of antihistamines is also of interest. Maietta (14, 15) has exploited such interactions in using the combined antigen-antihistaminic technique in shortening the treatment of hay fever.

These studies demonstrate that a wide variety of hydrocolloids and antihistamines form highly insoluble complexes which probably can serve as sustained-release forms of these drugs. Conceivably, soluble complexes which are probably formed in most of the systems studied also may be of pharmacological importance.

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Effectiveness of Antibacterial Agents Presently Employed in Ophthalmic Preparations as Preservatives Against *Pseudomonas aeruginosa*

By S. ROBERT KOHN†, LOUIS GERSHENFELD, and MARTIN BARR

Seven chemical substances or combinations of these substances presently employed as preservatives in ophthalmic solutions were studied to note their effectiveness as antibacterial agents against 13 different strains of *Pseudomonas aeruginosa*. New *in vitro* methods were devised. Among the latter were techniques to (*a*) differentiate between bacteriostatic and bactericidal activities and (*b*) determine the sterilizing time for each antibacterial agent. The methods presented here have several advantages over those previously employed and those now in use. An *in vivo* procedure was also employed in evaluating these chemical agents to note whether the findings were in agreement with the final results obtained in the *in vitro* studies. The following chemicals were examined: chlorobutanol, benzalkonium chloride, thimerosol, combinations of methyl and propylparaben, phenylmercuric nitrate, phenylethyl alcohol, and polymyxin B sulfate.

BECAUSE OF THE incidence and seriousness of *Pseudomonas aeruginosa* (*Ps. aeruginosa*) infections resulting from the use of contaminated ophthalmic solutions (1–11), various workers have critically investigated the antibacterial agents employed as preservatives in such preparations. The findings of these workers have been contradictory (12–18). The *in vitro* procedures employed in evaluating the effectiveness of the antibacterial agents have been challenged. The following observations are noted. There is need for methods which will determine the effectiveness of (*a*) antibacterial agents used as preservatives in ophthalmic solutions against *Ps. aeruginosa;* and (*b*) substances which are capable of inactivating or inhibiting the antibacterial action of the preservatives used.

The purpose of this paper is to report on studies which were performed in an effort to develop *in vitro* methods more effective than those employed at present and which will establish the efficiency of these antibacterial agents as preservatives in ophthalmic solutions against *Ps. aeruginosa.*

GENERAL CONSIDERATIONS

In devising methods for the evaluation of the effectiveness of antibacterial agents as preservatives in ophthalmic solutions, it is important to develop a technique which will determine the time required for such agents to produce sterility. Most methods which have been used to date did not always take this into consideration. Indeed, they usually measured only the bacteriostatic activity of preservatives.

Until recently, most workers employed a dilution technique to differentiate between the bactericidal and bacteriostatic activities of antibacterial agents. The basis for this procedure is the dilution of the

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preservative far below the concentration necessary to have a significant antibacterial effect. If growth of the bacteria occurred, after inoculation of this diluted solution into a subculture medium, the antibacterial agent was considered to possess bacteriostatic activity; if growth did not occur, then the antibacterial agent was reported as possessing bactericidal properties.

The above concept is not considered entirely valid at present. As Klarmann (19) has reported, in some of the dilution techniques, a quantity of the antibacterial agent may be transferred to the subculture medium in a concentration which will produce stasis, and some may become affixed to the cell walls or cells, initially producing stasis. Therefore, a bacteriostatic effect may be produced and misinterpreted for a bactericidal effect. Another disadvantage of the dilution technique is that living bacteria present may be diluted to an infinite concentration and may not grow in the subculture medium.

It is therefore apparent that an important factor in determining the antibacterial activity of preservatives in ophthalmic solutions is the complete inhibition or inactivation of the antibacterial agent in the evaluation procedure, a principle employed by Lawrence (16) and Riegelman, *et al.* (17).

Until recently, investigators had not attempted to correlate the results obtained using the *in vitro* procedures with an *in vivo* test. As pointed out by Riegelman, *et al.* (17), an *in vivo* test has several advantages and becomes an important consideration in procedures used for the evaluation of antibacterial agents employed as preservatives in ophthalmic solutions.

EXPERIMENTAL

The media employed in the experiments are listed in Table I.

General In Vitro Methods

To determine the effectiveness of presently employed antibacterial agents as preservatives for ophthalmic solutions against *Ps. aeruginosa*, four experimental *in vitro* procedures, each with a specific objective, were developed. These are described below.

Experiment I

Purpose.—As previously noted, it is important to distinguish between bacteriostatic and bactericidal activity in evaluating the effectiveness of chemical agents for use as preservatives in ophthalmic solutions. Various substances have been recommended as inactivating or neutralizing agents for the antibacterial activity of the chemicals employed. However, data concerning the effectiveness of inactivating agents are meager. Therefore, *Experiment I* was devised to determine and compare the effectiveness of various media for their ability to inhibit the activity of antibacterial agents against *Ps. aeruginosa*.

Preparation of the Inoculum.—Thirteen strains of *Ps. aeruginosa*, obtained from various sources, were identified by the usual methods including also the cytochrome oxidase test (20, 21), then maintained on agar slants. Two strains of *Ps. aeruginosa*, which were found more resistant to many of the antibacterial agents in preliminary studies were selected for use in this study. Prior to the day of the test, these strains were transferred for at least 3 consecutive days into 10 ml. of nutrient broth and incubated at 37° for 24 hours. On the day of the

Table I.—Media Employed

Medium I (pH 6.8)		
Nutrient broth, dehydrated[a,f]		0.8 Gm.
Purified water	*q.s. ad.*	100 ml.
Medium II (pH 6.8)		
Glycerin[b]		4 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium III (pH 6.8)		
Lecithin[c]		0.5 Gm.
Glycerin		4 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium IV (pH 6.7)		
Tween 80[d]		3 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium V (pH 6.7)		
Lecithin		0.5 Gm.
Tween 80		3 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium VI (pH 6.7)		
Tween 20[e]		3 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium VII (pH 6.7)		
Lecithin		0.5 Gm.
Tween 20		3 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium VIII (pH 7.1)		
Fluid thioglycollate medium, dehydrated[g]		2.93 Gm.
Purified water	*q.s. ad.*	100 ml.
Medium IX (pH 7.0)		
Tween 80		3 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium X (pH 7.0)		
Lecithin		0.5 Gm.
Tween 80		3 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium XI (pH 7.0)		
Tween 20		3 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium XII (pH 7.0)		
Lecithin		0.5 Gm.
Tween 20		3 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium XIII (pH 7.1)		
Glycerin		4 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium XIV (pH 7.1)		
Lecithin		0.5 Gm.
Glycerin		4 Gm.
Medium VIII	*q.s. ad.*	100 ml.
Medium XV (pH 6.7)		
Tween 80		5 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium XVI (pH 6.6)		
Tween 80		10 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium XVII (pH 6.7)		
Tween 20		5 Gm.
Medium I	*q.s. ad.*	100 ml.
Medium XVIII (pH 6.6)		
Tween 20		10 Gm.
Medium I	*q.s. ad.*	100 ml.

[a] Difco Laboratories, Inc., Detroit, Mich. [b] Colgate Palmolive Co., New York, N. Y. [c] Lecithin, (Ex Ovo Soluble) Pfansteihl Laboratories, Inc., Waukegan, Ill. [d] Polyoxyethylene (20) sorbitan mono-oleate, Atlas Powder Co., Wilmington, Del. [e] Polyoxyethylene (20) sorbitan monolaurate, Atlas Powder Co., Wilmington, Del. [f] All media containing nutrient broth, dehydrated, were sterilized by autoclaving at 121° for 15 minutes. [g] All media containing fluid thioglycollate medium dehydrated were autoclaved at 121° for 20 minutes.

test, the cultures were shaken by hand approximately 1 minute to break up clumps and allowed to stand for at least 15 minutes. Dilutions (1:10 and 1:1000) of the 24-hour cultures of the two strains were made using nutrient broth, and the approximate number of viable bacteria were determined by the pour-plate technique. The 1:10 and 1:1000 dilutions of the 24-hour cultures contained approximately 10^8 and 10^6 bacteria per ml., respectively, as noted after incubating the plates at 37° for 48 hours.

Preparation of the Antibacterial Agent–Medium Mixtures.—Four milliliters of each of the various test media was placed in culture tubes (approximately 15 cm. in length and 13 mm. inside diameter) and sterilized. Stock solutions of several concentrations of the antibacterial agents were prepared as aqueous solutions. One milliliter of a sterile stock solution of the antibacterial agent was added to a tube containing 4 ml. of the sterile medium. This was done for each concentration of the antibacterial agent tested and also for each medium being used. The tubes were then shaken by hand to insure a uniform mixture.

Preparation of the Controls.—Controls were employed to determine bacterial contamination (negative controls) and to determine whether the media were capable of supporting the growth of *Ps. aeruginosa* (positive controls). Media prepared with the antibacterial agent but without the presence of *Ps. aeruginosa* were employed as negative controls. Media prepared without the antibacterial agents but with *Ps. aeruginosa* were employed as positive controls.

Procedure.—One-tenth milliliter of the 1:10 dilution of each strain of *Ps. aeruginosa* was added separately to a tube containing a single concentration of the sterile antibacterial agent in the individual medium being tested. This was repeated for each concentration of the antibacterial agent in each medium being tested. Each tube was shaken by hand to insure an even distribution of the bacteria. After 1 hour of contact at 24°, 0.5 ml. was transferred into tubes containing 4.5 ml. of the subculture medium, and the mixture was shaken by hand. After 24 hours of contact at 24°, another transfer of 0.5 ml. was made into tubes containing 4.5 ml. of the subculture medium. The same procedure was repeated with the 1:1000 dilution of the strain used and with the two dilutions of the other strain. All tubes were incubated at 37° for a period of 7 days and observed for the presence or absence of growth. The procedure was repeated to verify the findings.

Experiment II

Purpose.—As previously indicated in the evaluation of the antibacterial activity of chemical agents intended for use as preservatives in ophthalmic solutions, an important consideration is the time required for such agents to kill the bacteria after contact (sterilizing time). *Experiment II* was therefore devised to determine whether the antibacterial agent is fast-acting or slow-acting. This experiment was also designed to show the necessity of developing subculture media containing suitable inactivating agents.

Preparation of the Inoculum.—A 1:10 dilution of each of the 24-hour-old cultures of the 13 strains of *Ps. aeruginosa* was used in this study. Each was prepared as described previously.

Preparation of the Media.—Tubes containing 4.5 ml. of each of the various test media described previously were prepared and sterilized.

Preparation of the Antibacterial Solutions.—Tubes containing 5 ml. of the sterile aqueous antibacterial solutions to be tested were prepared.

Preparation of Controls.—Controls were employed to determine bacterial contamination (negative controls) and to determine whether the media were capable of supporting the growth of *Ps. aeruginosa* (positive controls). Tubes containing 5 ml. of the antibacterial solutions, without the presence of *Ps. aeruginosa*, were employed as negative controls. Solutions prepared without the antibacterial agent but containing *Ps. aeruginosa* were employed as positive controls.

Procedures.—One-tenth milliliter of a 1:10 dilution of each strain of *Ps. aeruginosa* was separately added to a tube containing 5 ml. of the sterile antibacterial solution. This was repeated for all 13 strains. Each tube of the antibacterial agent-bacteria mixture was then shaken by hand. After 1-hour of contact at 24°, 0.5 ml. was transferred into the tube containing 4.5 ml. of the subculture medium. This was then shaken by hand. After 24 hours of contact at 24°, another transfer of 0.5 ml. was made into 4.5 ml. of the subculture medium. All subculture tubes were incubated at 37° for a period of 7 days and observed for the presence or absence of growth. The procedure was repeated to verify the findings.

Experiment III

Purpose.—The purpose of this experiment was similar to that of *Experiment II*, except that the exact sterilizing time (as noted under *Procedure*) was determined.

Preparation of the Inoculum.—Two dilutions (1:10 and 1:1000) of the 24-hour-old cultures of 13 different strains of *Ps. aeruginosa* were used in this experiment. They were prepared as previously described.

Preparation of the Medium.—Four and one-half milliliters of the medium, which had shown the maximum inactivation of the antibacterial agent being tested, was employed.

Preparation of the Antibacterial Solutions.—Tubes containing 5 ml. of the sterile antibacterial solution to be tested were prepared.

Preparation of Controls.—Negative and positive controls were prepared.

Procedure.—The procedure was similar to that employed in *Experiment II*, except for changes in the contact times. If the results of *Experiment II* showed the antibacterial agent to be effective against *Ps. aeruginosa* within 1 hour, *i.e.*, no growth in the subculture tubes containing the 1-hour transfer, then the agent was tested at contact times of 15, 30, 45, and 60 minutes. If the antibacterial agent was shown to be effective between 1 and 24 hours, *i.e.*, no growth in the subculture tubes containing the 24-hour transfers, tests were carried out at 3, 6, 9, and 12 hours of contact. If necessary 15, 18, and 21 hours of contact also were employed. If the antibacterial agent was shown to be ineffective after 24 hours of contact, further studies were not considered warranted.

Experiment IV

Purpose.—In *Experiment III*, the sterilizing time of each chemical agent employed as a preservative in ophthalmic solutions was determined against *Ps. aeruginosa*. Obviously, the sterilizing time must be based on the total destruction of all bacteria. In *Experiment III*, only 0.5 ml. of the antibacterial agent–bacteria mixtures was transferred to the subculture medium. The purpose of this experiment was to determine whether this volume of inoculum was as effective as would be transplants of larger volumes.

Preparation of the Inoculum.—The most resistant strains of *Ps. aeruginosa*, as noted in *Experiment III*, were selected and used in this experiment. A 1:10 and a 1:1000 dilution of the 24-hour-old cultures of the selected strains were used. They were prepared as described previously.

Preparation of the Medium.—The medium used in *Experiment III* was employed here. Four and one-half milliliters, 9 ml., 18 ml., and 45 ml. of the medium, respectively, were placed separately in appropriate containers and sterilized.

Preparation of the Antibacterial Solutions.—The concentration of the antibacterial solution used in this study depended upon the strains of *Ps. aeruginosa* which were selected. A series of four tubes containing 5 ml. of the sterile antibacterial solution was prepared for each dilution of each strain of *Ps. aeruginosa* employed in this experiment.

Preparation of the Controls.—Controls were prepared as described in *Experiment II*.

Procedure.—The contact time used in this experiment was the same as that which revealed no growth immediately following a contact time period which had shown growth in the subculture medium for the strains selected. On the day of the experiment, 0.1 ml. of each of the diluted strains was added separately to each tube in the series containing the antibacterial solution. This was repeated for each strain used in this experiment. Transfers were made from the series of tubes at the selected contact time, using a 0.5 ml., 1 ml., 2 ml., and 5 ml. inoculum into 4.5 ml., 9 ml., 18 ml., and 45 ml. of subculture medium, respectively. The containers of subculture medium–antibacterial agent–bacteria mixtures were shaken by hand. All containers of this subculture medium of the antibacterial agent–bacteria mixture were incubated at 37° for a period of 7 days and observed for the presence or absence of growth. The entire procedure was repeated to verify the findings.

Antibacterial Agents Studied

The antibacterial agents studied for their effectiveness as preservatives for ophthalmic solutions against *Ps. aeruginosa* were as follows: chlorobutanol,[1] benzalkonium chloride,[2] thimerosol,[3] combinations of methylparaben and propylparaben,[4] phenylmercuric nitrate,[5] phenylethyl alcohol,[6] and polymyxin B sulfate.[7] Aqueous solutions of these agents were prepared, sterilized by appropriate procedures, and assayed for content of active ingredient (22).

[1] Chlorobutanol, Hydrous, Merck and Co., Inc., Rahway, N. J.
[2] Zephiran Chloride, Winthrop Laboratories, Inc., New York, N. Y.
[3] Mann Fine Chemicals, Inc., New York, N. Y.
[4] Heyden Chemical Corp., New York, N. Y.
[5] Metalsalts Corp., Hawthorne, N. J.
[6] S. B. Penick and Co., Inc., New York, N. Y.
[7] Chas. Pfizer and Co., New York, N. Y.

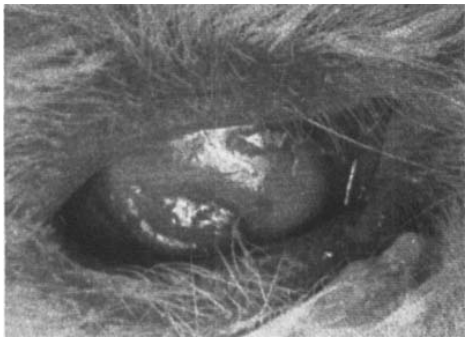

Fig. 1.—Appearance of the rabbit eye infected with *Ps. aeruginosa*.

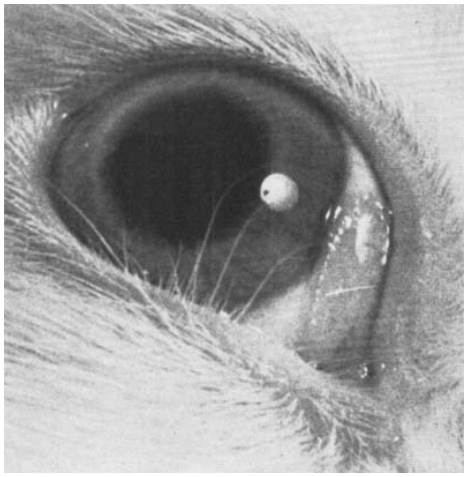

Fig. 2.—Appearance of the normal rabbit eye.

In Vivo Method

Purpose.—The purpose of the *in vivo* study is to prove that (*a*) the results obtained by employing *Experiment III* (*in vitro* method) for noting the efficiency of the antibacterial agents are valid, *i.e.*, the bacteria are incapable of growing in the subculture medium and accordingly are also incapable of producing an ocular infection *in vivo;* and (*b*) the inactivating media utilized in this study are adequate.

Preparation of the Inoculum.—The inoculum of *Ps. aeruginosa* was prepared as previously described. For the most part, the strains of *Ps. aeruginosa* which proved to be the most resistant to the antibacterial agents in the *in vitro* experiments were employed in this study. Occasionally an intermediate strain, with regard to resistance toward the antibacterial agent, and also a mixture of resistant strains were employed. The mixture of resistant strains was prepared on the day of the test by mixing 1 ml. of each of the respective 24-hour-old nutrient broth cultures in a sterile tube and

TABLE II.—HIGHEST CONCENTRATION OF PRESERVATIVES INACTIVATED BY VARIOUS MEDIA

Medium	Preservative	Chlorobutanol, %	Benzalkonium Chloride	Thimerosol	Methylparaben, %	Propylparaben, %	Phenylmercuric Nitrate	Phenylethyl Alcohol, %	Polymyxin B Sulfate, Units
I		0.4	1:30,000	[b]	0.14	0.02	[b]	0.2	31.25
II		...	1:30,000	...	...	...	[b]	...	15.6
III		...	1:2500	...	...	...	...	...	2000[a]
IV		...	1:1000[a]	[b]	...	...	...	...	31.25
V		...	1:1000[a]	[b]	...	...	1:50,000	...	31.25
VI		...	1:1000[a]	...	...	...	...	...	31.25
VII		...	1:1000[a]	...	...	...	...	...	31.25
VIII		...	...	1:20,000	...	...	1:6250	...	...
IX		...	...	1:20,000	...	...	1:6250	...	...
X		...	...	1:2500[a]	...	...	1:3125[a]	...	...
XI		...	...	1:20,000	...	...	1:6250	...	...
XII		...	...	1:2500[a]	...	...	1:3125[a]	...	...
XIII		...	...	1:20,000	...	...	1:6250	...	...
XIV		...	...	1:2500[a]	...	...	1:3125[a]	...	...
XV		0.5	...	...	0.2[a]	0.04[a]	...	0.5	...
XVI		0.6[a]	...	...	0.2[a]	0.04[a]	...	1.0[a]	...
XVII		0.6[a]	...	...	0.2[a]	0.04[a]	...	0.5	...
XVIII		0.6[a]	...	...	0.2[a]	0.04[a]	...	1.0[a]	...

[a] Highest concentration tested. [b] Growth was not evident in this medium when the preservative was in a concentration of 1:50,000 (the smallest concentration studied).

proceeding as when a single strain of *Ps. aeruginosa* was used.

Preparation of the Antibacterial Agent-Bacteria Mixtures.—One-tenth milliliter of the dilution of the strain or mixture of strains was added to 5 ml. of the sterile preservative solution being investigated. The tube was shaken by hand for approximately 15 seconds and allowed to remain at 24° for a specific contact time, each time period depending on the results obtained in the *in vitro* experiments for the antibacterial agent being studied.

Preparation of the Controls.—A sterile solution of the preservative was employed as a positive control, not only for accidental contamination, but also to detect any action and untoward reaction the preservative might have on the rabbit's eye. A dilution of *Ps. aeruginosa*, containing approximately 10,000 bacteria per ml., was employed as a negative control to determine pathogenicity of *Ps. aeruginosa* in the rabbit's eye.

Procedure.—Two contact time periods were employed for each antibacterial agent-bacteria mixture studied. The contact time periods were selected, as mentioned previously, from the findings of the *in vitro* experiments for the various antibacterial agents tested. The first contact time period selected was the longest period of time which still showed growth of the bacteria in the subculture medium; also the contact time period immediately following the first contact time period was chosen. Obviously, the latter contact time period would have shown no growth of the bacteria in the subculture medium. The first exposure period was employed to prove that when growth of *Ps. aeruginosa* occurs in the subculture medium, an infection could also be produced in the rabbit's eye. The second contact time period was utilized to fulfill the purpose of the *in vivo* experiment.

Usually six rabbits[8] were employed for each antibacterial agent studied. The rabbits were placed by hand in individual cages which were thoroughly cleaned with a disinfectant and live steam after each experiment. The antibacterial agent–bacteria mixture was injected intracorneally into the rabbit's eye. Approximately 0.01 ml. was injected from a one-quarter-ml. tuberculin syringe[9] graduated in 0.01 ml. One-quarter-inch 27-gauge needles[9] were employed. Approximately 5 minutes before each injection, two drops of a 2% butacaine sulfate[10] solution were placed in the conjunctival sacs of each rabbit.

At the first contact time period selected an intracorneal injection of the antibacterial agent–bacteria mixture was given into one eye of each of two rabbits. At the next contact time, another intracorneal injection was given into the other eye of each of the same two rabbits. A third rabbit received an injection of the sterile antibacterial solution (positive control) in one eye and an injection of the diluted bacteria (negative control) in the other eye. The rabbits were observed for a period of 7 days for evidence of ocular infections (see Fig. 1). Figure 1 shows the appearance of an eye infected with *Ps. aeruginosa* 5 days after the injection. Figure 2 shows the appearance of a normal eye. Most eyes infected with *Ps. aeruginosa* were completely closed within 7 days after the injection.

As a further control, swabs were periodically taken of any infected eyes to recover and identify the presence of *Ps. aeruginosa*.

The above procedure was repeated, employing another strain or a mixture of strains of *Ps. aeruginosa*.

RESULTS

From the data obtained from *Experiments I* and *II* employing the different antibacterial agents as preservatives (Tables II and III), the following media were selected to be employed as the inactivating media in *Experiments III* and *IV:* Medium XVIII for chlorobutanol, Medium V for benzalkonium chloride, Medium X for thimerosol, Medium XVIII for combinations of methylparaben and propylparaben, Medium X for phenylmercuric

[8] Male white New Zealand rabbits, Huntingdon Farms, Philadelphia, Pa.

[9] Becton, Dickinson and Co., Rutherford, N. J.

[10] Butyn Sulfate, Abbott Laboratories, Chicago, Ill.

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