Sterility Tests on Chemical Preparations for Parenteral Use*

By Bernard L. Blumberg

In a report on the sterility of ampul preparations made by the Council on Pharmacy and Chemistry of the American Medical Association (1), injectable ampul and other preparations were classified into four general groups. Group I included products of a biological nature which are obtained from plant and animal tissues and are very susceptible to bacterial contamination; group II consisted of bactericidal substances such as soluble mercury and other heavy metal salts; group III included glucosides and preparations containing sugars or nonantiseptic carbon compounds in which bacteria and molds may grow; and group IV represented inorganic and organic substances in which the growth of bacteria is not likely, but which are not bactericidal and therefore may contain viable bacteria or spores. Chemical preparations containing preservative in bacteriostatic concentration, and vegetable oil suspensions of insoluble mercury, bismuth and arsenic compounds were also relegated to this group. This report noted further that some of the firms producing ampuls in these groups were testing their products for sterility by the National Institute of Health method, some by other methods, and some were making no tests for sterility.

The increasing necessity for sterility tests for routine control purposes is apparent from the growing number of medicaments marketed in ampuls, and from the researches of Davis (2) and others. Their work with experimentally infected solutions of substances for parenteral administration has shown that such substances may be classified as non- or slightly germicidal, moderately germicidal, and strongly germicidal. Moreover, certain medicinal solutions which are nongermicidal and not conducive to spore germination may contain viable organisms after a 3-day fractional sterilization at 80° C. for 1 hr.

Directions for official sterility tests on liquids and solids appeared in U. S. P. XI and its Supplements, and N. F. VI (3). Apparently the prototype for these directions is the National Institute of Health sterility testing method, which is a well-established procedure for biological products. However, this method seems unnecessarily time consuming as a routine procedure for chemical products, especially those in group IV.

With respect to chemical ampuls, this author believes that the official sterility test may be considerably modified. The modified procedure should facilitate routine testing without sacrificing in any way the ultimate value of the test.

The purpose of this paper is to record the modifications which have been investigated. One series of experiments was designed to determine whether a broth prepared from a dehydrated infusion broth (Difco heart infusion broth) could be substituted for the official fresh infusion broth, and also whether the Durham fermentation tube could be substituted for the Smith fermentation tube. The second series of experiments presents the results obtained with a large number of commercial preparations.[1] Duplicate samples of these preparations were tested by the author, using a Smith tube–U. S. P. broth, and by Mr. J. W. Glaser, New York, N. Y., using a Durham tube–Difco broth. Practically identical methods of sampling and inoculating were used by both investigators. The two laboratories in which the tests were conducted were quite different in construction, and were some three or four miles apart.

EXPERIMENTAL

Cultures.—Staph. aureus, E. coli, B. subtilis and *Cl. novyi* were used for the study of comparative growth in Smith tube–U. S. P. broth, Smith tube–Difco broth, Durham tube–U. S. P. broth, and Durham tube–Difco broth. All cultures except

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Cl. novyi had been carried along as stock cultures for at least eighteen months, and had been checked occasionally for identity and purity. *Cl. novyi* had been obtained about six months previously from the A. T. C. C. and had been carried in pure culture. Stock cultures were maintained on beef extract broth agar (3 Gm. of beef extract, 5 Gm. of peptone, 5 Gm. of NaCl, 15 Gm. of agar and 1000 ml. of water; *p*H about 6.8) slants, except *Cl. novyi*, which was cultured in B. B. L. thioglycollate medium. Stock cultures which were used to inoculate initial subcultures were four to six weeks old. The initial subcultures were prepared by transferring a small quantity of growth from the respective stock cultures to tubes containing 10 cc. of beef extract broth (10 cc. of thioglycollate medium for *Cl. novyi*). After 24 hrs. incubation at 37° C., each subculture was again subcultured in 10 cc. of liquid medium as before, the transplant being made with a 4-mm. loop bent at an angle. This subculturing was repeated through three successive 24-hr., 37° periods, and the fourth subculture was then diluted after it had been incubated 12, 18, or 24 hrs. at 37° C., as noted in Table I. The macroscopic appearance of the liquid culture and a gram-stained smear served as checks on the culture before dilution.

Dilutions.—Dilutions were effected with sterile serological pipettes and sterile 0.8% saline solution contained in glass-stoppered flint glass dilution bottles. The volume of inoculum and dilution finally employed for each group of experiments are indicated in Table I. Preliminary experiments revealed that 10^{-5} dilutions (this is the dilution stated in the medium sensitivity test of the U. S. P. XI, Second Supplement (4) for *E. coli* and *Cl. novyi*) would produce growth in 100% of the tube–medium combinations noted above. Higher dilutions were necessary, therefore, in order to determine what number of positives any particular tube–medium combination would give, when all were inoculated under comparable conditions. An approximation of the number of organisms per cc. of the inoculating dilution was obtained in some cases by plate count. Three 1-cc. quantities of this dilution were plated on U. S. P. broth containing 1.5% agar, and three 1-cc. quantities on Difco broth containing 1.5% agar. Six plates were thus prepared for each organism except *Cl. novyi*. A preliminary attempt to plate the latter and incubate the plates anaerobically indicated that the procedure would seriously interfere with the rest of the inoculation time schedule.

Tubes and Inoculation.—The Smith tubes employed in these experiments were of the S. A. B. type and 35-cc. capacity. The Durham tubes consisted of a 6 × 1-in. outer tube and a 3 × 3/8-in. inverted inner tube, and contained 25 cc. of medium. Smith tubes were never covered with an anaerobic seal. Within ten minutes after inoculation, about 50% of all Durham tubes used were covered aseptically with a 1-in. layer of sterile high-viscosity mineral oil (Sonneborn Kaydol), or yellow petrolatum U. S. P. (see Table I). All tubes were glass capped. In every series of tubes inoculated, all four tube–medium combinations were represented. Inoculation of each four tubes in a

TABLE I

Culture	Age of Culture in Hrs. when Diluted	Incubation Time, Hrs.	Vol. of Inoculum, Cc.	Dilution	No. of Organisms in 6 Cc. of Dilution as Detd. by Plating	Smith Tubes–Difco Broth: No. of Tubes Seeded	Smith Tubes–Difco Broth: No. of Tubes Positive	Smith Tubes–U. S. P. Broth: No. of Tubes Seeded	Smith Tubes–U. S. P. Broth: No. of Tubes Positive	Durham Tubes–Difco Broth: No. of Tubes Seeded		Durham Tubes–Difco Broth: No. of Tubes Positive		Durham Tubes–U. S. P. Broth: No. of Tubes Seeded		Durham Tubes–U. S. P. Broth: No. of Tubes Positive	
										With Oil	Without Oil	With Oil	Without Oil	With Oil	Without Oil	With Oil	Without Oil
Cl. novyi	12	60[c]	1.0	10^{-6}	ND[a]	3	1	3	2	2	2	0	1	2	2	0	0
	12	60[c]	0.1	10^{-6}	ND	2	0	2	0	2	2	0	0	1	2	0	0
	18	48[c]	1.0	10^{-6}	ND	3	3	3	3	3	3	1	1	3	3	3	3
	24	116[d]	1.0	10^{-7}	ND	6	6	6	3	3[b]	3	3	3	3[b]	3	1	3
B. subtilis	12	36[c]	1.0	10^{-8}	ND	2	0	2	1	1	2	0	1	1	2	1	2
	12	36[c]	0.1	10^{-8}	ND	1	0	1	0	1	2	0	0	1	2	0	0
	12	48[c]	1.0	10^{-8}	1	3	2	3	0	3	3	1	1	3	3	0	1
	24	90[d]	1.0	10^{-7}	1	6	6	6	2	3[b]	3	0	2	3[b]	3	0	2
E. coli	12	36[c]	1.0	10^{-8}	ND	2	2	2	2	1	2	1	2	1	2	1	2
	12	36[c]	0.1	10^{-8}	ND	1	0	1	1	1	2	1	2	1	2	1	2
	12	48[c]	1.0	10^{-10}	1	3	0	3	0	3	3	0	0	3	3	2	0
	24	90[d]	1.0	10^{-9}	7	6	3	6	3	3[b]	3	2	2	3[b]	3	2	3
Staph. aureus	12	36[c]	1.0	10^{-8}	ND	2	2	2	2	1	2	1	2	1	2	1	2
	12	36[c]	0.1	10^{-8}	ND	1	0	1	0	1	2	1	1	1	2	0	1
	12	48[c]	1.0	10^{-9}	3	3	2	3	0	3	3	1	0	3	3	1	2
	24	90[d]	1.0	10^{-8}	123	6	6	6	6	3[b]	3	3	3	3[b]	3	3	3

[a] Not determined. [b] Solid Petrolatum. [c] Incubation temperature, 37° C. [d] Incubation temperature, 32° C.

series was carried out by delivering four 1-cc. quantities of dilution from a 5-cc. serological pipette; the order of pipetting was planned to minimize the variation in concentration of inoculum which might exist at different levels of the pipette. When the final dilution had been obtained, the time required to inoculate any comparative series was not more than fifteen minutes.

Media.—U. S. P. broth was prepared in a manner similar to the alternative method outlined in U. S. P. XI, Second Supplement (3). For each batch of broth, fresh beef hearts were purchased from local butcher shops. Difco broth was prepared according to directions stated on the label of the container. The concentration of dextrose in all media was 0.04%. Difco medium was always made fresh on the day required; U. S. P. broth was made every one to four weeks, and was sterilized in bulk (not more than 700–800 cc. to each container) at 15 lbs. for 20 min. All tubes of broth were inoculated shortly after they had cooled to room temperature. Sterility of medium was controlled in each experiment by leaving about 10% of the total number of tubes uninoculated. Approximately one-half of these control tubes were covered with oil or vaseline seal to check on the sterility of the sealing material and the method by which it was added. Occasional checks on both media prior to inoculation indicated that the *p*H was regularly 7.4–7.6. *p*H determinations were made with a Hellige glass standard comparator and bromothymol blue indicator.

Observations.—Observations were made over a 7-day period. The time of incubation in Table I represents the number of hours beyond which no more positives appeared. Tubes were regarded as positive when growth was characteristic macroscopically; some typical growth, positive tubes in every series were checked by gram-stained smears. Occasional tubes which did not appear typical were always smeared and confirmed microscopically. Tubes in which there was no macroscopic evidence of growth after seven days were considered negative. All results are tabulated in Table I.

DISCUSSION AND CONCLUSIONS

For determining the suitability of medium for sterility testing, the U. S. P. XI, Second Supplement (4), directs the use of 18–24-hr. broth cultures of *Cl. novyi* and *E. coli* diluted 10^{-5}. If the medium is satisfactory, 1-cc. inocula of these dilutions should grow. With this criterion as a basis, experiments were performed with cultures of *Staph. aureus, E. coli, B. subtilis and Cl. novyi.* The tube–medium combinations selected were Smith–U. S. P., Smith–Difco, Durham–U. S. P. and Durham–Difco. Preliminary tests indicated that all four combinations manifested growth in 100% of the tubes inoculated with 1 cc. of 10^{-5} dilution, so more extreme dilutions were employed. Experiments utilizing approximately 300 tubes were performed; the results on 247 of these are given in Table I. Although any one tube–medium combination shows a somewhat higher or lower number of positive growths than another (see Table I), it seems reasonable to assume that the random distribution of the organism per cc. of inoculum may have been as contributory to the final figures as the sensitivity of the tube–medium. Final conclusions on this point would depend upon a series of experiments which could be statistically interpreted.

From the point of view of routine testing, some of the obvious advantages of a Durham tube–dehydrated broth setup are: The Durham tube is much less expensive, it can be cleaned and handled more conveniently, it does not require expensive special racks, and it occupies less room in the incubator; the dehydrated medium is readily available, it can be prepared fresh and quickly when required, and it probably gives a broth of more standard composition than the fresh infusion. The following disadvantages of such a setup have been suggested: lack of ability of the Durham tube to support the growth of microaerophils and anaerobes without special precautions, inability of dehydrated medium to support the growth of bacterial contaminants in chemical ampuls and expensiveness of dehydrated medium.

There are counterconsiderations for each of these points. The experiments reported here indicate that the Durham tube–Difco medium will, without special precautions, support the growth of organisms representative of possible contaminants in chemical ampuls to a degree comparable to the Smith tube–U. S. P. broth. The material expense of dehydrated medium is more than that of the fresh infusion. However, the time saved in preparation, aside from other advantages previously noted, largely offsets this expense. This factor seems especially important in laboratories using not more than one to two liters of medium weekly.

When ampul products are biological in nature, one may assume that contamination with anaerobes or fastidious pathogens is not improbable. Therefore a tube–medium combination which would provide optimum conditions for these organisms is highly desirable. On the other hand, this author has not found any references, with perhaps the exception of Gershenfeld's (5), indicating that such organisms are present in chemicals or chemical ampuls. If they do occur, it appears questionable whether the official tube–medium would enhance the possibilities of detecting them. A brief survey of the abstract literature for contaminants in stock chemicals and chemical ampuls reveals that the organisms which have been isolated (6, 7, 8) are saprophytic cocci and rods. One investigation (8) of 133 samples, of which 44 were found to contain bacteria, states that anaerobic and pathogenic bacteria, tetanus bacillus and hemolytic streptococcus were absent. The present writer has isolated several cultures from contaminated commercial samples. All of these manifested copious growth when isolated and subcultured aerobically on ordinary beef extract liquid and solid media.

As previously noted, studies have been made to compare the results obtained on commercial samples when duplicates were tested by the official tube–medium and the Durham–Difco setup. These studies, covering more than 80 various samples to date, show that the latter compares favorably with the former. These experiments will be submitted for publication in the near future.

SUMMARY

A published classification of injectable substances into one biological and three chemical groups has been reviewed and considered in relation to the present official sterility test for liquids.

The equivalence of a tube–medium setup more convenient for routine sterility testing than the official tube–medium has been discussed, and the experiments relative thereto have been detailed.

For sterility tests on certain types of chemical ampuls, the experiments reported here, together with information published by other investigators, suggest that the use of a Durham tube–dehydrated medium may be satisfactory. In view of the many variables to be considered in experiments of this type, cooperative research on this problem would be desirable.

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Digitalis purpurea Naturalized in Newfoundland

*By M. L. Fernald**

It may be of importance to know that in portions of southern Newfoundland *Digitalis purpurea* is so thoroughly naturalized as to be an aggressive weed of roadsides, sandy embankments and shores. In 1910, the late Professor Karl M. Wiegand and I found it in great profusion near the mouth of Spruce Brook, on the shores of St. George Pond, slightly north of Stephenville Crossing (Spruce Brook Camps are well known to sportsmen). Our label of August 19, 1910, reads: "Escaped to banks of streams and lake (said to have spread for 5 miles around Spruce Brook)." An herbarium specimen collected by Dr. Perley Spalding in 1926 shows that it was still about Spruce Brook. In southeastern Newfoundland it is also becoming abundant. A label accompanying a specimen received from Mrs. Agnes M. Ayer in 1928 reads: "Established over several miles about Salmonier." It is evident that the cool and relatively moist summer climate of southern Newfoundland is favorable to the plant. Possibly this is a region which would supply a considerable bulk of digitalis already growing and where its cultivation would meet with immediate success. I suggest investigation of the present supply about Spruce Brook, where it is conspicuously abundant.

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