Table III—Effect of Continuous Administration of Pentobarbital (25 mg./kg. i.p.) on Development of Tolerance

No. Injections	Injection Interval, Days[a]	Mean S.T., min.[b]	Tolerance Index, %
1	1	46.4 (8)	
2	2	26.7 (8)	173.0[c]
3–7	3–7	—	
8	8	36.0 (8)	128[c]
9	9	31.8 (8)	145[c]
10–14	10–14	—	
15	15	38.2 (8)	121[c]
16	16	41.6 (8)	111

[a] Injections were given daily to the same animals for 16 days. [b] The sleeping time was determined on 1, 2, 8, 9, 15, and 16 days. Sample size is given in parentheses. [c] $p < 0.05$.

prolonged by hepatic injury (7). Plaa *et al.* found a positive relationship between the doses of several hepatotoxins and the prolongation of pentobarbital sleeping time (7). Singh and Boyd found that tannic acid, which causes centrilobular liver necrosis, prolonged the sleeping time of thiopental (8). This effect became evident at 72 hr. after the administration of tannic acid. Balazs and Grice also reported that administration of carbon tetrachloride can prolong the sleeping time of pentobarbital (9).

Pentobarbital is metabolized in the liver (3). Conney *et al.* have shown that pentobarbital is an enzyme inducer (10). One explanation of the development of tolerance to pentobarbital is that pentobarbital possibly stimulates its own metabolizing enzyme and thus results in increased pentobarbital metabolism on repeated administration and decreased sleeping time (Tables I–III). This leads the authors to suggest that in the development of tolerance to pentobarbital, the liver possibly is involved.

Clinical Signs—During the development of tolerance to pentobarbital, neither water consumption nor urine excretion was affected. However, on the day after the tolerance had been induced, a significant increase ($p < 0.05$) was noticeable in water consumption and urine excretion. Singh reported similar clinical findings with thiopental also (11).

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Sterility Testing of Insulin by Membrane Filtration: A Collaborative Study

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Abstract □ A membrane filtration procedure was devised for testing the sterility of insulin zinc suspensions solubilized in ascorbic acid diluting fluid. A collaborative study showed that the filtration procedure afforded significant improvements over the direct method of sterility testing.

Keyphrases □ Insulin, sterility—membrane filtration □ Membrane filtration—sterility testing, insulin zinc suspensions □ Sterility testing—insulin, membrane filtration □ Collaborative study—membrane filtration, insulin sterility

In 1941 the Federal Food, Drug, and Cosmetic Act was amended to establish a certification service to ensure the safety and efficacy of insulin-containing drugs by testing each lot prior to distribution. The official analytical methods and standards of quality and purity are described in the USP (1), the NF (2), and the *Code of Federal Regulations* (3). Since insulin must be administered by parenteral injection, these official compendia require all insulin preparations to be sterile.

The official USP XVII/NF XII method for the sterility testing of insulin requires that 20 containers from each "filling operation" be tested in thioglycollate broth for detecting bacteria and in Sabouraud fluid medium for molds and yeasts. The solution or suspension of insulin is transferred with a sterile syringe and needle directly to tubes of media. Tubes containing thioglycollate medium are incubated for not less than 7 days at 30–32° and those containing fluid Sabouraud for not less than 10 days at 22–25°. After incubation the media are examined for the presence or absence of microbial growth.

The principal objection to the USP XVII/NF XII method is that a precipitate is formed by the insulin suspensions in the culture media. Macroscopically the precipitate is indistinguishable from microbial growth;

Table I—Recovery of Microorganisms from Insulin Zinc Suspension Preserved with 0.1% Methylparaben Tested at 0 Time and after 7 Days at 25°[a]

Microorganisms	Estimated No. Cells/ml.	Insulin				0.1% Peptone Solution			
		0 Time		7 Days		0 Time		7 Days	
		USP	MF	USP	MF	USP	MF	USP	MF
Saccharomyces cerevisiae ATCC 9763	15	+	+	+	+	+	+	+	+
Aspergillus niger ATCC 6275 (spores)	20	+	+	+	+	+	+	+	+
Bacillus subtilis ATCC 6633 (spores)	10	+	+	+	+	+	+	+	+
Bacillus circulans PCI 260 (spores)	10	+	+	+	+	+	+	+	+
Corynebacterium acnes PCI 1502	20	+	+	−	−	+	+	+	+
Pseudomonas aeruginosa ATCC 9027	15	+	+	−	−	+	+	+	+
Salmonella sp. PCI 431	10	+	+	−	−	+	+	+	+
Staphylococcus aureus ATCC 6538P	10	+	+	−	−	+	+	+	+

[a] MF = Membrane filtration method; USP = direct method; − = no growth; + = growth.

therefore, subcultures are necessary to determine whether viable microorganisms are present. Because the additional work required for these subcultures is time-consuming and expensive and could lead to false positive results due to adventitious contamination, a method was sought to improve the test.

EXPERIMENTAL

The membrane filtration sterility test method described for antibiotics by Bowman (4) is easily applied to insulin solutions, because they filter readily and leave no residue on the membrane. However, the suspensions (*e.g.*, protamine zinc insulin suspension, isophane insulin suspension, and insulin zinc suspension) are not amenable to the filtration sterility test, because the crystals are insoluble in the peptone diluting fluid used in the procedure.

Solubilization Studies—Because insulin suspensions presented problems in filtering, various procedures were tried for solubilizing them. When the pH was lowered from the normal of approximately 7.2 to 3.2 ± 0.2, the suspended material immediately dissolved at room temperature and the resultant solutions could then be filtered quickly, without leaving any visible residue on the membrane. However, when 1 *N* hydrochloric acid was used to lower the pH, it was toxic to some vegetative organisms.

Preliminary investigation indicated that ascorbic acid would be suitable for solubilizing insulin suspensions without adversely affecting the viability of microorganisms that might be present. A 1% ascorbic acid solution (AA diluting fluid) was prepared in 0.1% peptone (w/v) solution (pH 3.2 ± 0.2). Protamine zinc insulin suspensions were solubilized immediately and insulin zinc suspensions were solubilized in approximately 1 min. with the AA diluting fluid. The membranes were assayed by a radioimmuno assay for residual insulin (5). Less than 1 unit of insulin was retained by a membrane used to filter 1600 units of insulin (6).

Survival Studies—To assess the ability of organisms to survive in insulin suspensions, survival studies were performed. Vials of sterile insulin containing 0.1% methylparaben as a preservative were inoculated with vegetative bacteria or yeasts or with spores of bacteria or molds. Vials containing peptone water were used as controls. All were tested for sterility by the USP/NF method and by the proposed membrane filtration test at zero time and after 1 week at room temperature. Growth of the added microorganisms was ob-

Table II—Results of a Collaborative Study Comparing the Direct Method (DM) to the Membrane Filtration Method (MF) for the Sterility Testing of Insulin Suspensions (80 units/ml.)[a,b]

Test Organisms	Collaborator No.	Insulin								0.1% Peptone Solution Controls							
		1–5 CPU				50–100 CPU				1–5 CPU				50–100 CPU			
		DM		MF		DM		MF		DM		MF		DM		MF	
		FTM	SBCD	FTM	SBCD	FTM	SBCD	FTM	SBCD	FTM	SBCD	FTM	SBCD	FTM	SBCD	FTM	SBCD
Salmonella sp. PCI 431	1	Neg.	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	2	7	7	7	Neg.	7	7	7	7	7	7	7	7	7	7	7	7
Bacillus subtilis ATCC 6633 (spores)	1	Neg.	7	Neg.	7	7	7	7	7	7	7	Neg.	7	7	7	7	7
	2	Neg.	7	7	7	7	7	Neg.	7	Neg.	7	Neg.	7	7	7	Neg.	7
Staphylococcus aureus ATCC 6538P	1	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	2	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
Aspergillus niger ATCC 6275 (spores)	1	10	Neg.	7	7	10	7	7	7	7	Neg.	Neg.	7	7	7	7	7
	2	10	10	Neg.	7	7	10	7	7	7	10	Neg.	7	7	7	7	7
Bacillus circulans PCI 260 (spores)	1	7	Neg.	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	2	7	Neg.	7	Neg.	7	10	7	7	7	10	Neg.	Neg.	7	10	7	7
Bacillus sp. PCI 261 (spores)	1	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
	2	7	7	7	10	7	7	7	7	Neg.	7	Neg.	Neg.	7	7	7	7
Saccharomyces cerevisiae[c] ATCC 9763	1	7	Neg.	7	7	7	Neg.	7	7	7	Neg.	7	7	7	Neg.	7	7
	2	Neg.	7	7	7	7	7	7	7	Neg.	7	10	7	7	7	7	7
Candida albicans[c] ATCC 10231	1	14	Neg.	7	7	14	14	7	7	7	Neg.	7	7	7	14	7	7
	2	Neg.	7	7	7	7	7	7	7	Neg.	7	7	7	7	7	7	7

[a] Results are given as the maximum number of days incubation required for recovery of microorganisms. [b] Definitions and conditions as follows: CPU, colony-producing units; FTM, fluid thioglycollate medium incubated at 32–35°; SBCD, soybean-casein digest medium incubated at 22–25°. [c] Subcultures were necessary to determine whether viable microorganisms were present or absent when the direct method was used.

tained from all the suspensions at zero time. As shown in Table I, only the yeast and the sporing microorganisms survived in insulin for 7 days. In a similar study on globin insulin preserved with phenol, Sykes and Hooper (7) found that vegetative bacteria were killed by components other than preservatives (such as protamine) while spores of bacteria and molds survived.

The results obtained in these survival studies indicated that a filtration sterility test could be applied to insulin suspension and solutions. After some preliminary studies in collaboration with two manufacturers of insulin, it was decided that a definitive collaborative study should be conducted to establish the validity of the filtration method before proposing its inclusion in the USP and NF.

Collaborative Study on the Sterility Testing of Insulin—The procedure used was essentially that used for antibiotics (8) except that a separate membrane was used for each medium, soybean-casein digest broth was substituted for liquid Sabouraud medium, and the membrane was washed only once with 0.1% peptone (w/v) solution. These changes were based on the understanding that USP XVIII (9) and NF XIII (10) will include a similar membrane filtration sterility test as an alternative test for sterile preparations that are amenable to filtration.

Cultures of the eight microorganisms used in the study were supplied by the authors. Dilutions of inocula were selected to simulate low levels of contamination that might possibly be encountered in a contaminated insulin preparation.

The following directions (11) were followed by the two collaborating laboratories: aseptically add 39 ml. of insulin suspension preserved with 0.1% methylparaben to each of 80 sterile 60-ml. (2-oz.) prescription bottles. To each of another 80 bottles, add 39 ml. of 0.1% peptone solution. Prepare a dilution of one culture to yield five cells per ml., then aseptically add 1 ml. of this dilution to each of five bottles of insulin suspension and to five bottles of the control peptone solution. Prepare another dilution of the same culture to yield approximately 100 cells per ml., and add 1 ml. to each of another five bottles of insulin suspension and of peptone solution, respectively.

Perform a USP XVII/NF XII direct sterility test on the contents of two of the bottles of each dilution of microorganisms. From one bottle transfer 2-ml. portions to each of 20 tubes containing 40 ml. of thioglycollate medium and from the other bottle transfer 2 ml. to each of 20 tubes containing 40 ml. of soybean-casein digest medium.

Perform the proposed membrane filtration test, described here and in USP XVIII. using two more bottles of each dilution of microorganisms in the test fluids. These two bottles simulate the two pooled vessels (prior to filtration) to be described in the USP XVIII method.

As a control on the inoculum, filter the contents of the remaining bottle through a membrane filter; but instead of transferring the membrane to fluid media, place it on the surface of a soybean-casein digest agar plate.

Read all tests after incubation at the appropriate temperature for 7, 10, and 14 days.

Repeat this procedure for each of the other seven microorganisms.

Thus, for each microorganism, there is a total of 10 bottles for each testing fluid, five containing 5 cells per bottle and five containing 100 cells per bottle.

Proposed Membrane Filtration Method—Aseptically transfer the required volumes from each of 20 containers either directly to each of two sterile filter funnels or to each of two sterile flasks for pooling prior to transfer. [For required volume see the table in the chapter on Sterility Tests (9) in USP XVIII which describes the ratio of product to container content.] If the sample is a suspension, solubilize it by pouring the pooled insulin from a flask into 200–400 ml. of freshly prepared 1.0% ascorbic acid USP in 0.1% (w/v) peptone solution. (If the sample is a solution, omit this solubilization step.) After solubilization is complete, filter the solution through a bacteriological membrane filter. All air entering the system must be passed through air filters capable of removing microorganisms. Filter one 100-ml. portion of 0.1% peptone solution through the membrane to remove residual insulin. Transfer the entire membrane to a sterile 38 × 200-mm. (outside dimensions) test tube containing approximately 90 ml. of sterile thioglycollate medium. Repeat this procedure for the pooled insulin in the second flask, and transfer the second membrane to a tube containing approximately 90 ml. of fluid soybean-casein digest medium. Incubate the tubes for 7 days at 32 and 25°, respectively, and then examine for macroscopic evidence of growth, *i.e.*, the development of turbidity. The batch meets the requirements of the test if growth is absent in all tubes. If growth is observed in any tube, the lot fails to meet the requirements of the test for sterility, unless it can be demonstrated by retests or by other means that the test was invalid for causes unrelated to the product.

RESULTS AND CONCLUSIONS

As shown in Table II, both the high and low levels of microorganisms were recovered by the USP XVII/NF XII direct method and by the proposed membrane method. The inoculum control proved that the added test microorganisms were within the desired range of either 100 cells or 5 cells per 40 ml. of the sample. In each individual test by membrane filtration the microorganisms grew in one of the two media within 7 days. However, the USP/NF direct method required 10 days of incubation for the visual detection of *Aspergillus niger*, and the growth of *Saccharomyces cerevisiae* and *Candida albicans* could be determined only by subculture. Both collaborators agreed that the proposed membrane filtration method using AA diluting fluid to solubilize zinc insulin suspension is acceptable for sterility testing. Since USP XVIII (9) and NF XIII (10) are expected to recognize both the direct and the membrane filtration methods, either may be acceptable for the sterility testing of insulin after these compendia become official. However, several advantages accrue from the use of the filtration test. The incubation time is shortened from 14 to 7 days, the necessity for subculturing turbid broth tubes to determine the presence or absence of microbial growth is eliminated, and the volume of medium required for testing is drastically reduced.

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