Figure 5 demonstrates that the apparent affinity of the Ca^{2+} carrier for respiration-generated, high-energy intermediates is as high or higher than that of the ADP phosphorylation reactions. Observations which have been made with mitochondria from tissues of vertebrates demonstrate that Ca^{2+} acts in preference to ADP in stimulating state 4 respiration (Chance, 1965).

The present investigations lead to the conclusion that blowfly flight muscle mitochondria do possess Ca^{2+} carriers. The experiments also suggest that Ca^{2+} transport occurs in an efficent energy transduction process.

Acknowledgment

The author wishes to thank Mr. William L. Strauss for his excellent technical assistance.

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Perturbation of Liposomal and Planar Lipid Bilayer Membranes by Bacitracin–Cation Complex†

Ruby I. MacDonald, Robert C. MacDonald,* and Neal W. Cornell‡

ABSTRACT: The antibiotic bacitracin at concentrations between 10^{-3} and 10^{-2} M causes the release of trapped, low molecular weight marker from artificial lipid vesicles or liposomes. This antiliposome effect is a paradigm of the antibacterial effect on these grounds. (1) Both actions are specifically enhanced by cadmium or zinc. (2) The spectrophotometrically detected formation of the presumably biologically active divalent cation-bacitracin complex exhibits a pH dependence which is also characteristic of complex-induced liposome lysis. (3) Microbiologically active concentrations of bacitracin and $CdCl_2$—*i.e., ca.* 10^{-5} M—lower the conductance of and induce instability in planar lipid bilayer membranes. As determined by microelectrophoresis, exposure to bacitracin alone does not materially change the negative surface charge density of lipid vesicles. In the presence of the antibiotic and cadmium—but not calcium—however, the liposomal ζ potential is significantly more positive. The function of cadmium or zinc with respect to the antimicrobial effect of bacitracin, therefore, appears to be the promotion of the cell-antibiotic interaction. Cadmium apparently does not enhance the surfactant property of the antibiotic, insofar as it has little influence on the critical micellar concentration of bacitracin.

The mechanism(s) by which the polypeptide bacitracin exerts its antibacterial effect has remained a matter for speculation. Although the antibiotic was once thought to interfere primarily with cell wall synthesis, bacterial protoplasts lacking cell walls have been shown to be as susceptible to bacitracin as bacteria possessing cell walls (Snoke and Cornell, 1965). An alternative mode of action is suggested by the observation that both lysis and inhibition of growth of *Staphylococcus aureus* by bacitracin were enhanced in the presence of zinc (Smith and Weinberg, 1962). As either zinc or cadmium is required for biologic activity of bacitracin (see Weinberg, 1967), membrane damage, as a precursor to lysis, might be central to its antibacterial action. This hypothesis is supported by the finding that bacitracin markedly increased the efflux of potassium from cells recently exposed to the antibiotic (Hancock and Fitz-James, 1964).

The question thus arises as to whether the cell membrane *per se* can be affected by bacitracin, *i.e.*, whether bacitracin's effect on membranes could be exclusive of its effect(s) on other cell components. A study of bacitracin–liposome interaction was undertaken to answer this question. The liposome or smectic mesophase of a lipid(s), which consists of concentric, bilayered shells of lipid separated by aqueous compartments, has become a standard reagent for assessing the membrane-dam-

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aging capacity of a given agent (see Bangham, 1972). Such lipid vesicles are easily loaded with impermeant markers, the release of which then provides an indication of membrane injury. In this way, liposomes have been used to demonstrate, for example, complement lysis (Haxby *et al.*, 1968; Lachmann *et al.*, 1970), melittin or bee venom toxin lysis (Sessa *et al.*, 1969), and polyene antibiotic lysis (Weissmann and Sessa, 1967; Kinsky *et al.*, 1968). As reported here, the bacitracin-Cd complex did cause lipid membranes to release previously trapped marker. The divalent cation enhancement of and pH dependence of the antiliposomal action suggest a real similarity between this effect on dye-containing lipid vesicles and the effect on bacteria.

Complementary to the liposome experiments, assessments of the resistances and stabilities of planar lipid bilayer membranes—also a standard technique for studying membrane perturbing agents (see Mueller and Rudin, 1969)—were performed in the presence of bacitracin and/or cadmium. By this means, physiologically active concentrations of the antibiotic-cation complex were demonstrated to affect simple lipid membranes. Electrophoretic and surface tension data, furthermore, indicate that cadmium enables bacitracin to interact with lipid membranes but does not significantly enhance the surfactant property of bacitracin.

Materials and Methods

Effect of Bacitracin on Liposomes. Standard procedures were followed for the loading of liposomes with impermeant marker (Weissmann and Sessa, 1967; Haxby *et al.*, 1968). Lipids dissolved in organic solvent (diisostearoyllecithin prepared according to Johnson *et al.*, 1973; cholesterol from Matheson Coleman and Bell; stearylamine from K & K Laboratories, Inc.; and dicetyl phosphate from K & K Laboratories, Inc.) were combined in the following molar ratios: 5.2 diisostearoyllecithin:3.7 cholesterol:1 stearylamine, 5.2 diisostearoyllecithin:3.7 cholesterol:1 dicetyl phosphate, 13.5 diisostearoyllecithin:1.85cholesterol:1 dicetyl phosphate, 5.2 diisostearoyllecithin:3.7 cholesterol, and 13.5 diisostearoyllecithin:1.85cholesterol. These mixtures were then dried on a rotary evaporator, suspended in 0.07 M NaCl + 0.14 M *p*-nitroanilinesulfonate (p-NAS)[1] (which had been recrystallized from alcohol and adjusted to pH 7), and incubated for 30 min at 37° with intermittent vortexing. Sonication of some of these preparations led to an expected decrease in dye content but did not affect the experimental results. Precautions against autoxidation of lecithin were unnecessary since diisostearoyllecithin is nonautoxidizable. Finally, untrapped marker was removed by dialysis against three changes of 0.14 M NaCl-0.01 M Tris-HCl (pH 7.4) at room temperature over a 2-hr period. The liposomes were stored at 4°.

To our knowledge, p-NAS has not been employed previously as an indicator of liposome leakiness. It was therefore necessary to determine that: (1) the optical density of p-NAS peaks at 370 mμ (where its extinction coefficient is 1.2×10^4) and is linear at that wavelength from 10^{-5} to 10^{-4} M; (2) at room temperature the equilibration of p-NAS between a 0.2-ml volume in a bag of cellulose dialysis tubing and the 1.5-ml volume surrounding this bag, as it sits in a plexiglas well, is achieved 60 min after the addition of the marker to the dialysis bag; (3) the egress of p-NAS from the dialysis bag is proportional to the added p-NAS from equilibrium concentrations of 10^{-5}-10^{-4} M (this relationship holds in the presence of 0.14 M NaCl but not in its absence; with the latter condition, deviation from linearity is observed at lower concentrations of the p-NAS marker.); (4) bacitracin, $CdCl_2$, and liposomes without marker do not significantly influence the efflux of free p-NAS from dialysis bags.

The suitability of p-NAS having been established, the effect of bacitracin on the release of this marker from liposomes was determined as follows. Liposomes containing p-NAS were added to dialysis bags at a final lipid concentration of about 2 mM and in a final volume of 0.2 or 0.3 ml. The solution in each dialysis bag was the same as that of the 1.5 ml in its surrounding well—*i.e.*, composed of a buffer—either 0.01 M Tris-HCl (pH 7.4) or 0.005 M sodium cacodylate at varying pH—and salt—0.14 M NaCl with or without divalent cation of appropriate kind and concentration. At zero time bacitracin was injected into certain dialysis bags. At 15-, 30-, 60-, and 120-min intervals thereafter, the well contents was removed and the OD 370 mμ measured. Each experiment was performed on three different occasions. Although purified bacitracin (N. W. Cornell and K. Yanagimoto, unpublished results) was used in most of the experiments described, commercial bacitracin (Sigma Chemical Co., St. Louis, Mo.) gave the same results.

Electrophoresis of Liposomes. Microelectrophoresis was performed as described by Bangham *et al.* (1958). Unsonicated dispersions of diisostearoyllecithin and cholesterol in a molar ratio of 7:2 were incubated in 0.005 M Tris-HCl (pH 7.5) for 30 min at 37° at a total lipid concentration of 0.8 mg/ml. Various additives were mixed with liposome samples prior to electrophoresis at room temperature. The ζ potential of red blood cells was determined to ensure that the apparatus was working properly.

Surface Tension Measurements. The hanging plate method of Wilhelmy (see Davies and Rideal, 1963) was used to measure surface tension. Solutions of purified bacitracin and/or $CdCl_2$ were mixed and added to Teflon wells with a capacity of approximately 1 ml, and a platinum plate, hanging from an electrobalance, was dipped into each of the wells. The platinum plate was flamed to a red glow whenever a solution of a different composition was to be tested.

Effect of Bacitracin on Planar Lipid Bilayers. Bilayer membranes were formed across a *ca.* 1-mm hole in a Teflon partition of a Teflon-glass cell, using a brush or Teflon paddle. A 25-mV potential was impressed across the membrane with a battery-potentiometer circuit connected to calomel electrodes. The resulting current was measured with a picoammeter and recorded on a strip chart.

Results

Bacitracin-Cd Complex Causes Liposome Leakiness. Figure 1 indicates the time course of p-NAS release from diisostearoyllecithin-cholesterol liposomes exposed to various combinations of additives. All results are expressed as per cent p-NAS released from the vesicles—100% corresponding to the OD units expected had the liposomes been completely solubilized with Triton. The base line value after 120 min in the absence or presence of $CdCl_2$ alone is about 13% in Figure 1 and probably represents marker which was released very slowly subsequent to preparative dialysis of the liposomes. Accordingly, little increase in the p-NAS levels occurred during the experiment between 60 and 120 min.

In the absence of $CdCl_2$, 10^{-3} and 10^{-2} M bacitracin caused some escape of p-NAS, but the amounts were significantly less than those freed by 10^{-3} M bacitracin plus 10^{-2} M $CdCl_2$ and 10^{-2} M bacitracin plus 10^{-3} M $CdCl_2$, respectively. At each time point seen in Figure 1, both concentrations of $CdCl_2$ enhanced the antibiotic-dependent release of marker by a factor

[1] Abbreviation used is: p-NAS, *p*-nitroanilinesulfonate.

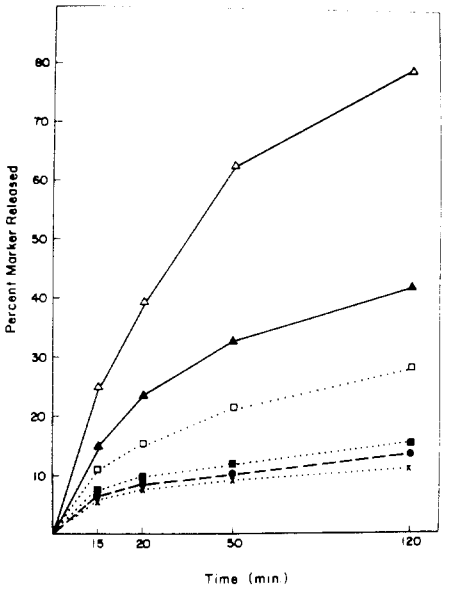

FIGURE 1: Effect of various combinations of agents on the release of p-NAS marker from lecithin-cholesterol liposomes. As described in the Materials and Methods section of the text, marker-containing liposomes were exposed to various agents in dialysis bags, and the amounts of marker freed thereby and equilibrated with the compartments surrounding the dialysis bags were measured spectrophotometrically at the time intervals shown. (●) No addition; (×) 10^{-2} M $CdCl_2$; bacitracin alone (■) 10^{-3} M, (□) 10^{-2} M, and bacitracin + $CdCl_2$ (▲) 10^{-3} M bacitracin + 10^{-2} M $CdCl_2$, (△) 10^{-2} M bacitracin + 10^{-3} M $CdCl_2$.

of nearly 2 after 60 and 120 min. If the appropriate base line or "without additive(s)" value is subtracted from each "with additive(s)" value on the assumption that the former is invariant, the synergistic nature of the bacitracin-Cd relationship is striking. Although this synergism displays some quantitative fluctuation from trial to trial, it is qualitatively reproducible. Incidentally, the lysis of liposomes by Triton was found to be unaffected by $CdCl_2$.

Specificity of the Divalent Cation Requirement. The specificity of the divalent cation requirement for the antibacterial activity of bacitracin is well established (see Weinberg, 1967). To evaluate the relevance of the bacitracin-Cd induced release of p-NAS from liposomes for the *in vivo* effect of the complex, different divalent cations were substituted for $CdCl_2$. The liposome lysing action of bacitracin was facilitated by divalent cations in the following order of effectiveness: Cd (58%), Zn (39%), Mn (27%), Ca (13%) (Figure 2). Since in this experiment bacitracin without divalent cation freed 24% of the dye, calcium actually depressed the effect of bacitracin. The values for samples with any one of the cations in the absence of bacitracin (not shown) were the same as for the base line, untreated sample. These results are in close agreement with those of Adler and Snoke (1962) who claimed Cd to be more potent than Zn in promoting the antimicrobial activity of bacitracin and with those of Snoke and Cornell (1965) who reported that either Cd or Zn was necessary for lysis of protoplasts of *Bacillus licheniformis* and *Micrococcus lysodeikticus*. Other divalent cations were inactive in these respects.

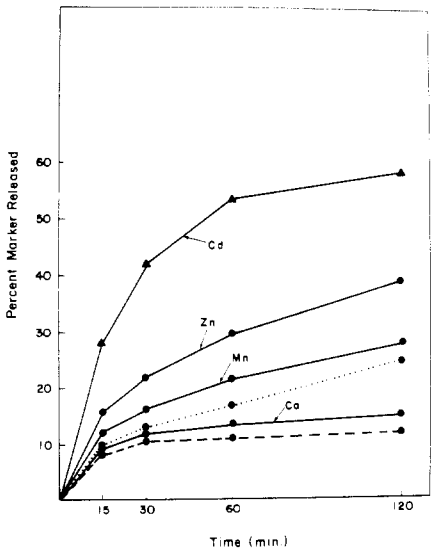

FIGURE 2: Specificity of divalent cation requirement for bacitracin-facilitated lysis of lecithin-cholesterol liposomes: (- - -) no additions, (. . . .) 10^{-3} M bacitracin without divalent cation, and (——) 10^{-3} M bacitracin with 10^{-2} M divalent cation. Solid line curves are labeled as to the relevant divalent cations.

TABLE I: Effect of Bacitracin and/or Cd^{2+} on the Per Cent Release of p-NAS from Diisostearoyllecithin-Cholesterol from Liposomes in the Presence of NO_3^- or Cl^-.[a]

Cd Concentration (M)	NO_3^-	Cl^-
	With 10^{-2} M Bacitracin	
10^{-2}	41.5	45.8
10^{-3}	35.7	47.9
10^{-4}	17.3	17.3
None	15.1	
	Without Bacitracin	
10^{-2}	7.34	5.62
10^{-3}	7.78	6.05
10^{-4}	8.3	5.62

[a] Procedure described in Materials and Methods.

In some experiments Cl^- was replaced by NO_3^- with the anticipation that anion specificity might also manifest itself. The NO_3^- containing samples, however, gave the same values as the Cl^- containing samples after 2 hr of incubation (Table I).

pH Dependence. The Cd- or Zn-bacitracin complex can be detected by its characteristic absorbance at 254 mμ. The complete conversion of bacitracin to this spectrophotometrically measured and presumably biologically active form requires a tenfold molar excess of divalent cation (Craig *et al.*, 1969; N. W. Cornell and K. Yanagimoto, unpublished results). Furthermore, complex formation is pH dependent with an optimum near neutrality for concentrations of both antibiotic and divalent cation of about 10^{-4} M. These observations parallel the pH dependence of the effect of the complex on liposomes in that liposome sensitivity was minimal at pH 5.5 (17% in Figure 3A), moderate at pH 6.5 (24% in Figure 3B), and maximal at pH 7.5 (35% in Figure 3C). It may be noted that the amount of p-NAS released at pH 5.5 is significant, whereas the bacitracin divalent cation complex was not detected in the presence of molar equivalents of cation and antibiotic at pH 5.5 (Craig *et al.*, 1969; Cornell and Guiney, 1970; N. W. Cornell and K. Yanagimoto, unpublished results). When the spectrophotometric determination was repeated with a 10:1 molar ratio of Cd-bacitracin, however, substantial complex formation could be

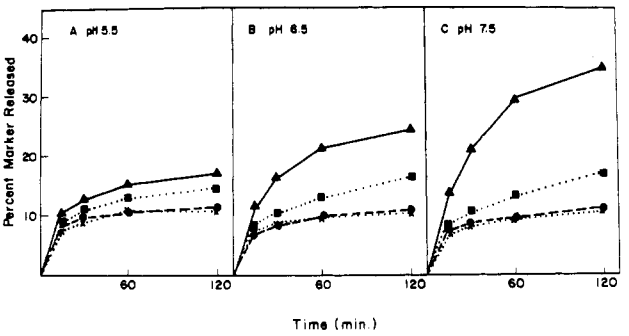

FIGURE 3: Influence of pH on the effects of 10^{-3} M bacitracin and/or 10^{-2} M $CdCl_2$ on the release of p-NAS marker from lecithin-cholesterol liposomes: (X) no additions, (●) $CdCl_2$, (■) bacitracin, and (▲) $CdCl_2$ + bacitracin.

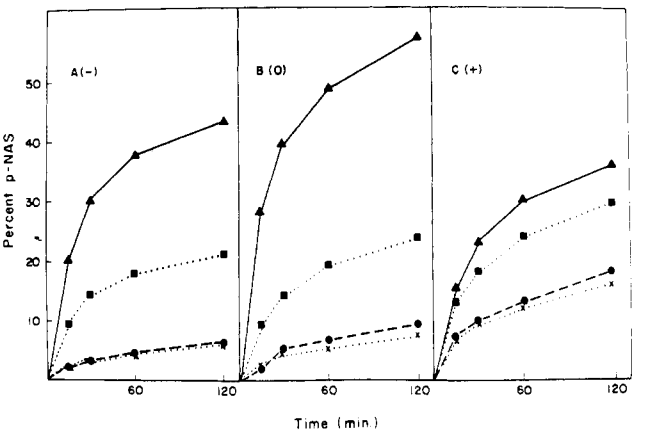

FIGURE 4: Response of liposomes bearing different charge signs to 10^{-3} M bacitracin and/or 10^{-2} M $CdCl_2$. Uncharged liposomes (Figure 4B) were composed of lecithin and cholesterol. Liposomes were rendered negatively charged (Figure 4A) by the inclusion of dicetyl phosphate, whereas liposomes were made positively charged (Figure 4C) by the inclusion of stearylamine. (●) no additions, (X) $CdCl_2$ alone, (■) bacitracin alone, and (▲) both $CdCl_2$ and bacitracin.

measured (unpublished results). Since the molar ratio of Cd-bacitracin in the liposome assay was 10:1, rather than 1:1, the release of p-NAS elicited by Cd-bacitracin at pH 5.5 appears reasonable.

Lipid Substrate Requirements. Liposomes of different charge sign were compared with respect to their response to bacitracin-Cd (Figures 4A-C). Vesicles negatively charged with dicetyl phosphate, uncharged, and positively charged with stearylamine all reacted specifically to the complex but to different extents. Although the total marker released was substantial in each case (negative ones released 43%, neutral ones released 57%, and positive ones released 36%), the net differences attributable to bacitracin-Cd were as follows: 22% (−), 34% (0), and 6% (+). In other words, the presence of Cd augmented the solely bacitracin-dependent p-NAS release (*i.e.*, base line having been discounted) by factors of 2.47 (−), 3.43 (0), and 1.63 (+).

Incorporation of cholesterol in liposomes enhances their sensitivity to the polyene antibiotic filipin (Kinsky *et al.*, 1968). As might be expected from this finding, filipin does not harm organisms lacking cholesterol. Since bacitracin affects cholesterol-free bacteria, on the other hand, liposomes without cholesterol should be as sensitive to bacitracin as liposomes with cholesterol. Figure 5A and B show this to be the case.

Effect of Bacitracin-Cd on Surface Charge Density of Liposomes. The surface charge density of diisostearoyllecithin-cholesterol liposomes in 0.005 M Tris-HCl (pH 7.5), as indicated in Table II, varies from −10.6 to −13.0 mV. A small amount of fatty acid contamination (about 4 e^-/100,000 Å^2) is therefore in evidence, since lecithin is uncharged between pH 3 and pH 9.5 (Bangham, 1968). Cadmium and calcium ions both neutralize this charge, but—most significantly—only in the presence of cadmium and bacitracin is a net positive potential seen. In contrast, when calcium and bacitracin are combined, the ζ potential remains zero, a finding in accord with other data showing that calcium cannot promote the interaction of bacitracin and lipid vesicles. The potential acquired by liposomes in the presence of bacitracin and cadmium is calculated, according to double layer theory, to be equivalent to about one positive electronic charge per 50 lipid molecules at the surface of the liposome. Without information about the charge residing on the bacitracin-cadmium complex, however, one cannot calculate the surface concentration of complexes. The simple demonstration of a significant positive potential, nevertheless, is clear evidence that the bacitracin-cadmium complex has an affinity for lipid surfaces not exhibited by either component alone or by bacitracin and a noncomplexing ion, *i.e.*, calcium.

Critical Micellar Concentration of Bacitracin. According to surface tension measurements of bacitracin-containing solutions (Figure 6), the critical micellar concentration is in the region of 10^{-5} M, at which level the surface tension is 48 dynes/cm, slightly higher than the 45 dynes/cm obtained by Helms and Weinberg (1963). Bacitracin-Cd gives a somewhat lower surface tension—*i.e.*, about 43 dynes/cm. It should be noted that the maximum solubility of Zn-bacitracin in water (see Merck Index of Chemicals and Drugs, 1960) is in the region of 0.004 M, at least 10 times greater than the highest concentration employed here.

Effect of Bacitracin-Cd on Planar Lipid Bilayers. Although bacitracin-Cd induced substantial leakage of p-NAS from liposomes, the sensitivity of the assay seems rather low in that the concentrations required—*i.e.*, 10^{-3} M—are considerably higher than are normally used for antibiotic purposes—*i.e.*,

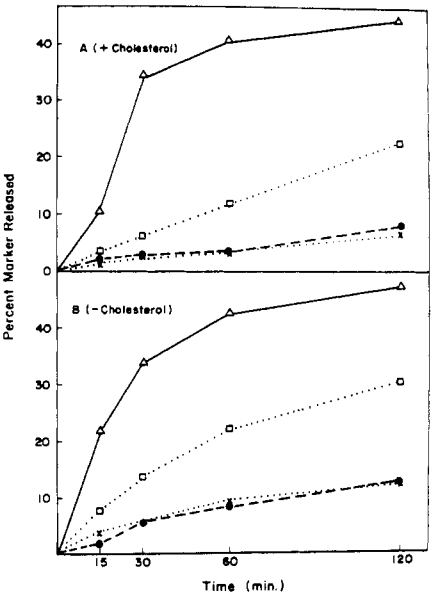

FIGURE 5: Liposomes in Figure 5A contained cholesterol in addition to lecithin, whereas liposomes in Figure 5B were composed of lecithin alone. Both kinds of vesicles were affected most drastically by 10^{-2} M bacitracin + 10^{-2} M $CdCl_2$ (△). (□) Bacitracin alone, (X) $CdCl_2$ alone, and (●) no additions.

TABLE II: Effects of Bacitracin, Cadmium, and Calcium on the ζ Potential (mV) of Diisostearoyllecithin–Cholesterol Liposomes in 0.005 M Tris (pH 7.5).[a]

Additives	Expt 1	Expt 2	Expt 3
None	−10.6 ± 0.7	−13.0 ± 1.0	−10.23 ± 0.9
10^{-3} M bacitracin	−8.1 ± 0.5	−10.3 ± 0.7	−10.0 ± 1.0
10^{-2} M $CdCl_2$	0	0	0
10^{-2} M $CaCl_2$	0	0	
10^{-3} M bacitracin + 10^{-2} M $CdCl_2$	+12.7 ± 0.5	+12.4 ± 0.4	+13.8 ± 0.6
10^{-3} M bacitracin + 10^{-2} M $CaCl_2$	0	0	

[a] Procedure described in Materials and Methods.

10^{-5} M. Electrical conductance measurements of planar lipid bilayer membranes, on the other hand, proved to be much more sensitive in revealing perturbations of the nonpolar core of these membranes.

Several membrane compositions were tested. The most sensitive was diisostearoyllecithin–cholesterol, 4:1 (w/w), as a 2% solution in hexadecane as the spreading solvent. In the presence of bacitracin at about 5×10^{-5} M and $CdCl_2$ at 5×10^{-4} M, membranes could be formed and would thin, at least partially, to the "black" stage; their conductance, however, increased by about tenfold during the course of thinning and the membranes invariably ruptured within a few minutes.

In another series of experiments with a 2:1 wt. ratio of diisostearoyllecithin–dehydrocholesterol in decane, the effect of cadmium was particularly dramatic. In the presence of 5×10^{-4} M bacitracin and 4×10^{-4} M $CdCl_2$, the membrane conductance rose by a factor of 10–100 shortly after formation and rupture occurred within a few minutes (Figure 7). With bacitracin alone at this concentration, the conductance is the same as that of an unmodified membrane. If Cd is then added to such a membrane, it responds by breaking almost immediately (Figure 7, lower curve). In the absence of bacitracin, cadmium ion has no effect.

It should be noted that Mueller and Rudin in their extensive survey of possible ionophores (1969) observed no effect of bacitracin on the conductance of planar bilayers. The sharp contrast between their findings and ours might well reflect the strict requirement for divalent cation, although the suggestion is presumptuous in the absence of more information on their experimental conditions.

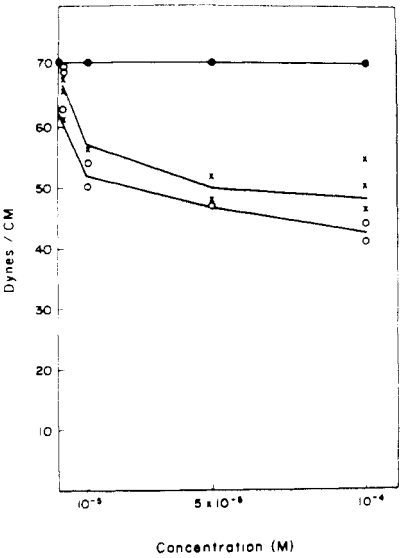

FIGURE 6: Comparison of the critical micellar concentrations of bacitracin (X) and of the bacitracin–Cd complex (O), as determined by the Wilhelmy hanging plate method; (●) the surface tension of water.

Discussion

The results presented here unequivocally demonstrate that bacitracin in combination with $CdCl_2$ or with $ZnCl_2$ can interact with purely lipid membranes so as to increase their permeability to a small molecule. In preliminary experiments human red blood cells exposed to the complex also sustained permeability changes as evidenced by their release of hemoglobin. Bacitracin alone or cadmium alone were ineffective in this respect. Although further study is necessary for the understanding of this type of hemolysis, it is clear that bacitracin can cause what amounts to morbidity of an artificial or a natural cell which lacks genetic machinery, a protein synthesizing system, and an energy generating mechanism. Therefore, the

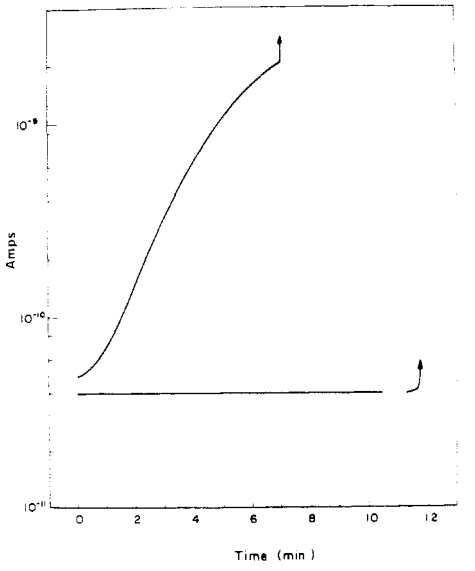

FIGURE 7: Effect of bacitracin ± Cd^{2+} on conductance of planar lipid bilayer membranes. The upper curve depicts the conductance of a membrane formed in 5×10^{-4} M bacitracin and 4×10^{-4} M $CdCl_2$. The membrane broke 7 min after this measurement was made. The lower curve represents the conductance of a membrane formed in 10^{-4} M bacitracin alone. The solution bathing one side of the membrane was made 3.5×10^{-4} M in $CdCl_2$ at 10.5 min and the membrane broke shortly thereafter. The gap in the lower curve indicates stirring time. Both membranes consisted of diisostearoyllecithin–dehydrocholesterol, 2:1 (w/w) in decane.

membrane lesion initiated by the complex, as described here, is not secondary to other kinds of lesions.

Certain characteristics of the effect on artificial cells, furthermore, suggest that the antimicrobial activity of bacitracin could follow from its ability to inflict membrane damage. First, like the bactericidal effect (see Weinberg, 1967), the antiliposome—as well as the hemolytic—effect is enhanced by the presence of specific divalent cations—*i.e.*, Cd or Zn. Since replacement of $CdCl_2$ with $Cd(NO_3)_2$ did not alter the results, specificity is probably dictated by the divalent cation, rather than the cation-anion complex. Second, the pH dependence of the *in vitro* effect correlates well with the pH dependence of the formation of the presumably biologically active bacitracin-divalent cation complex (Craig *et al.*, 1969; Cornell and Guiney, 1970). Third, liposome sensitivity to bacitracin is not dependent upon the presence of cholesterol. This finding is expected since bacterial membranes contain no cholesterol, but it seems at variance with the hypothesis that binding of bacitracin to C_{55}-isoprenyl pyrophosphate in the bacterial cell membrane is essential to the *in vivo* action of the antibiotic (Stone and Strominger, 1971; Storm and Strominger, 1973, 1974). The interaction of C_{55}-isoprenyl pyrophosphate and bacitracin, however, has not yet been shown to have the same specific divalent cation requirement as the bactericidal effect.

With respect to its solubility properties, bacitracin is somewhat of a curiosity among the numerous membrane-active antibiotics in that most of those commonly studied (*e.g.*, valinomycin, amphotericin, gramicidin, and nigericin—see Jain, 1972) are insoluble in polar solvents but fairly soluble in nonpolar solvents (Merck Index of Chemicals and Drugs, 1960). The reverse is true of bacitracin. Furthermore, a comparison of solubility data on bacitracin and zinc-bacitracin reveals that free bacitracin generally has a higher polar-nonpolar partition coefficient than zinc-bacitracin (Merck Index of Chemicals and Drugs, 1960). On the premise that one essential component of a membrane-disruptive property is hydrophobicity, one would predict that zinc and cadmium should specifically enhance this property of bacitracin by facilitating its interaction with lipid membranes. In fact, cadmium and bacitracin together—but neither alone nor calcium and bacitracin together—endow diisostearoyllecithin-cholesterol liposomes with a positive surface potential. Thus, cadmium, but not calcium, enables bacitracin to form a complex with the liposomes. The lesser sensitivity of positively charged liposomes as opposed to negatively or "uncharged" ones to bacitracin-Cd (Figure 4) may be due, in part, to the repulsion of like charges on liposome and divalent cation which would discourage their interaction.

A second necessary component of a membrane disruptive property is the capacity to exert surface pressure either directly or indirectly. In their analysis of the mode of action of various hemolysins, Schulman *et al.* (1955) have distinguished direct from indirect mechanisms on the basis of surface tension measurements. The "direct" lysins depress the surface tension of water to less than that achieved by the membrane itself, whereas the "indirect" lysins would be less potent in this respect. Since bacitracin *per se* or in combination with cadmium induces a minimal surface tension of 43 dynes/cm, which is much short of that produced by lecithin—*i.e.*, about 25 dynes/cm—it cannot directly exert the critical pressure. Two possibilities remain: (1) bacitracin-Cd interacts with one or more of the molecules in the membrane so as to cause that molecule(s) to exert the critical pressure; (2) bacitracin-Cd causes permeability changes which create osmotic pressure sufficient for lysis.

Results of the experiments on planar bilayer membranes indicate quite clearly that these simple lipid membranes are sufficiently perturbed by concentrations of bacitracin-Cd near the usual antibiotic concentrations so that membrane resistance, stability, or both are drastically reduced. At least two inconsequential factors, therefore, may account for the apparently low sensitivity of the liposome assay. First, since the concentration given for the liposome assay is that of bacitracin in the dialysis bag and since bacitracin is dialyzable, the final concentration of bacitracin should be somewhat lower than 10^{-3} M. Second and more importantly, the marker-containing compartment or "interstitial volume" of a hand-shaken lipid dispersion is only 10–12% of the total volume of the liposomes (Bangham *et al.*, 1967) so that a liposome target should give less response than a bacterial target with a much larger interstitial volume.

In principle it is possible to determine the nature of the charge carrier in membranes that have been modified but remain intact. We were unsuccessful in such attempts because the effect of bacitracin-Cd is sharply concentration dependent and quite drastic. Below a threshold concentration there is practically no effect, whereas above it rapid rupture ensues. In the most sensitive membranes, this threshold concentration corresponds approximately to the critical micellar concentration of the complex, so micelle formation—in addition to a specific divalent cation—may be necessary for the interaction of bacitracin with lipid membranes.

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The Nonequivalence of the Phosphorus Atoms in Cardiolipin

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ABSTRACT: Cardiolipin possesses two nonequivalent phosphorus atoms. This conclusion, based on symmetry considerations, is consistent with the available ^{31}P nuclear magnetic resonance (T. O. Henderson, T. Glonek, and T. Myers (1974), *Biochemistry 13,* 623) and phospholipase D hydrolysis data (A. N. Tucker and D. C. White (1971), *J. Bacteriol. 108,* 1058), both sets of data being difficult to rationalize on alternate grounds. Predictions concerning expected chemical reactions of cardiolipin, based on generally applicable symmetry arguments, are advanced.

In a recent application of ^{31}P nuclear magnetic resonance (nmr) spectroscopy two distinct resonances were observed for the two phosphorus atoms in cardiolipin (**1**) (Henderson *et al.,* 1974). The observed resonance doubling was interpreted in terms of an intramolecular hydrogen bond with one of the two "equivalent" phosphorus atoms, this bond undergoing slow exchange (on the nmr time scale) between the two otherwise "equivalent" phosphorus atoms. This explanation was employed to rationalize observed ^{31}P chemical shifts of monophosphorylated derivatives.

In seemingly unrelated experiments (White and Tucker, 1969; Short and White, 1970; Tucker and White, 1971) it has been noted that different metabolic turnover rates exist for the two phosphorus atoms in cardiolipins. Explanations of biphasic metabolic rates have centered on "pools" of materials with differing degrees of availability to metabolic processes.

We should like to pose an explanation for both the nmr results and the metabolic studies based on symmetry arguments. Symmetry considerations demand that the two phosphate moieties in cardiolipins be recognized as intrinsically nonequivalent; the nonequivalence in turn demands that the chemical and physical properties of the two groups be different.

Stereochemical Definitions and Nomenclature

The general interrelations of symmetry, stereochemistry, and nmr spectroscopy have been enunciated previously (Mislow, 1966; Mislow and Raban, 1967; Jacobus *et al.,* 1968; Jacobus and Raban, 1969; Jacobus, 1971). These relationships will be concisely reiterated here since they have bearing on both the previously mentioned nmr and metabolic studies.

Nuclei or groups of nuclei within molecules which can be interconverted by a rotational or rotational-reflectional symmetry operation[1] are equivalent in every sense under all conditions. Such nuclei are termed homotopic.

Nuclei or groups of nuclei within molecules which can only be interconverted by a reflection symmetry operation, *i.e.,* which bear a mirror image relationship one to the other, are termed enantiotopic. The environments of such nuclei or groups of nuclei are enantiomeric to one another.

Those nuclei or groups of nuclei which possess the same connectivity[2] but which cannot be interconverted by any symmetry operation are termed diastereotopic. Such nuclei reside in diastereomeric environments and bear the consequences of these environments, *i.e.,* they are nonidentical in every sense.

Homotopic nuclei are equivalent under all conditions; such nuclei must react with chemical reagents (achiral or chiral) at identical rates as they must also interact with solvent molecules (achiral or chiral) in identical manners. Homotopic nuclei are magnetically equivalent (isochronous) in achiral or chiral solvents, giving rise to single resonances in the absence of spin-spin coupling.

Enantiotopic nuclei or groups of nuclei are chemically and magnetically indistinguishable under achiral conditions; under chiral conditions such nuclei or groups of nuclei must react at different rates and must be chemical shift nonequivalent (anisochronous). Such nuclei or groups under chiral conditions exhibit "resonance doubling," this phenomenon being the basis of optical purity determinations employing chiral solvents (Pirkle and Beare, 1968) or chiral shift reagents (Whitesides and Lewis, 1971).

Diastereotopic nuclei or groups of nuclei are chemically and magnetically nonequivalent under all conditions. Such nuclei or groups of nuclei must react at different rates with any reagent and must be chemical shift nonequivalent in achiral or chiral solvents.

As will be demonstrated for cardiolipin, the application of symmetry considerations to accurately assess nmr spectroscopic and kinetic data on chiral systems is mandatory.

Biosynthesis of Cardiolipin

Two distinct pathways for the biosynthesis of cardiolipins

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[1] A symmetry operation is a manipulation (either real or imaginary) of an object such that the object after the manipulation is indistinguishable from the original object. Symmetry operations are of three types: rotation, reflection, and reflection rotation (Mislow, 1966).

[2] Like nuclei or like groups of nuclei bonded to the same atom or to identical groups of intervening nuclei possess the same connectivity.