lungs, and these effects were inhibited by diethylcarbamazine but not by indomethacin. The injection of PAF into lungs perfused with physiological salt solution stimulated a lung cell to produce leukotrienes. Since the isolated rat lung responds to the injection of synthetic leukotrienes C_4 and D_4 with vasoconstriction (*18*), the vasoconstriction and edema after PAF injection may be due in part to the action of these leukotrienes.

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Inhibitory Action of Gossypol on Enzymes and Growth of *Trypanosoma cruzi*

Abstract. *Gossypol, a phenolic compound isolated from the cotton plant, is a powerful inhibitor of nicotinamide adenine dinucleotide–linked enzymes (α-hydroxyacid dehydrogenase and malate dehydrogenase) of* Trypanosoma cruzi, *the parasite that causes Chagas' disease. Parasites at the epimastigote stage that were incubated for 5 minutes with 100 micromolar gossypol were completely immobilized. Concentrations of gossypol as low as 0.01 micromolar markedly reduced the growth rate of* T. cruzi *in culture.*

There is evidence (*1*) that gossypol (1,1′, 6,6′, 7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-(2,2′-binaphthalene)-8,8′-dicarboxaldehyde) is an active male contraceptive agent. Oral administration of this compound produces oligospermia and azoospermia in humans and other species. Studies by Lee and Malling (*2*) suggest that gossypol acts by inhibiting selectively the lactate dehydrogenase (E.C. 1.1.1.27) isozyme X, which plays an important role in sperm metabolism. Isozyme X (LDH X or C_4) is associated with spermatozoa and spermatogenic cells (*3*). On the basis of its substrate specificity and subcellular distribution, LDH X has been postulated to integrate a shuttle system transferring H from cytosol to mitochondria (*4*). It therefore appears that LDH X is related to metabolic processes that provide energy for motility and survival of spermatozoa.

Studies in our laboratory (*5*) also indicate that gossypol inhibits the activity of human LDH X. We found that the compound is a noncompetitive inhibitor of LDH X with respect to pyruvate (inhibition constant, K_i, 11.0 μM) and is an uncompetitive inhibitor with respect to reduced nicotinamide adenine dinucleotide (NADH) (K_i = 7.0 μM). These studies prompted us to investigate the action of gossypol on the α-hydroxyacid dehydrogenase and other oxidoreductases of the flagellate protozoan *Trypanosoma cruzi*. Our interest was justified by the fact that *T. cruzi* is the agent of Chagas' disease, for which there is no satisfactory drug.

Cultured epimastigotes of *T. cruzi* contain an NAD-linked oxidoreductase designated α-hydroxyacid dehydrogenase (*6*). The enzyme is present as two isozymes with different properties (*7*). The substrate specificity of these isozymes is similar to that of LDH X; in particular, isozyme II exhibits a spectrum of substrates almost identical to that demonstrated for mouse isozyme X (*7*).

The parasites were cultured and extracts were prepared as described previously (*7*). The isozyme II of α-hydroxyacid dehydrogenase and malate dehydrogenase (E.C. 1.1.1.37) were partially purified from aqueous extracts of *T. cruzi* by chromatography on a column of DEAE cellulose and by precipitation with ammonium sulfate (*7*). Succinate dehydrogenase (E.C. 1.3.99.1) was obtained by following the method of Weeger *et al.* (*8*), except that the epimastigotes were suspended in 100 m*M* phosphate buffer, *p*H 7.4, homogenized, and then centrifuged at 10,000*g* for 30 minutes. The pellet was washed three times with distilled water and finally suspended in 20 m*M* phosphate buffer, 1 m*M* EDTA, *p*H 7.6. We then continued the procedure as described by Weeger *et al.*

Table 1. Effect of gossypol on oxidoreductases of *T. cruzi*. The K_m values were calculated by Lineweaver-Burk plots. The velocity, *V*, is expressed as a percentage, taking as 100 percent the maximum activity without gossypol. The K_i values were calculated by using various concentrations of substrates at a constant inhibitor concentration.

Enzyme	Substrate	K_m (m*M*)	*V*	K_i (μM)
α-Hydroxy-acid dehydro-genase	α-Ketoisocaproate	0.41	100	
	α-Ketoisocaproate plus gossypol (0.5 μM)	0.41	59.57	0.73
	NADH	0.026	100	
	NADH plus gossypol (4.0 μM)	0.014	53.28	1.85
Malate dehydro-genase	Oxaloacetate	0.21	100	
	Oxaloacetate plus gossypol (0.3 μM)	0.21	55.5	0.37
	NADH	0.066	100	
	NADH plus gossypol (0.16 μM)	0.036	52.8	0.21
Glutamate dehydro-genase	α-Ketoglutarate	2.7	100	
	α-Ketoglutarate plus gossypol (5 μM)	2.7	41.35	3.5
	NADPH	0.015	100	
	NADPH plus gossypol (11.1 μM)	0.0072	45.26	10.3

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(*8*). Glutamate dehydrogenase–NADP (E.C. 1.4.1.4) was assayed in crude homogenates according to the method of Juan *et al.* (*9*). α-Hydroxyacid dehydrogenase activity was determined by using α-ketoisocaproate, a preferred substrate for isozyme II (*7*); malate dehydrogenase was assayed by the method of Yoshida (*10*); and succinate dehydrogenase was assayed according to Singer (*11*). All enzyme assays were carried out at 37°C.

Studies of inhibition were conducted by incubating the mixture containing the enzyme with gossypol–acetic acid for 10 minutes at room temperature, and then starting the reaction by addition of substrates. Values for the Michaelis constant, velocity (K_m, K_m', V, V'), and K_i were calculated from the data we obtained (Table 1).

Gossypol proved to be a powerful inhibitor of α-hydroxyacid dehydrogenase and malate dehydrogenase. Gossypol acted as a noncompetitive inhibitor with respect to the substrates α-ketoisocaproate and oxaloacetate and as an uncompetitive inhibitor with respect to the coenzyme NADH. The values of K_i shown in Table 1 were obtained with partially purified enzymes. The results were similar for crude extracts.

The same type of inhibition was demonstrated for glutamate dehydrogenase–NADP. The K_i values, especially with respect to the coenzyme, were higher than those observed for the NAD-linked enzymes (Table 1). Succinate dehydrogenase, a flavoprotein, was not affected by gossypol at concentrations up to 100 μ*M*.

The effect of gossypol on oxidoreductases may result in a blockage of energy-providing metabolic pathways in *T. cruzi*. It is possible that the α-hydroxyacid dehydrogenase in the parasite fulfills a role similar to that assigned to LDH X in spermatozoa. To establish whether this blockage could affect the motility of whole parasites, we conducted studies with cultured epimastigotes diluted with the liquid phase of the medium to a concentration of 1×10^6 parasites per milliliter. We then added gossypol to the suspension at concentrations of 25, 50, and 100 μ*M*. The number of motile forms was counted in a Malassez chamber. After 5 minutes of incubation, the preparations with 25 μ*M* gossypol contained 50 percent motile parasites; those with 50 μ*M* gossypol contained only 18 percent motile forms compared to controls; and in preparations with 100 μ*M* gossypol, immobilization was almost total.

We also investigated the effect of gossypol on *T. cruzi* cultures. The compound was diluted in ethanol and added to the medium up to four final concentrations: 0.01, 0.5, 5.0, and 25.0 μ*M*. Each culture flask was inoculated with 2.4×10^6 parasites. Cells in the liquid phase of the medium were counted in a Malassez chamber every 2 days up to day 10. Ethanol did not affect cultures of the parasite. The growth curves of cultures containing an equivalent volume of ethanol (10 μl/ml) and no gossypol were the same as those that contained no ethanol. The effect of gossypol, however, was striking (Table 2). With a 25 μ*M* concentration of gossypol, growth was inhibited almost completely and only a few, rounded epimastigotes with very reduced motility could be observed. With 5.0 μ*M* gossypol, the action was also intense; about 50 percent of the epimastigotes in the medium were rounded forms. The inhibitory action of gossypol on growth was marked even at concentrations as low as 0.01 μ*M* (Table 2). At this concentration, about 20 percent of the parasites were rounded forms. We have not yet determined whether the trypomastigote and amastigote stages of the parasite are also affected by gossypol, nor have we studied the effect of the compound on the course of experimental infections of *T. cruzi* in mammalian cells in vitro or in vivo.

Table 2. Effect of gossypol on growth of *T. cruzi* in culture. Results are expressed in percentages, taking as 100 percent the parasite count of control cultures without gossypol added to the medium. Parasite counts were performed in duplicate. The values are means of two experiments.

Gossypol concentration (μ*M*)	Time in culture (days)				
	2	4	6	8	10
25	5.9	4.0	0.1	0.3	0.3
5	26.0	25.0	25.0	8.0	2.0
0.5	57.0	40.0	24.0	17.0	11.0
0.01	59.0	51.0	36.0	23.0	13.0

The effectiveness of orally administered gossypol as an antifertility agent in humans, producing deleterious effects on seminiferous epithelium, demonstrates that it can traverse the blood-testis barrier. This indicates that the drug penetrates cellular membranes and other permeability barriers. Available clinical studies (*12*) suggest that the compound is fairly well tolerated by humans at effective doses and that spermatogenesis is completely restored when the treatment is discontinued.

The K_i values for gossypol with human LDH X are higher than those determined for the α-hydroxyacid dehydrogenase from *T. cruzi*. It has been reported that gossypol neither accumulates in the testis nor attains higher concentrations in this tissue than in the rest of the body (*12*). If the action of gossypol is indeed due to its inhibitory effect on LDH X, it could be assumed that a dose sufficient to block spermatogenesis in humans could also be toxic for the parasite.

These findings encourage studies on the possible value of gossypol as a therapeutic agent for Chagas' disease and other trypanosomiases.

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