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Interaction of Duramycin with Artificial and Natural Membranes†

J. Navarro,‡ J. Chabot, K. Sherrill, R. Aneja,§ S. A. Zahler, and E. Racker*
Section of Biochemistry, Molecular and Cell Biology and Section of Genetics and Development, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

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ABSTRACT: Duramycin is a polypeptide antibiotic (molecular weight 2012) obtained from culture filtrates of *Streptomyces cinnamomeus* forma azacoluta. In this work, we show that low concentrations of duramycin induced aggregation of lipid vesicles containing unsaturated phosphatidylethanolamine and unsaturated monogalactosyl diglyceride, and of sarcoplasmic reticulum vesicles from rabbit skeletal muscle. Furthermore, duramycin inhibited the ATP-dependent Ca^{2+} uptake in sarcoplasmic reticulum vesicles without affecting the hydrolysis of ATP or the permeability of Ca^{2+}. Also, duramycin only inhibited the bacteriorhodopsin proton pump reconstituted into phospholipid vesicles containing phosphatidylethanolamine. We have isolated a duramycin-resistant strain of *Bacillus subtilis* and have mapped the location of duramycin resistance. In this strain, the secretion of protons and influx of calcium were resistant to duramycin, and its lipid composition was profoundly different from that of the parent strain. No phosphatidylethanolamine was detected in the resistant strain. Our findings are consistent with the idea that duramycin recognizes a particular membrane conformation determined by the presence of phosphatidylethanolamine or monogalactosyl diglyceride.

Duramycin is a polypeptide antibiotic (M_r 2012) obtained from culture filtrates of *Streptomyces cinnamomeus* forma azacoluta (NRRL B-1699). It is active against Gram-positive bacteria, some yeasts, and fungi (Shotwell et al., 1958). It was shown recently that duramycin inhibits at low concentrations (5 μg/100 μg of protein) the chloride transporter and at higher concentrations (20 μg/100 μg of protein) the proton pump of clathrin-coated vesicles (Stone et al., 1984). It also permeabilizes intact cells (Racker et al., 1984). At very high concentrations (150 μg/100 μg of protein), the (Na^+, K^+)-ATPase was significantly inhibited, and reversal was observed by lipid mixtures containing phosphatidylethanolamine (Nakamura & Racker, 1984).

In this paper, we describe experiments on the interaction of duramycin with artificial liposomes, with the Ca^{2+} pump of sarcoplasmic reticulum, and with the proton pump of vesicles reconstituted with bacteriorhodopsin and the secretion of protons and uptake of calcium in duramycin-sensitive and duramycin-resistant *Bacillus subtilis*. Our data suggest that

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* Address correspondence to this author at the Section of Biochemistry, Molecular and Cell Biology, Cornell University.
‡ Supported by a postdoctoral fellowship from the Muscular Dystrophy Association. Present address: Department of Physiology, Boston University School of Medicine, Boston, MA 02118.
§ Present address: Nutri-med Biotech, Cornell University, Research Park, Langmuir Lab, Ithaca, NY 14850.

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duramycin recognizes a specific membrane conformation determined by the presence of phosphatidylethanolamine.

Materials and Methods

Synthetic and natural phospholipids were obtained from Avanti (Birmingham, AL). Monogalactosyl diglyceride and digalactosyl diglyceride were purchased from Applied Science Laboratories, Inc. (State College, PA). Thin-layer chromatography (TLC)[1] was carried out on silica gel layers (particle siie 6–10 μm, no binder, with phosphor, 250-μm thickness Adsorbil Plus P Prekotes soft layer from AllTech Associates). All solvents were HPLC grade. Triethylamine (>99%) was purchased from Fluka. Duramycin was supplied by Dr. O. Shotwell (Northern Regional Research Center, U.S. Department of Agriculture, Peoria, IL). A23187 was a gift from Dr. R. Hamill, E. Lilly Co. All other reagents were obtained from Sigma (St. Louis, MO).

Preparation of Lipid Vesicles. Lipid vesicles were prepared by cholate dialysis. Lipids in chloroform solution were first dried under a stream of nitrogen and lyophilized for 2 h and then solubilized with 1% potassium cholate at a weight ratio of detergent to lipid of 1:1 in the presence of 10 mM Tris-HCl (pH 7.4) and 0.15 M KCl. This mixture was sonicated to clarity in a bath-type sonicator (special cylindrical ultrasonic tank and G 122 SP1 generator, Laboratory Supplies Co., Inc., Hicksville, NY). The final concentration of lipids was 10 mg/mL. One milliter of solubilized lipids was dialyzed against 500 mL of 10 mM Tris-HCl (pH 7.4) and 0.15 M KCl at 0 °C for 24 h.

Turbidity Measurements. Duramycin-induced turbidity of liposomes was measured in a Cary 19 spectrophotometer by monitoring the changes in optical density at 400 nm as described (Rowe, 1982). Liposomes were diluted with 10 mM Tris-HCl (pH 7.4) and 0.15 M KCl to a final concentration of 300 μg of lipid/mL. Duramycin was added to the lipid vesicles, and the changes in optical density were recorded as a function of time.

Ca^{2+} Uptake and ATP Hydrolysis in Sarcoplasmic Reticulum Vesicles. Sarcoplasmic reticulm vesicles were prepared as described (MacLennan, 1970). ATP-dependent Ca^{2+} uptake in sarcoplasmic reticulum was determined by the Dowex–Tris column method (Gasko et al., 1976). The incubation mixture in a final volume of 0.25 mL contained 20 mM Tris–maleate (pH 7.0), 100 mM KCl, 10 mM $MgCl_2$, 2 mM potassium oxalate, 50 μM $^{45}CaCl_2$ (2 μCi/μmol), and 25 μg of protein. The reaction was initiated by addition of ATP to a final concentration of 1 mM, at 20–22 °C. The reaction was terminated by passing an aliquot (100 μL) of the incubation mixture through a Dowex 50X (100 mesh) column which was washed with the equilibration buffer containing 10 mM Tris-HCl (pH 7.4) and 0.25 M sucrose. By this procedure, the extravesicular Ca^{2+} was adsorbed to the column, and the radioactivity of the emerging sarcoplasmic reticulum vesicles was measured to determine Ca^{2+} uptake. ATP hydrolysis was determined in parallel experiments in the same incubation mixture containing [γ-^{32}P]ATP instead of $^{45}Ca^{2+}$.

[1] Abbreviations: DOPE (PE in figures), dioleoylphosphatidylethanolamine; MGDG, monogalactosyl diglyceride; DOPC (PC in figures), dioleoylphosphatidylcholine; SR, sarcoplasmic reticulum; TLC, thin-layer chromatography; DG, diglyceride; DPG, diphosphatidylglycerol; DOPG (PG in figures), dioleoylphosphatidylglycerol; DGuDG, diglucosyl diglyceride; lysyl-PG, lysylphosphatidylglycerol; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PI, phosphatidylinositol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Table I: Location of the *durR1* Mutation by PBS1 Transduction of the Donor CU3681 *durR1* and the Recipient CU3692 *cysA14 aroI906*

selection	phenotypes[a]	no. of transductants tested
Cys$^+$	Cys$^+$ Dur^R Aro$^-$	38
	Cys$^+$ Dur^A Aro$^-$	58
		96[b]
Aro$^+$	Cys$^+$ Dur^R Aro$^+$	10
	Cys$^-$ Dur^R Aro$^+$	43
	Cys$^-$ Dur^S Aro$^+$	46
		99[b]

[a] Dur^R or Dur^S indicates the ability or inability, respectively, to grow on TBAB + 90 μg of duramycin mL^{-1}. The data suggest the gene order *cysA-durR-aroI*. [b] Total.

The reaction was initiated by addition of 1 mM [γ-^{32}P]ATP (0.9 μCi/μmol) and terminated by mixing a 50-μL aliquot with 200 μL of 20 mM ammonium molybdate in 2.6 M H_2SO_4. After extraction with 1 mL of benzene/isobutyl alcohol (1:1), an aliquot of the organic phase was taken for the determination of [^{32}P]P_i released. ATP hydrolysis was calculated by subtracting the ATP hydrolysis in the presence of Ca^{2+} from that in the absence of Ca^{2+} and presence of 1 mM EGTA in the incubation mixture.

Freeze–Fracture Electron Microscopy. Drops of samples were placed between thin copper plates and frozen in liquid nitrogen/solid nitrogen. Copper sandwiches were mounted in a complementary fracture apparatus and fractured in a Balzer's freeze etch device when the vacuum was better than 2×10^{-6} torr. Fractures were done when the sample temperature was −150 °C. A carbon/platinum replica was formed. Replicas were cleaned with sodium hypochlorite, placed on coated copper grids, and examined in a Phillips 300 electron microscope. Most samples had 30% glycerol as a cryoprotectant.

Growth of Bacteria and Genetic Analyses. B. subtilis strain CU184, a prototrophic derivative of strain 168 (Spizizen, 1958), was grown in antibiotic medium 3 (Difco), pH 7.0, to late logarithmic phase at 37 °C in a gyrorotating shaker. One-tenth milliliter of the culture was plated on a tryptose blood agar base (Difco; TBAB) plate containing 90 μg of duramycin mL^{-1}. One of the spontaneous mutants capable of growth in the presence of 200 μg mL^{-1} antibiotic was purified and labeled CU3681; the mutuation is called *durR1*. The parent strain, CU184, is unable to grow in the presence of 4 μg of duramycin mL^{-1}. Like its parent strain, CU3681 was immune to bacteriophage SP*βc1* and sensitive to bacteriophage *ϕ*3T*c4*.

Preliminary mapping data [using methods described in Ward & Zahler (1973)] suggested that the *durR1* mutation is linked by transduction with phage PBS1 to the *cysA14* marker. An experiment that demonstrated the location of *durR1* between *cysA14* and *aroI906* on the *B. subtilis* chromosome at about 20 °C on the map (Henner & Hoch, 1980) is shown in Table I.

Lipid Analysis of Duramycin-Resistant and -Sensitive B. subtilis. Lipids were extracted from *B. subtilis* according to Bligh & Dyer (1959) as modified by Lillich & White (1971) and identified by a thin-layer chromatography method developed by one of us (R.A.). Silica gel plates were preconditioned with chloroform/methanol/triethylamine/water (62:27:12:3) and dried in a fume hood with an air stream. The lipids were spotted and developed with the same solvent mixture. The plates were dried again in the fume hood and then at 150 °C for 5–10 min and then sprayed for detection

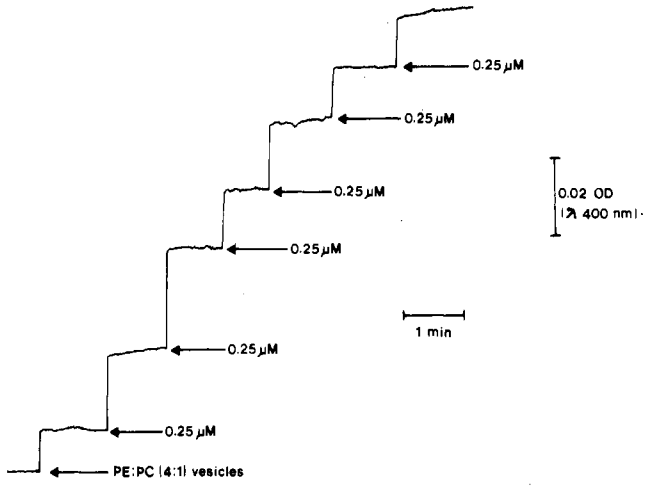

FIGURE 1: Duramycin-induced turbidity in DOPE–DOPC vesicles. Phospholipid vesicles were prepared and analyzed as described under Materials and Methods. The final concentration of lipids was 300 μg/mL at a DOPE:DOPC ratio of 4:1. Duramycin was added sequentially directly to the cuvette containing the liposomes.

of amino groups (ninhydrin), phosphate (Dittmer & Lester, 1964), and carbon by charring. Purified samples were reexamined by cochromatography with reference samples, and lysyl-PG was identified as described (Bishop et al., 1977; Houtsmüller & Van Deenen, 1965).

Results and Discussion

Duramycin-Induced Aggregation of Lipid Vesicles Containing DOPE or MGDG. We observed visually that duramycin added to crude soybean phospholipid vesicles induced turbidity. In order to analyze this phenomenon quantitatively, we monitored the changes in optical density at 400 nm in vesicles of defined lipid composition. As shown in Figure 1, duramycin induced increasing turbidity when added sequentially to liposomes prepared with dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) at a ratio of 4:1. There was no apparent effect on vesicles containing dioleoylphosphatidylcholine, phosphatidylserine, phosphatidic acid, or cardiolipin in the absence of DOPE or MGDG (Figure 2). Similar results were obtained when lipid vesicles were prepared by sonication in the absence of potassium cholate. DOPE and MGDG are chemically and structurally different lipids; however, both lipids upon hydration tend to adopt hexagonal II structures (Cullis et al., 1982; Shipley et al. 1973). Since our data show that duramycin induces aggregation only of liposomes containing the above lipids, we suggest that duramycin recognizes a lipid configuration formed by either DOPE or MGDG rather than a particular chemical group such as $-NH_2$. The molecular details of that lipid conformation are unknown. Alternatively, it is also possible that the relative larger size of the head group of lipids tending to form bilayers (e.g., phosphatidylcholine) prevents the interaction of duramycin with the membrane.

Duramycin-induced turbidity in suspensions of lipid vesicles could have arisen from either aggregation or fusion of the vesicles. Freeze–fracture electron microscopy of vesicles containing DOPE revealed that duramycin induced aggregation rather than fusion (Figure 3A,B). It should be noted that at the concentration required for aggregation, duramcyin did not induce leakage of [^{3}H]glucose from [^{3}H]glucose-loaded DOPE vesicles (data not shown).

Inhibition by Duramycin of ΔpH Generation in DOPE–Bacteriorhodopsin Vesicles. Since duramycin seems to interact with membranes containing DOPE, we have explored the

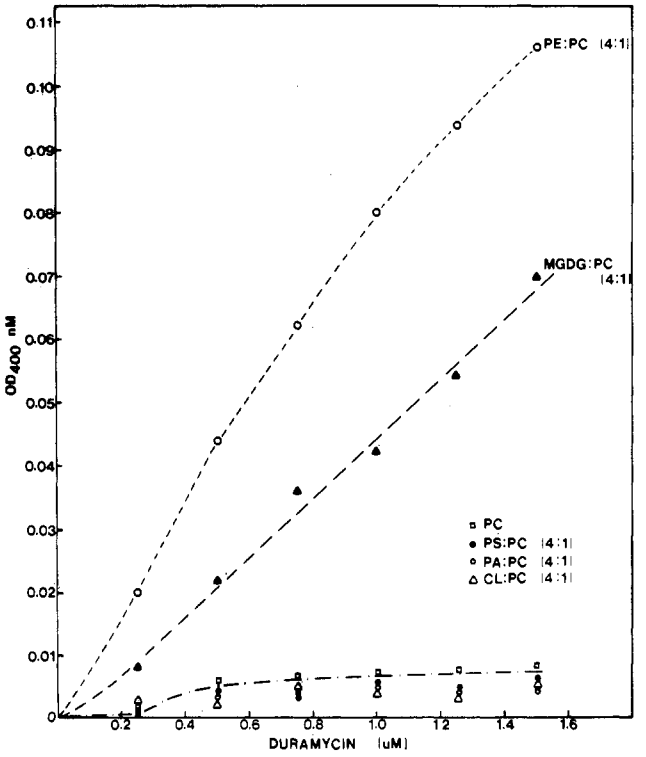

FIGURE 2: Duramycin-induced turbidity in vesicles made with several lipids. Experimental conditions were as described in the legend of Figure 1.

Table II: Effect of Phospholipid Composition on the Duramycin Sensitivity of the Reconstituted Bacteriorhodopsin Proton Pump[a]

vesicles	extent of proton pumping (nA of H^+)
DOPC	8.7
+25 μM duramycin	8.7
+25 μM additional duramycin	8.6
DOPC/DOPE (1:4)	11.2
+12.5 μM duramycin	6.1
+12.5 μM additional duramycin	4.3

[a] Bacteriorhodopsin (15 μg) was reconstituted into phospholipid vesicles by the octyl glucoside dilution method (Racker et al., 1979). The phospholipids used were (a) dioleoylphosphatidylcholine, 4% sonicated in 0.15 M KCl, and (b) dioleoyl-PC + dioleoyl-PE (1:4), 2% in 0.15 M KCl sonicated to almost clarity. The final octyl glucoside concentration was 0.75% with 100 μg of total phospholipids in a final volume of 40 μL at 0.15 M KCl. After 3-min incubation at 25 °C, the reconstituted vesicles were diluted into 1 mL of 0.15 M KCl and tested as described for light-driven proton translocation (Racker & Stoeckenius, 1974). Duramycin was added sequentially at the indicated concentrations.

sensitivity to duramycin of bacteriorhodopsin vesicles reconstituted by octyl glucoside dilution (Racker et al., 1979) with either DOPC or DOPC plus DOPE. DOPE- and DOPC-reconstituted vesicles revealed similar proton pump activity. Duramycin induced 50% inhibition of ΔpH generation in DOPE vesicles at 12.5 μM, whereas DOPC vesicles were unaffected even at 25 μM concentrations of duramycin (Table II).

Inhibition of the Ca^{2+} Pump of Sarcoplasmic Reticulum (SR) Vesicles. Hidalgo et al. (1982) have shown that modification of amino groups of phosphatidylethanolamine in sarcoplasmic reticulum with fluorescamine inhibited the rate of Ca^{2+} transport without affecting the rate of ATP hydrolysis and the Ca^{2+} loading. Recently, we have shown that lipids tending to adopt hexagonal II structures (DOPE or MGDG) are required for efficient coupling of ATP hydrolysis to Ca^{2+}

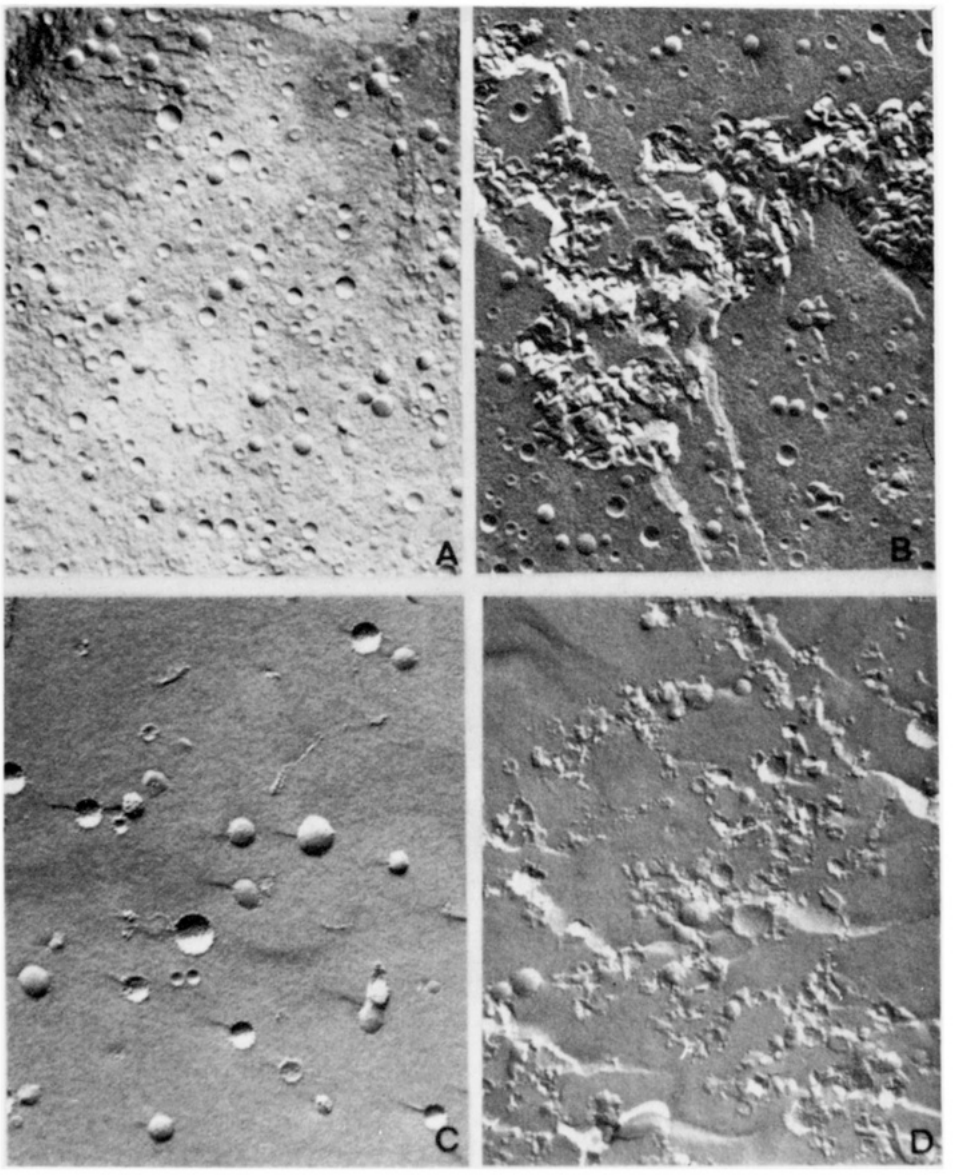

FIGURE 3: Freeze–fracture electron microscopy of reconstituted DOPE–DOPC vesicles and sarcoplasmic reticulum vesicles. (A) DOPE–DOPC vesicles at a weight ratio of 4:1 and a final concentration of lipid of 20 mg/mL. (B) DOPE–DOPC vesicles treated with duramycin (50 nmol/mg of lipid). (C) Sarcoplasmic reticulum vesicles (30 mg/mL). (D) Sarcoplasmic reticulum vesicles treated with duramycin (50 nmol/mg of lipid).

transport by the Ca^{2+}-ATPase (Navarro et al., 1984). These findings and the observation with duramycin described above led us to explore the effect of duramycin on the Ca^{2+} pump of sarcoplasmic reticulum. As shown in Figure 4, the ATP-dependent Ca^{2+} uptake was strongly inhibited by micromolar concentrations of duramycin, whereas the Ca^{2+}-dependent ATP hydrolysis was almost unaffected. Furthermore, duramycin reduced the Ca^{2+} loading capacity of sarcoplasmic reticulum vesicles. In order to rule out Ca^{2+} leakage induced by duramycin, we have measured efflux from $^{45}Ca^{2+}$-loaded sarcoplasmic reticulum vesicles. Figure 5 shows that duramycin up to 6 μM had little or no effect on the passive permeability for Ca^{2+}. In contrast, the "Ca^{2+} ionophore" A23187 induced a rapid efflux of Ca^{2+}.

As previously desribed (Baskin & Deamer, 1969), freeze–fracture electron microscopy of SR vesicles revealed a cytoplasmic face containing high-density particles and a luminal face with a low number of particles (Figure 3C). Duramycin-induced aggregation of SR vesicles was similar to that observed ith artificial vesicles containing DOPE or MGDG (Figure 3B,D). However, duramycin-induced aggregation of SR vesicles may or may not be associated with the inhibition of calcium transport.

Our results suggest that the interaction of SR vesicles with duramycin perturb the lipid–protein interaction required for the coupling of hydrolysis of ATP to Ca^{2+} transport in SR vesicles. Thus, duramycin may interact with the phosphatidylethanolamine–transport protein complex rather than with the lipid itself.

Lipid Composition, Proton Secretion, and Ca^{2+} Transport in Duramycin-Sensitive and -Resistant Mutants of B. subtilis. These studies were carried out with the idea that resistant mutants to duramycin may also arise from changes in the membrane lipid composition. We have isolated a duramycin-resistant mutant of *B. subtilis* which is capable of growth at high concentrations of duramycin (200 μg/mL). In TLC, the lipids from *B. subtilis* strain CU184 (duramycin-sensitive) were resolved into six spots (1–6, Figure 6, lane C) Spot 1 was negative for phosphate and ninhydrin and had the R_f of diglyceride (DG). Spot 2 was negative for ninhydrin and positive for phosphate and had the R_f of diphosphatidylglycerol (cardiolipin) (DPG). Spot 3 was negative for ninhydrin and

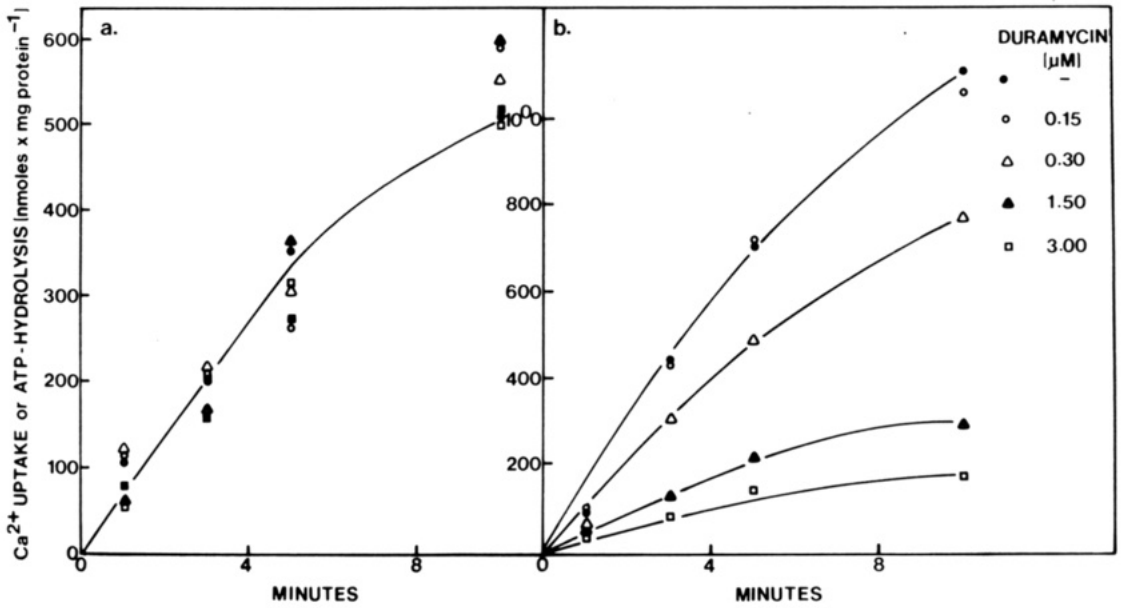

FIGURE 4: Effect of duramycin on Ca^{2+} uptake and ATP hydrolysis in sarcoplasmic reticulum vesicles. (a) Time course of the Ca^{2+}-dependent ATP hydrolysis. (b) Time course of the ATP-dependent Ca^{2+} uptake.

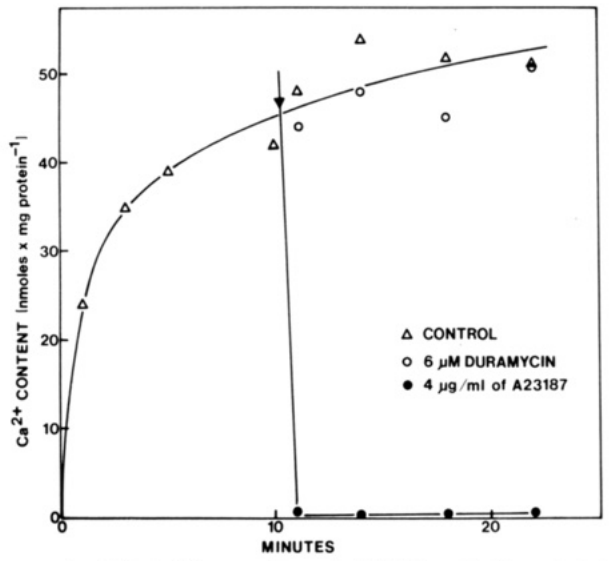

FIGURE 5: Effect of duramycin and A23187 on Ca^{2+} content in sarcoplasmic reticulum vesicles. Arrow indicates the time that duramycin or A23187 was added to the incubation mixture.

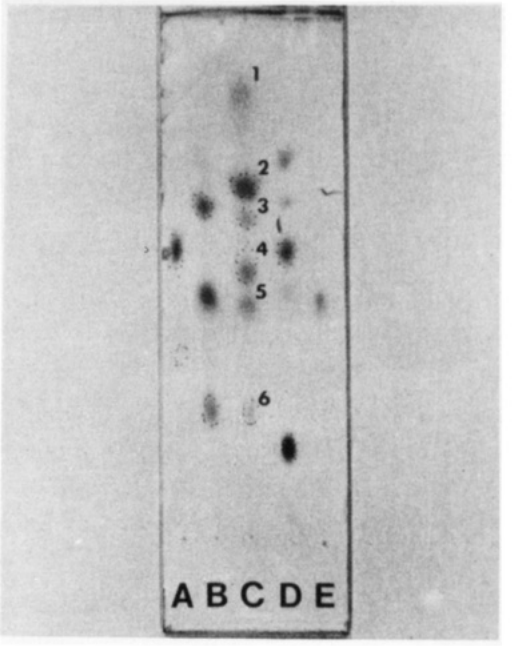

FIGURE 6: Lipid composition of *B. subtilis* CU184 and a duramycin-resistant mutant. Thin-layer chromatogram of the lipids extracted from *B. subtilis* strain CU184. (Lane B) Lipids of duramycin-resistant strain; (lane C) lipids of duramycin-sensitive strain. Other lanes contain, in decreasing R_f order, reference lipids (lane A) PE and lyso-PE, (lane D) DPG, PG, PE, PI, and PC, and (lane E) digalactosyl diglyceride.

positive for phosphate and had the R_f of phosphatidylglycerol (PG). Spot 4 was positive for ninhydrin and for phosphate and had the R_f of phosphatidylethanolamine (PE). Spot 5 was negative for ninhydrin and for phosphate and had the R_f of diglucosyl diglyceride (DGuDG). Spot 6 was positive for ninhydrin and for phosphate and had the R_f of lysyl-phosphatidylglycerol (Lysyl-PG). In the lipids from the duramcyin-resistant strain (lane B), spot 4 corresponding to PE was absent, and only faint spots corresponding to 1 (DG) and 2 (DPG) were present. PG, DGuDG, and lysyl-PG (corresponding to spots 3, 5, and 6, respectively) comprised the major lipids.

Table III shows that the rate of proton secretion in duramycin-resitant mutants was insensitive to very high concentrations (25 μM) of duramycin, whereas the wild-type *B. subtilis* was very sensitive to low concentrations (0.25 μM) of duramycin. A similar pattern of inhibition was found when we analyzed the effect of duramycin on Ca^{2+} influx in intact cells (Figure 7). Our findings suggest that the inhibitory action of duramycin on the proton secretion and Ca^{2+} influx in *B. subtilis* may be related to the lipid composition of the membrane.

Table III: Effect of Duramycin on Proton Secretion in Duramycin-Resistant and -Sensitive *B. subtilis*[a]

resistant *B. subtilis* CU3681		sensitive *B. subtilis* CU184	
additions	nA of H^+/min	additions	nA of H^+/min
none	11.8	none	6.8
duramycin (2 μM)	11.8	duramycin (0.10 μM)	4.2
duramycin (50 μM)	9.2	duramycin (0.25 μM)	2.0

[a] The incubation mixture contained 150 mM NaCl, 20 mM glucose, and 660 μg (wet weight) of *B. subtilis* in a final volume of 1 mL. Proton secretion was measured with a pH electrode at pH 6.3–6.5.

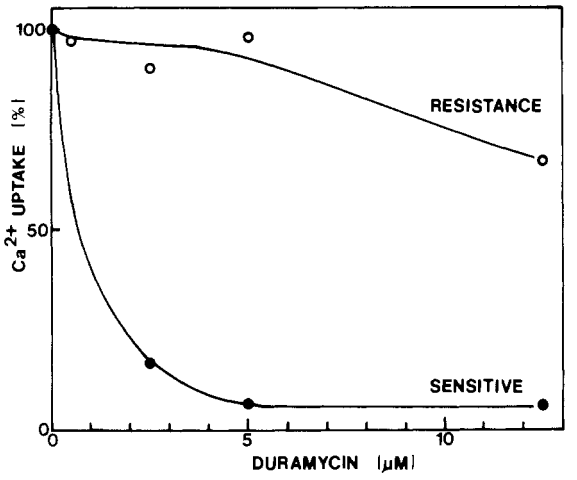

FIGURE 7: Effect of duramycin on Ca^{2+} uptake in duramycin-sensitive and -resistant *B. subtilis*. The incubation mixture contained 15 mM glycylglycine buffer (pH 7.0), 0.1 M NaCl, 50 μM CCCP, 10 μM $^{45}CaCl_2$ (100 μCi/μmol), and 150 μg (wet weight) of *B. subtilis* in a final volume of 500 μL. The reaction mixture was incubated at room temperature (20–22 °C) for 10 min. The transport was terminated by passing an aliquot of the reaction mixture through Dowex–Tris columns as described under Materials and Methods. 100% accumulation in 10 min was 217 and 220 pmol of Ca^{2+}/mg of wet weight for the wild-type and duramycin-resistant *B. subtilis*, respectively.

The protoplast membrane of *Bacillus subtilis* strain Marburg has been reported (Bishop et al., 1977; Kusaka & Kitahara, 1967) to contain diphosphatidylglycerol (cardiolipin) (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), diglucosyl diglyceride (DGuDG), and lysyl-phosphatidylglycerol (lysyl-PG) (Bishop et al., 1977). In the present study, the same five polar lipids were identified in *B. subtilis* strain CU184 (duramycin-sensitive). The relative amounts are estimated in the order DPG > PE > DGuDG > PG > lysyl-PG. In contrast, PE was absent from the lipids of duramycin-resistant *B. subtilis* strain CU3681, and DPG was present as a very minor component; the relative amounts are estimates in the order PG > DGuDG > lysyl-PE >>> DPG.

It has been reported that the lipid composition of the cytoplasmic membrane of *B. subtilis* varies in response to the composition of the growth medium (Bishop et al., 1977; Kusaka & Kitahara, 1967). Changes were reported in the quantitative amounts of the constituents by growing the bacteria in media without or with glucose; this resulted in a change of lipids from DPG > PE > PG > lysyl-PG to PE > lysyl-PG > DPG > PG and in some reduction in lipid-bound sugar content. Changes analogous to those found between the duramycin-sensitive and duramycin-resistant strains have been observed previously in phosphate- and magnesium-limited cultures of *B. subtilis* at neutral pH (Minnikin & Abdolrahimzadeh, 1974; Minnikin et al., 1972). It is therefore quite possible that the mutation involves a transport process and that the lipid changes are secondary.

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Registry No. DOPC, 10015-85-7; DOPE, 2462-63-7; Ca, 7440-70-2; H^+, 12408-02-5.

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