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MEMBRANE STRUCTURAL/FUNCTIONAL PERTURBATIONS INDUCED BY GOSSYPOL

EFFECTS ON MEMBRANE ORDER, LIPOSOME PERMEABILITY, AND INSULIN-SENSITIVE HEXOSE TRANSPORT

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Abstract—The effects of gossypol on membrane structure and membrane-associated functions were studied to explore possible reasons for the ability of gossypol to disrupt cellular processes, many of which involve intracellular and plasma membranes. The experiments reported here measured the effects of gossypol on membrane order, permeability, and hexose transport. Electron spin resonance (ESR) studies of I(12,3) nitroxide fatty acid spin-labeled unilamellar liposomes showed that exposure to 0.05 to 4 mM gossypol caused a dose-dependent increase in the polarity-corrected order parameter (*S*), indicating reduced motional freedom of the spin probe after exposure to gossypol. This observation is consistent with the idea that gossypol causes an ordering or "condensing" of the membrane lipid matrix. Gossypol-induced changes in order parameter in phosphatidylcholine:cholesterol liposomes varied depending on the liposome composition. Liposomes exposed to gossypol also showed increasing permeability to glycerol as the gossypol:phospholipid ratio increased up to 10 mole %. Higher concentrations of gossypol were less effective at enhancing permeability. In addition, basal and insulin-stimulated 2-deoxy-D-[^{3}H]glucose transport were inhibited in freshly isolated rat adipocytes incubated with gossypol at 37°. Half-maximal inhibition occurred at approximately 0.2 mM for uptake in both the presence and absence of 40 ng/ml insulin. Microscopic observation of the cells under low power (40×) confirmed that diminished hexose transport was not simply due to breakage of the adipocyte plasma membrane, resulting in a decrease in intact cell population and decreased accumulation of label in the gossypol-treated cells. Gossypol produced no significant changes in numbers of intact cells or gross morphology at the concentrations tested. We suggest that ordering and increased permeability of the lipid regions of plasma and subcellular membranes may contribute to some of the toxic and pharmacologic properties of gossypol. Our results also support the idea that gossypol may exert more pronounced effects in cells that are most sensitive to variations in availability of glucose substrates for energy metabolism.

Gossypol [(2,2′-binaphthalene)-8,8′-dicarboxaldehyde - 1,1′,6,6′,7,7′ - hexahydroxy - 5,5′ - diisopropyl-3,3′-dimethyl, Fig. 1. inset], a compound extracted from the pigment glands of cotton plants and other species of the genus *Gossypium* (fam. *Malvaceae*), exerts a great variety of effects in cells and tissues [1]. Much of the current interest in gossypol is due to its ability to suppress male reproductive function and its potential as a reversible male contraceptive drug [2–4]. Gossypol also has been investigated for use in the treatment of cancer [5, 6], gynecological disorders such as endometriosis and menopausal bleeding [7], herpes simplex Type 2 viral infections [8], and trypanosomal infections such as Chagas' disease [9]. Recognition of the potential pharmacologic value of gossypol has prompted many investigations of its underlying mechanism of action and potential toxicity at pharmacologic doses; however, the consequences of long-term ingestion of pharmacologically useful doses of gossypol are not known.

Gossypol is highly lipophilic, and cell fractionation studies have shown that this compound associates

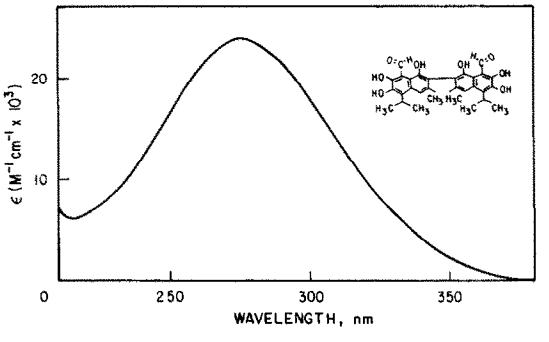

Fig. 1. Absorption spectrum of gossypol acetic acid (25 μM) in absolute ethanol. Inset shows structure of gossypol.

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with microsomal, mitochondrial and, to a lesser extent, nuclear and plasma membranes [10]. Low concentrations of gossypol alter many functions associated with mitochondrial and other cell membranes. For example, oxidative phosphorylation and electron transport [11, 12], Na^+, K^+- and Ca^{2+}, Mg^{2+}-ATPases [13, 14], adenylate cyclase [15], and cyclic adenosine 3′,5′-monophosphate phosphodiesterase [16] are all inhibited by micromolar concentrations of gossypol. Both membrane-bound acrosin and the conversion of proacrosin are inhibited by gossypol [17]. Although several of the enzyme kinetic studies just cited reported using pure or semi-purified enzyme preparations, most measurements of enzyme activity have been made using intact sperm or other cell homogenates.

Given the expanding list of enzymes known to be affected by gossypol, many of which are associated with membranes, some nonspecific interaction of gossypol with biological membranes might explain its myriad effects. For example, Reyes *et al.* [18] have reported that gossypol decreases interfacial membrane potential and increases conductance at physiological pH. Although their findings were not experimentally correlated with membrane function, their results provide evidence that, in addition to any direct action that gossypol may have on specific enzyme proteins, nonspecific physicochemical alterations of the membrane bilayer may also contribute to the ability of gossypol to interfere with many different biological processes.

The purpose of the experiments reported here was to investigate further the effects of gossypol on membrane structure and function, specifically membrane fluid properties (order), permeability, and hexose transport. Electron spin resonance (ESR) studies were conducted to provide information about the effects of gossypol on membrane order, using the I(12,3)-nitroxide stearate spin-labeled fatty acid probe incorporated into liposome preparations. The order parameter (S) of the spin probe is sensitive to the membrane lipid "fluidity" or, more accurately, the flexibility of the incorporated probe about the normal to the membrane surface. The effects of gossypol on membrane permeability were also studied. A spectrophotometric method was used to observe the effects of gossypol on the swelling rate of liposomes placed in a hypertonic glycerol solution. Changes in the liposome shape (or, more accurately, forward angle light scattering ability) reflected changes in liposome permeability. In addition, both basal and insulin stimulated 2-deoxy-[^{3}H]glucose transport by freshly isolated rat epididymal adipocytes in the presence and absence of gossypol were investigated. Whereas gossypol is well known to inhibit glycolytic enzymes [19–22], given that gossypol affected membrane lipid structure and permeability, we also wanted to explore the possibility that gossypol might also alter hexose uptake by the plasma membrane.

MATERIALS AND METHODS

ESR spin-label studies. Pure egg phosphatidylcholine (PC) liposomes and PC liposomes containing different proportions of cholesterol were prepared in 25 mM 3-[*N*-morpholino]-propanesulfonic acid (MOPS) buffer containing 100 mM NaCl, 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA), pH 7.4. The mixed lipids, dissolved in ethanol, were dried to a thin film under a stream of nitrogen. The above buffer was added, and the tubes were capped under nitrogen and gently sonicated under low power [23]. The final total lipid concentration was 4 mM. Lipids and other reagents were purchased from the Sigma Chemical Co., St. Louis, MO.

Spin-label measurements were also performed using unilamellar liposomes prepared by the reverse phase evaporation technique [24] in parallel with the swelling rate (permeability) studies, as described below.

The nitroxide fatty acid spin label I(12,3), 2-(3-carboxypropyl)-4′,4′-dimethyl-2-tridecyl-3-oxazolidinyloxyl (Syva, Palo Alta, CA) was incorporated into liposomes at room temperature as described in Sauerheber *et al.* [25]. Electron spin resonance (ESR) spectra were recorded with a Varian E-104A Century Series ESR spectrometer equipped with a variable temperature control unit that regulated the sample cavity to 37 ± 0.1°. In all cases, duplicate unexpanded spectra with "magnified wings" were recorded as follows. First, the "unexpanded" spectrum was recorded with a 100 gauss field sweep, 5×10^4 receiver gain, 4 min scan time, and 1 sec time constant. The outer wings were then "magnified" by recording with a 100 gauss field sweep, 5×10^5 receiver gain, 16 min scan time, and 1 sec time constant [26]. The order parameters S, $S(T_\parallel)$, and $S(T_\perp)$ were calculated from the outer and inner hyperfine splittings $2T_\parallel$ and $2T_\perp$ as indicated in Gordon and Sauerheber [26]. S, $S(T_\parallel)$, and $S(T_\perp)$ were employed here to monitor changes in the "fluidity" (or, more accurately, the flexibility of probe molecules) of I(12,3)-labeled lipid vesicles. S requires both hyperfine splittings and, at magnetically dilute concentrations, corrects for small differences in polarity between the membrane and reference crystal. Although $S(T_\parallel)$ and $S(T_\perp)$ do not include corrections for polarity contributions, these expressions have been useful approximate measures of the fluidity in those cases where only one hyperfine splitting is usable [26]. The isotropic hyperfine coupling constant for the probe in the membrane, a_N', where $a_N' = 1/3\ (T_\parallel + 2T_\perp)$, is sensitive to the polarity of the spin-label environment.

The probe:membrane lipid ratio was fixed in any given experiment determining the relative effects of gossypol. This ratio was approximately 6 nmoles label:280 nmoles total lipid, a low probe:membrane ratio according to criteria established earlier [25], but not necessarily completely free of interactions among probe radicals [27].

Gossypol acetic acid was obtained from the U.S. Department of Agriculture, New Orleans, LA (Lot No. GS 19-3). Purity was reported to be 95%. Gossypol was stored with desiccant in the dark at ≤4°.

Liposome swelling rate (membrane permeability) studies. Unilamellar liposomes were prepared by the reverse phase evaporation technique [24] in an approximate concentration of 0.5 μM. The liposomes

were comprised of 96% 1-palmitoyl-2-oleyl-phosphatidylcholine (PC) and 4% phosphatidylserine (PS) (from bovine liver, Avanti Biochemicals, Birmingham, AL) and were prepared from a starting mixture containing 70 μmoles total lipid and 1 μCi 1-palmitoyl 2-[^{14}C]arachidonyl PC tracer (New England Nuclear, Boston, MA). The lipids were taken up in 3 ml chloroform in a 10-ml flask, to which was added 1.3 ml of 10 mM MOPS buffer, pH 7.4, containing 1 mM EGTA, 50 mM KCl, and 5% Ficoll 70 (Pharmacia, Uppsala, Sweden). The admixture was probe-sonicated at medium power on an ice bath under nitrogen for 30 sec. The milky emulsion was then rotary evaporated at 33° with a controlled nitrogen atmosphere under reduced pressure for 4 hr, after which time the liposomes were washed in 50 vol. of a buffer containing 50 mM KCl, and 10 mM MOPS, pH 7.4. The unilamellar liposomes were pelleted at 10,000 *g* for 10 min and then subjected to a second washing cycle. Liposome yield was estimated by counting the [^{14}C] washings in a Searle liquid scintillation spectrometer using 10 ml Scint A fluid (Packard Instruments, Downers Grove, IL). Typical yields were 80–90%.

Gossypol/liposome mixtures were made by adding 10 μl of concentrated solutions of gossypol dissolved in dimethyl formamide:methanol (1:2) to 1-ml aliquots of 8.4 mM liposome suspension and left to equilibrate for 2 hr with gentle agitation in the dark under a nitrogen atmosphere at 37°. Liposomes were pelleted at 10,000 *g* and resuspended in the same volume of buffer. Gossypol/lipid molar ratios in the liposomes were obtained by measurement of [^{14}C] in an aliquot of liposomes, and by direct estimation of gossypol absorbance from an aliquot of liposomes added to 1 ml ethanol ($\varepsilon_{275\,nm}$ = 24,000).

Permeability (liposome swelling rate) studies were performed according to the spectrophotometric method described in De Gier *et al.* [28]. Aliquots (30 μl) of gossypol/liposome suspensions were rapidly added, with stirring, to 720 μl of 150 mM glycerol in pH 7.4 MOPS containing 50 mM KCl. The degree of light scatter at 500 nm increased proportionately to the swelling of the vesicles after introduction into the concentrated glycerol solution. Forward-angle light scattering was quantitated by initial transmittance changes using a Cary 219 spectrophotometer. The reference cuvette contained a sample of lysosomes in the absence of glycerol. Gossypol acetic acid itself does not absorb appreciably at 500 nm (see Fig. 1).

To obtain a direct correlation of the effect of gossypol on liposome permeability with effect on liposomal membrane motional properties, aliquots of each gossypol:liposome suspension were also labeled with the I(12,3) spin probe for ESR order parameter determinations.

Hexose uptake in intact adipocytes exposed to gossypol. Basal and insulin-stimulated uptake of 2-deoxy-D-[^{3}H]glucose (New England Nuclear) were measured essentially according to established methods [29, 30]. Adipocytes were prepared from untreated rat epididymal adipose tissue by collagenase digestion in 25 mM MOPS buffer containing 4% bovine serum albumin (physiologic buffer) as described by Hyslop *et al.* [31]. Cells were washed in 25 mM MOPS buffer containing 1% bovine serum albumin.

The structural and functional integrity of the isolated adipocytes prepared for our studies was examined routinely. Cells were counted and sized and found to exhibit average diameters comparable to cells observed in intact adipose tissue. The preparations were also examined for overall D-glucose consumption as in Olefsky [30] and Sauerheber *et al.* [32], both in the absence and presence of insulin. Uptake of glucose from the incubation medium by freshly isolated cells was cell concentration dependent and exhibited an influx rate comparable to the 2-deoxy-D-[^{3}H]glucose uptake in the hexose transport assays.

Aliquots of adipocyte suspension (170 μl, 0.2 to 0.8×10^5 cells) were first preincubated at 37° in physiologic buffer (0.25 ml total volume) for 10 min. Labeled 2-deoxy-D-[^{3}H]glucose was then added so that the final labeled hexose concentration was 0.1 mM (specific activity: 4.0 mCi/mmole). Uptake of hormone was allowed to proceed for 10 min. Adipocytes were removed by centrifugation through dinonyl phthalate, and the cells were counted for [^{3}H]. Uptake of 2-deoxy-D-[^{3}H]glucose was linear over the 10 min assay period in all experiments reported here and reflected the rate of transport of hexose into the cells. Adipocyte [^{3}H] values obtained in assays using cells treated with a known glucose transport inhibitor (50 μM cytochalasin B) were subtracted in order to correct for nonspecific diffusion and traces of label in the buffer trapped in spaces between the washed cells [30]. Under our conditions, approximately 15–20% of apparent uptake is due primarily to trapping of labeled hexose in the centrifuged cell pellet and some diffusion over the 10-min assay period.

The effects of gossypol on hexose transport were observed by performing parallel experiments in the presence and absence of different concentrations of the agent. The desired amounts of gossypol dissolved in ethanol were added to sample tubes and evaporated to dryness under nitrogen gas. Adipocytes were then added to the sample tubes containing gossypol, hand vortexed, and allowed to equilibrate for 15 min at 37° prior to assay for hexose uptake. Cell viability was determined before and after incubation by light microscopic examination at 40×.

RESULTS

Effects on I(12,3)-labeled liposome order. Exposure of labeled PC liposomes to gossypol in physiologic buffer (pH 7.4) caused a concentration-dependent increase in the order parameters S, $S(T_{\parallel})$, and $S(T_{\perp})$ (Fig. 2). This effect was similar to the well-known "condensing" or "rigidizing" effect of cholesterol that occurs in liposomes incubated above major gel–liquid transition temperatures [33]. Gossypol concentrations employed in the experiments shown in Fig. 2 ranged from a drug:lipid molar ratio of 0.25:1 to 1:1, corresponding to 1–4 mM aqueous concentrations of gossypol incubated with 4 mM lipid. Nearly a 1% increase in S, similar to that which would be seen with a 1° temperature decrease in the membrane, was observed with a drug:PC molar ratio

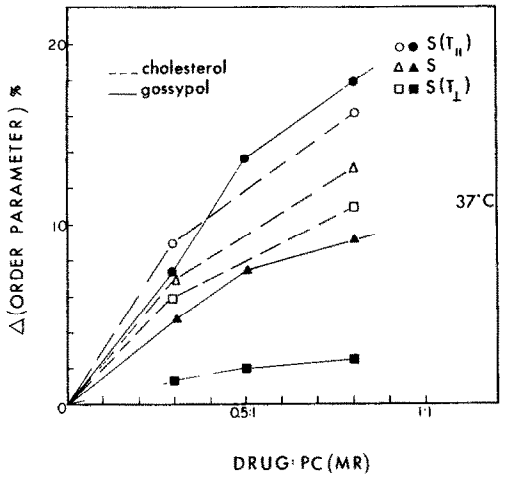

Fig. 2. Gossypol and cholesterol effects on order parameters of I(12,3)-labeled phosphatidylcholine (PC) liposomes at 37°. Aliquots of gossypol in ethanol were dried and vortexed with PC liposomes (4 mM) suspended in 25 mM MOPS buffer, pH 7.4, at the molar ratios (MR) indicated, where 0.5:1 MR = 1 gossypol molecule:2 phospholipid molecules. The probe:lipid molar ratio was 5:280.

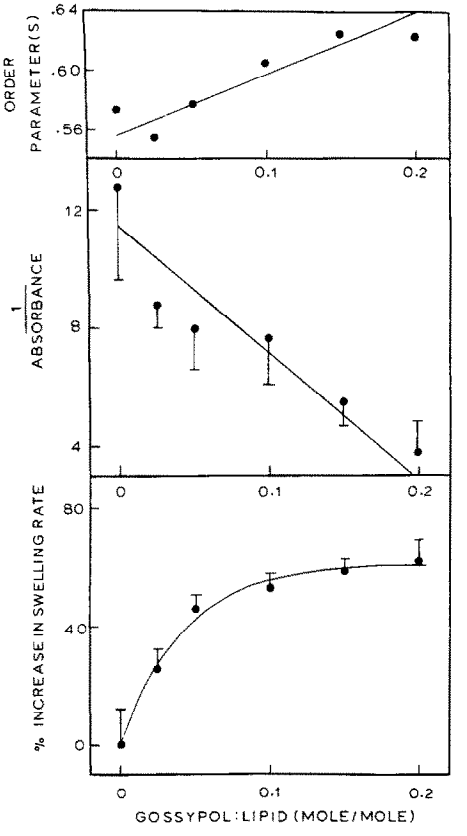

Fig. 3. Correlation of the effects of gossypol on order parameter (S) and swelling rate (permeability) of phosphatidylcholine:phosphatidylserine (PC:PS) liposomes. The concentration of total lipid (96% PC:4% PS) in each case was 8.4 mM. Aliquots of gossypol in ethanol were dried and vortexed with PC liposomes in 25 mM MOPS buffer, pH 7.4, at the molar ratios indicated. Lines drawn in the top and middle panels were determined by linear regression [34]. Top panel: PC:PS liposomes exposed to various concentrations of gossypol were subsequently labeled with I(12,3), and the order parameters were calculated from duplicate ESR spectra. The probe:lipid molar ratio was 5:280. The correlation coefficient of the regression line (r) = 0.99. Middle panel: Initial reciprocal absorbance at 500 nm of PC:PS liposomes exposed to gossypol. Gossypol itself does not absorb at 500 nm. Therefore, the shape of the plot was due to the forward angle scattering ability of the liposomes exposed to various concentrations of gossypol. Values represent mean ± standard deviation of duplicate measurements. The correlation coefficient of the regression line (r) = 0.99. Bottom panel: Percentage increase in swelling rate (permeability) resulting from glycerol diffusion into PC:PS liposomes previously exposed to gossypol. Liposome swelling rate was monitored at 500 nm and expressed as the percentage increase above control liposome swelling rate in the absence of gossypol. Increasing percentage change in swelling rate seen with gossypol concentrations up to 10 mole % reflects increasing liposome permeability to 150 mM glycerol subsequent to gossypol exposure. Values represent mean ± standard deviation of duplicate measurements.

of about 0.1:1 (0.1 mM gossypol). Gossypol also increased the order parameters of liposomes containing both phosphatidylcholine and cholesterol (PC:C) (Table 1) and phosphatidylcholine:phosphatidylserine (PC:PS) liposomes (Fig. 3, top panel). Gossypol-induced changes in order parameter in PC:C liposomes varied depending on the liposome composition (Table 1). A range of 0.025:1 to 0.2:1 gossypol:lipid molar ratios was examined using the PC:PS liposome system, corresponding to 0.21 to 1.68 mM gossypol concentrations added to the 8.4 mM liposome suspensions.

Addition of gossypol to aqueous PC liposome suspensions also caused a dramatic increase in the a_N' value of the labeled membrane, whereas cholesterol produced a smaller effect, as shown in Table 2. The pH of the MOPS buffer was unchanged after addition of 4 mM gossypol acetic acid. Moreover, decreases in pH that could occur upon introduction of strongly acidic compounds would be expected to *decrease* the membrane order parameter and a_N', rather than increase these parameters as was observed with gossypol. Therefore, the effects of gossypol on the ESR spectra could not be attributed merely to altered probe partitioning in the membrane due to a change in pH of the solvent medium.

Effects on liposome permeability. The gossypol-induced percentage increase in swelling rate (expressed in terms of fractional increase above swelling rate of unexposed control liposomes) as a function of the total gossypol:lipid molar ratio is depicted in Fig. 3, bottom panel. Gossypol concentrations up to 10 mole % (corresponding to 0.84 mM gossypol added to the 8.4 mM liposome suspension) caused a dose-dependent increase in liposome permeability to glycerol, as indexed by initial swelling rate values. Above 10 mole %, gossypol was less effective at inducing permeability changes. Gossypol exerted no time-dependent effects on absorbance (light scattering) in the absence of glycerol.

Table 1. Percentage increases in order parameters of I(12,3)-labeled phosphatidylcholine:cholesterol (PC:C) liposomes after exposure to gossypol at 37°

Liposome system*	Molar ratio	Δ (order parameter) % $S(T_{\parallel})$	S	$S(T_{\perp})$
PC	—	13.7	7.5	2.4
PC:C	1.0:0.3	12.4	5.3	0.0
PC:C	1.0:0.75	12.4	0.6	−0.5

* Liposomes were prepared by cosonication and then labeled at a probe:lipid ratio of 6:280. Duplicate ESR spectra were recorded for each system both before and after mixing gossypol with labeled liposomes at a drug:total lipid ratio of 0.5:1 (2 mM gossypol:4 mM total lipid).

To ascertain whether gossypol was associating with the liposomes in a stoichiometric manner over the range of concentrations tested, ESR order parameter measurements were made under similar conditions as the permeability studies. The data in Fig. 3, top panel, show a linear increase in the ordering of the liposomes. Also, the reciprocal initial absorbance of the gossypol:liposome mixture was recorded for each mixture and plotted as a function of added gossypol (shown in Fig. 3, middle panel). Since gossypol itself does not absorb at 500 nm, the linear increase in absorbance following gossypol addition suggests that gossypol was influencing the liposomes in a concentration-dependent manner over the entire range of gossypol concentrations tested in these permeability studies. The increase in initial absorbance of the liposomes following equilibration with gossypol may possibly result from a decrease in average size, or some other physical alteration in the liposome structure. This was not investigated further.

In any case, the data taken together strongly suggest that the biphasic effect of gossypol on liposome permeability over the range of concentrations studied is unlikely to be due to nonlinear partitioning of gossypol in the membrane lipid phase.

Effects on basal and insulin-stimulated hexose transport. Addition of gossypol to adipocyte suspensions caused a progressive, dose-dependent inhibition of the uptake of 2-deoxy-D-[^{3}H]glucose, both in the presence and absence of insulin (Fig. 4). The inhibition was independent of the order of addition of gossypol and insulin (data not shown), and was characterized by a depression of both the basal and maximally stimulated activities. The percent inhibition of both basal and insulin-stimulated hexose uptake appeared to be similar at 0.25 mM gossypol. Half-maximal inhibition occurred at approximately 200 μM and was essentially complete at $\geq$500 μM gossypol concentrations. Microscopic examination of adipocytes before and after the hexose transport assays revealed no significant changes in gross morphology or numbers of intact cells per sample.

Table 2. Gossypol and cholesterol effects on the isotropic hyperfine coupling constants, a_N', determined from I(12,3)-labeled phosphatidylcholine (PC) liposomes at 37°

Molar ratio* Gossypol:PC	Cholesterol:PC	a_N'	Δ (a_N') %
0:1	—	15.04	—
0.25:1	—	15.24	1.3 ± 0.8
0.5:1	—	15.38	2.5 ± 1.0
1:1	—	15.58	3.9 ± 0.9
—	0:1	15.00	—
—	0.3:1	15.12	0.8 ± 0.9
—	0.75:1	15.30	2.0 ± 0.7

* Aliquots of gossypol in ethanol were dried and vortexed with PC liposomes at the molar ratios indicated prior to labeling. Cholesterol was incorporated into liposomes by cosonicating prior to labeling. A concentration of 4 mM total lipid was used in each sample, and the probe:lipid ratio was 5:280. Values were determined from duplicate spectra for each condition.

DISCUSSION

Even slight alterations in the structural and functional integrity of lipid regions of plasma and subcellular membranes caused by exposure to exogenous agents can lead to irreversible cell damage or death. At what point these alterations are great enough to produce physiologic manifestations of toxicity *in vivo* is not yet known. The following discussion of the biological consequences of the lipid-

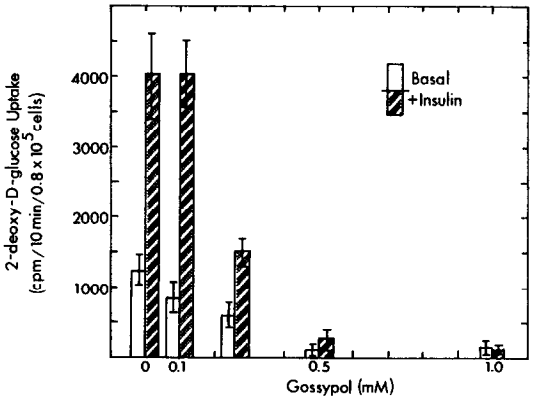

Fig. 4. Gossypol inhibition of 2-deoxy-D-[^{3}H]glucose transport by adipocytes in the absence and presence of 40 ng/ml insulin. Data represent mean ± standard deviation of results from three different adipocyte preparations, each batch examined over the gossypol concentration range shown. Microscopic (40×) observation of cells after gossypol exposure revealed no gross morphologic abnormalities.

rigidizing property of gossypol and effects on membrane permeability and hexose transport considers current understanding of the various roles of membrane lipids in the maintenance of normal cell functions. We also relate our findings to previously reported toxic and pharmacologic effects of gossypol.

Exposure to gossypol resulted in an increased lipid order (rigidity) of all I(12,3)-labeled liposomal membranes examined in these experiments. The magnitude of the rigidizing effect was dependent upon the concentration of gossypol added to aqueous liposome suspensions over a range of 0.05 to 4 mM, and was analogous to the well-characterized condensing effect of cholesterol [33]. In addition to direct interaction with enzyme molecules themselves, membrane rigidizing and fluidizing agents are capable of altering cellular biochemical processes through alternative mechanisms: (a) by disrupting the fluid matrix surrounding and supporting membrane-bound functional proteins, or (b) by inducing membrane permeability changes resulting in altered cellular homeostasis so that both membrane-bound and soluble enzyme activities are impaired [35, 36]. For instance, although the precise mechanism of inhibition may not be understood completely, membrane-associated enzymes such as Na^+,K^+- and Ca^{2+},Mg^{2+}-ATPases, 3′,5′-nucleotidase, and adenylate cyclase are all known to be inhibited when the environment of the membrane in which they are located becomes more ordered [35, 36].

The order and permeability experiments reported here used membranes comprised only of lipids. Thus, while gossypol may indeed have direct effects on functional and structural protein molecules, for example through Schiffs base condensation reactions with amino groups [1], our observations support the idea that alterations in the physical properties of lipid regions of the membrane may also explain the ability of gossypol to alter the activity of such a diversity of membrane-associated functions [11–17].

Using the I(12,3) spin probe, we also observed that gossypol caused an ordering effect in both rat liver plasma membranes and adipocyte ghost membranes (data not shown). Therefore, this membrane ordering effect is not an artifact observed only in model membranes. The possibility that changes in order parameter were due merely to a direct interaction between gossypol and the probe cannot be ruled out completely. However, this seems unlikely since ESR results in the experiments shown in Figs. 2 and 3 were similar regardless of order of addition of gossypol and label. In addition, the ability of both spin label and fluorescent probes to report similar order parameter changes due to exposure to chemical agents has been well documented [37–39]. Differences in order are believed to reflect physical, structural features of the membrane rather than merely differing tendencies of the drug to interact directly with the probe.

Calculation of the polarity-uncorrected order parameters S, $S(T)$ and $S(T_\perp)$ and the isotropic hyperfine coupling constant a_N' at physiological pH (7.4) shows that, in addition to increasing membrane order, gossypol also increases either the membrane polarity or probe clustering in the membrane, and possibly both [25]. Previous studies of I(12,3)-labeled membranes have indicated that $2T_\perp$ is not only sensitive to the polarity of the spin-label environment but also increases with the probe concentration at high probe:lipid ratios, while $2T_\parallel$ remains virtually unchanged [25, 40]. Accordingly, S and $S(T_\perp)$ decrease while $S(T_\parallel)$ remains constant with increasing probe concentration due to enhanced radical interactions between probe molecules in the membrane [40]. Since Fig. 2 of the current experiments shows that the slopes of the S, $S(T_\parallel)$, and $S(T_\perp)$ gossypol curves are not parallel, gossypol may either increase the polarity of the membrane or enhance probe–probe interactions. With a pK_a of 7.2 [1], gossypol tends to be ionized under most physiological conditions. An increase in the polarity of the membrane environment would be consistent with earlier observations by Reyes *et al.* [18] that gossypol alters the electrochemical properties of PC membrane bilayers. It would also substantiate earlier studies by the same author showing that membrane dielectric constants and dipole potential may be modified by compounds (e.g. benzyl alcohol and chloroform) that alter fluid properties of membranes [41]. We also note with interest that Reyes *et al.* [18] observed, as we did, that the magnitude of the electrochemical effects of gossypol in membranes is dependent on the lipid composition of the membrane, with liposomes comprised only of cholesterol showing very little effect as compared with those containing no cholesterol.

In experiments designed to better understand the responses of liposomes of varying lipid composition to the membrane ordering effect of gossypol, we found differing effects on percentage changes S, $S(T_\perp)$, and a_N' in liposomes containing various relative proportions of cholesterol. A similar phenomenon has been observed with other chemical agents such as chlorpromazine, cannabinol, and pentobarbital [39, 42]. Several possible explanations for our results are as follows: (a) modulation of the effect of gossypol may be due to differences in solubility in different liposome systems; (b) gossypol and cholesterol may occupy the same domains in the membrane, leaving gossypol little opportunity for insertion into the cholesterol-rich domains; (c) gossypol may bind either more efficiently or exclusively to the fatty acid or other non-cholesterol lipid components of membranes than it does to cholesterol; and (d) membranes containing cholesterol may distribute the fatty acid probe differently from membranes not containing cholesterol, since cholesterol-containing membranes are more ordered and provide an apparently more polar environment for the probe [33]. With regard to this last possibility, for example, gossypol-induced changes in S and $S(T_\perp)$ may not be as dramatic in cholesterol-containing membranes because of the counteracting effects of probe clustering in these membranes that would tend to decrease the values of the order parameters.

Detailed quantitative comparison of the role of membrane composition in modulating the effects of gossypol requires that the solubility of gossypol in each membrane be known. In addition, the reversibility of gossypol effects on different membrane components must be studied in greater detail in order to determine which of the above possibilities best

explains the interaction of gossypol with membranes. Apparently, enzyme activity can be at least partially restored in some *in vitro* preparations comprised of natural membranes and model liposomes by repeated washing [14, 17, 18], although some studies have reported irreversible gossypol binding to certain target molecules [17, 21]. Regardless of the precise mechanism of interaction with membranes, however, given equivalent circulating concentrations of gossypol, our studies show that membranes containing different PC:C ratios may be differentially susceptible to the perturbing effects of gossypol.

In our experiments, the condensing effect of gossypol in PC:PS membranes was accompanied by an increase in membrane permeability to glycerol. This result was not surprising, considering that many of the signs of acute gossypol toxicity seen in laboratory animals (e.g. mitochondrial swelling, increased red blood cell hemolysis, widespread organ congestion and edema [1]) suggest that a degeneration of cell membranes at all levels of organization may be occurring with acute or prolonged exposure to gossypol *in vivo*. It is conceivable that gossypol-induced increases in membrane permeability are associated with all of these observed imbalances in cellular homeostasis. It is notable that a *decrease* in membrane permeability is more typically (although not always) associated with membrane-ordering agents [43]; thus, gossypol may be a unique tool for investigation of the mechanistic relationship between membrane order and permeability.

Not surprisingly, in addition to ordering membranes, gossypol also inhibited the membrane-bound glucose transport system in adipocytes (for other examples of chemicals with similar ability see Refs. 31, 44 and 45). Haspel *et al.* [46] had reported previously that hexose uptake by erthrocytes is not affected by 0.01 to 0.05 mM concentrations of gossypol. Our experiments showed that concentrations as low as 0.25 mM inhibited adipocyte hexose transport, so gossypol evidently does inhibit glucose uptake in cells if present in sufficient concentrations in susceptible cells. Gossypol also inhibits sugar metabolism in sperm [19–21], and our work shows that this may be at least partially due to inhibited glucose uptake of sperm. These cells are known to be extremely sensitive to alterations in carbohydrate metabolism [47].

Data showing that hexose uptake is inhibited by gossypol also may be related to reports showing the effects of gossypol on tumor cells [6] and tumor cell metabolism [48]. These cells are also particularly sensitive to agents that interfere with energy metabolism, and are more dependent on glycolysis for ATP synthesis than are normal cells [49].

All of these studies considered together show that, in addition to previously reported direct biochemical effects, gossypol also condenses the lipid regions of membranes and causes alterations in membrane permeability and transport at concentrations ranging from 50 to 400 μM under the conditions reported. Generalized electrochemical effects on membranes have been reported by others at even lower gossypol concentrations in the range of 0.7 to 2 μM [18]. Inhibition of membrane-associated enzyme activities has been noted usually in the range of 5–500 μM concentrations [11–17]. Taking into consideration all existing data collected under a variety of different experimental conditions, it appears that the membrane structural effects of gossypol may indeed at least contribute to disruption of membrane-associated functions. However, further quantitative comparison of the relative contribution of each of these various mechanisms in explaining the observed pharmacologic and toxic effects of gossypol should be undertaken with caution. As we and others [18] have observed, results of gossypol experiments can differ significantly if the membrane lipid composition is allowed to vary under otherwise controlled experimental conditions. Parallel membrane structural/functional experiments using standardized membrane preparations should be conducted in order to make meaningful quantitative structure/function correlations.

Animal experiments indicate that the tissue level of gossypol in the testes of infertile rats is estimated to be 0.016 mM [22], although the relative partitioning between different tissue regions and different cell types complicates direct comparison with other tissues. Furthermore, gossypol is likely to reach higher levels than this in other organs, particularly liver, spleen, and kidney [2, 50] due to its lipophilic nature and long tissue retention times. Although the *in vivo* consequences of exposure to chronic low levels of membrane perturbing agents have not been well characterized, it will be important to focus attention on possible generalized toxicity to membrane-related functions, especially if gossypol is authorized for chronic use. More information on typical human tissue levels in volunteers consuming gossypol would be very useful for designing and interpreting results of these and other *in vitro* studies.

In summary, nonspecific perturbation of plasma and subcellular membranes by gossypol may relate to its ability to disrupt many cell processes. In view of the important role that membranes play in regulating cell function, and the increasing amount of evidence showing that gossypol disrupts membrane-associated functions, further investigation of the solubility and effects of gossypol in different model and natural membranes should be encouraged.

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