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Molecular Mechanisms of Gossypol Action on Lipid Membranes*

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Gossypol, an aldehyde extracted from cotton plants, produces both general toxic and antifertility effects in mammals. The cellular mechanisms by which gossypol exerts these effects are not understood. In this study, we have characterized the interactions of gossypol with lipid monolayer and bilayer membranes in order to assess if the drug acts by modifying the electrochemical properties of membranes. The charged form of gossypol binds to monolayers of different lipid compositions with apparent dissociation constants ranging from 0.7 to 2 μM. Binding of charged gossypol decreases the interfacial potential by 80–235 mV, the magnitude of this decrease being dependent upon the lipid composition. Gossypol also induces a conductance in phospholipid bilayer membranes. The relation between steady-state bilayer conductance *versus* gossypol concentration indicates that the current-carrying species is a single molecule of gossypol. The increase in bilayer conductance is accompanied by an increase in proton permeability. These changes induced by gossypol in model membranes can account for the mitochondrial uncoupling effects of this molecule and may be responsible for the inhibitory effects of gossypol on several membrane transport systems.

Gossypol, a disesquiterpene aldehyde extracted from cotton plants, produces both general toxic and antifertility effects in mammals. These actions appear to be both species and dose dependent (for a review, see Zatuchini and Osborn, 1981). Thus, in man and rat, ingestion of 20 mg/day and 10–20 mg/kg/day of gossypol, respectively, for several weeks produces antifertility effects on testicular function with no apparent general toxicity. The cellular mechanisms by which gossypol exerts its toxic and antifertility effects are not well understood. Gossypol has been shown to affect the activities of some membrane-bound mitochondrial enzymes, to uncouple mitochondrial oxidative phosphorylation, to inhibit the (Na^+-K^+)-ATPase, and to inhibit anion transport in red blood cells (Abou-Donia and Dieckert, 1974; Tso *et al.*, 1982; Adeyemo *et al.*, 1982; Haspel *et al.*, 1982). These actions of gossypol presumably are exerted at the level of cell membranes. The effects of gossypol on membrane function can be attributed either to specific interactions with membrane proteins or to binding and modification of the properties of the lipid bilayer matrix. Thus, a detailed characterization of the interactions of this compound with lipid membranes is needed to estimate the relative contribution of gossypol acting as a protein reagent or as a lipid bilayer modifier in its toxic and antifertility actions.

In the present study, we have characterized the interactions of gossypol with lipid monolayer and lipid bilayer membranes. We show that gossypol binds strongly to monolayers of different lipid compositions. Binding of gossypol to lipid monolayers and bilayers decreases the interfacial potential of these membranes. We further show that gossypol also induces an electrical conductance in phospholipid bilayers. This increase in bilayer conductance is accompanied by an increase in proton permeability. All these changes induced by gossypol in model membranes can explain the effects of gossypol as an uncoupler of mitochondrial oxidative phosphorylation and could be responsible for the inhibitory effects of gossypol on several membrane transport systems (*e.g.* anion transport in erythrocytes; (Na^+-K^+)-ATPase). The properties of the gossypol-induced conductance in lipid bilayers and the binding of this compound to lipid vesicles are consistent with a model where gossypol acts as a proton carrier in biological membranes.

MATERIALS AND METHODS

Isolation of Rat Liver Cells—Hepatocytes were isolated from rats previously starved for 24 h using a method modified from Berry and Friend (1969). The perfusion solutions of modified Krebs-Henseleit buffers were supplemented with 20 mM glucose, 15 mM Hepes[1] (pH 7.4), and bovine serum albumin (2 mg/ml). Calcium choride (1.6 mM) was included in the collagenase solution (80 units/ml, Worthington). The original cell suspension was incubated in Krebs-Henseleit medium supplemented with 20 mM glucose, 15 mM Hepes (pH 7.4), bovine serum albumin (2.5 mg/ml) at 37 °C with 95% O_2, 5% CO_2 for 20 min. The cells were then filtered through a 74-μm nylon mesh screen and thrice subjected to differential centrifugations (each 2 min, 50 × *g*) in a refrigerated centrifuge (4 °C). Trypan blue exclusion values were always >92% after a 4-min exposure to the dye (2.5 mM) at 37 °C under 95% O_2, 5% CO_2.

Preparation of Rat Liver Mitochondria—Rat liver mitochondria were prepared using a homogenization and differential centrifugation technique in a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes/KOH (pH 7.4), 2 mM EGTA, and 0.5 mg/ml of defatted bovine serum albumin (Pedersen *et al.*, 1978). Freshly prepared mitochondria had respiratory control ratios between 5 and 6 in glutamate/malate and high potassium medium at 37 °C. Mitochondrial ADP/oxygen ratios in glutamate/malate and succinate were 2.9 and 1.9, respectively.

Oxygen Consumption Measurements—Oxygen consumption measurements were performed polarographically in a sealed glass chamber (0.9 ml) surrounded by a water jacket held at 37 °C. Isolated intact cells were preincubated for 15 min in modified Krebs-Henseleit buffer at 37 °C with 95% O_2, 5% CO_2 supply before being transferred to the O_2 consumption chamber. QO_2 values were obtained from the slope of the O_2 tension *versus* time record between 15 and 60 s after each

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[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

experimental addition to the QO_2 chamber. Oxygen consumption of isolated mitochondria was measured by addition of an aliquot of the organelle suspension to the O_2 consumption chamber containing medium preincubated at 37 °C with 95% O_2, 5% CO_2.

Interfacial Potential Measurements in Lipid Monolayers—The interfacial potential of lipid monolayers and the changes induced by gossypol were measured at room temperature (20 ± 2 °C) in a two-compartment chamber as described by Reyes *et al.* (1983). An excess of lipids (10 μl, 10 mg/ml) was added in chloroform solution to the air/water interface to obtain a saturated monolayer. The experiments were performed at constant monolayer area (20 cm^2). The subphase was composed of NaCl at different concentrations and buffered at different pH values as described in the text. The concentration of gossypol was varied by addition of microliter volumes of a concentrated ethanolic solution of gossypol to the subphase of the monolayer-free compartment. The subphase was stirred with one magnetic bar positioned under a partition that separated the surface of the monolayer and monolayer-free compartments. Control experiments showed that ethanol at the concentrations used (<0.3%, v/v) had no effect on the surface potential of the lipid monolayers.

ζ Potential Measurements—The ζ potential of multilamellar phosphatidylcholine vesicles in the absence and presence of gossypol was estimated from electrophoretic mobility measurements. The multilamellar PC vesicles were prepared by evaporating to dryness 1 ml of a 10 mg/ml chloroform solution of PC in a N_2 atmosphere, addition of 1 ml of the corresponding buffer, and 2 min of vortexing. The measurements of the electrophoretic mobility were made in a commercially available cylindrical microelectrophoresis apparatus (Rank Brothers, Bottisham, Cambridge, United Kingdom) based on a design by Bangham *et al.* (1958). The apparatus was calibrated with fresh human erythrocytes and from their known electrophoretic mobilities at the ionic concentrations of 14.5 and 145 mM NaCl (Seaman and Heard, 1960). All the measurements were performed at 25 °C. The ζ potential was calculated from the Helmholtz-Smoluchowski equation as described by McLaughlin *et al.* (1975).

Lipid Bilayer Experiments—The bilayer membranes were formed at room temperature (20 ± 2 °C) according to the Montal and Mueller technique (1972). The phospholipid was spread on the surface of an electrolyte solution using 5 μl of a 10 mg/ml solution of the lipids in pentane. The ~0.5-mm round aperture in the Teflon partition separating the two aqueous compartments was pretreated with a 2% solution of squalene in pentane. Unless otherwise indicated, the electrolyte solutions were symmetrical and consisted of 0.1 M NaCl buffered with 50 mM phosphate. Gossypol and CCCP were added from concentrated ethanolic solutions to the aqueous phases bathing the membrane. Control experiments showed that ethanol, at the concentrations used (<0.5%, v/v), had no effect on either the bare membrane conductance or capacitance, or on the CCCP-induced conductance.

The system of measuring the electrical properties of the membranes has been described in detail by Alvarez and Latorre (1978). The capacitance of the membranes was measured by applying a 20-Hz, 2-mV peak-to-peak triangular voltage wave form. The area of the membrane was estimated from the capacitance value and the known value of the specific capacitance of the phosphatidylethanolamine bilayer (0.68 microfaraday/cm^2; Reyes and Latorre, 1979). The zero-voltage conductance induced by gossypol and CCCP were calculated from steady-state current-voltage curves recorded directly on a X-Y recorder 10 min after symmetrical addition of the compounds to the aqueous phase.

Duration of the Experiments with Lipid Monolayers and Lipid Bilayers—The changes in interfacial potential and conductance induced by gossypol on lipid monolayers and lipid bilayers reach a steady level about 7–10 min after addition of gossypol to the aqueous solution and remain stable for at least 45 min. When adding successive concentrations of gossypol, the experimental measurements were normally completed within 30–40 min.

Sources of Lipids and Chemicals—Bacterial phosphatidylethanolamine, soybean phosphatidylcholine, diphytanoyl phosphatidylcholine, and cardiolipin were obtained from Avanti Biochemicals (Birmingham, AL). Cholesterol was purchased from Applied Science Laboratories Inc. (State College, PA). Gossypol acetic acid (Peking batch 1) in powder form was supplied by the Rockefeller Foundation, New York, NY. Prior to distribution, the gossypol had been purified by high-pressure liquid chromatography (99.8% purity) by the Institute Materia Medica, Chinese Academy of Medical Sciences, Beijing, China. Gossypol-free acid was obtained from Sigma. CCCP was obtained from Calbiochem-Behring. Pentane and chloroform (Spectro grade) were purchased from Fisher. Squalene was obtained from Eastman Organic Chemicals (Rochester, NY).

Gossypol acetate crystals were stored with desiccant at −20 °C. The solutions of gossypol in ethanol were prepared fresh every week and kept at −20 °C. Under these conditions, the chemical half-life of gossypol in ethanolic solution is >100 days (Nomeir and Abou-Donia, 1982).

RESULTS

Tautomeric Equilibrium of Gossypol in Aqueous Solution—The gossypol molecule presents three tautomeric forms in aqueous solution (Berardi and Goldblatt, 1980). Two of these tautomers are the keto and enol forms of the aldehyde and hydroxyls in positions 8, 8′ and 7, 7′, respectively (Fig. 1). This keto-enol tautomerism predicts that gossypol should have acidic protons that could combine with a base to give an enolate anion. The acid dissociation constant of this acid-base equilibrium of gossypol in aqueous solution was estimated using the following method. Ten ml of a 0.97 mM NaOH solution were titrated with consecutive aliquots of a 10 mM HCl solution in the presence and absence of 50 μM gossypol and 0.1 M NaCl at 20 °C. The titration curves obtained under these different experimental conditions are shown in Fig. 2. A gossypol-induced inflection in the titration curves was observed with a mid-titration point at an average pH of 6.5. The solubility of gossypol in aqueous solution is greatly reduced at pH values lower than 6.5. For instance, gossypol is soluble up to approximately 2 μM at pH 4.7.[2] For this reason, the buffering capacity given by gossypol in solution, manifested as an inflection in the titration curves, decreases at acidic pH. This effect of solubility would set the true pK_a value of gossypol in aqueous solution at a value less than 6.5.

Interaction of Gossypol with PC Lipid Monolayers—Spreading of a PC monolayer on a clean air/electrolyte solution interface at pH 7.2 induced a change in the interfacial potential ($\Delta\Psi$) of about 400 mV (air phase positive) (Fig. 3). Addition of gossypol to the subphase of the monolayer produced a decrease in the monolayer interfacial potential ($\Delta\Delta\Psi$). This drug-induced change in the monolayer interfacial potential was partially reversible (60%) when the subphase of the monolayer (15 ml) was washed successively three times with

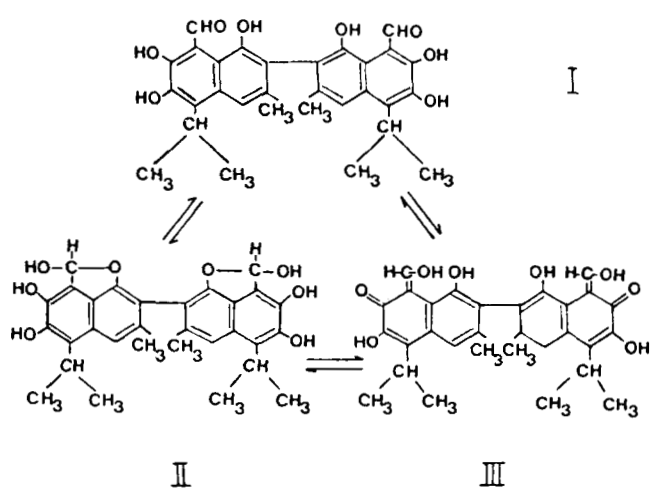

FIG. 1. **Tautomeric structures of gossypol (1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl[2,2′-binaphthalene]-8,8′-dicarboxaldehyde) in aqueous solution.**

[2] J. Reyes, J. Allen, N. Tanphaichitr, A. R. Bellvé, and D. J. Benos, unpublished observations.

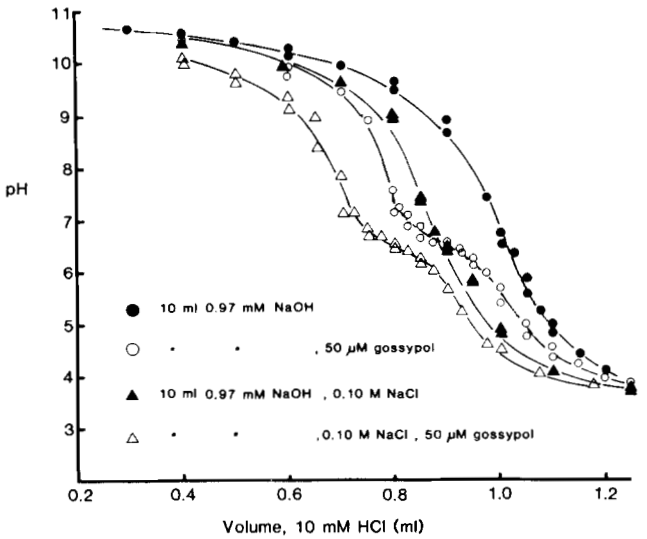

FIG. 2. **Titration curves of 50 μM gossypol in aqueous solution.**

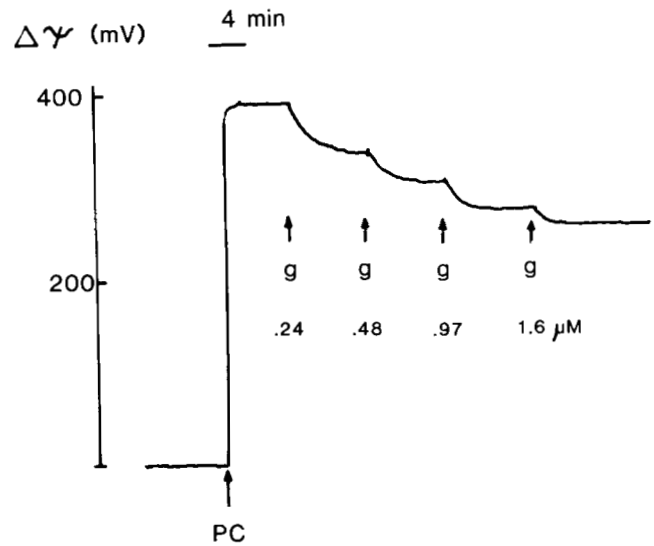

FIG. 3. **Time course of interfacial potential (ΔΨ) changes of a PC monolayer produced by stepwise increments in the concentration of gossypol (*g*) in the electrolyte subphase.** The *arrow* labeled *PC* denotes the addition of 10 μl (10 mg/ml) of phosphatidylcholine in chloroform to the air/electrolyte interface. The subphase electrolyte solution consisted of 0.10 M NaCl buffered to pH 7.2 with 1 mM phosphate at 20 ± 1 °C.

20 ml of gossypol-free buffer (data not shown).

pH Dependence of the Effect of Gossypol on PC Monolayer Interfacial Potentials—Addition of gossypol up to 1.4 μM to the subphase of a PC monolayer at pH 4.2 (0.1 M NaCl, 2 mM acetate) produced less than a 10-mV change in the interfacial potential of the monolayer. A change of the subphase pH from 4.2 to 7.0 by addition of a predetermined amount of NaOH in the presence of gossypol induced a 120-mV decrease in the monolayer interfacial potential (data not shown). Control experiments showed that similar pH changes produced no effects on the interfacial potentials of PC monolayers in the absence of gossypol (see also Papahadjopoulos, 1968). The dose-effect relationship of gossypol on PC monolayer interfacial potentials at different subphase pH values is presented in Fig. 4*a*. These results demonstrate that the tautomeric form(s) of gossypol present below pH 5 either do not bind to PC monolayers or that these tautomers have electrical properties different from the tautomeric species present at neutral or basic pH. Moreover, measurements of the changes in interfacial potential of PC monolayers at 2 μM gossypol showed a mid-titration point at pH 5.5–6.0 (Fig. 4*b*).

Modulation by Lipid Composition of the Binding of Gossypol to Lipid Monolayers—The changes induced by gossypol on the interfacial potential of lipid monolayers made with different lipid compositions are depicted in Fig. 5. These results show that the lipid composition modulates the magnitude of the changes in ΔΨ that gossypol produces on monolayers. The lipid compositions selected in these studies represent the different compositions of plasma and inner mitochondrial membranes in mammalian species. Gossypol produced the largest change in ΔΨ in monolayers comprised of at least 50% PC. Addition of either cholesterol or cardiolipin to PC or PC-PE monolayers depressed the magnitude of the gossypol-induced change in interfacial potential at all drug concentrations.

The ΔΔΨ of lipid monolayers induced by the adsorption of increasing concentrations of gossypol exhibits a saturation curve, thereby permitting the derivation of some operationally defined binding and maximal effect parameters. On this basis, the data obtained for PC and PE monolayers can be presented in a single reciprocal plot as shown in Fig. 6 ($-\Delta\Delta\Psi$ *versus* $-\Delta\Delta\Psi$/[gossypol]; Eadie, 1942). Only the data below −100 mV were used in these analyses since at these concentrations the gossypol-induced double-layer potential is ≤6 mV (see below). A linear regression of these points gives the apparent dissociation parameter (M_d^{app}, slope) and the maximum change in the interfacial potential ($-\Delta\Delta\Psi_{max}$, *y* intercept) of the effect of gossypol in lipid monolayers. The apparent dissociations and maximum interfacial potential changes of gossypol for different types of lipid monolayers obtained using the mentioned procedure are shown in Table I. The M_d^{app} and $-\Delta\Delta\Psi_{max}$ calculated in this way also contain the contributions of gossypol-induced double-layer potentials and permanent double-layer potentials of the monolayers. The apparent dissociation constants for different lipids fall in the range 0.7–2.0 μM gossypol. From these results, we can conclude that gossypol will bind to any lipid membrane in the organism when present at micromolar plasma concentrations. However, the magnitude of the interfacial potential change, and hence the effects that such changes can produce on membrane function, will depend on the lipid composition of the membrane.

Binding of Negatively Charged Gossypol Molecules to PC Vesicles—The ζ potential induced by gossypol on multilamellar PC vesicles was estimated from the electrophoretic mobility at 25 °C. The vesicles were incubated with different concentrations of gossypol at room temperature for 10 min and then transferred to the microelectrophoretic apparatus. Fig. 7*a* shows curves of ζ potential changes of the vesicles *versus* aqueous gossypol concentration at different pH values. At 1 mM NaCl, decreasing pH from 8.5 to 5.0 produced a decreased change in ζ potential by gossypol. This pH dependence of the gossypol-induced change in ζ potential of PC vesicles is similar to gossypol's influence on lipid monolayer interfacial potentials (see Fig. 4). A mid-titration point for the induced ζ potentials exists at around pH 5.5–6.0 (Fig. 7*b*). At constant pH, increasing salt concentration from 1 to 100 mM decreased the gossypol-induced change in ζ potential. The gossypol-induced negative ζ potentials in PC vesicles at 100 mM NaCl and pH 7.6 were 4, 12, and 24 mV at 1, 2, and 4 μM gossypol, respectively.

Gossypol-induced Conductance in Lipid Bilayer Membranes—Addition of gossypol to the aqueous phases bathing a PE membrane increased the bilayer conductance. An ex-

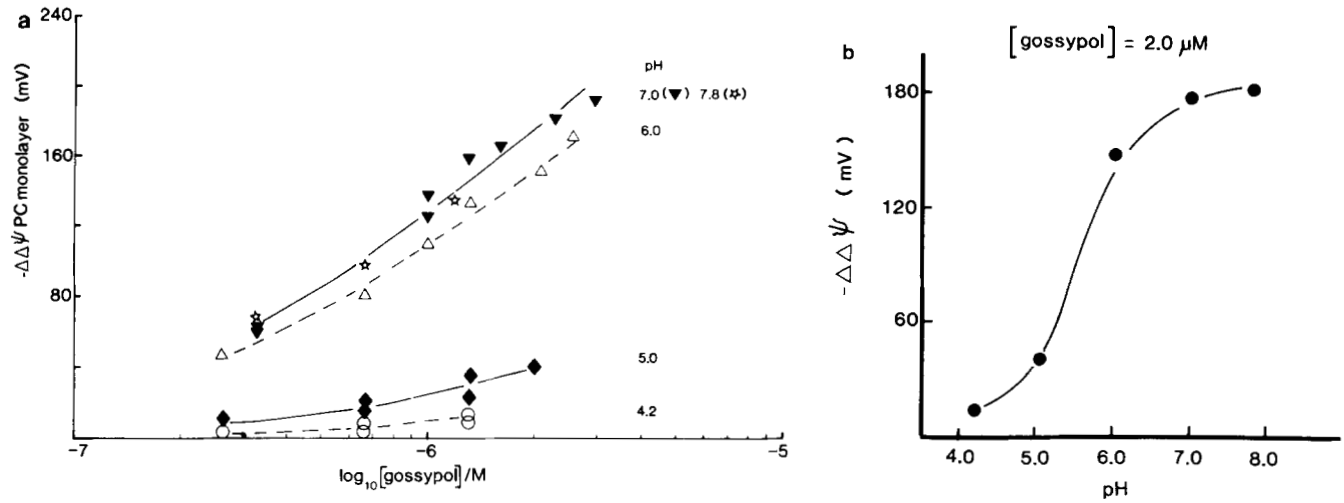

FIG. 4. **pH dependence of gossypol effect on monolayer interfacial potentials.** *a*, log dose-response curves of gossypol-induced changes in phosphatidylcholine monolayer interfacial potentials ($-\Delta\Delta\Psi$) *versus* gossypol concentration at different subphase pH values. In all experiments, the subphase consisted of 0.1 M NaCl and either 0.5 mM phosphate (pH 7.8, 7.0, and 6.0) or 0.5 mM acetate (pH 5.0 and 4.2). Gossypol-free acid (Sigma) at pH 7.0 produces similar changes in PC monolayer interfacial potential as compared with gossypol/acetic acid (data not shown). *b*, interfacial potential change induced by 2 μM gossypol concentration on PC monolayers and at different pH values of the electrolyte solution. Other experimental conditions are the same as in *a*.

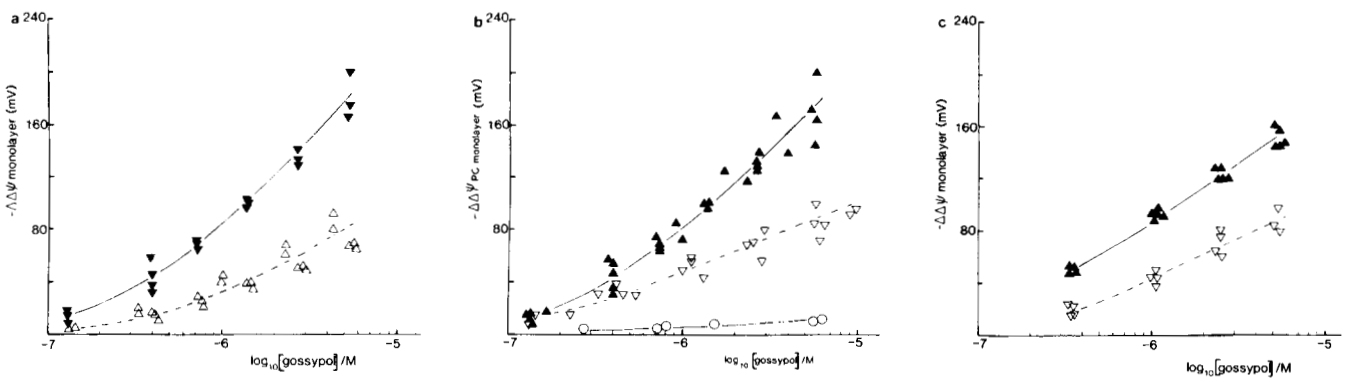

FIG. 5. **Lipid composition dependence of the effect of gossypol on monolayer interfacial potentials.** *a*, phosphatidylcholine (▼) and phosphatidylethanolamine (△) monolayers; *b*, PC (▲), cholesterol (○), and PC/cholesterol (▽, molar ratio 0.5:0.5) monolayers; *c*, PC/PE (▲, molar ratio 0.52:0.48) and PC/PE/cardiolipin (▽, molar ratio 0.4:0.4:0.2) monolayers.

ample of the current-voltage characteristics of the gossypol-modified membrane at pH 7.1 is shown in Fig. 8*a*. A supralinear current-voltage relationship with no evidence of current saturation was observed at least up to an applied voltage of ± 150 mV. Fig. 8*b* shows the concentrations and pH dependency of the zero-voltage, gossypol-induced conductance in PE bilayers. At low drug concentrations, the conductance exhibited a linear relationship with the aqueous concentration of gossypol, the plots having a slope close to 1. However, at high gossypol concentrations, the zero-voltage conductance reached a saturation level at approximately 1 microsiemen/cm^2. The drug-induced bilayer conductance at 0.16 μM gossypol was pH dependent and had a maximum at a pH of around 6.0 (Fig. 9).

Membrane Potentials of Gossypol-treated Bilayers under a pH Gradient—Table II shows the values of zero-current membrane potentials/decade of proton gradient across a membrane treated with 1.5 μM gossypol. The values of the potentials were obtained from current-voltage curves of gossypol-treated PE membranes under a 0.3–0.4 unit pH gradient across the bilayer. The presence of a pH gradient produced a parallel shift in the current-voltage curves of gossypol-treated PE bilayers. Below pH 5.1, the membrane potentials of gossypol-treated PE bilayers under a pH gradient were close to the predicted Nernstian potential (58 mV). These results are consistent with a model where gossypol is acting as a proton carrier in lipid bilayers (see McLaughlin and Dilger, 1980).

DISCUSSION

A likely cellular site of action of gossypol as an antifertility agent, and presumably also the site for its toxic effects, is some organelle membrane, probably the plasma and/or inner mitochondrial membrane (Abou-Donia and Dieckert, 1974; Tso *et al.*, 1982; Adeyemo *et al.*, 1982; Haspel *et al.*, 1982). Whether gossypol exerts its actions by directly binding and modifying membrane proteins or through a modification of the properties of the lipid matrix of the membranes, which in turn modifies protein function, is not clear at present. In order to evaluate the role of gossypol in modifying membrane lipids and the biological consequences of these actions, we have characterized in detail the interactions of gossypol with lipid membranes. It is appropriate to discuss the actions of gossypol on membranes in the following sequence: (*a*) acid-base equilibrium and binding of neutral and charged forms of

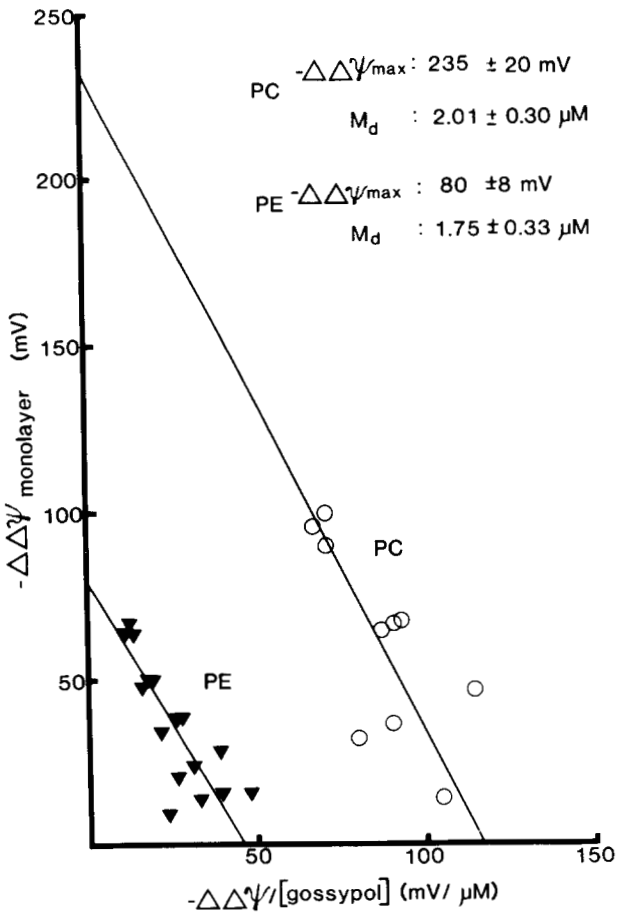

FIG. 6. **Single reciprocal plot of changes in interfacial potential ($-\Delta\Delta\Psi$) of PC and PE monolayers *versus* $-\Delta\Delta\Psi$/[gossypol].** The data were taken from Fig. 5*a*, and the lines were fit by linear regression analysis. The slope of the regression line yields the apparent dissociation parameter of gossypol binding to the monolayer, M_d^{app}, and the *y* intercept gives the maximal change in interfacial potential ($-\Delta\Delta\Psi$).

TABLE I

Adsorption parameters of gossypol to phospholipid monolayers

X represents molar fraction.

	M_d^{app}	$-\Delta\Delta\Psi$
	μM	*mV*
PC	2.01 ± 0.23	235 ± 17
PE	1.75 ± 0.33	80 ± 8
PC/cholesterol (X_{chol}: 0.5)	0.72 ± 0.11	90 ± 6
PC/PE/cardiolipin (*X*: 0.4, 0.4, 0.2)	1.50 ± 0.30	111 ± 12

gossypol to lipids; (*b*) changes in the surface potential of lipid membranes induced by the drug; (*c*) biological consequences of gossypol-induced alterations of membrane interfacial potentials; and (*d*) the uncoupling actions of gossypol.

Acid-Base Equilibrium and Binding of the Neutral and Charged Forms of Gossypol to Lipids—Gossypol presents an acid-base equilibrium in aqueous solutions. As determined from direct titration curves, the pK_a for this acid-base equilibrium in aqueous solution is approximately 6.0 (Fig. 2). Thus, below pH 5, >90% of the gossypol molecules are in the undissociated form. The charged and uncharged forms of gossypol have very different effects on lipid monolayers. The uncharged form of gossypol (pH 4.2) does not significantly modify the interfacial potential of PC monolayers up to a concentration of 2 μM in the aqueous phase (Fig. 4). By contrast, in the same concentration range, the negatively charged form of gossypol (pH ≥6) induces large changes in the interfacial potential of the monolayers. Direct evidence of the binding of a negatively charged form of gossypol is observed from the ζ potential measurements of PC vesicles. In agreement with the data in PC monolayers, the binding of negatively charged gossypol molecules to PC vesicles is also a function of pH (Fig. 7). As predicted from the acid-base equilibrium of gossypol, the mid-titration region of the ζ potential induction by gossypol is at about pH 5.5–6.0. We can conclude that at physiological pH, it is primarily the charged (deprotonated) form of gossypol that binds and induces changes in the interfacial potentials of biological membranes.

Changes in Surface Potential Induced by Gossypol in Lipid Membranes—Most of the biological effects described for gossypol are observed when the drug is in micromolar concentrations in the aqueous phase. At these concentrations, gossypol binds to lipids and modifies the interfacial potential of lipid monolayers. This induction of changes in the interfacial potential also has been observed in phospholipid bilayers. PE bilayers treated with 1 μM CCCP at pH 7.0 yield a zero-voltage bilayer conductance of 11.4 μS/cm². Symmetrical addition of 1 μM gossypol to the aqueous phases bathing the membrane decreases the zero-voltage bilayer conductance to 2.5 microsiemens/cm² (data not shown). Using the formula $\Delta\Delta\Psi = RT/zF\ln(G_0^{CCCP,gossypol}/G_0^{CCCP})$ (*e.g.* Melnik *et al.*, 1977), it can be estimated that gossypol induces a 40-mV decrease in the interfacial potential of PE bilayers. This prediction is in agreement with the interfacial potential change produced by gossypol in PE monolayers at the same concentration (*cf.* Fig. 5*a*).

The interfacial potential change produced by gossypol in lipid membranes is correlated clearly with the appearance of the negatively charged form of the molecule. This interfacial potential change can in principle arise from the adsorption of gossypol molecules to the solution/lipid interface and the production of one of the following electrostatic phenomena: 1) adsorption of negatively charged gossypol molecules to the solution/membrane interface with induction of a negative, diffuse double-layer potential; and 2) adsorption of negatively charged gossypol molecules within a low dielectric region of the membrane with induction of both a negative diffuse double-layer potential and an internal electrostatic potential change (boundary and/or dipole potential) (McLaughlin, 1977). The second possibility seems the most likely. Thus, negatively charged gossypol molecules do bind and induce a negative ζ potential in PC vesicles. Furthermore, the diffuse double-layer potential at the solution/lipid interface obtained from the ζ potential measurements in PC vesicles and corrected for the existence of a plane of shear at 2 Å from the interface (Eisenberg *et al.*, 1979) can account only for about 10% of the total potential change produced by gossypol in PC monolayers. This finding indicates that gossypol is also producing a change in the internal electrostatic potential of the lipid monolayer.

The maximal effect of gossypol on the interfacial potentials of the monolayers is also a function of the lipid composition of the membranes. No further information on the molecular basis of these differences in maximal interfacial potential changes in different lipid monolayers can be obtained from these results. Independent determinations of the contribution of diffuse double-layer potential and internal electrostatic potential changes for each type of lipid would be necessary in order to further analyze the cause of these differences. Fur-

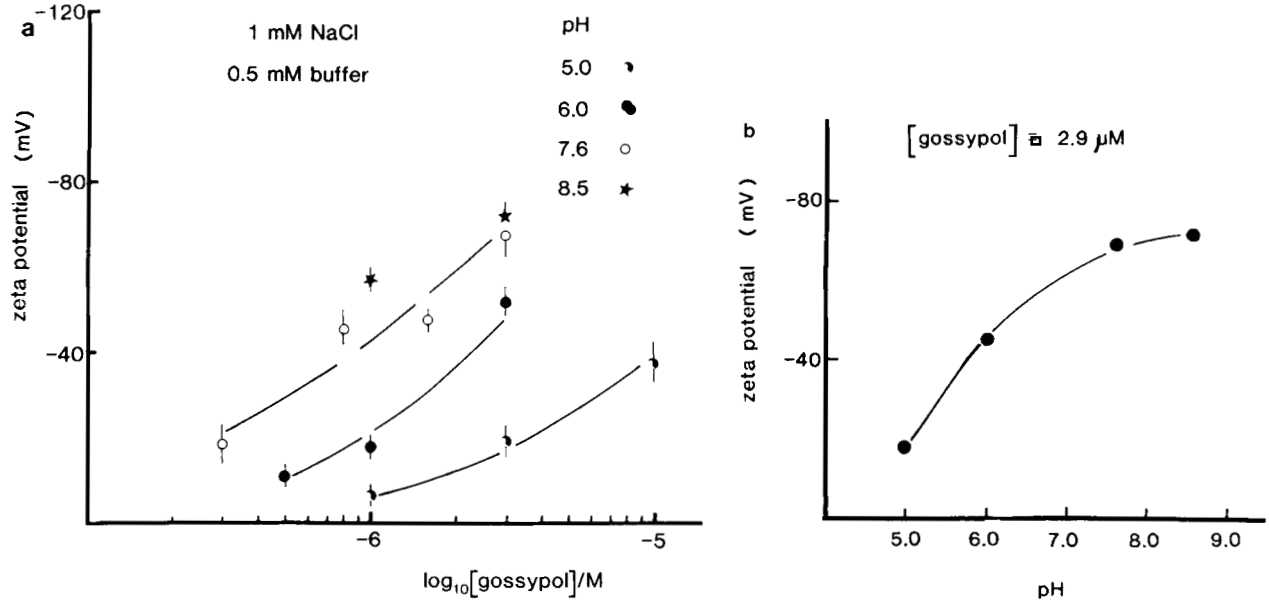

FIG. 7. **Effect of gossypol on lipid vesicle ζ potential.** *a*, Log dose-response curves of measured ζ potentials of multilamellar phosphatidylcholine vesicles *versus* gossypol concentration at different values of pH. *b*, ζ potentials of multilamellar PC vesicles at 2.9 μM gossypol concentration and at different pH values of the electrolyte solution.

thermore, the changes in internal electrostatic potential are a function of both the density of gossypol molecules bound to the interface and the location of gossypol in the interfacial region (*e.g.* Reyes *et al.*, 1983).

Possible Biological Consequences of the Interfacial Potential Changes Induced by Gossypol—Most of the effects of gossypol on biological membranes described in the literature are evident at concentrations in the aqueous phase of about 10 μM. It is at this concentration that gossypol decreases the interfacial potential of lipid membranes by 80–160 mV (Fig. 5). Can these potential changes modify protein functions in biological systems? A familiar example where changes in potential gradients applied on membrane proteins produce dramatic changes in function comes from observations on membrane excitability. A 30–40-mV change in the transmembrane potential gradient can shift, almost completely, the state of the tetrodotoxin-sensitive, voltage-dependent Na^+ channel from the "off" to the "on" state and vice versa (Hodgkin and Huxley, 1952). This transmembrane potential change implies, for a 30-Å dielectric thickness, a change in the membrane electric field of $1.0–1.3 \times 10^5$ V/cm. Although these membrane transport proteins have differentiated domains that are highly sensitive to voltage, the electric field calculated above can help estimate the extent to which membrane proteins respond to these types of forces. If the change in internal electrostatic potential observed in lipid monolayers and lipid bilayers occurs in an adsorbed layer of 10 Å (*e.g.* Melnik *et al.*, 1977), the regional change in electric field caused by adsorption of gossypol would be about $8–16 \times 10^5$ V/cm, about 8–16 times the electric field changes that are known to influence excitable membrane proteins. Furthermore, experimental and theoretical evidence in model systems has clearly shown that membrane interfacial potentials can influence both carrier- and channel-mediated ion transport (*e.g.* Jordan, 1983). It is reasonable, then, to expect that the functions of membrane proteins might change when these compounds bind to either the solution/lipid interface, lipid/protein interfaces, or to hydrophilic/hydrophobic interfacial domains in membrane proteins. In fact, several molecules that are known to modify the internal electrostatic potential of lipid bilayers and monolayers also effect a wide variety of membrane protein functions (see Reyes *et al.*, 1983; Reyes and Benos, 1984, for a discussion). The involvement of changes in interfacial potentials as causal factors in the modification of membrane protein functions mentioned above has not been definitely proved. However, it is tempting to include gossypol among those compounds that through interaction with polar/apolar interfaces and modification of the electrostatic potential seem to produce dramatic changes in enzymatic and transport properties of biological membranes.

Uncoupling Actions of Gossypol—Gossypol uncouples oxidative phosphorylation in liver and sperm mitochondria (Abou-Donia and Dieckert, 1974; Tso and Lee, 1981; Shepu *et al.*, 1983). Two hypotheses for the mechanism of action of mitochondrial uncouplers have been proposed in the literature. First, Mitchell's chemiosmotic hypothesis (Mitchell, 1961) associates uncoupling action with the ability of compounds to collapse the proton electrochemical gradient across the inner mitochondrial membrane (see McLaughlin and Dilger, 1980; Terada, 1981, for reviews). Second, the capacities of certain uncouplers, like 2-azido-4-nitrophenol and 2-nitro-4-azidocarbonyl cyanide phenylhydrazone, to interact with high-affinity binding sites in the inner mitochondrial membrane and the uncoupling properties of picrate have supported the hypotheses that some uncouplers might exert their actions by directly interacting with inner mitochondrial proteins (*e.g.* Hanstein, 1976). The aldehyde groups in gossypol are known to react directly with free amino groups in proteins (Tanksley *et al.*, 1970). Thus, it is important to determine the extent to which the uncoupling effects of gossypol are a result of its action as a chemical agent that can collapse the inner mitochondrial membrane proton electrochemical gradient.

Gossypol has several properties that suggest the molecule acts as a proton and charge carrier across the lipid bilayer of the inner mitochondrial membrane. First, gossypol is a weak acid with a pK_a in aqueous solution of about 5.5–6.0. Second, the π-π electron interactions in the molecule make it likely that the charge in anionic gossypol is distributed in the aromatic rings in such a way that the Born radius is increased as compared to the nonaromatic analog. In this way, the

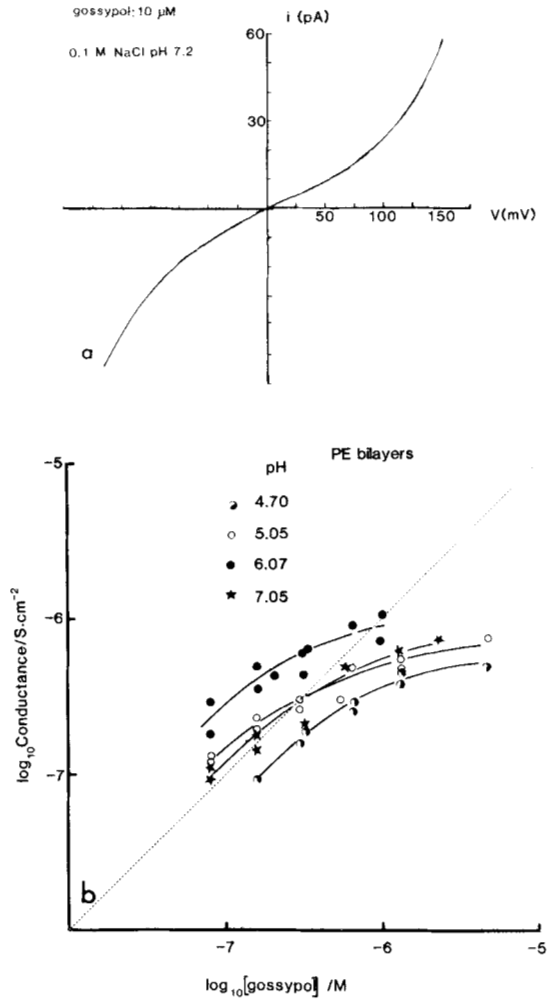

FIG. 8. **The effect of gossypol on the conductance of solvent-free planar lipid bilayer membranes.** *a*, current-voltage relation of a phosphatidylethanolamine bilayer in the presence of 10 μM gossypol in both compartments. Membrane voltage was changed by applying a triangular ramp at 200 mV/min. *b*, Log-log plot of bilayer membrane zero-voltage conductance *versus* gossypol concentration at different values of bathing solution pH. The solution bathing the bilayer consisted of 0.1 M NaCl and either 50 mM phosphate (pH 5.5–7.1) or 50 mM phosphate/citrate (pH 4.1–5.1). The *broken line* is for reference purposes and has a slope of 1.

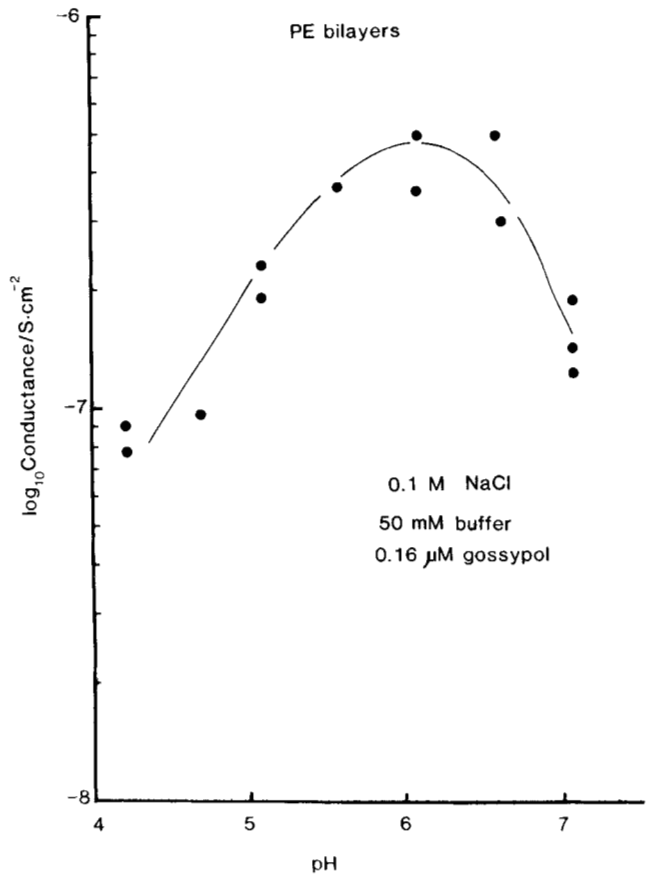

FIG. 9. **Zero-voltage conductance of PE bilayers *versus* pH at 0.16 μM symmetrical gossypol concentration.**

TABLE II

Gossypol-treated/Phosphatidylethanolamine bilayer potentials induced by a pH gradient

The membrane potentials of PE bilayers treated with gossypol (1.5 μM) induced by 0.3–0.4 pH unit gradients are the average of at least two different membranes. The pH values listed are on the low side of the pH gradient.

pH	$V_{i=0}$/pH unit
	mV
4.7	57
5.1	57
6.1	42
7.1	46

energy for partition of gossypol in the membrane would be decreased (Parsegian, 1969; Neumcke and Läuger, 1969). Third, the molecule contains a hydrophobic domain that would favor the partition and permeability of both the negatively charged and uncharged forms of gossypol in lipid bilayers. The data presented in this report confirm these predictions, because of the following. 1) Gossypol can bind to lipid monolayers and lipid bilayers and modify their electrical properties when present in micromolar and submicromolar concentrations in the aqueous phases. 2) The negatively charged form of the molecule binds to phospholipid bilayers. This conclusion is supported by at least two independent experimental results. First, the ζ potential measurements with PC vesicles show the existence of a concentration-dependent, gossypol-induced surface charge on phospholipid vesicles, indicating that negatively charged gossypol molecules are binding to the solution/membrane interface. Second, the effects of gossypol on lipid monolayers are correlated with the deprotonation of gossypol and can be explained as the binding of the negatively charged molecule to the monolayer/solution interface. 3) The negatively charged gossypol molecule can cross phospholipid bilayers. This conclusion is supported by the following observations. (*a*) The induction of a concentration-dependent, gossypol-induced conductance in lipid bilayers indicates that a charged species is permeable through the membrane; and (*b*) the pH dependence of the zero-voltage gossypol-induced conductance between pH 4.5 and 5.5 results from the appearance of the anionic gossypol species in solution (see LeBlanc, 1971). From the following equation,

$$G_0 = (z^2F^2/RT) \cdot P_m^{G^-} C_a^{G^-}$$

in which P_m is the overall membrane permeability to anionic gossypol, C_a is the aqueous concentration of negatively charged gossypol, and R, T, z, and F have their usual meanings, it is possible to estimate that the overall membrane permeability to negatively charged gossypol is $>10^{-4}$ cm/s.

Thus, all the above-mentioned experimental evidence is consistent with a model in which the negatively charged form

of gossypol binds to the membrane/electrolyte interface and then can permeate across the membrane. These results do not provide direct evidence that the uncharged form of gossypol can permeate lipid bilayers. However, the pH dependence of the zero-voltage conductance of the gossypol-treated membranes and the transmembrane potential developed under a pH gradient are consistent with a model in which gossypol can induce proton and charge permeation in lipid membranes. Furthermore, the direct dependence of bilayer conductance with gossypol concentration in the low concentration range (slope ~1) strongly suggests that gossypol can act as a monomolecular proton carrier (see Neumcke and Bamberg, 1973, for a review).

An unexpected property of the gossypol-treated lipid bilayers is the development of a diffusion potential in the presence of a NaCl gradient (10–100 mM). This diffusion potential is evidenced by a "salt-induced" shift in the current *versus* voltage curves of the bilayers, equivalent to about 15 mV/decade of salt gradient (data not shown). This special property of gossypol resembles compounds like nigericin which have been shown to increase both proton and cation permeability in lipid membranes (*e.g.* Pressman, 1976).

The evidence therefore suggests that gossypol acts as an uncoupler of oxidative phosphorylation through characteristic interactions with phospholipid membranes and by its action as a proton and charge carrier across membranes.

Biological Effects of Gossypol As an Uncoupler—The proton and ion transport properties of gossypol predict that the molecule acts by collapsing proton and/or ion gradients in a dose-dependent fashion in all cells and epithelia in the organism. If, as proposed in the literature (*e.g.* Shepu *et al.*, 1983), gossypol is acting as an antifertility agent through this type of uncoupling mechanism, an obvious question arises concerning the molecule's differential effect on Sertoli and germinal cells as compared to other cell types. Two hypotheses can be proposed to explain the drug's apparent differential action.

1) Access of gossypol into somatic cells is restricted as compared to Sertoli and germinal cells due to the permeability barrier of the cell plasma membrane.

There are no direct measurements reported on the relative rate of entry of gossypol into germinal or somatic cells. However, an estimation of the rate of entry of gossypol into cells can be obtained (Benos *et al.*, 1983) by considering the kinetics of gossypol diffusion across membranes and the known values of the permeabilities of the charged and uncharged forms of the molecule. The rate of gossypol entry into the cells can be estimated using the following equation (Benos *et al.*, 1983):

$$d/dt\left(\frac{C_i^{G^-} \cdot V_c}{A}\right) = \left(\frac{P^{GH} \cdot C_o^{tot}}{1 + 10^{(pK_a - pH_i)}}\right) - (P^{GH} - 10^{(pK_a - pH_i)})C_i^{G^-}$$

where C^{tot} is the total external concentration of gossypol, $C_i^{G^-}$ is the intracellular charged gossypol concentration, P^{GH} is the permeability coefficient of the uncharged form of gossypol, V_c is the cellular volume, A is the area, and pK_a and pH have their usual meanings. This equation is derived from Fick's first diffusion law and the Henderson-Hasselbalch equation, assuming $P^{GH} \gg P^{G^-}$ and that the acid-base equilibrium is much faster than the transmembrane diffusion of gossypol. Times for 90% equilibration in the intracellular aqueous compartment of a cell with a geometry similar to the hepatocyte and using uncharged gossypol permeability coefficients of 10^{-4} and 10^{-3} cm/s are obtained after 5.0 and 0.5 min, respectively (*e.g.* McLaughlin and Dilger, 1980; Benos *et al.*, 1983). Because there is no evidence of the existence of specific uptake systems for gossypol in body organs (Shepu *et al.*, 1983), it is very likely that gossypol is distributed at equilibrium throughout the body compartments in the time scale of whole-organism experiments.

Another piece of evidence suggesting that gossypol equilibrates rapidly in the intracellular compartment can be derived from oxygen consumption (Qo_2) measurements using liver cells and isolated liver mitochondria. If the intracellular concentration of gossypol is significantly below the equilibrium concentration when the Qo_2 measurements are performed (30–120 s), the dose-response curve of isolated, intact liver cells Qo_2 *versus* gossypol concentration should be shifted toward higher gossypol concentrations compared to those obtained for isolated mitochondria (Fig. 10). At similar protein concentrations, the mid-response point of isolated liver cells and isolated mitochondria dose-response curves are superimposable within experimental error. This result strongly suggests that the time for 90% equilibration of gossypol within the cell compartment is <5 min.

These contentions indicate, based on the time scale of the *in vivo* antifertility experiments, that the differential effects of gossypol on Sertoli and germinal cells are not due to exclusion of the molecule from the cytoplasmic compartment of somatic cells.

2) Mitochondria exhibit a preferential sensitivity to gossypol uncoupling in Sertoli and germinal cells.

The dose-response curves of Qo_2 *versus* gossypol concentration of liver cells, liver cell mitochondria, sperm cells, sperm cell mitochondria, and testicular mitochondria do not seem to differ markedly from one another (Fig. 10; also see Abou-Donia and Dieckert, 1974; Tso and Lee, 1981; Shepu *et al.*, 1983). Evidence showing that gossypol interacts preferentially with mitochondria of Sertoli and germinal cells is provided by comparative studies on the mitochondrial uptake of rhodamine-123, a cationic fluorescent dye (Tanphaichitr and Bellvé, 1984). Gossypol prevents the specific mitochon-

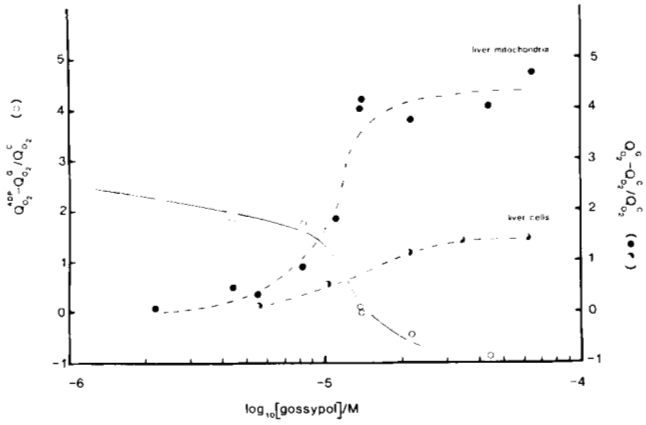

FIG. 10. **Fractional increase in the oxygen consumption rate (Qo_2) of isolated liver cells (◑) and liver mitochondria (●) as a function of the gossypol concentration added to the medium.** The symbols Qo_2 and Qo_2^C represent the rates of oxygen consumption in the presence and absence of gossypol, respectively. The cells were suspended in Krebs-Henseleit buffer supplemented with 20 mM glucose at 37 °C previously gassed with 95% O_2, 5% CO_2. Liver cells were present at a concentration of 2 mg of protein/ml. Mitochondria were suspended in a mannitol/sucrose medium with 5 mM inorganic phosphate, 10 mM succinate (pH 7.4). Liver mitochondria were present at a concentration of 2 mg of protein/ml. After the addition of gossypol to liver mitochondria, the isolated organelles were exposed to 0.6 mM ADP, and the new rate of oxygen consumption was measured (Qo_2^{ADP}). The ratio $(Qo_2^{ADP} - Qo_2^G)/Qo_2^C$ (○) is an estimation of the degree of coupling of mitochondrial oxidative phosphorylation at each gossypol concentration.

drial accumulation of the dye when Sertoli cells and epididymal spermatozoa are exposed to 10 μM concentrations of the drug. By contrast, at this or even 5-fold higher concentrations of gossypol, the mitochondrial rhodamine-123 uptake by a variety of other cell types was not altered. The cationic fluorescent dyes are most probably monitoring inner mitochondrial membrane potential (Johnson *et al.*, 1981). However, since this membrane potential can be negative even in the presence of maximally uncoupling concentrations of proton ionophores (Scarpa, 1979), the relation of the above phenomena to gossypol uncoupling of mitochondrial oxidative phosphorylation and to the antifertility effects of gossypol has yet to be established.

In conclusion, the binding properties and interactions of gossypol with lipid membranes can explain most of the reported acute effects of this molecule on biological membranes. However, there is no evidence to state that these membrane effects can explain the apparent preferential actions of gossypol on the functions of Sertoli and germinal cells.

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