

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, thimerosal, 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

| | | |
|---|--|---|
| Nutrient broth, Purified water | Medium I (pH 6.8) dehydrated ^{a,f} | 0.8 Gm. |
| | <i>q. s. ad.</i> | 100 ml. |
| Glycerin ^b Medium I | Medium II (pH 6.8) <i>q. s. ad.</i> | 4 Gm. 100 ml. |
| | Lecithin ^c Glycerin Medium I | Medium III (pH 6.8) <i>q. s. ad.</i> |
| Tween 80 ^d Medium I | | Medium IV (pH 6.7) <i>q. s. ad.</i> |
| | Lecithin Tween 80 Medium I | Medium V (pH 6.7) <i>q. s. ad.</i> |
| Tween 20 ^e Medium I | | Medium VI (pH 6.7) <i>q. s. ad.</i> |
| | Lecithin Tween 20 Medium I | Medium VII (pH 6.7) <i>q. s. ad.</i> |
| Fluid thioglycollate medium, dehydrated ^g Purified water | | Medium VIII (pH 7.1) <i>q. s. ad.</i> |
| | Tween 80 Medium VIII | Medium IX (pH 7.0) <i>q. s. ad.</i> |
| Lecithin Tween 80 Medium VIII | | Medium X (pH 7.0) <i>q. s. ad.</i> |
| | Tween 20 Medium VIII | Medium XI (pH 7.0) <i>q. s. ad.</i> |
| Lecithin Tween 20 Medium VIII | | Medium XII (pH 7.0) <i>q. s. ad.</i> |
| | Glycerin Medium VIII | Medium XIII (pH 7.1) <i>q. s. ad.</i> |
| Lecithin Glycerin Medium VIII | | Medium XIV (pH 7.1) <i>q. s. ad.</i> |
| | Tween 80 Medium I | Medium XV (pH 6.7) <i>q. s. ad.</i> |
| Tween 80 Medium I | | Medium XVI (pH 6.6) <i>q. s. ad.</i> |
| | Tween 20 Medium I | Medium XVII (pH 6.7) <i>q. s. ad.</i> |
| Tween 20 Medium I | | Medium XVIII (pH 6.6) <i>q. s. ad.</i> |

^a Difco Laboratories, Inc., Detroit, Mich. ^b Colgate Palmolive Co., New York, N. Y. ^c Lecithin, (Ex Ovo Soluble) Pfanstielh Laboratories, Inc., Waukegan, Ill. ^d Polyoxyethylene (20) sorbitan mono-oleate, Atlas Powder Co., Wilmington, Del. ^e Polyoxyethylene (20) sorbitan mono-laurate, 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 thimerosal,³ combinations of methylparaben and propylparaben,⁴ phenylmercuric nitrate,⁵ phenylethyl alcohol,⁶ and polymyxin B sulfate.⁷ Aqueous solutions of these



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

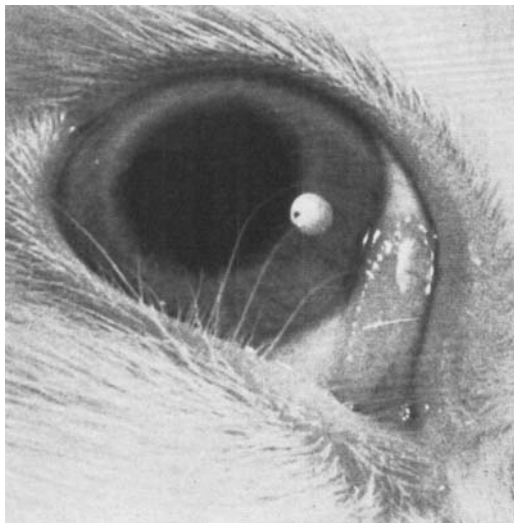


Fig. 2.—Appearance of the normal rabbit eye.

agents were prepared, sterilized by appropriate procedures, and assayed for content of active ingredient (22).

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

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.

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

| Preservative | Chlorobutanol, % | Benzalkonium Chloride | Thimerosol | Methylparaben, % | Propylparaben, % | Phenylmercuric Nitrate | Phenylethyl Alcohol, % | Polymyxin B Sulfate, Units |
|--------------|------------------|-----------------------|---------------------|------------------|-------------------|------------------------|------------------------|----------------------------|
| Medium I | 0.4 | 1:30,000 | <i>b</i> | 0.14 | 0.02 | <i>b</i> | 0.2 | 31.25 |
| II | ... | 1:30,000 | ... | ... | ... | <i>b</i> | ... | 15.6 |
| III | ... | 1:2500 | ... | ... | ... | ... | ... | 2000 ^a |
| IV | ... | 1:1000 ^a | <i>b</i> | ... | ... | ... | ... | 31.25 |
| V | ... | 1:1000 ^a | <i>b</i> | ... | ... | 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 rabbits⁸ 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 syringe⁹ graduated in 0.01 ml. One-quarter-inch 27-gauge needles⁹ were employed. Approximately 5 minutes before each injection, two drops of a 2% butacaine sulfate¹⁰ 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.

nitrate, and Medium III for polymyxin B sulfate.

Media XVI and XVIII were found to be the best neutralizing media for phenylethyl alcohol in *Experiment I*. Since the sterilizing time for phenylethyl alcohol was found to be greater than 24 hours, further experiments were not carried out on this chemical in *Experiment II*.

In Table IV a summary of the results obtained in *Experiment III* is presented. The sterilizing times which are listed against *Ps. aeruginosa* for each of the chemical agents are those which have been obtained under severe *in vitro* test conditions (dilution of bacterial inoculum was 1:10). They indicate the time required to kill all of the strains of *Ps. aeruginosa* employed in this study.

The results of *Experiment IV* are not recorded here, but in all cases they indicate that 0.5 ml. was adequate as the volume of antibacterial agent-bacteria mixture used as the transfer in *Experiments II and III*.

The results of the *in vivo* experiments indicate that they are in agreement in all instances with the *in vitro* findings of *Experiment III*, i.e., those solutions which produced growth following transfer into subculture media produced ocular infections; those which did not produce growth did not produce ocular infections.

DISCUSSION

In the evaluation of the effectiveness of antibacterial agents used as preservatives in ophthalmic

solutions against *Ps. aeruginosa*, the time required for such an agent to kill the bacteria must be known. The experiments employed in these studies determine the sterilizing time for each antibacterial agent against *Ps. aeruginosa*. In addition, other important factors are evident in the experiments previously described which are not contained in those procedures in current use or those employed in the past.

One advantage is that the procedures do not rely upon a dilution technique to differentiate between bacteriostatic and bactericidal effects of the antibacterial agents being tested. Inactivating agents were employed for this purpose. The latter, incorporated in the subculture media, function by inactivating or neutralizing the antibacterial properties of the chemical agents employed for their preservative activity.

Another advantage of the test procedures employed in this study is that they establish the inactivating or neutralizing ability of the subculture media toward the antibacterial activity of the chemical agents studied. Such data have not been reported previously by the various workers in the field.

In the evaluation of the effectiveness of antibacterial preservatives, it is not only important to determine the time required for sterilization, but it is also necessary to determine whether the bactericidal effect was complete. A method which evaluates the destruction of bacteria but neglects to determine whether a total kill has occurred may

TABLE III.—NUMBER OF STRAINS OF *Ps. aeruginosa* WHICH DISPLAYED GROWTH IN BEST TEST MEDIA AFTER CONTACT WITH PRESERVATIVES^a

| Preservative | Contact Time, hr. | Medium | | | | | | |
|-------------------------------------|-------------------|--------|----|-----|------|-------|----|-----|
| | | I | XV | XVI | XVII | XVIII | | |
| Chlorobutanol, 0.5% | 1 | 13 | 13 | 13 | 13 | 13 | | |
| | 24 | 0 | 0 | 0 | 0 | 0 | | |
| Benzalkonium chloride, 1:5000 | 0.5 | Medium | | | | | | |
| | | I | II | III | IV | V | VI | VII |
| | 1 | 2 | 1 | 1 | 3 | 4 | 3 | 4 |
| | 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Thimerosol, 1:5000 | 1 | Medium | | | | | | |
| | | I | IV | V | VIII | IX | | |
| | 1 | 0 | 0 | 0 | 3 | 3 | | |
| | 24 | 0 | 0 | 0 | 0 | 0 | | |
| Thimerosol, 1:5000 | 1 | Medium | | | | | | |
| | | X | XI | XII | XIII | XIV | | |
| | 1 | 13 | 3 | 13 | 3 | 3 | | |
| | 24 | 0 | 0 | 0 | 0 | 0 | | |
| Methylparaben, 0.2% | 1 | Medium | | | | | | |
| | | I | XV | XVI | XVII | XVIII | | |
| | 1 | 2 | 3 | 6 | 3 | 6 | | |
| Propylparaben, 0.04% | 24 | 0 | 0 | 0 | 0 | 0 | | |
| Phenylmercuric nitrate, 1:10,000 | 1 | Medium | | | | | | |
| | | I | IV | V | VIII | IX | | |
| | 1 | 0 | 0 | 0 | 0 | 3 | | |
| | 24 | 0 | 0 | 0 | 0 | 0 | | |
| Phenylmercuric nitrate, 1:10,000 | 1 | Medium | | | | | | |
| | | X | XI | XII | XIII | XIV | | |
| | 1 | 7 | 2 | 6 | 0 | 2 | | |
| | 24 | 0 | 0 | 0 | 0 | 0 | | |
| Phenylethyl alcohol, 0.5% | 1 | Medium | | | | | | |
| | | I | XV | XVI | XVII | XVIII | | |
| | 1 | 13 | 13 | 13 | 13 | 13 | | |
| | 24 | 13 | 13 | 13 | 13 | 13 | | |
| Polymyxin B sulfate, 1000 units/ml. | 1 | Medium | | | | | | |
| | | I | II | III | IV | V | VI | VII |
| | 1 | 0 | 0 | 13 | 0 | 0 | 0 | 0 |
| | 24 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

^a Thirteen strains tested.

prove to be in error. In the experiments employed in this investigation, the adequacy of the volume of the antibacterial agent-bacteria mixture transferred to the subculture media has been evaluated. This has not usually been taken into consideration.

It was noted that the results of the *in vivo* test agreed at all times with the results of the *in vitro* procedures. This was additional evidence of the effectiveness of the inactivating media employed for the various antibacterial chemical agents under study.

An antibacterial agent intended for use as a preservative in ophthalmic solutions should have quick-acting bactericidal activity. There is no exact definition for the term *quick-acting*. In this discussion, an antibacterial agent will arbitrarily be considered to possess *quick-acting* bactericidal activity against *Ps. aeruginosa* if the sterilizing time of that agent was 1 hour or less under the extreme test conditions employed in this study.

Chlorobutanol is not a quick-acting antibacterial preservative against *Ps. aeruginosa*. A 12-hour period of contact was required for a 0.5% solution of chlorobutanol, which is the concentration most commonly employed to produce a bactericidal effect in marketed multiple-dose ophthalmic solutions. Lawrence (16) and Riegelman, *et al.* (17), also reported chlorobutanol to be slowly bactericidal against *Ps. aeruginosa*. A 10% Tween 20 nutrient broth medium was found the most effective inhibiting medium for chlorobutanol. The probable mechanism of action whereby Tween 20 acts is probably a binding or association reaction between the chlorobutanol and the Tween.

Of all the antibacterial agents investigated in this study, benzalkonium chloride, in a concentration of 1:5000, revealed the quickest-acting bactericidal activity against *Ps. aeruginosa*. The results obtained for benzalkonium chloride are in agreement with those of Lawrence (16), but they disagree with those of Riegelman, *et al.* (17), who reported a longer sterilizing time against *Ps. aeruginosa*. This may be due to the strains of *Ps. aeruginosa* employed. The subculture medium found to have the greatest neutralizing activity against benzalkonium chloride contained polysorbate 80 (Tween 80) and lecithin as the inactivating agents. Both of these compounds

inactivate the antibacterial activity of benzalkonium chloride; however, the mechanisms of inactivation are different. The possible mechanism of action of the polysorbate 80 is a binding or association reaction with the benzalkonium chloride, whereas the negative charge of lecithin is probably responsible for the inactivating role it plays.

The results indicate that thimerosol, in the highest concentration (1:5000) usually employed as an antibacterial preservative in marketed ophthalmic solutions, is too slow-acting in its bactericidal effect (6 hours) against *Ps. aeruginosa*. The inactivating medium employed to neutralize the antibacterial action of thimerosol was fluid thioglycollate medium containing polysorbate 80 and lecithin. The former, by itself, has had general acceptance as the inactivating medium for the antibacterial organic mercurials, there being an interaction between the mercurial ion and the -SH groups of the thioglycollic acid. However, it was found to be less effective than the combination of substances employed in our inactivating medium.

A combination of 0.2% of methylparaben and 0.04% propylparaben possessed a sterilizing time of 3 hours against *Ps. aeruginosa*. However, this concentration is not usually employed in ophthalmic solutions. In a concentration more commonly employed, 0.18% methylparaben and 0.02% propylparaben, the sterilization time required was 6 hours. A Tween 20-nutrient broth medium was employed as the inactivating medium for the antibacterial activity of the parabens. Tween 20 as the major inactivator probably functions by the same mechanism of action as that discussed under chlorobutanol.

The results indicate that phenylmercuric nitrate acts too slowly as a bactericide against *Ps. aeruginosa* for use as an antibacterial preservative in ophthalmic solutions, the sterilizing time being 6 hours. The findings in *Experiments I and II* indicated that polysorbate 80 and lecithin in fluid thioglycollate medium was most effective in neutralizing the antibacterial activity of phenylmercuric nitrate and even better than fluid thioglycollate medium by itself. This observation was also made by Riegelman, *et al.* (17). The increase in the inactivating ability of fluid thioglycollate medium when it contains lecithin solubilized by polysorbate 80 is probably because of the interaction of the oppositely charged molecules of lecithin and phenylmercuric nitrate.

A 0.5% solution of phenylethyl alcohol did not have sterilizing power when in contact with *Ps. aeruginosa* for a period of 24 hours and must therefore be considered to be a very inferior antibacterial preservative against this organism.

Polymyxin B sulfate was found to be only slowly bactericidal against *Ps. aeruginosa* and is therefore unsuitable as an antibacterial preservative in multiple-dose ophthalmic solutions. This conclusion does not agree with that of Riegelman, *et al.* (17). However, Riegelman's results could not be duplicated when employing his technique, the inactivating medium used by him and his workers, and utilizing the strains of *Ps. aeruginosa* employed in this investigation. A possible explanation for this discrepancy may be the resistance of the strains of *Ps. aeruginosa* employed toward polymyxin B sulfate. Attention is directed to the fact that Riegelman, *et al.* (17),

TABLE IV.—STERILIZING TIMES AGAINST *Ps. aeruginosa* (10⁸) FOR CHEMICAL AGENTS COMMONLY EMPLOYED AS PRESERVATIVES IN OPHTHALMIC SOLUTIONS

| Chemical Agents | Concn. | Sterilizing Time |
|---------------------------------|----------------|------------------|
| Chlorobutanol | 0.7% | 9 hr. |
| Chlorobutanol | 0.5% | 12 hr. |
| Benzalkonium chloride | 1:5000 | 45 min. |
| Benzalkonium chloride | 1:10,000 | 9 hr. |
| Thimerosol | 1:5000 | 6 hr. |
| Thimerosol | 1:10,000 | 9 hr. |
| Methylparaben and propylparaben | 0.2% | |
| | 0.04% | 3 hr. |
| Methylparaben and propylparaben | 0.18% | |
| | 0.02% | 6 hr. |
| Phenylmercuric nitrate | 1:10,000 | 6 hr. |
| Phenylmercuric nitrate | 1:20,000 | 6 hr. |
| Phenylethyl alcohol | 0.5% | ^a |
| Polymyxin B sulfate | 2000 units/ml. | 12 hr. |
| Polymyxin B sulfate | 1000 units/ml. | 18 hr. |

^a Sterilization not effected in 24 hours.

did not employ many strains of *Ps. aeruginosa* in their experiments. It was observed that lecithin, suspended with the aid of glycerin in nutrient broth, was most effective in inactivating polymyxin B sulfate. The possible mechanism of action is similar to that given under benzalkonium chloride. It was also noted that nutrient broth containing lecithin solubilized by either Tween 20 or Tween 80 was not as effective as the above-mentioned inactivating medium. This observation was also noted by Riegelman, *et al.* (17), and Bliss and Worth (23). A possible explanation may be that the effective charge of the lecithin molecule is reduced when it is solubilized by the Tweens. This reduction in the inhibiting action of lecithin by the Tweens was not observed with benzalkonium chloride. The Tweens, by themselves, were capable of reducing the antibacterial action of benzalkonium chloride, while they had no such effect on polymyxin B sulfate.

SUMMARY

1. Various *in vitro* studies were carried out to determine the antibacterial effectiveness of various chemical agents to be used as preservatives in ophthalmic solutions against 13 strains of *Ps. aeruginosa*. Methods were devised to (a) differentiate between the bacteriostatic and bactericidal properties of the chemical preservatives by formulating suitable inactivating media, (b) determine the sterilizing time for each chemical preservative, and (c) determine whether the volume of inoculum taken for sampling in the experiment was adequate.

2. An *in vivo* procedure was employed in evaluating the chemical agents to note whether findings would be in agreement with the final results obtained in the *in vitro* studies.

3. The following antibacterial agents used or recommended for use in ophthalmic solutions were thoroughly investigated: chlorobutanol, benzalkonium chloride, thimerosal, combinations of methylparaben and propylparaben, phenylmercuric nitrate, phenylethyl alcohol, and polymyxin B sulfate. Of these, only benzalkonium chloride possessed a sterilizing time against *Ps. aeruginosa* of less than 1 hour.

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Effect of Certain Drugs on Perfused Human Placentas II

Vasodilators

By HENRY P. CIUCHTA and RONALD F. GAUTIERI

Perfusion studies conducted on full term human placentas produced the following results: marked vasodilation with isosorbide dinitrate, isoproterenol hydrochloride, nitroglycerin and papaverine hydrochloride, slight vasodilation, and vasoconstriction with cyclandelate and isoxsuprine hydrochloride. Except for alcohol, which produced a slight decrease in the volume flow rate through the placenta, the solvents employed with these drugs had no effect on placental vasculature.

DURING THE PAST 40 years the action of various agents on placental vasculature has

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been studied by numerous investigators, including Kosakae (1), Ueda (2), von Euler (3), Schmitt (4), Eliasson and Astrom (5), and Astrom and Samelius (6). The results of these experiments are rather inconsistent. This undoubtedly is partly due to the employment of different experimental procedures and the inability at times to obtain placentas within a short time after delivery. More than likely, the