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Incorporation of Gossypol and Formation of Its Protein Conjugates in Mouse Transformed Sertoli (TM4) Cells

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Mitochondria of both mouse transformed Sertoli (TM4) cells and primary-cultured rat and mouse Sertoli cells were shown to be preferentially affected by gossypol (Tanphaichitr et al, 1984). To investigate whether this selective effect was due to a greater ability of TM4 cells to accumulate gossypol, TM4 and other somatic cell lines, including dog (MDCK) and kangaroo (PtK2) kidney epithelial cells, rat embryo fibroblasts (Rat-1) and mouse BALB/c 3T3 fibroblasts, were incubated with [^{14}C]gossypol and the incorporated specific activity of the drug was assessed. The results indicate that TM4 cells accumulated [^{14}C]gossypol at the highest level. Incorporated [^{14}C]gossypol appeared to bind to TM4 cell macromolecules and remained in the dialysis tubing after extensive dialysis. Characterization of these gossypol-conjugated proteins by SDS-polyacrylamide gel electrophoresis followed by fluorography revealed that these proteins had apparent M_r's of 92,500, 70,000, 63,200, 60,000, 58,100, 54,000, 52,000, 50,000, 47,500, 40,000, 37,000, 35,000, 30,000, 20,000, and 14,500 daltons. Conjugation of these proteins with gossypol may result in macromolecular dysfunction and abnormal structure.

Key words: gossypol, TM4 cells, protein conjugates.

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Gossypol(1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl[2,2'-binaphthalene]-8,8'-dicarboxaldehyde) is a yellow compound that is derived from cottonseeds (Abou-Donia, 1976). It has been used as a male contraceptive in mainland China and some developing countries (Prasad and Diczfalusy, 1981; Zatuchni and Osborn, 1981; Sang, 1983; Segal, 1985). Gossypol exerts its antifertility action by interfering with spermatogenesis, as demonstrated in both humans and experimental animals (National Coordinating Group, 1978; Chang et al, 1980; Prasad and Diczfalusy, 1981). By ingesting 20 mg of the drug daily for 2 months, these men became azoospermic (Natl Coord Group, 1978). Experiments entailing forcefeeding the rat a single dose of [^{14}C]gossypol (20 μCi/7.5 mg) indicate

that most of the radiolabeled drug initially accumulated in the liver and then is excreted into the feces. However, about 10% of the drug remains in the body, primarily in the testis, 9 days after administration (Natl Coord Group, 1978). Isolation of testicular organelles from these gossypol-treated rats reveals that the drug was incorporated into the mitochondria (Jensen et al, 1982; Xue et al, 1983), which may result in a metabolic imbalance that in turn leads to cessation of spermatogenesis. It is unclear which testicular cells are most susceptible to gossypol treatment, and if its effect results from their greater ability to incorporate the drug.

Gossypol has been shown to bind to macromolecules and sequentially to perturb their biologic functions (Abou-Donia, 1976). Gossypol bound to artificial phospholipid membranes decreases membrane potential (Reyes et al, 1984). Prevention of lipid peroxidation has also been observed in human spermatozoa and erythrocyte membranes exposed to gossypol (Sheriff et al, 1986). DNA and DNA polymerase have been shown to bind to gossypol, leading to a decrease in DNA synthesis (Rosenberg et al, 1986). In addition, other proteins have been demonstrated to interact with gossypol, probably through strong noncovalent interaction or covalent bonding between the aldehyde group of gossypol and the amine group of proteins (Lyman et al, 1959; Cater and Lyman, 1969). Gossypol bound to band 3 of red blood cells causes inhibition of cell function on inorganic anion exchange (Haspel et al, 1985). On the other hand, stoichiometric interaction between albumin and gossypol mitigates the drug's adverse action in cells cultured in albumin- or serum-containing media (Haspel et al, 1984; Tanphaichitr et al, 1984).

TM4 cells are established cultured cells that are derived from an immature mouse testis. These cells express some properties typical of normal Sertoli cells. They contain tripartite nucleoli, proliferate in response to FSH, secrete low levels of ABP and plasminogen activator and form aggregated balls when plated onto the myoid cell layer (Mather et al, 1982). On the other hand, TM4 cells possess some properties characteristic of transformed cells. These include a 10-h cell cycle in comparison to the 24 hours of primary Sertoli cell cultures, when grown in medium containing fetal calf serum, epidermal growth factor (EGF), insulin and transferrin. Unlike the primary cultures of Sertoli cells, TM4cells continue to proliferate with an approximate 22-h cell cycle in serum-free conditions. The ability of TM4 cells to grow in a defined medium is advantageous when studying the *in vitro* effects of gossypol since the drug binds avidly to albumin and probably also to other serum proteins (Haspel et al, 1985). Our previous studies show that TM4 cells behave in a manner similar to primary cultures of Sertoli cells, being more susceptible to gossypol than are other cultured somatic cells. This adverse effect of gossypol on TM4 cells is more selective for their mitochondria (Tanphaichitr et al, 1984; Robinson et al, 1986). Using [^{14}C]gossypol, we have attempted to answer the question of whether the observed selective effects of gossypol on TM4 cells are due to the greater ability of these cells to incorporate the drug compared to other somatic cell lines cultured under the same condition. Additionally, we have examined the interaction of incorporated gossypol with TM4 proteins.

Materials and Methods

Materials

Ham's F-12 medium was purchased from M.A. Bioproducts (Walkersville, MD) and Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum, gentamycin and penicillin-streptomycin solution (5,000 units penicillin: 5,000 μg streptomycin/ml) were obtained from Grand Island Biological Co. (Grand Island, NY). Insulin, transferrin and EGF were provided by Collaborative Research (Cambridge, MA). HEPES and 2-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) was obtained from the United States Biochemical Corp. (Cleveland, OH). Tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), acrylamide, N,N'-methylene-bis-acrylamide (BIS), glycine amino-acetic acid, ammonium persulfate, a low molecular weight standard protein kit for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie Brilliant Blue R-250 and BioRad Protein Assay Reagent were purchased from BioRad Laboratories (Richmond, CA). The EN^{3}HANCE fluorographic solution was an NEN product (EI DuPont de Nemours & Co., Boston, MA). Insta-gel scintillation fluid was obtained from Packard Instrumental Company (Downers Grove, IL). [^{14}C]gossypol ([1,1'-^{14}C]gossypol) (specific activity = 1.67 mCi/mmol; 1 Ci = 3.7 × 10^{10} becquerel) was a generous gift from Dr. Martin Sonenberg, the Sloan-Kettering Division, Cornell University graduate School of Medical Sciences, New York, NY. Its synthesis was described in detail previously (Haspel et al, 1984). A transformed Sertoli cell line, TM4, was provided by Dr. Jennifer P. Mather, the Population Council, New York, NY.

Cellular Incorporation of [^{14}C]Gossypol

TM4 cells were seeded and cultured at 37 C, 5% CO_2, in 6-well culture plates (9.6 cm^2/well) in 2.5 ml of a mixture of Ham's F-12 and DMEM (1:1, v/v), supplemented with

2% fetal calf serum, HEPES (15 mM), $NaHCO_3$ (1.2 g/l), gentamycin (10 mg/l), insulin (10 μg/ml), transferrin (5 μg/ml) and EGF (1 ng/ml) (Mather et al, 1982). On reaching confluence (2.3×10^6 cells/well), the cells were released from the substratum by trypsinization (Mather et al, 1982) and diluted in fresh medium containing all supplements except fetal calf serum. Approximately 0.6×10^6 cells were placed in each well. Within 2 days in this supplemented medium without fetal calf serum, these cell cultures reached confluence. The cell monolayer was washed twice in the same medium and incubated for 1 hour at 37 C, 5% CO_2. Various amounts of [^{14}C]gossypol, freshly made in ethanol as a stock solution, were then added to the cell cultures. At the end of an indicated time period, the culture plate was chilled on ice, and the culture medium was removed rapidly. The cells were then washed successively in prechilled (4 C) phosphate-buffered saline (PBS), and finally dissolved in 0.3 N NaOH. Incorporation of [^{14}C]gossypol was also investigated in other nonreproductive somatic cell lines, including mouse BALB/c 3T3 fibroblasts, rat embryo fibroblasts (Rat-1) and dog (MDCK) and kangaroo rat (PtK2) kidney epithelial cells. These cells were seeded in 6-well culture plates in the same manner as the TM4 cells. However, all of these cell lines were cultured until reaching confluence in DMEM supplemented with 10% fetal calf serum, 2% glutamine and 1% penicillin-streptomycin. The amount of fetal serum in the medium was then reduced to only 2% at the time of subculturing, and the cells were passed and cultured until reaching confluence using the same medium for 2 more days. The spent medium was replaced with fresh medium containing all supplements and 2% fetal calf serum. Radiolabeling of all cells was then performed as described above. For comparison, TM4 cells were cultured in a similar manner to other cell lines. At the end of the labeling period, cells were washed and lysed in NaOH as described above.

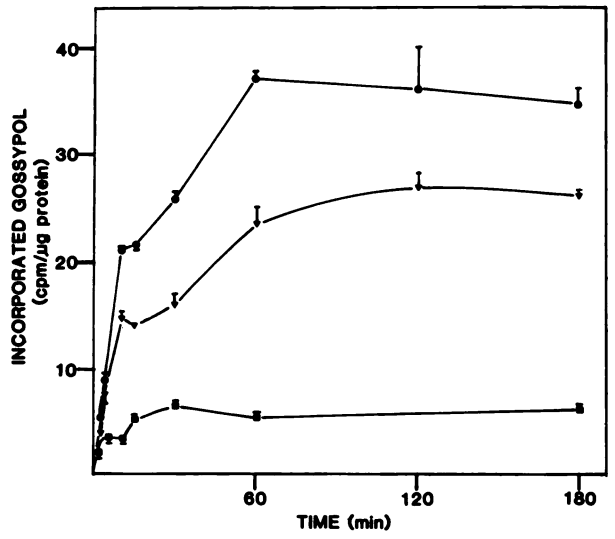

Fig. 1. Kinetics of the incorporation of [^{14}C]gossypol at 2.5, 5 and 10μg/ml into TM4 cells cultured in the absence of fetal calf serum. (■—■) = 2.5 μg/ml, (▼—▼) = 5 μg/ml and (●—●) = 10 μg/ml of [^{14}C]gossypol. Data are means ± standard errors of means (SEM) of five replicates, and are representative of three similar experiments.

The radioactivity of a 500-μl aliquot of the NaOH-cell lysate was quantified in a Beckman L-S230 scintillation counter using 4 ml of Instagel preacidified with 0.048 M glacial acetic acid as a liquid scintillation fluid. BioRad Protein Assay Reagent was then used to measure the amount of proteins in this cell lysate. Specific activity was expressed as cpm/μg protein.

Assessment of Gossypol-protein Conjugates in TM4 Cells

The radioactive cell lysate from the five replicate wells was pooled and assessed for its specific activity. The pooled lysate was then dialyzed against distilled water for 2 days in a dialysis tubing with the retention M_r cutoff of 10,000 daltons. Specific activity of the solution remaining in the tubing was reassessed.

In a parallel experiment, TM4 cells cultured in the defined medium with all supplements except fetal calf serum were incubated with [^{14}C]gossypol (10 μg/ml) for 1 hr as described above. Cells were then lysed with SDS sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% mercaptoethanol and 10% glycerol) and subjected to SDS-PAGE (10 to 18% polyacrylamide, 14.7×0.075 cm^3) (Laemmli, 1970). Standard proteins with known molecular weights were coelectrophoresed with TM4 cell lysate proteins. Gels were stained for protein bands with 0.125% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid.

[^{14}C]gossypol-protein bands were detected by fluorography. The unstained gel was fixed for 1 hour in 10% (w/v) trichloroacetic acid, 10% (v/v) glacial acetic acid and 30% (v/v) methanol. The gel was then transferred into the EN^{3}HANCE solution and incubated at room temperature for another hour. After precipitation of the fluor in the gel by cold water, the gel was dried *in vacuo* and apposed to Kodak X-AR5 x-ray film. The film was exposed at −80 C for 42 days followed by development in a Kodak M6 AW RP X-OMAT processor.

Results

Figure 1 shows the kinetics of cellular incorporation of [^{14}C]gossypol (2.5, 5 and 10 μg/ml) by TM4 cells cultured in the absence of fetal calf serum. The incorporation pattern at all of these concentrations was biphasic; the level of incorporated [^{14}C]gossypol reached the first plateau within the first 15 min, then rose again and attained the second plateau within 60 min. The second plateau was about twice as high as the first in the three experiments with different [^{14}C]gossypol concentrations. Presumably, the incorporation patterns were not affected by the fetal calf serum remaining on the cell surface since the cultures were maintained in the medium without fetal calf serum for 2 days followed by washing in

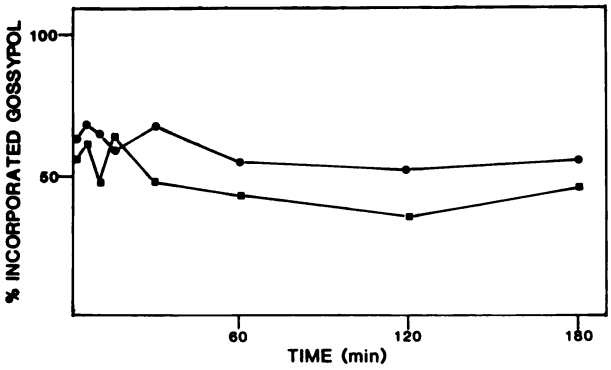

Fig. 2. Percent specific activity of [^{14}C]gossypol-bound TM4 cell macromolecules as a function of drug treatment time. NaOH lysates from TM4 cells exposed to 5 (■—■) and 10 (●—●) μg/ml of [^{14}C]gossypol for various time periods were dialyzed against distilled water. Data presented are representative of three similar experiments.

the same defined medium prior to addition of [^{14}C]gossypol. Moreover, TM4 cells previously cultured in 2% fetal calf serum but then extensively washed in the defined medium had similar levels of [^{14}C]gossypol incorporation as the TM4 cells cultured for 2 days in the absence of fetal calf serum (data not shown). The incorporation of radioactive gossypol occurred only in active and living TM4 cells. Neither overconfluent TM4 cells nor the cells pretreated with methanol or 10% trichloroacetic acid for 5 min incorporated [^{14}C]gossypol above the blank value (data not shown).

Previous reports have described the binding of gossypol to macromolecules such as proteins and phospholipids (Lyman et al, 1959; Cater and Lyman, 1969; Abou-Donia, 1976; Sato et al, 1983). To investigate this phenomenon in TM4 cells exposed to [^{14}C]gossypol the cell lysate in 0.3 N NaOH was dialyzed against water for 2 days in a dialysis tubing with a retention M_r cutoff of 10,000 daltons. Under this condition, < 5% of free [^{14}C]gossypol dissolved in 0.3 N NaOH remained in the dialysis tubing (data not shown). By contrast, about 50 to 60% of the specific activity of the TM4 cell lysates remained in the tubing at all the various times of [^{14}C]gossypol treatment (Fig. 2). The decreased specific activity of the dialyzed cell lysate was probably due to a release of some unbound radioactive material from the tubing. On the other hand, the amount of proteins appeared the same in the tubing before and after the procedure. The results suggest that the radioactive drug or its metabolite interacted with cellular macromolecules with significant affinity that withstood this prolonged dialysis.

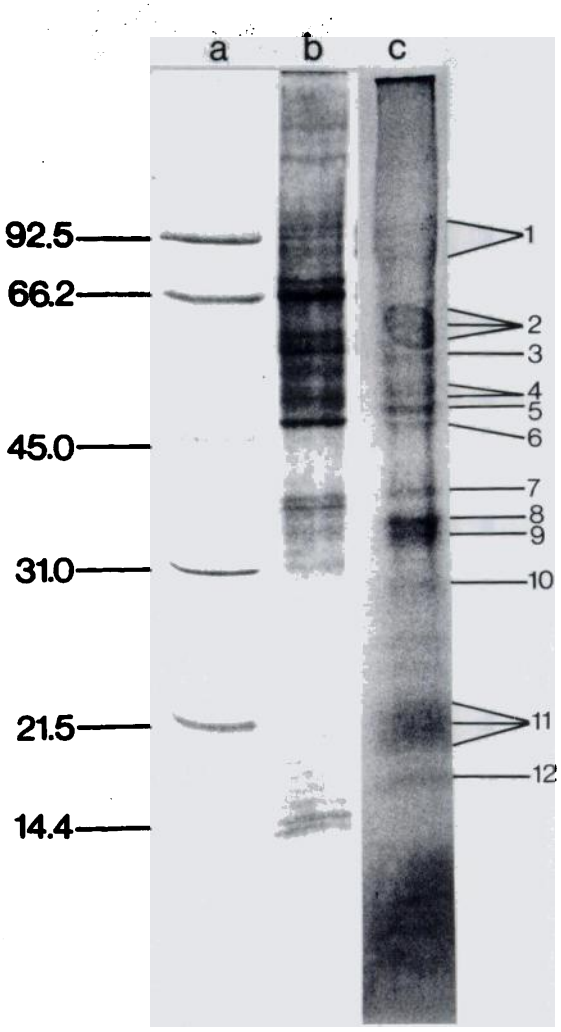

Fig. 3. Electrophoretogram and fluorogram of proteins and [^{14}C]gossypol-conjugated proteins of TM4 cells. Lane (a) displays a set of standard proteins whose M_r's are shown on the left. Lane (b) illustrates proteins extracted from [^{14}C]gossypol-labeled TM4 cells. Proteins in both lanes (a) and (b) were stained with Coomassie Brilliant Blue. Lane (c) is the fluorogram of [^{14}C]gossypol-conjugated proteins. Twelve radioactive bands of M_r's 92.5, 64.4, 58.1, 53, 50, 47.5, 40, 37, 35, 30, 20, and 14.5 kdaltons were observed.

Cellular proteins were one type of TM4 macromolecule that interacted with [^{14}C]gossypol (Fig. 3). In this study, TM4 cells were incubated with 10 μg/ml of [^{14}C]gossypol for 1 hour, followed by extraction of their proteins by a buffer containing SDS. These proteins were subjected to PAGE and then fluorography. Figure 3 displays protein bands 1 to 12 that contained the radioactive gossypol or

its metabolite. Bands 3 and 5 to 9 were discrete, with M_r's of 58,100, 50,000, 47,500, 40,000, 37,000, and 35,000 daltons, respectively. Band 2 was a triplet of M_r's 70,000, 63,200 and 60,000 daltons, whereas Band 4 was a doublet of M_r's of 54,000 and 52,000. On the other hand, Bands 1 and 10 to 12 were diffuse, with average M_r's of 92,500, 30,000, 20,000 and 14,500 daltons, respectively. Significantly, the conjugation of the proteins and the radiolabeled drug withstood the SDS treatment, suggesting that the interaction probably involved covalent bonding.

A more selective effect of gossypol on TM4 cells could be attributed to a greater ability of the cells to incorporate the drug. This possibility was investigated by comparing the level of incorporated [^{14}C]gossypol of mouse transformed Sertoli TM4 cells with those of dog (MDCK) and kangaroo rat (PtK2) kidney epithelial and mouse BALB/c fibroblast ($3T_3$) cells. Unlike TM4 cells, these cells did not survive in culture medium without fetal calf serum.

The experiments were, therefore, performed in medium containing 2% fetal calf serum, although these other cell cultures normally included 10% fetal calf serum. It was possible, however, that residual fetal calf serum in the MDCK, PtK2 and 3T3 cells may have affected the cellular incorporation of [^{14}C]gossypol. To ensure the removal of excess fetal calf serum, the cells were cultured in the presence of 2% fetal calf serum for 2 days and then washed in the 2% medium before radioactive gossypol was added. Twenty-five μg/ml of gossypol was chosen for labeling all cell types since it is the approximate EC_{50} for populations of PtK2, MDCK and $3T_3$ cells that still incorporate rhodamine 123 into their mitochondria after a 5-hour gossypol treatment (Tanphaichitr et al, 1984). In addition, TM4 cells cultured in the presence of 2% fetal calf serum were incubated with 7.5 μg/ml of [^{14}C]gossypol, which is the EC_{50} of TM4 cells. Figure 4 shows that when 25 μg/ml of [^{14}C]gossypol were added to medium containing 2% of fetal calf serum, TM4 incorporated more label than the other somatic cells. Similar to the study performed in the absence of fetal calf serum, the kinetics of radioactive incorporation by TM4 cells cultured in the presence of 2% fetal calf serum and exposed to 25 μg/ml of [^{14}C]gossypol was biphasic. Drug accumulation peaked first at 10 min, declined slightly, then rose in the second phase and finally reached a plateau at 30 min. PtK2 and MDCK cells showed similar kinetic patterns of [^{14}C]gossypol incorporation, but their maximum levels of incorporation were about one half that of the TM4 cells. By contrast, at all time points studied, $3T_3$ cells incorporated [^{14}C]gossypol at only 10% of the maximum level of TM4 cells. Rat embryo fibroblasts (Rat-1) showed a similar pattern of [^{14}C]gossypol accumulation as that observed in $3T_3$ cells (data not shown). When the concentration of [^{14}C]gossypol was decreased to 7.5 μg/ml, the kinetics of TM4 drug incorporation were much slower than at 25 μg/ml; at 7.5 μg/ml, the maximum [^{14}C]gossypol labeling was half of that observed at 25 μg/ml. Significantly, all of these cell lines had similar amounts of protein/culture well (ie, 200 to 225 μg/well); therefore, the different specific activities of incorporated [^{14}C]gossypol were probably not due to different numbers of cellular macromolecules. It was evident that all cell lines were alive under these [^{14}C]gossypol labeling conditions, since they were able to hydrolyze fluorescein diacetate to acetate and fluorescein (Tanphaichitr et al, 1984).

Discussion

In this study, we have shown that TM4 cells could actively incorporate [^{14}C]gossypol (> 5 μg/ml). The maximum level of incorporation was attained within 60 min after addition of [^{14}C]gossypol. The rapid accumulation of the compound into the cells is not surprising in view of gossypol's lipophilic property. However, the maximum incorporation levels were not commensurate at all concentrations of [^{14}C]gossypol studied. At a low level (2.5 μg/ml) of the drug, a significant amount of gossypol may bind to proteins or macromolecules secreted by TM4 cells, which may result in decreased drug transportation into the cells. On the other hand, at a high concentration (10 μg/ml), diffusion of the drug into the cells may have reached saturation. In addition, at high concentrations the drug may have some adverse effects on membrane permeability.

The efficiency of [^{14}C]gossypol incorporation was also investigated in other somatic established cell lines, including BALB/c $3T_3$, Rat-1, MDCK and PtK2 cells. Since these cells could not be cultured in a defined medium, their cultures were supplemented with a minimal level of fetal calf serum (2%). For comparative purposes, TM4 cells were also cultured in medium containing 2% fetal calf serum. At 25 μg/ml of [^{14}C]gossypol, TM4 cells incorporated the radioactive drug at the highest level (Fig. 4). This result may explain why TM4 cell mitochondria are particularly susceptible to adverse actions of the drug (Tanphaichitr et al, 1984; Robinson et al, 1986). Interestingly, at 25 μg/ml of gossypol, the mitochon-

drial functions of PtK2 and 3T3 cells, as assessed by rhodamine 123 accumulation, were similarly perturbed. However, PtK2 cells significantly incorporated the radiolabeled drug, while 3T3 cells did not. The results suggest that mitochondrial impairment in 3T3 cells may not derive from a direct effect of gossypol. Rather, the drug may be loosely bound to the plasma membrane, which then may disturb the transport of certain ions such as Ca+2 that may be necessary for maintaining transmembrane mitochondrial potential (Tzagoloff, 1982). Alternatively, [^{14}C]gossypol may have been converted in 3T3 cells into a metabolite that possessed no radioactive aldehyde groups (eg, apogossypol) but still had adverse effects on the mitochondria. On the other hand, a significant amount of [^{14}C]gossypol was incorporated into both Kangaroo rat and dog kidney epithelial cells (PtK2 and MDCK), where the accumulated drug or its metabolite may exert direct effects on the mitochondria. Possibly, drug incorporation may also occur *in vivo* in human kidney epithelial cells and may cause the hypokalemia observed in some males who have taken gossypol (Qian, 1985). It should be noted that while 25 μg/ml of gossypol is the EC_{50} for PtK2 cells positive for mitochondrial rhodamine 123 accumulation, 7.5 μg/ml is the corresponding EC_{50} of TM4 cells similarly cultured in 2% fetal calf serum (Tanphaichitr et al, 1984). At their respective EC_{50} gossypol concentrations, both types of cell incorporated the radiolabeled drug at the same level. The results suggest that a similar mechanism of drug action may have occurred in the two cell types.

Formation of covalent conjugates of TM4 cell proteins with gossypol or its aldehyde-containing metabolite (Fig. 3) is of particular interest. Presumably, this covalent bonding is through Schiff's base formation between the gossypol's aldehyde group and the protein amine group. Since each gossypol molecule contains two aldehyde groups and, in general, more than one amine group exists in a protein molecule, multimers of conjugates between proteins and gossypol or gossypol's metabolites may have resulted from this interaction. It should be noted that some minor protein bands shown by Coomassie Brilliant Blue R-250 staining were major conjugates of [^{14}C]gossypol or its radioactive metabolites (ie, Bands 8 to 9, Fig. 3). Due to the limited amount of [^{14}C]gossypol available, it was not feasible to perform the same experiment in other cell lines. However, it should be noted that a similar retention in the dialysis tubing of [^{14}C]gossypol or its radioactive metabolite was observed when the NaOH lysates of PtK2 and MDCK cells exposed to [^{14}C]gossypol (25 μg/ml) were extensively dialyzed against water (see Fig. 4). The results imply that macromolecules conjugated with gossypol or its metabolites also formed in these two kidney epithelial cell lines. The covalent conjugates of gossypol cellular macromolecules have been described in sea urchin spermatozoa, although the nature of these macromolecules has not been characterized (Sato et al, 1983).

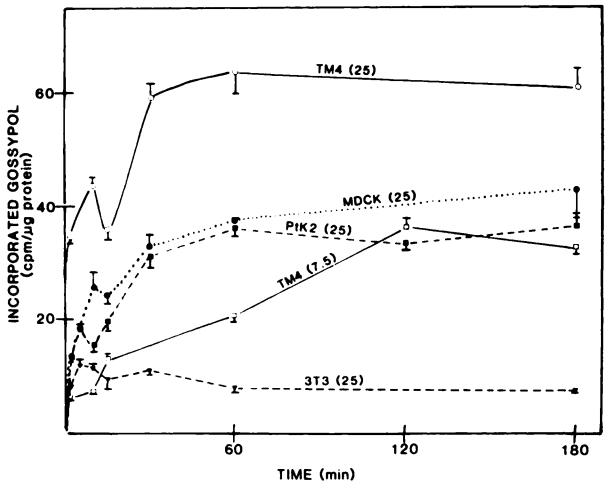

Fig. 4. Kinetics of the incorporation of [^{14}C]gossypol into TM4, MDCK, PtK2 and 3T3 cells cultured in the presence of 2% fetal calf serum. (□—□), (o—o) = TM4 cells treated with 7.5 and 25 μg/ml of [^{14}C]gossypol, respectively. (●---●), (■---■) and (▼---▼) = MDCK, PtK2 and 3T3 cells, respectively, treated with 25 μg/ml of the radioactive drug. Data are means ± SEM of five replicates, and are representative of three similar experiments.

In summary, we have shown that TM4 cells incorporated [^{14}C]gossypol preferentially in comparison to other somatic nonreproductive cells. The radioactive drug or its metabolites were able to conjugate proteins and other macromolecules. Conceivably, protein cross-linking induced by gossypol would lead to dysfunction and/or an abnormal structure of these macromolecules, similar to results from glucose-derived cross-linked collagens and other structural proteins in chronic diabetic animals and men (Brownlee et al, 1986). Our *in situ* autoradiographic study at the light microscopic level shows that the grains of [^{14}C]gossypol or its radioactive metabolite are accumulated mainly in the TM4 cell cytoplasm (Tanphaichitr, unpublished results). Further studies are needed to determine whether the mitochondria are the cell organelles that

preferentially accumulate gossypol (or its metabolites), since they have previously been shown to be the target of gossypol in TM4 cells (Tanphaichitr et al, 1984; Robinson et al, 1986). Other studies should also investigate whether the protein conjugates of gossypol or its metabolites play a role in the drug's adverse effects on the target organelles. In addition, it remains to be seen if these protein conjugates exist in primary cultures of Sertoli cells and *in vivo* in animals treated with gossypol.

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