyacrylamide disc electrophoresis (Green et al., 1967; Green and Perdue, 1966; Salton, 1967 *a*, *b*; Rottem and Razin, 1967). Some of the general features of the lipid components of cell membranes are listed in Table II.

Membranes usually contain one or two phospholipids which account for the major portion of their total phospholipid content. Phosphatidylcholine (lecithin), which is so commonly encountered in higher organisms, is comparatively rare in bacteria (Hilderbrand and Law, 1964; Kates, 1966). In bacteria, the membrane phospholipids show species and strain specificities

and are susceptible to the influence of the cultural conditions employed during growth (Kates, 1966; op den Kamp et al., 1967). Of considerable relevance to the discussion of the physical chemistry of hemolysis is the widely accepted observation that sterols are uniformly absent from bacteria and blue-green algae (Wright, 1961). The *Mycoplasma* group of bacteria, although unable to synthesize cholesterol, will incorporate sterols into the membrane structures and are dependent on an exogenous supply of sterol for growth (Razin et al., 1963). The presence of sterols in the membranes of yeast, fungi, and mycoplasmas has been correlated with the sensitivity of these organisms to the polyene antibiotics, and, conversely, their absence from the bacteria and blue-green algae can adequately explain their resistance (Lampen, 1966).

The lack of sterols in bacterial membranes, as we shall see from the discussion to follow, has no immediate bearing on their sensitivity or resistance to anionic and cationic surface-active agents. As would be anticipated from

TABLE II

FEATURES OF THE LIPID CONSTITUENTS OF CELL MEMBRANES

Animal cell membranes	Phospholipids, sterols
Yeast and fungal cell membranes	Phospholipids, sterols
Bacterial membranes (Gram-positive)	Phospholipids (species-specific), sterols absent, carotenoids in some
Bacterial envelopes (Gram-negative)	Phospholipids, OH-fatty acids in lipopolysaccharides, sterols absent
Mycoplasma membranes	Phospholipids, sterol incorporated from growth medium

the monolayer penetration studies of Pethica and Anderson (1953), surface-active compounds could penetrate into phospholipid layers of the bacterial membrane and account for the germicidal and lytic action on these organisms. It is evident from the studies with bacteria and the investigations of Kondo and Tomizawa (1966) that interactions with phospholipids can also play an important role in cell lysis.

ACTION OF LYTIC AGENTS ON BACTERIA

Domagk's investigations in 1935 established the powerful bactericidal properties of the cationic, surface-active quaternary ammonium compounds, and since that time there have been numerous studies of their effects on bacterial cells. Lysis or clearing of bacterial cell suspensions exposed to either anionic or cationic surface-active agents, if it occurs at all, is usually a late event in the interaction and is rarely as dramatic as the lysis seen with erythrocytes or animal cells (e.g. chick fibroblasts; see Table I). Unlike mammalian cells, bacteria possess a highly cross-linked peptidoglycan outer wall, which is responsible for the rigidity of the cell. In Gram-positive bacteria, the wall

is a robust structure usually 200–400 A in thickness, and in some instances it may exceed these dimensions. This outer wall thus acts as a protective "corset," preventing the breakdown or osmotic explosion of the more plastic protoplast membrane when the cell encounters environments of low tonicity

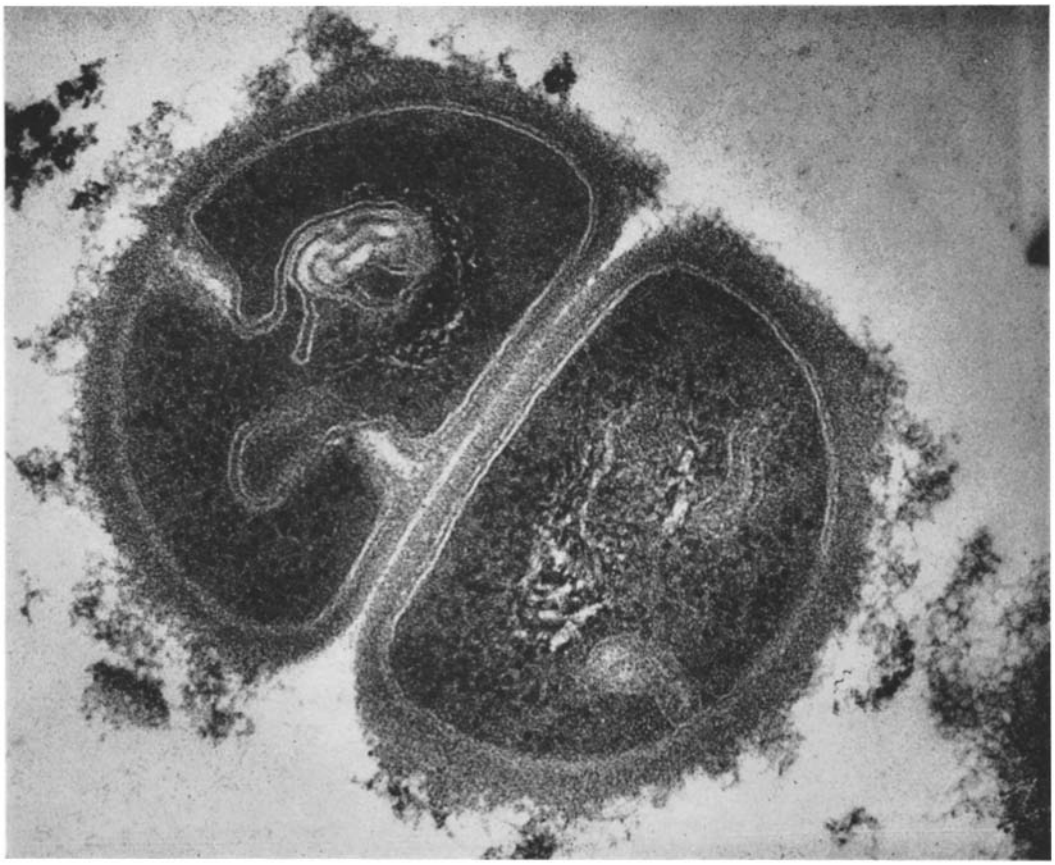

FIGURE 6. Electron micrograph of a thin section of *M. lysodeikticus*, illustrating the presence of the thick, outer cell wall, underlying plasma membrane, and internal membranes and structures. × 137,500. Preparation through the courtesy of Dr. John H. Freer.

(Salton, 1964). The Gram-negative bacteria have a complex envelope structure which includes a rigid peptidoglycan layer as well as a more "labile" outer lipopolysaccharide component anchored to wall. Thus, bacteria are not dependent on a single, relatively fragile membrane for their cell surface boundary. However, it is of interest that when the cell wall is selectively removed in an isotonic medium with a wall-degrading enzyme, the resultant protoplasts

behave much more like erythrocytes and exhibit marked sensitivity to lysis with surfactants.

The anatomy of the surface structure of the Gram-positive organism *Micrococcus lysodeikticus* is illustrated in the thin section of the cells presented in Fig. 6, and these are contrasted in Fig. 7 with the appearance of a stabilized

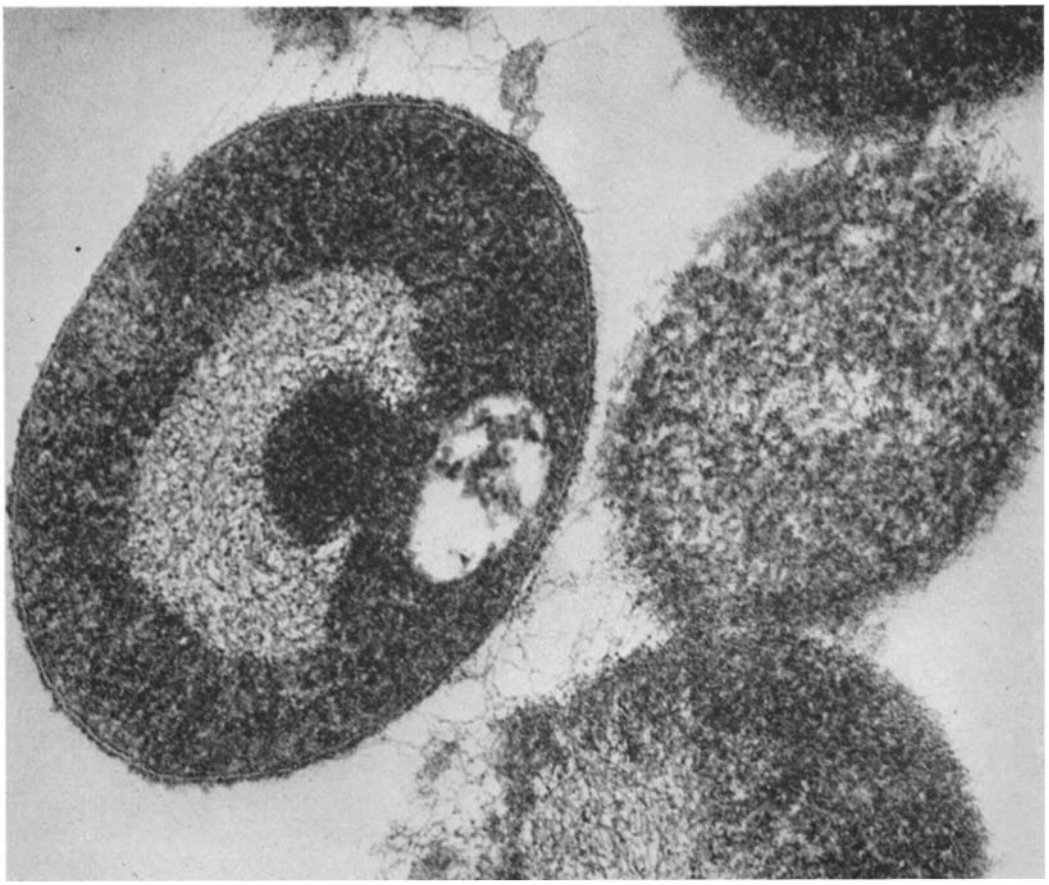

FIGURE 7. Electron micrograph of a thin section of the protoplast of *M. lysodeikticus* formed by digestion of the cell wall with lysozyme in the presence of 1.0 M sucrose. × 122,000. Preparation through the courtesy of Dr. John H. Freer.

protoplast formed by removing the cell wall with the specific wall-degrading enzyme lysozyme. The complex multilayered appearance of the envelope (outer wall layers + underlying plasma membrane) of the Gram-negative organism *Escherichia coli* is shown in the thin section of the cells illustrated in Fig. 8.

In terms of the surface structure of bacterial cells, some of the essential

features relevant to the consideration of the interaction between the cell and surface-active agents are listed in Table III for the Gram-positive organisms and in Table IV for Gram-negative bacteria. It can be concluded from leakage studies and work with the isolated walls that the wall is not the primary

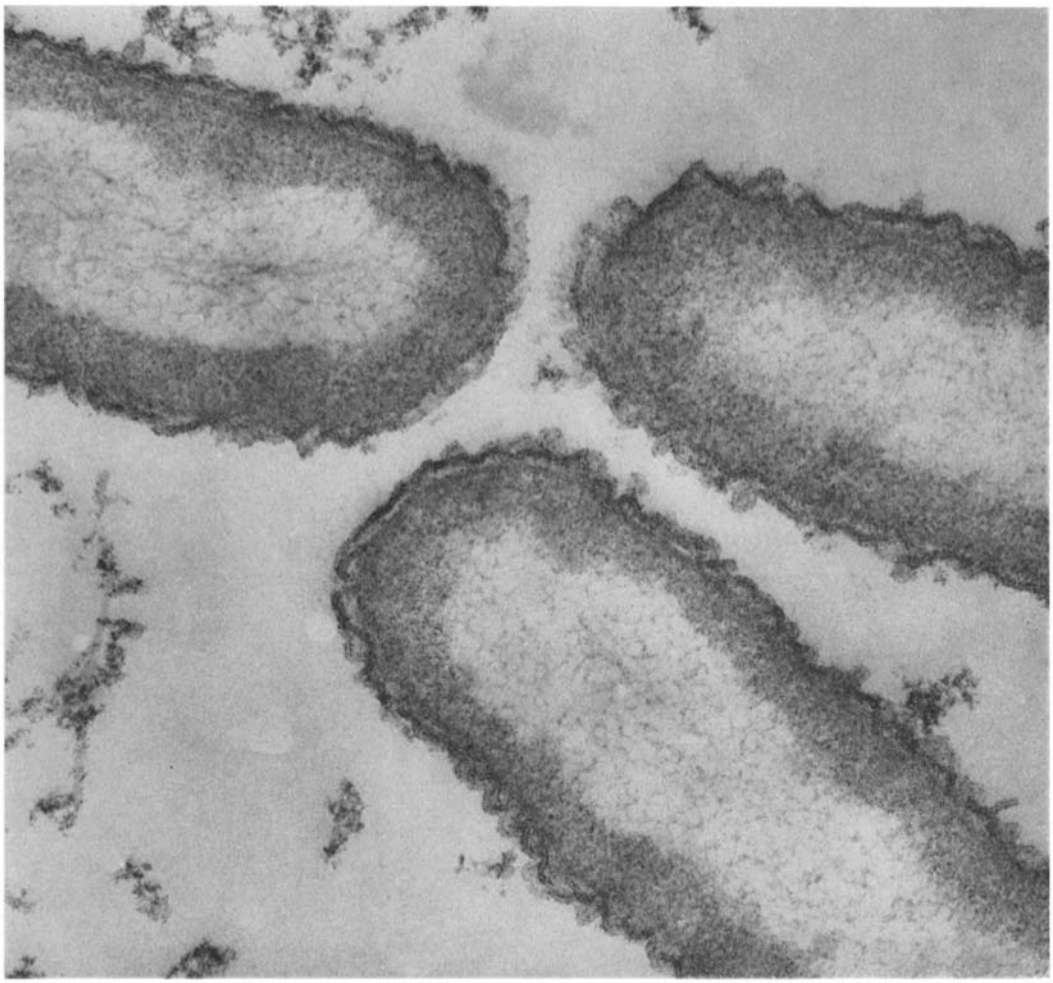

FIGURE 8. Electron micrograph of a thin section of *E. coli*, showing the multiple-layered outer envelope structure and the internal organization of the cell. × 104,500. Preparation through the courtesy of Dr. John H. Freer.

target for the bactericidal action of lytic agents. In the Gram-negative organisms, the interaction between the surface-active compounds and the lipopolysaccharides could conceivably release the enzymes retained in the periplasmic region (Neu and Heppel, 1964) before they penetrate to the site of

TABLE III

SURFACE STRUCTURES IN RELATION TO THE ACTION OF LYTIC AGENTS ON GRAM-POSITIVE BACTERIA

Cell wall	Cell membrane
Permeation of molecules up to about 100,000 mol wt	Lipid-protein structure with high affinity for surface-active lytic agents
Generally carries negatively charged polymers, e.g. teichoic acid, unsubstituted muramic acid residues in some peptidoglycans	Membrane readily dissociated by wide variety of lytic agents
Free amino groups in wall peptidoglycans	
A rigid covalently bonded structure, not dissociable by lytic agents	
Degraded enzymatically by specific enzymes, e.g. lysozyme, lysostaphin	

the membrane. However, the general sequence of events for the interaction between both groups of bacteria and surface-active agents can be identified as indicated in Table V. Aspects of the mode of action of surface-active agents, including ionic "detergents" and polypeptide antibiotics, have been discussed in detail in earlier publications (Schulman et al., 1955; Newton, 1958).

One of the earliest detectable effects, other than adsorption to the cell surface (McQuillen, 1950), is the release of "metabolic pool" constituents such as inorganic phosphate, amino acids, purines, and pyrimidines (Hotchkiss, 1946; Gale and Taylor, 1947; Salton, 1951). The relationship between germicidal action of cetyltrimethylammonium bromide (CTAB) and the time course of release of cellular solutes from *Staphylococcus aureus* is shown in Fig. 9 (Salton, 1951). The rapid initiation of the release of cellular constituents normally retained within the cell by its osmotic barrier indicates the speed with which these agents disorganize the plasma membrane, resulting in an irreversible permeability change and gross leakage from the cells. Other Gram-positive bacteria, such as *Streptococcus faecalis*, show an even faster release of intra-

TABLE IV

SURFACE STRUCTURES IN RELATION TO THE ACTION OF LYTIC AGENTS ON GRAM-NEGATIVE BACTERIA

Cell envelope	Structure
"Wall" *	Complex structure of organized lipopolysaccharides, lipid, and protein, susceptible to dissociation by surface-active agents
	Rigid layer of peptidoglycan, not dissociable by lytic agents
Membrane	Fragments probably derived from plasma membrane, lipid, and protein components

* External "wall" in Gram-negative bacteria can act as a barrier to penetration of small molecules, e.g. actinomycin D, SDS, and crystal violet.

TABLE V

SEQUENCE OF EVENTS IN THE ACTION OF LYTIC AGENTS ON BACTERIA

1. Adsorption and penetration of porous wall
2. Interaction with lipid-protein membrane; reaction with hydrophobic proteins, oriented lipids; membrane disorganization
3. Leakage of low molecular weight metabolites, e.g. amino acids, purines, pyrimidines, nucleotides, ions
4. Degradation of proteins, nucleic acids
5. Lysis due to wall-degrading autolytic enzymes

cellular solutes, as shown in Fig. 10. Although the intracellular pool of metabolites in Gram-negative bacteria is generally lower, the leakage of inorganic phosphate and ultraviolet-absorbing constituents is also demonstrable after exposure to CTAB, as illustrated in Fig. 11.

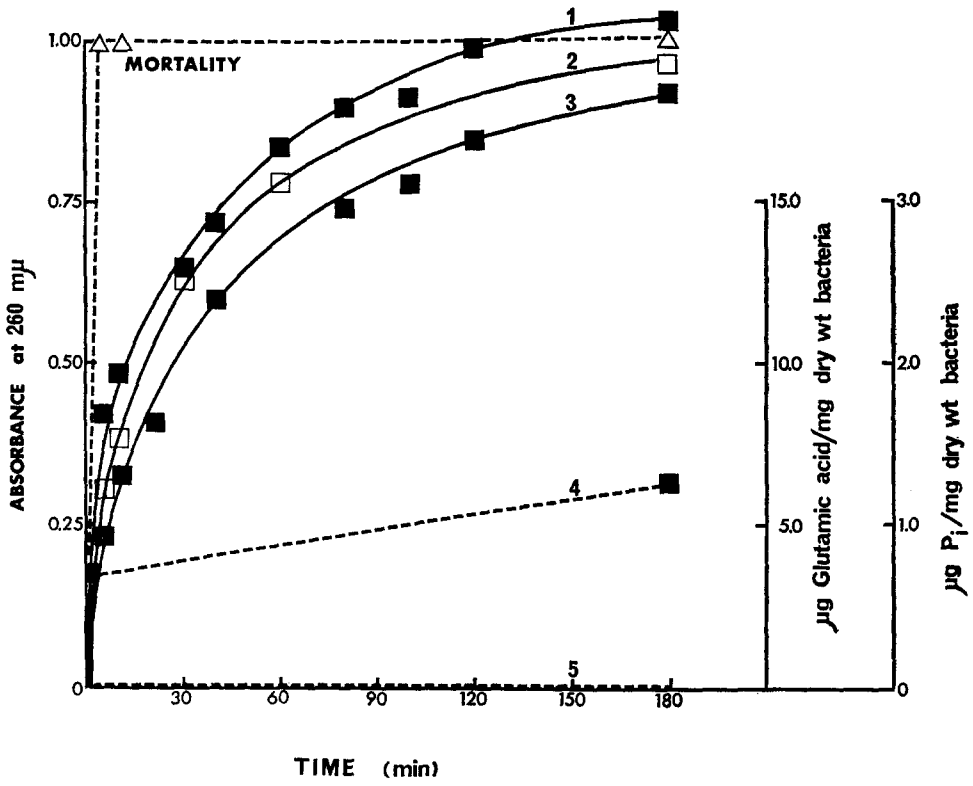

FIGURE 9. The action of cetyltrimethylammonium bromide on *S. aureus*, showing the germicidal activity (mortality curve, △- - -△) and the time course for the leakage of 260 mμ-absorbing material (curve 1), free glutamic acid (curve 2), and inorganic phosphate (P_i) (curve 3). The release of 260 mμ-absorbing material (curve 4) and inorganic phosphate and free glutamic acid (curve 5) is shown for the untreated bacteria. Data from Salton (1951).

From the evidence available through these leakage studies, it was concluded that the antibacterial effects of surface-active compounds such as CTAB could be adequately explained by an interaction between surface-active molecules and components of the plasma membrane (Salton, 1951). Indeed, a quantitative relationship was observed for the proportion of cells killed, the amount of CTAB used, and the quantity of solutes released within 5 min exposure to the surface-active agent (Salton, 1951). Thus, the primary effect of surface-

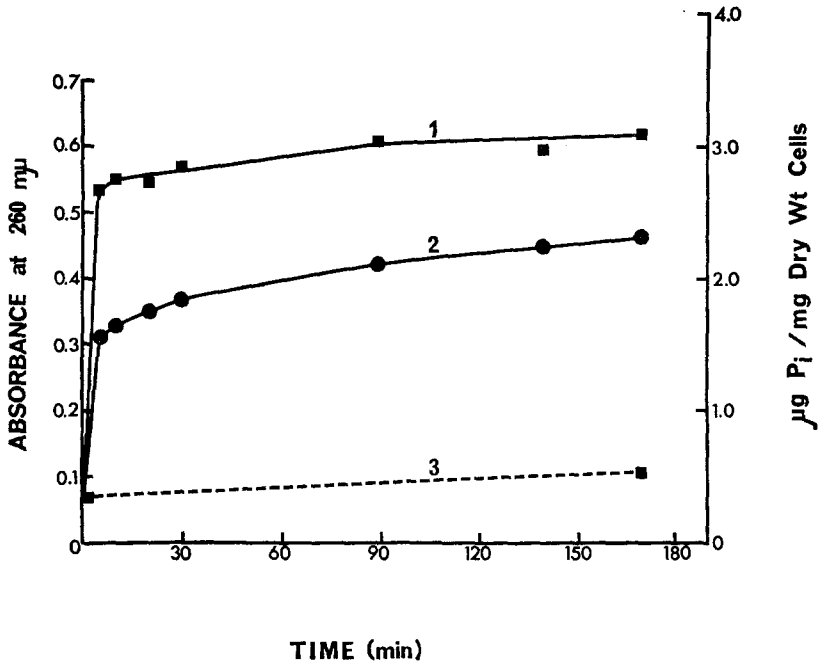

FIGURE 10. The time course for the release of inorganic phosphate, P_i (curve 1), and 260 mμ-absorbing material (curve 2) from *S. faecalis* treated with cetyltrimethylammonium bromide at 20°C. The leakage of 260 mμ-absorbing material from untreated cells is shown in curve 3. Unpublished data of Salton.

active agents on bacteria appears to involve membrane damage and the initiation of leakage. It is of interest that the permeability changes allowing the outward diffusion of solutes also permit the entry of certain substrates unable to penetrate whole cells (Newton, 1958). Gross lysis may or may not ensue, and will be dependent on the degradation of wall and cellular structures by the cell's autolytic enzyme systems.

Studies of the adsorption or uptake of surface-active agents by bacterial cells and isolated walls or envelopes (*E. coli*) have indicated a high affinity for these compounds, as illustrated in Fig. 12 (Salton, 1957). The number of molecules taken up by each bacterial cell at saturation levels is far in excess of a

surface monolayer and therefore suggests that CTAB is bound to wall, membrane, and intracellular proteins (Salton, 1951). Even when just sufficient surfactant is added to kill 99.9% of the cells, the area occupied by the number of molecules taken up per cell would exceed that required for a monolayer of CTAB on the cell surface. It is thus likely that sufficient CTAB is taken up to saturate ionized sites on the wall and still leave enough for reaction with and disorganization of the membrane. Specific localization of surface-active agents in the membrane has not been undertaken, but with the fluorescent-labeled polypeptide antibiotic, polymyxin, Newton (1956) was able to show its asso-

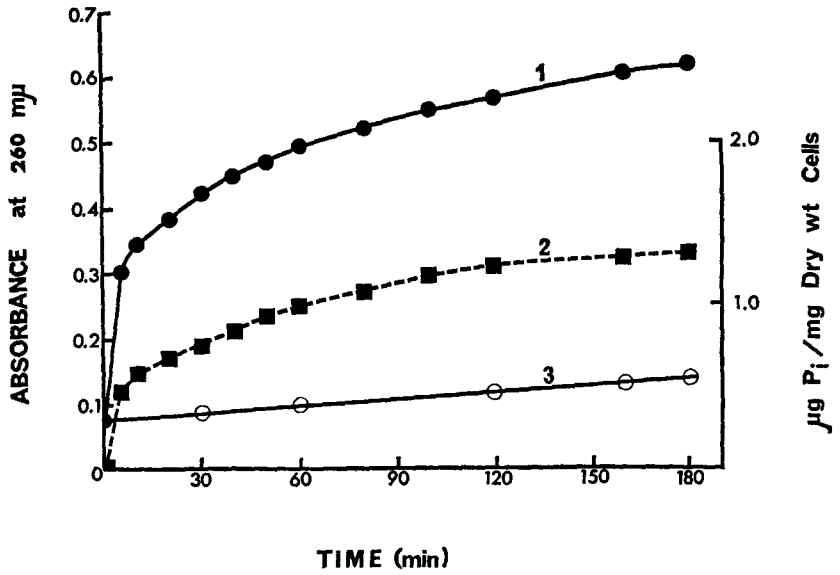

FIGURE 11. The time course for the release of 260 mμ-absorbing material (curve 1) and inorganic phosphate, P_i (curve 2), from the Gram-negative organism *E. coli* treated with cetyltrimethylammonium bromide at 20°C. The leakage of 260 mμ-absorbing material from the untreated cells is shown in curve 3. Data from Salton (1951).

ciation with the cell membrane or envelope in the case of Gram-negative organisms.

THE ACTION OF LYTIC AGENTS ON BACTERIAL PROTOPLASTS, ISOLATED MEMBRANES, AND ENVELOPES

Our knowledge of the action of lytic agents on bacteria was further extended following the demonstration by Weibull (1953) that stable protoplasts could be isolated from bacterial cells when the wall was removed by digestion with lysozyme in the presence of osmotic stabilizers such as sucrose or polyethylene glycol. On removal of the rigid cell wall, the protoplasts of *Bacillus megaterium*

exhibited marked susceptibility to lysis with SDS, as illustrated in Fig. 13 (Salton, 1957). Gilby and Few (1957) also demonstrated the lysis of *M. lysodeikticus* protoplasts by SDS, sodium dodecyl sulfonate, and certain cationic surface-active compounds.

The use of cell wall–degrading enzymes has greatly facilitated the isolation and characterization of membranes from Gram-positive bacteria. Investigations of the chemical composition of these membranes have established that they are essentially lipid-protein structures, as shown by the selected data

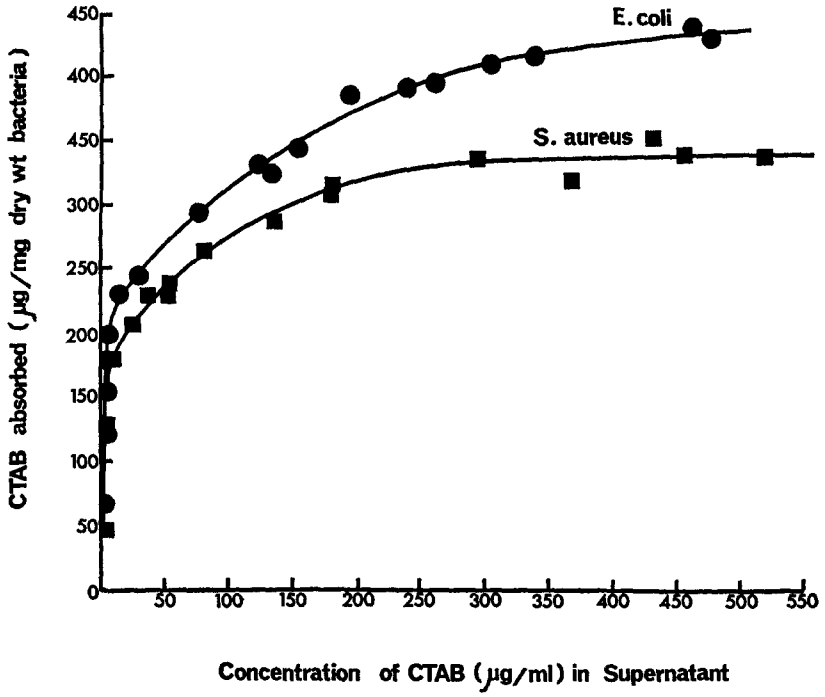

FIGURE 12. The adsorption of cetyltrimethylammonium bromide (CTAB) by washed cells of *S. aureus* and *E. coli* plotted as a function of the CTAB concentration in the supernatant fluid. Data from Salton (1951).

presented in Table VI. There is now a great deal of information on the chemical composition of bacterial membranes and their lipid, phospholipid, and fatty acid constituents. No attempt will be made to summarize these studies here, and the reader is referred to recent reviews on these topics (Kates, 1966; O'Leary, 1967; Salton, 1967 *a*).

As would be anticipated from the studies of lytic action on bacterial protoplasts, anionic surface-active compounds of the long-chain alcohol sulfate series are very effective in dissociating isolated cell membranes. Washed suspensions of membranes prepared from *M. lysodeikticus* were treated with the

C_{10}, C_{12}, and C_{14} alcohol sulfates, and the results, summarized in Table VII, show a rapid dissociation as determined by the reduction in turbidity of the membrane suspensions. The greater effectiveness of the C_{12} and C_{14} compounds is in general agreement with the biological activity of these compounds on bacteria and their hemolytic activities.

Of immediate relevance to the lytic activity of surface-active compounds are the observations on the dissociation of isolated membranes from Gram-positive bacteria (Salton and Netschey, 1965), envelopes (or "walls") from Gram-negative organisms (Shafa and Salton, 1960), and the membranes from *Mycoplasma* species (Razin et al., 1965). The disaggregation of the membranes of *M. lysodeikticus* by SDS and by a nonionic product, Nonidet P.40, was more effective than that observed with dodecyltrimethylammonium bromide (Salton and Netschey, 1965). Examination of the products in the ultracentrifuge in both our studies (Salton and Netschey, 1965) and those of Razin et al. (1965) suggested a surprising degree of apparent homogeneity, with components possessing sedimentation coefficients of about 2–3S. Although there is some doubt as to what the physicochemical behavior of the dissociated products means in terms of the size of lipid-protein associations in the native cell membrane (Engelman, Terry, and Morowitz, 1967; Salton, 1967 *b*), at least these studies have established that surface-active agents break down the membranes into relatively small lipid-protein-detergent aggregates.

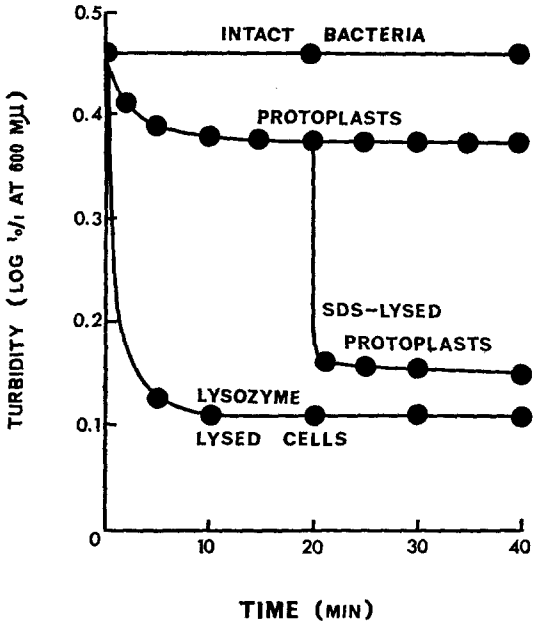

FIGURE 13. The lytic action of sodium dodecyl sulfate (SDS) on protoplasts of *B. megaterium* formed by the action of lysozyme in 0.15 M sucrose. The lysis of the cells by lysozyme in the absence of an osmotic stabilizer is also shown. SDS has no lytic action on intact cells under these conditions. Data from Salton (1957).

With the more complex envelopes of Gram-negative bacteria, previously

TABLE VI

CHEMICAL COMPOSITION OF MEMBRANES ISOLATED FROM GRAM-POSITIVE BACTERIA*

Organism	Protein	Total lipid
	%	%
Bacillus species	58–75	20–28
M. lysodeikticus	65–68‡	23–26
	49§	
Sarcina lutea	57‡	23
S. aureus	69‡	30
	73§	

* Data from Salton (1967 *a*, 1968).
‡ Biuret method.
§ Lowry method.

referred to as "walls" (Shafa and Salton, 1960), anionic surface-active agents effect a rapid dissociation of the isolated structures. Again, for a homologous series of alcohol sulfates of alkyl chain length from C_{10} to C_{16}, the C_{14} homologue was the most effective dissociating agent, as illustrated in Fig. 14. It will be recalled from the discussion of the structure of Gram-negative envelopes above that they possess a rigid peptidoglycan, lipopolysaccharide, and residual elements of the plasma membrane. The ease with which the envelope preparations were disaggregated by the anionic compounds was surprising in view of the presence of the rigid peptidoglycan structure (Shafa and Salton, 1960), but, as later found by Weidel, Frank, and Leutgeb (1963), the loss of rigidity of this layer could be attributed to the activity of enzymes

TABLE VII

DISSOCIATION OF ISOLATED MEMBRANES OF *M. LYSODEIKTICUS* BY ALCOHOL SULFATES*

Membranes were suspended in 0.05 M Tris buffer, pH 7.4, at 25°C.

Chain length, alcohol sulfate	Concentration	Turbidity reduction (10 min)
	mM	%
C_{14}	2	86
	1	82
	0.3	60
C_{12}	2.3	80
	1.2	76
	0.4	60
C_{10}	2.6	61
	1.3	32
	0.4	9

* M. R. J. Salton. Unpublished data.

degrading the peptidoglycan during the isolation of the envelopes. Such findings emphasize the extent to which autolytic enzymes in bacteria can contribute to the breakdown of the surface structures, and, as already mentioned, they undoubtedly play a role in secondary lytic processes in bacteria. It should also be pointed out that the other major macromolecular component of the envelopes of Gram-negative bacteria, the lipopolysaccharide complex, is also susceptible to dissociation with SDS, giving products with sedimentation

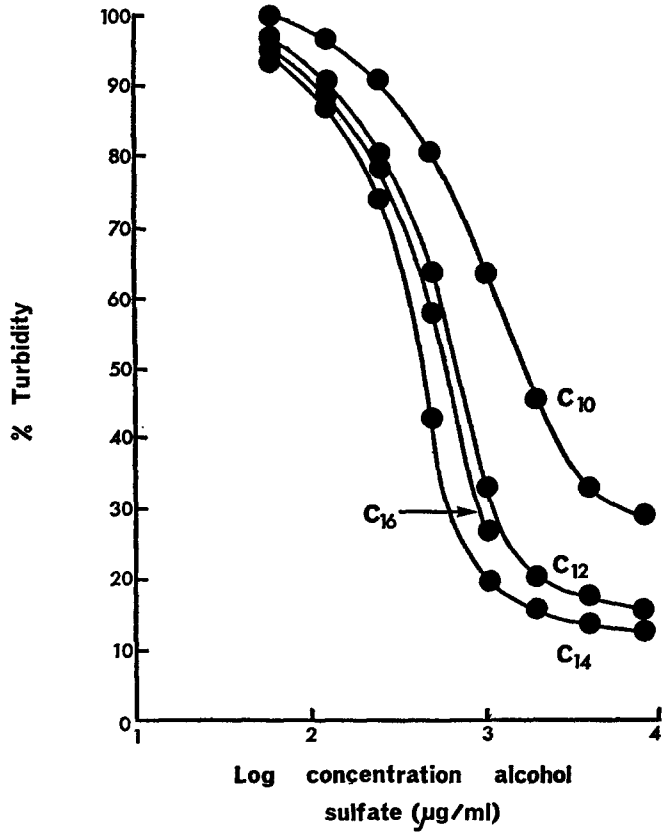

FIGURE 14. The influence of chain length of alcohol sulfates on the dissolution of isolated envelopes ("walls") of the Gram-negative organism *Salmonella gallinarum*. Data from Shafa and Salton (1960).

coefficients (3S) close to those observed for the membrane products (Oroszlan and Mora, 1963; Ribi et al., 1966). The lipopolysaccharides also contribute to the turbidity of isolated bacterial envelopes, and loss of optical density of the suspensions due to dissociation of this component is to be expected.

CONCLUSIONS

There is now abundant experimental evidence that biological responses such as hemolytic activity, lysis of animal cells and bacterial protoplasts, and germi-

cidal action of surface-active compounds can be correlated with the physical chemistry of these substances. Moreover, the order of effectiveness of homologous series of lytic agents is generally preserved when the dissociation of isolated membrane structures is studied. Detailed differences in responses are to be anticipated, as they may reflect the specific differences in the various types of lipids now known to exist in cell membranes.

In conclusion, it is clear that we still do not know very much about the specific arrangement of protein and lipid molecules in cell surface membranes, and, until more is known about the molecular architecture of these structures, we will be unable to decide upon the relative importance of the interaction between surfactant and protein or surface-active agent and lipid. These considerations apply to surface membranes of mammalian and microbial cells alike. The monolayer technique has given us much information about lipid interactions with biologically active agents and may eventually be extended into the field of lipid-protein and protein-protein interactions, now that methods for the resolution of these hydrophobic proteins from cell membranes are receiving closer attention. When this is achieved, it should then be possible to set down the sequence of events following the action of lytic agents on cell surfaces and ultimately assign an order to the relative importance of their interactions with lipid and protein components of the membranes.

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Discussion

Dr. Blank: I would like to call upon Professor Booij from the University of Leiden. Perhaps, Dr. Booij, you might wish to comment on the chain length phenomenon that Dr. Salton has spoken about, especially in view of your results with the penetration of micelles and how chain length affects this process.

Dr. H. L. Booij—Many years ago we studied the influence of a series of soaps of different chain lengths on a colloid chemical model, an oleate coacervate (BOOIJ, H. L., and H. G. BUNGENBERG DE JONG. 1949. *Biochim. Biophys. Acta*, 3:242). If you add potassium chloride in increasing concentration to an oleate solution, a separation into two phases appears at a certain (high) concentration. This means that under the influence of KCl large flat micelles of the bilayer type are formed. These micelles make contact here and there, thus forming a separate, colloid-rich, liquid layer, the coacervate. This system is very interesting because it shows contraction or expansion under the influence of various organic substances. Straight-chain fatty acid anions of varying chain lengths (C_6–C_{22}) were added to the system. In most cases we find a swelling action, which suggests that the oleate bilayers are slightly disorganized (see Fig. 1, Discussion).

Soaps with short chains will have but small activity, as these soaps are highly soluble in water. They have little tendency to enter the micelle structure. Then we see an increasing activity; a maximum is reached at about 11 carbon atoms. This is followed by minimal activity at 15–16 carbon atoms. Soaps with longer chains again show pronounced activity. The explanation seems to be that the soap bilayers are the result of interplay between two opposing factors: a repulsing force between the charged carboxyl groups, and an attractive force between the carbon chains. A short carbon chain, as well as a too long chain, does not fit nicely into the oleate pattern (the "effective length" of the oleate ion is less than 18 carbon atoms) and tends to disturb the micelles.

In coacervates of charged surfactants with branched chains, the minimum does not appear. Here the core of the bilayer is much less regular than in the oleate coacervate. From the fact that—as far as this has been studied—we do not find a minimum when a homologous series of lytic agents is added to living cells, it might be concluded that biological membranes do not have a highly ordered lipid core. This is

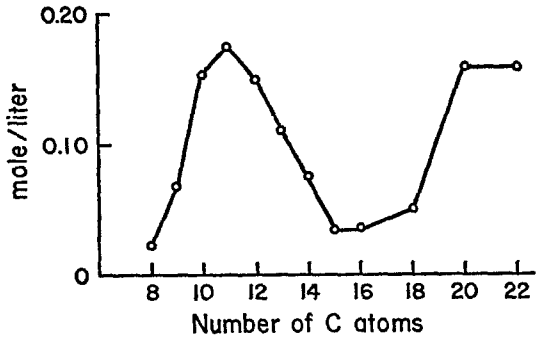

FIGURE 1. Swelling activity (expressed as the shift of the KCl curve caused by 0.5 mM added substance) of fatty acid anions of an oleate coacervate. *Figure reprinted by permission from Biochimica et Biophysica Acta, 1949, 1:84.*

not surprising, as the lipids of biological membranes contain all kinds of saturated and unsaturated carbon chains and, in many cases, ring structures such as cholesterol.

Still I have the feeling that it is worthwhile to study the influence of homologous series of lytic agents on biological membranes, especially in the case of low over-all permeability. These studies may give information on the question whether the core of biomembranes is highly ordered or not.

Dr. Gabriel J. Gasic—Usually investigators forget to mention that in animal cells there also exists a coat outside the cell membrane, but I did not expect such an omission from the very interesting presentation by Dr. Salton. Would you say that the coat in animal cells is in some way analogous to that of the cell wall in bacteria?

Dr. Salton: I kept my remarks strictly to cells that either grow in suspension or can be obtained in suspension. I realize that in mammalian cells there are a number of external components and also lots of things that are responsible for cell adhesion, a phenomenon that we do not see very often in the bacterial system. I was trying to emphasize that there is a basic similarity between the relatively naked types of cells, such as erythrocytes, and the bacterial protoplasts, but not to ignore completely the existence of other structures on the surface of other types of cells.

Dr. Blank: Is there a threshold associated with this phemomenon? In other words, are there concentrations of surfactant that are not effective in lysis?

Dr. Salton: Usually, with the bacteria, germicidal concentrations have been studied and not "threshold" concentrations. That has not been looked at very carefully so far as lysis of isolated protoplasts is concerned. What has been looked at has been the relationship between leakage and the amount of surface-active agent that will kill a given weight of bacteria, and there is a strict quantitative relationship between these. There doesn't appear to be a threshold value as far as one can see.

For a given ratio of detergent to bacterial cells, one can detect within 5 min a quantitative relationship between the amount of materials that leak out and the proportion of cells killed. So in this respect the threshold is not apparent. We do not know of any threshold effect with protoplasts.

Dr. Blank: I was thinking that if there were a threshold effect, perhaps this might give one a probe to measure how effective a pump might be to oppose the leakage.

The floor is open to discussion of all the papers presented in this session, and those on the bilayer. I know that Dr. Rand had a question.

Dr. Rand: In reply to Dr. Mysels, he is quite right in saying that we probably are measuring dilational viscosity. The viscosity is extremely high in terms of poises, and perhaps even similar to that of water, which is a completely hydrogen-bonded substance. May I ask Dr. Mysels if he has any idea of the dilational viscosity of a bilayer of lipid?

Dr. Mysels: No, I have no idea about dilational viscosity of a lipid bilayer. In fact, there are few measurements in the field. During the intermission, Dr. Scriven called me down for not having pointed out that those measurements are quite difficult, because the dilational and the shear effects tend to get mixed up. For monolayers, dilational viscosity can run into hundreds of poises very easily, and I would expect them to be quite high for bilayers, because essentially it is the difficulty of replenishment from the bulk that determines the viscosity. The harder it is for new molecules to restore the original surface tension, the longer the time lag, and the greater the viscosity during the stretching process.

Dr. Rand: In a monolayer you can work with a surface to which you are neither adding nor subtracting molecules.

Dr. Mysels: Yes, and therefore in a bilayer areal viscosity would tend to go to infinity; that is, the surface tension becomes simply elastic, and this is what has been found for soap films. The system is simply elastic, as I mentioned in my paper.

Dr. Rand: The other point I would like to mention is a correlation which I did not really get a chance to expand on yesterday and which may help to answer a question before the last paper about the probable presence of pores in membranes. Let us consider the model system. If the black membrane does in fact have the structure of the bilayer which I showed yesterday, and I think that is a pretty easy assumption to take, then the configuration of the hydrocarbon chains is liquid, which means that they are in thermal motion and thrashing about, and the spaces are opening and closing all the time. Dr. Finkelstein mentioned yesterday that the addition of cholesterol to the lipid of a black membrane decreases its permeability to water, and I showed that the addition of cholesterol decreases the surface area occupied by the phospho-

lipid molecules. This means that without cholesterol the thermal motion of a phospholipid hydrocarbon chain results in its having available to it 30 A^2 projected onto the surface of the lipid layer. In the presence of cholesterol, this thermal motion is inhibited somewhat, and one hydrocarbon chain occupies only 25 A^2. Presumably, therefore, there are statistically fewer or perhaps smaller "pores" opening and closing because of the presence of cholesterol, thus inhibiting the passage of water molecules through the layer. To my mind this is an important correlation between its structure and function, assuming that these two structures are the same.

This brings me to a question that I would like to ask the black membrane people. In the bulk system, without cholesterol, if you go below the melting point of the hydrocarbon chains, they become highly ordered and rigid. What happens with black membranes below the Kraft point or the melting point of the hydrocarbon chains? I would expect radical changes in the permeability properties, because the chains are no longer liquid. The presence of cholesterol in the phospholipids keeps the membrane liquid, or the hydrocarbon chains highly disordered, even at low temperatures. Does the presence of cholesterol help to form stable black membranes at low temperatures?

Dr. Finkelstein: You are quite right. It is very hard to make membranes from saturated lipids, that is, at room temperature; but we were able to make a few that lasted long enough to get water permeability measurements on them, and it is true that the water permeability came down by about a factor of 2.5 from what it was in membranes formed from the unsaturated form. Again, you can attribute this to the condensing of the saturated chains. I think there's a dual effect: there is a decrease in the solubility of water in this phase, and also the diffusion coefficient of water is reduced once it is in such a packed phase.

Dr. Thomson has told me that at 60°C they can make films readily with saturated lecithin. Now at 60°C the chains are melted, and you are back to the state, at least roughly, you were in with the unsaturated chains at room temperature.