Table I—Effects of Various Parabens and *p*-Hydroxybenzoic Acid on Phenol Formation in the Presence of Single or Combined Cell Suspension as Enzyme Source[a]

Microorganisms Added (Strain)	Substrate, 12 μmoles (1.2 m*M*)					
	No Substrate	*p*-Hydroxy-benzoic Acid	Methyl-paraben	Ethyl-paraben	Propyl-paraben	Butyl-paraben
3	0	11.4 12.2	—	0	—	—
4	0	0	—	0	—	—
5	0	0	—	0	—	—
3 and 4	0	7.4 10.0	—	9.4 9.0	—	—
3 and 5	0	9.9 9.7	—	9.4 9.3	—	—
4 and 5	0	0	—	0	—	—
3, 4, and 5	0	8.3 9.8	8.5 9.7	9.1 9.9	8.8 9.6	8.9 9.3

[a] Each figure in the table shows the amount of phenol formed (micromoles). Reaction conditions were those given in Fig. 1, except that each viable cell count of strains 3, 4, and 5 added was 5.9×10^{10}, 1.3×10^{10}, and 2.1×10^{10}, respectively, in a final volume of 10 ml.

has also been studied intensively by many investigators (6–10), who found that it catalyzes direct splitting of alanine from tyrosine to form phenol. In our studies with tyrosine as the substrate in place of alkylparabens, however, phenol was not produced.

Various alkylparabens are used or prescribed to prevent proliferation of contaminated microorganisms in pharmaceutical preparations. However, *P. aeruginosa* (NCTC 7244) was found to utilize alkylparabens as a carbon source and to grow well in an eye drop solution containing them (11). Furthermore, as described in the present communication, microorganisms such as strain 3 *(K. aerogenes)* metabolize *p*-hydroxybenzoic acid into phenol under restricted nutritional conditions.

Pseudomonas or *Klebsiella* species and other organisms have been isolated from various nonsterile pharmaceutical or cosmetic preparations and the production environment (12). It also has been shown that serious spoilage of drugs is brought about by these microorganisms. Although the mechanism of deterioration has not been elucidated from the standpoint of bacteriological metabolism, this communication presents a typical example concerning drug deterioration by microorganisms.

(1) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 846.

(2) A. Sadamatsu, T. Kina, T. Ariyoshi, and E. Takabatake, *Shokuhin-Eisei Zasshi*, **7**, 50(1966).

(3) D. J. W. Grant and J. C. Patel, *Anal. Biochem.*, **28**, 139(1969).

(4) F. W. Ochynski, *Anal. Chem.*, **85**, 278(1960).

(5) J. C. Patel and D. J. W. Grant, *Antonie von Leeuwenhoek; J. Microbiol. Serol.*, **35**, 53(1969).

(6) Y. Kakihara and K. Ichihara, *Med. J. Osaka Univ.*, **3**, 497(1953).

(7) M. Uchida, Y. Takemoto, Y. Kakihara, and K. Ichihara, *ibid.*, **3**, 509(1953).

(8) H. Yoshimatsu, *ibid.*, **9**, 727(1957).

(9) H. Kumagai, H. Yamada, H. Matsui, H. Ohkishi, and K. Ogata, *J. Biol. Chem.*, **245**, 1767(1970).

(10) *Ibid.*, **245**, 1773(1970).

(11) W. B. Hugo and J. H. S. Foster, *J. Pharm. Pharmacol.*, **16**, 209(1964).

(12) C. W. Bruch, *Drug Cosmet. Ind.*, **Oct. 1972**, 51.

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Received December 4, 1974.
Accepted for publication February 25, 1975.

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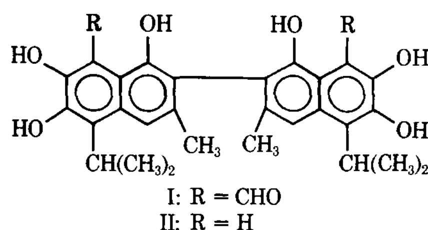
Antiviral Activity of Gossypol and Apogossypol

Keyphrases ☐ Gossypol—screened for antiviral activity ☐ Apogossypol—screened for antiviral activity ☐ Antiviral activity—gossypol and apogossypol

To the Editor:

The pigment gland of the cotton seed contains an array of organic compounds including gossypol (I), which has been demonstrated to have antibacterial (1), antiviral (2–4), and antitumor (5) activities as well as mammalian toxicity (6, 7). Apogossypol (II), which has lower mammalian toxicity, is formed by deformylation of gossypol with base (8). The following report of preliminary findings establishes that apogossypol retains the potent antiviral activity of gossypol.

Gossypol can inactivate influenza virus infectivity *in vitro*, and influenza virus inactivated by treatment with gossypol was given to mice with a resultant 96–100% protection rate (2–4). Since little is known about either the mechanism of action or the activity spectrum of gossypol, an investigation of the *in vitro* inactivation of viruses by this compound and the less toxic apogossypol was initiated.



The toxicity of gossypol[1] and apogossypol[1] for HEp-2 cell cultures was determined to delineate the concentrations of gossypol that could be utilized in the antiviral assays. The HEp-2 cell cultures were incubated for 1 hr with varying concentrations (0.5–20 μg/ml) of gossypol in Hank's balanced salt solution; this solution then was removed and replaced with maintenance medium (Eagle's minimum essential medium containing 2% heat inactivated fetal calf serum). The cell cultures were observed for cytopathic effect and cell viability.

Gossypol concentrations of 5 μg/ml (9.6×10^{-6} *M*) and lower were not toxic for the HEp-2 cells, whereas cells incubated with gossypol at concentrations of 10 μg/ml and higher exhibited a rapid toxic effect accompanied by a decrease in cell viability. Under these conditions, apogossypol gave no indication of toxicity to HEp-2 cells at a concentration of 50 μg/ml (1.14×10^{-4} *M*).

The antiviral activity of gossypol was assayed against polio virus, parainfluenza-3 virus, and herpes simplex virus, and the antiviral activity of apogossypol was determined against poliovirus and herpes simplex. Virus suspensions in Hank's balanced salt solution were incubated for 30 min at 25° with gossypol or apogossypol; samples then were removed at intervals and assayed for infectivity by determining the number of plaque-forming units per milliliter (10). Parainfluenza-3 and herpes simplex were inactivated by incubation with apogossypol; however, there was no apparent activity exerted against poliovirus by either agent. As shown in Fig. 1, the potency of apogossypol against herpes simplex is somewhat less than that of gossypol.

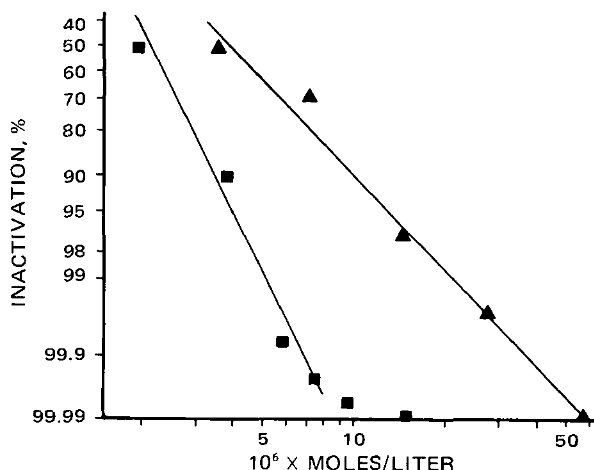


Figure 1—*Dose-response curves for gossypol (■) and apogossypol (▲). Data for gossypol are averages of two independent determinations (three replicates per determination). Data for apogossypol are averages of four independent determinations (three replicates per determination).*

Although the compounds have similar structures, the nature of the viral inactivation differs. This difference is reflected by the different slopes of the dose–response lines and the ability to inactivate the virus totally. Apogossypol can inactivate the virus completely (no plaque-forming units observed) at concentrations above 12.5 μg/ml (2.86×10^{-5} *M*) with no apparent toxicity to the HEp-2 cell cultures. Gossypol can decrease the number of plaque-forming units by a factor of 10^5, but the concentration required is also toxic to the HEp-2 cells. This toxicity probably accounts for the nonlinearity of the dose–response line at gossypol concentrations greater than 10^{-5} *M*.

Since the host cell would tolerate concentrations of gossypol and apogossypol that were antiviral *in vitro*, experiments were performed to determine whether treatment with gossypol could affect intracellular replication. Cell monolayers were infected with herpes simplex to produce approximately 100 plaques/plate. At the indicated intervals, 1 ml of Hank's balanced salt solution containing 5 μg of gossypol was added to each of three infected monolayers and incubated at 25° for 1 hr. Figure 2 shows that the antiviral effect of gossypol was apparent only during the initial stages of adsorption. Incubation with gossypol did not alter the result of infection after the virus entered the cell[2]. Similar results were observed using apogossypol in a concentration of 25 μg/ml.

These experiments demonstrate that:

1. Apogossypol is considerably (at least 10-fold) less toxic to HEp-2 cells in culture than is gossypol.
2. Apogossypol and gossypol both inactivate the enveloped virus parainfluenza-3 and herpes simplex *in vitro*.
3. The nonenveloped poliovirus is not affected by either agent.
4. Incubation of infected cells with gossypol or apogossypol does not alter subsequent plaque formation, indicating that the antiviral effect does not occur within the cell.

The molecular mechanism of viral inactivation by these compounds has not been determined. The presence of the reactive aldehyde substituents, which might react with free amino groups of proteins, has been postulated to be of importance in the biological activity of gossypol, including the mammalian toxicity (6). Likewise, previous investigators showed that gossypol inhibits the conversion of pepsinogen to pepsin by forming an imine with the ϵ-amino group of a lysine residue in pepsinogen (11–13). While a simi-

[1] Samples of gossypol (as the acetic acid complex) were provided by Dr. Walter Pons of the U.S. Department of Agriculture and by Dr. Harold Lewis of Cotton Inc. These samples were converted to free gossypol and recrystallized three times from chloroform, mp 199–200° [lit. (9) 200°]. Apogossypol was synthesized by the method of Clark (8) and stored in sealed (vacuum) tubes until assayed.

[2] The time required for the virus to enter the cell was established in a similar experiment using antiherpes serum rather than gossypol or apogossypol to inactivate the virus.

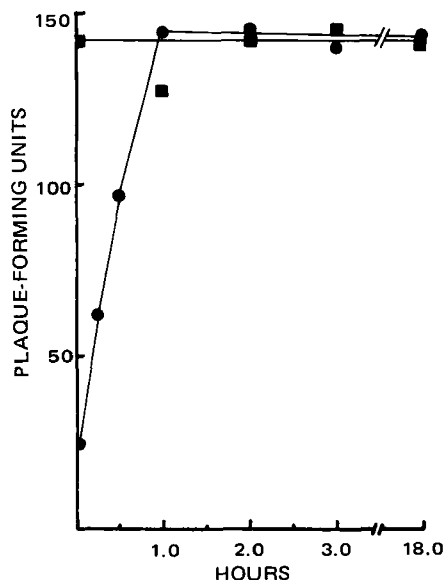


Figure 2—*Plot of time between inoculation with herpes simplex and treatment with gossypol (5 µg/ml)* versus *the plaque-forming units observed. Key:* ■, *control; and* ●, *gossypol treated.*

lar mechanism may be operative in viral inactivation, it does not appear likely due to the good activity of apogossypol.

An alternative mechanism of antiviral activity could be that these compounds bind to the virion envelope and cause the subsequent destruction of the integrity or loss of the envelope. If the membrane is the site of action, the basis for the selective toxicity to the viral particle might result from the structural differences between the envelope membrane of herpes and the outer membrane of the host cell (14). An alternative basis of the selective toxicity to the viral particle might be that the host cell has the capacity to repair membrane damage or inactivate gossypol and apogossypol. The viral particle, which is metabolically dormant, would not be expected to have either of these capabilities.

(1) P. Margalith, *Appl. Microbiol.*, **15,** 952(1967).
(2) L. V. Goryunova and S. A. Vichkanova, *Farmakol. Toksikol.*, **32,** 615; through *Chem. Abstr.*, **72,** 1885r(1970).
(3) S. A. Vichkanova and L. V. Goryunova, *Antibiotiki*, **13,** 828(1968).
(4) S. A. Vichkanova, A. I. Oifa, and L. V. Goryunova, *ibid.*, **15,** 1071(1970); through *Chem. Abstr.*, **74,** 86042(1971).
(5) E. M. Vermel, *Acta Unio Int. Cancrum*, **20,** 211(1964).
(6) J. E. Albrecht, A. J. Clawson, L. C. Ulberg, and F. H. Smith, *J. Anim. Sci.*, **27,** 976(1968).
(7) F. H. Smith and A. J. Clawson, *J. Amer. Oil Chem. Soc.*, **47,** 443(1970).
(8) E. P. Clark, *J. Biol. Chem.*, **78,** 159(1928).
(9) R. Adams, T. A. Geissman, and J. D. Edwards, *Chem. Rev.*, **60,** 555(1960).
(10) J. F. Flanagan, *J. Virol.*, **1,** 583(1967).
(11) T. H. Finlay, E. D. Dharmagrongatama, and G. E. Perlmann, *J. Biol. Chem.*, **248,** 4827(1973).
(12) T. D. Tanksley, H. Neumann, M. Lyman, C. N. Pace, and J. M. Prescott, *ibid.*, **245,** 6456(1970).
(13) R. C. Wong, Y. Nakagawa, and G. E. Perlmann, *ibid.*, **247,** 1625(1972).
(14) E. K. Wagner, *Amer. Sci.*, **62,** 584(1974).

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Received February 3, 1975.
Accepted for publication March 6, 1975.
Supported by the U.S. Department of Agriculture with funds made available through Cotton Inc.
We are indebted to Mr. Charles Carney and Mrs. Francis Byrd for technical assistance.
ˣ To whom inquiries should be directed.

Demonstration of Claimed "Controlled-Release" Properties of a Drug Formulation

Keyphrases □ Controlled-release properties of drug formulations—claimed *versus* actual, blood level profiles compared to "plain-release" blood level profiles □ Timed-release formulations—claimed *versus* actual "controlled-release" properties, blood level profiles compared to "plain-release" blood level profiles

To the Editor:

The concept of a "controlled-release" formulation of a drug is, by now, a familiar one. The basic idea is that of delaying or prolonging the *in vivo* release of an orally administered drug. Controlled-release formulations are often termed "slow release," "sustained release," or "delayed release." The advantages of such formulations are obvious; a sustained-release formulation, for example, which prolongs blood levels over a greater period and eliminates high peaks of drug concentration, allows the possibilities of less frequent dosing and the elimination of side effects related to peaks in the blood level profile.

Two questions might reasonably be asked concerning a purportedly controlled-release formulation. First, is it efficacious in the dosage schedule recom-

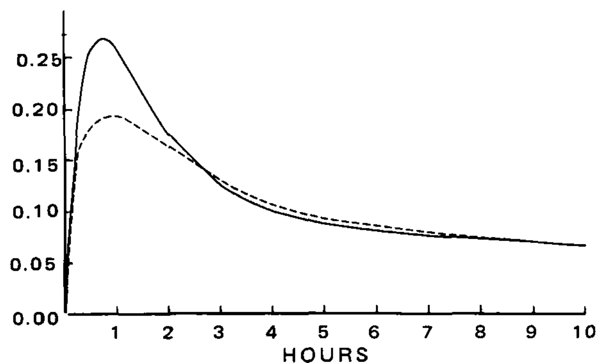

Figure 1—*Illustrative blood level curves. Key:* – – –, *controlled-release formulation; and* —, *plain-release formulation.*