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Renal Transport of Gossypol in the Rabbit (42063)

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Abstract. The present work was carried out to investigate the transport characteristics of gossypol, a toxic weak organic acid (pK = 7.2) contained in cottonseed, into the rabbit renal cortical slice. The uptake of gossypol increased linearly during a 2-hr incubation after which it leveled off with the average slice-to-medium concentration ratio (S/M) slightly above 20. In the presence of metabolic inhibitors, the S/M gossypol leveled off at about 9, suggesting an extensive binding of gossypol to tissue proteins. The uptake of gossypol was significantly inhibited by *p*-aminohippurate (PAH), probenecid, ouabain, and DIDS, all of which are known inhibitors of renal organic anion transport. However, the gossypol uptake was not affected by tetraethylammonium (TEA), a prototypical organic cation. Kinetic studies indicated that the apparent K_m for gossypol transport is 0.28 mM, and also that probenecid inhibits gossypol transport in a competitive manner. It is concluded that gossypol is transported by the renal tubule through the classic organic anion system.

Gossypol[2,2′-binaphthylene)-8,8′-dicarboxaldehyde-1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl], a toxic substance in cottonseed, is a recently developed oral male contraceptive (1). This compound is a weak organic acid with a pK value of 7.2 and accumulates in both the liver and kidneys at a very high concentration in rats (2). Evidently, the liver appears to be the major excretory organ for gossypol by secreting this compound into the bile probably via an active organic anion transport system. In contrast, a very small portion of the ingested gossypol is excreted via urine, and it was speculated that gossypol was filtered and actively secreted by the tubular mechanism for organic acids and subsequently reabsorbed through nonionic diffusion by virtue of its high lipid solubility (2). However, the speculation regarding the hepatic and renal handling of gossypol has never been critically tested.

Recently, Haspel *et al.* (3) found that gossypol is a potent inhibitor of inorganic anion exchange in human erythrocytes while it does not affect hexose transport, cation transport, or Na–K-ATPase activity. Since many specific inhibitors of red cell anion exchange have been shown to specifically interact with the renal organic anion transport system (4–6), the effect of gossypol on organic ion transport in the rabbit kidney slice was investigated in our laboratory (7). It was found that the slice uptake of *p*-aminohippurate (PAH), a prototypical organic anion, is significantly inhibited (by 30%) when the medium concentration of gossypol is raised to 10^{-4} M. However, gossypol at the latter concentration also significantly decreased the slice oxygen consumption (30%), Mg-ATPase (70%), and Na–K-ATPase (90%) activities of renal cortical microsomes. These findings strongly suggest that gossypol inhibits PAH uptake as the result of nonspecific nephrotoxicity rather than through its specific interaction with the organic anion transport system.

The present investigation was undertaken to directly study the transport characteristics of gossypol itself by the rabbit kidney cortical slice. The results obtained from this study clearly indicate that there is an active transport component of gossypol, which competitively interacts with the classic organic anion transport system.

Methods. New Zealand white rabbits (weighing approximately 2 kg) were killed by cervical dislocation, and the kidneys were removed and immediately perfused through the renal artery with ice-cold isotonic saline (NaCl, 140 mM; KCl, 10 mM; and $CaCl_2$, 1.5 mM) to remove as much blood as possible.

A Stadie-Riggs microtome was used to make thin renal cortical slices (0.4–0.5 mm). The slices were stored briefly in an ice-cold

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modified Cross-Taggart medium before incubation. The composition of the medium was 140 m*M* NaCl, 10 m*M* KCl, 1.5 m*M* $CaCl_2$, and 5.0 m*M* Na phosphate (pH 7.4). The renal slices (approximately 150 mg wet wt) were incubated in 30-ml beakers containing 4 ml of Cross-Taggart medium containing various concentrations of cold gossypol (as specified under Results) and a trace amount of ^{14}CHO-gossypol (the specific activity 1.68 mCi/m*M*, prepared by Dr. J. F. Ren and Dr. W. Watanabe of the Memorial Sloan–Kettering Cancer Center). Unless stated otherwise, the incubations were carried out in a Dubnoff metabolic shaker (25°C) with a 100% oxygen atmosphere (8). The duration of incubation varied with experimental series and is specified under Results.

Following incubation, the slices were quickly removed, blotted, and weighed. The tissue was solubilized in 1 *N* NaOH and then neutralized with HCl. Aliquots of the medium were prepared similarly. Both tissue and media were assayed for ^{14}C activity by using standard liquid scintillation counting technique (9). In brief, samples were added to 10 ml Aquasol (New England Nuclear) or ACS (Amersham/Searle) for liquid scintillation spectrometry. Quench corrections were performed with the appropriate set of standards for each type of sample composition. The gossypol uptake data are given as either the rate (μmole/g hr) or the slice-to-medium (S/M) ratio: the tissue concentration of gossypol (mole/g wet tissue) divided by that of the medium (mole/ml medium).

Since gossypol is insoluble in water, it was dissolved in ethanol and then added to the appropriate solution to attain desired concentrations ranging from 10^{-7} to 10^{-4} *M*. All solutions contained 0.5% ethanol, at which concentration organic ion transport is not affected (7).

The data were analyzed statistically by the unpaired Student *t* test, and the level of probability to denote significance was chosen as $P < 0.05$.

Gossypol, labeled and unlabeled, was kindly provided by Dr. M. Sonnenberg and Dr. H. Haspel of Memorial Sloan–Kettering Cancer Institute. DIDS was obtained from Pierce Chemical, Rockford, Illinois. All other inhibitors and reagents were obtained from Sigma Chemical, St. Louis, Missouri. All reagents were of the highest purity available.

Results. The time course of radioactivity uptake was determined over a period of 240 min in the absence of (control) and presence of metabolic inhibitors (1 m*M* iodoacetamide, 1 m*M* 2,4-dinitrophenol, and nitrogen) and the results are shown in Fig. 1. The concentration of gossypol in the incubation medium was 10^{-6} *M*. In the absence of metabolic inhibitors, the S/M ratio for gossypol increased almost linearly during the first 2 hr after which it leveled off at about 20. In the presence of metabolic inhibitors, the S/M value followed a similar time course but leveled off at about 9 after 2 hr. In other words, the passive uptake of gossypol is very large and contributed to 40–50% of the total uptake. Similar results were obtained with a gossypol concentration of 10^{-5} (data not shown).

In the second series of experiments, the effects of various chemical agents on the uptake of gossypol were examined (Table I). Agents that are known to inhibit the active transport of organic anions (8, 10, 11), such as PAH, probenecid, ouabain, and metabolic inhibitors, significantly decreased the S/M gossypol. On the other hand, acetate, an agent known to stimulate the transport of PAH through yet unknown mechanisms (8),

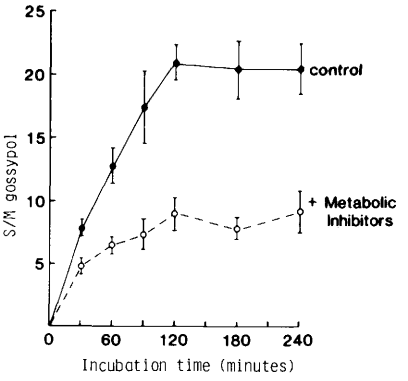

FIG. 1. S/M gossypol in the absence (control) and presence of metabolic inhibitors as a function of incubation time. The concentration of gossypol in the medium was 10^{-6} *M*, and "metabolic inhibitors" consisted of 1 m*M* iodoacetamide, 1 m*M* 2,4-dinitrophenol, and nitrogen bubbling. Each point represents the mean (±SE) of three experiments.

TABLE I. EFFECTS OF VARIOUS CHEMICAL AGENTS ON GOSSYPOL UPTAKE

Agents (n)	S/M gossypol
Control (4)	8.00 ± 0.64
10 mM acetate (4)	8.30 ± 1.15
1 mM tetraethyl ammonium (4)	8.62 ± 0.29
1 mM p-aminohippurate (4)	5.57 ± 0.66*
1 mM probenecid (5)	5.20 ± 0.64*
1 mM ouabain (4)	5.85 ± 0.32*
1 mM DIDS (2)[a]	6.83
Metabolic inhibition (5)[b]	3.76 ± 0.20*

Note. Each value represents mean (±SE) obtained at the end of 30-min incubation in a medium containing 10^{-5} M gossypol.

[a] 4,4'-Diisothiocyano-2,2'-disulfonic stilbene.

[b] A combination of 1 mM iodoacetamide, 1 mM 2,4-dinitrophenol, and incubation in a nitrogen atmosphere.

* Represents significant difference from control ($P < 0.05$).

had no significant effect on gossypol uptake. In a limited number of experiments, an inhibition of gossypol uptake was observed in the presence of 1 mM DIDS which is known to inhibit PAH transport (5). However, the uptake of gossypol was not affected by 1 mM tetraethylammonium (TEA) which is known to be transported by an entirely different mechanism (12). These results are consistent with the view that the active component of gossypol uptake into the slice is mediated by the classic organic anion transport mechanism.

In the third series of studies, kinetic experiments were carried out in order to determine the nature of the inhibition of gossypol uptake by probenecid, a classic competitive inhibitor of organic anion transport in the kidney. The initial rate of active gossypol transport was measured in the absence and presence of 1 mM probenecid, by subtracting the passive transport component (determined in the presence of metabolic inhibitors) from the total uptake. The initial rate of transport was assessed by incubating the slices for 30 min (Fig. 1). The concentrations of gossypol in the medium were set at 25, 50, 75, 100, and 150 μM. A Lineweaver–Burk plot of the data indicates that the transport of gossypol is inhibited by probenecid in a competitive manner (Fig. 2). The apparent K_m for gossypol transport was 0.28 mM while the V_{max} was 3.33 μmole/g hr and the K_i for probenecid 0.59 mM.

Discussion. Although it has been postulated by Abou-Donia (2) that gossypol, a weak organic acid with pK of 7.2, is secreted by the liver and kidneys through an organic anion transport system, the supporting data have been lacking. Previous studies conducted in our laboratory indicated that gossypol indeed significantly inhibited the renal slice uptake of PAH, but this phenomenon was observed only when the medium concentration of gossypol was raised to 10^{-4} M at which other cellular functions (e.g., oxygen consumption, Na–K- and Mg-ATPase activities) were also significantly affected (7). Therefore, we were not able to establish that gossypol inhibits PAH transport by interacting with the organic anion transport system at the membrane level.

The results obtained from the present work clearly provide support for the notion that gossypol is transported into the renal cortical slice via the same organic anion transport system that is responsible for the uptake of PAH. The transport of gossypol was affected by well-known anion transport inhibitors such as probenecid, DIDS, PAH, and ouabain but not by organic cations such as TEA (Table I). Moreover, probenecid inhibited the transport of gossypol in a competitive manner

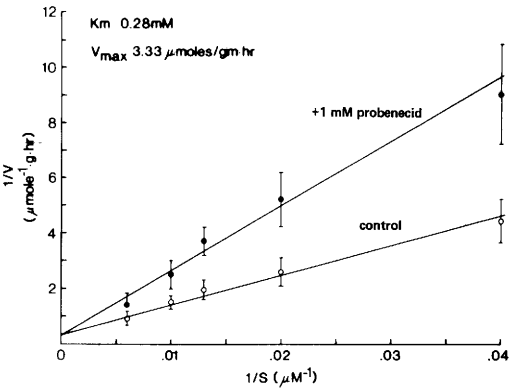

FIG. 2. Lineweaver–Burk analysis of data obtained for transport of gossypol in the absence (control) and presence of 1 mM probenecid in the incubation medium. "V" represents the rate of active transport of gossypol (corrected for the passive component), and "S" the concentration of gossypol in the medium. Each point represents the mean (±SE) of four experiments.

(Fig. 2). Since the hepatic cells are known to have an organic anion transport system similar to that found in proximal tubule (13), the present findings suggest that gossypol may well be secreted by the liver into the bile by a similar mechanism.

It should be emphasized that nothing is known of the specific catabolism of gossypol and therefore the slice concentration of "gossypol" could indeed include metabolites. However, the specific aim of this work was to assess the membrane transport of gossypol and since accumulation of radioactivity was specifically inhibited by the classical transport inhibitors listed above, the metabolism of gossypol will not influence our conclusions.

Although the kidney is able to actively secrete gossypol the liver appears to be the major excretory organ for gossypol (2). This phenomenon may be related to the fact that gossypol extensively binds to plasma proteins (14). In the course of studying the urinary and biliary excretions of phenol red derivatives (organic anions) in the dog, Kim and Hong (15) found that compounds with a high degree of protein binding are predominantly excreted in the bile rather than in urine, and vice versa. However, we cannot rule out a possibility that the very low rate of urinary excretion of ingested gossypol is also due to the extensive reabsorption of filtered and secreted gossypol through nonionic diffusion by virtue of its high lipid solubility (2).

In the presence of metabolic inhibitors, the gossypol uptake by renal slices continued to increase with time, reaching a steady-state S/M level of approximately 9.0 (Fig. 1). This may be attributed to a high degree of tissue protein binding, as might be expected in view of the extensive plasma protein binding of gossypol (14). In addition, metabolic conversions of gossypol within the tubular cell to unconjugated metabolites, glucuronides, and sulfates (16) would also contribute to the high passive S/M level. In this connection, it is of interest to note that the inhibitory constant (K_i) of probenecid for the competitive inhibition of gossypol uptake (Fig. 2) was 0.59 mM. The latter value is one order of magnitude higher than the K_m value for probenecid uptake (17) and K_i value for probenecid when it was used to competitively inhibit the slice uptake of PAH (18). Even a higher K_i (approximately 2.0 mM) for probenecid was observed when the latter substance was used as a competitive inhibitor of bromcresol green, a phenol red derivative with an extremely high degree of protein binding (18). The reason for these discrepancies is not clear at present, but these results indicate that kinetic data should be interpreted with caution when a competitor and/or transport substrate has a high degree of protein binding.

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