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The Sterility Testing of Pharmaceuticals

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The administration of drugs by parenteral routes has required the development of meaningful sterility tests to be used in the analytical control of these pharmaceuticals. Proom (1) postulated that sterility tests should either demonstrate the absence of microorganisms or provide an estimate of the actual numbers and types of organisms present. The latter data are intended to show that there are insufficient numbers or types of organisms to be dangerous to the consumer or to actually degrade the product. Sterility tests appeared in the *British Pharmacopoeia* for the first time in 1932; before then the Regulations issued under their Therapeutic Substances Act of 1925 specified sterility tests for certain vaccines, toxins, serums, and similar products as well as for insulin and arsphenamine (2). Sterility tests were first introduced into the *United States Pharmacopeia* when USP XI became official on June 1, 1936. In the same year, the Sixth Edition of the *National Formulary* (*NF*) also included a sterility test for ampul solutions.

Both the USP and the NF are recognized as official compendia by the Federal Food Drug, and Cosmetic Act and by comparable laws of the individual states of the United States. The Congress recognized these compendia as sources of standards for strength, quality, and purity of drugs moving in interstate commerce. Since the first sterility tests were introduced, the compendia have been active in fostering new procedures and adopting changes that have increased the sensitivity of the tests.

In addition to the official compendia, two Federal agencies promulgate regulations governing the sterility testing of pharmaceuticals. At the turn of the century the first federal legislation was enacted to provide for the production of vaccines under government license. Under this law, the Hygienic Laboratory of the Public Health Service was created. As the list of vaccines grew and was augmented by serums, toxins, antitoxins, and blood products, the agency responsible for supervising production and establishing the purity and potency standards for them also grew (3). The sterility tests for these products, known as "biologics," are described in Public Health Service Regulations, which issue from the Division of Biologics Standards of the National Institutes of Health, Bethesda, Maryland, Title 42, part 73.73 (4). In 1945 Congress gave the Food and Drug Administration (FDA) authority to set standards for antibiotics. This authority included the testing and certification of each batch prior to distribution. For the purpose of administering the certification program, standards of potency and purity of antibotics are established under Title 21 of the Code of Federal Regulations and are published in the *Federal Register* (5). They include a sterility test procedure for each antibiotic required to be sterile. For both biologics and antibiotics, the compendial monographs conform to the appropriate regulations of the regulatory agency. Both agencies have contributed to advances in sterility test methodology, and members of their staffs have cooperated with the revisers of the compendia in performing investigational studies on procedures, media, temperatures, and time of incubation.

The first sterility test described in the compendia was applied only to sterile liquids. Only one medium, a beef-

peptone broth, was used. Half the tubes of this medium were inoculated with 5 drops (0.2 ml.) of the liquid; the other half with 20 drops (0.8 ml.). After the inoculated tubes were incubated for 7 days at 37°, they were examined for the presence or absence of microbial growth. When USP XII became official in 1942, it provided for a test to detect aerobic as well as anaerobic microorganisms in sterile solids and liquids and also included procedures for the inactivation of certain preservatives.

USP XIII introduced the use of a clear broth medium, fluid thioglycollate, for the cultivation of aerobic, microaerophilic, and anaerobic bacteria, and a honey medium for the recovery of molds and yeasts. Tests of samples in fluid thioglycollate medium were incubated for 7 days at 37°, whereas cultures of material in honey medium were incubated for 15 days at 22–25°. USP XIII also included for the first time a brief description of the laboratory area to be used for performing sterility tests. It stressed the importance of having qualified personnel, trained in aseptic techniques, to perform the tests.

In USP XIV (1950) the incubation temperature for thioglycollate was lowered from 37° to 32–35° and Sabouraud Liquid Medium (modified) containing a peptone and dextrose replaced the honey medium for molds and yeasts. In USP XV fluid thioglycollate was still the medium for bacteria, but Sabouraud Liquid Medium (modified) was changed to USP Fluid Sabouraud Medium, which specified the use of two peptones (pancreatic digest of casein and peptic digest of animal tissue) and dextrose.

The sterility tests described in USP XVI and XVII remained essentially the same as in USP XV; however, according to the galley proof of the chapter on Sterility Testing, major changes are expected in the forthcoming USP XVIII. The sterility tests in the *National Formulary* have generally been comparable to those of the USP. Therefore, it is anticipated that NF XIII, which will be published in 1970, –or a Supplement to NF XIII– will also include similar modifications.

The history of the sterility tests in the USP and NF since 1936 shows that official methods are constantly changing. The changes, which are also common to official compendia of other countries, reflect improved techniques, procedures, and media for detecting microorganisms from any item or medicament required to be sterile.

PRINCIPLES OF STERILITY TESTING

The general principles governing the design and interpretation of sterility tests were expounded by Bryce (6). He elaborated on the limitations of the methods of testing described by various official compendia. These limitations derive from two practically insoluble problems. The first is that of adequate sampling, and the second is the inability to cultivate all viable microorganisms that may be present. He stated that pharmacopeias imply that sterility is the state of being free from living organisms of all types. This concept is simple enough, but unfortunately it is unreal, being incapable of experimental verification. Bryce concluded that the sterility test is, in fact, a test for only certain contaminant organisms. In addition, he said that since the test attempts to infer the state of the whole from the result of an examination of the part, it is essentially a statistical operation. The forthcoming USP XVIII will recognize the difficulties of the experimental verification of the sterile state. Therefore, it will probably explain the objectives of the sterility test as well as the limitations. It is likely to be expressed as follows: The objective of the sterilization process is to make the article safe for use, but the sterility tests may be expected to reveal only that living organisms have been removed or destroyed to the extent that they no longer multiply in appropriate culture media under favorable conditions. Interpretation of the results of sterility tests must allow for the possibility that the degree of contamination is of a low order of magnitude. Confidence in the results of the tests with respect to a given lot of articles is based upon knowledge that the lot has been subjected to a sterilization procedure of proven effectiveness. Sykes (2) pointed out that it is clearly a prerequisite that before a preparation is submitted to a test for sterility, it must have been subjected to such a treatment as can be reliably expected to yield a sterile product. The exclusive purpose of the test is to check that the approved sterilization process has been carried out satisfactorily; the test cannot of itself check that the process is satisfactory.

SAMPLING PROCEDURES

The proper sample size and sampling procedure for the sterility test has been the subject of much debate and discussion among manufacturers and control authorities of parenteral drugs. The relative merits of sampling schemes based on constant sample size, regardless of lot size, versus those based on proportional samples, were reviewed by Bryce (6). The statistical properties of sampling plans have been dealt with by Knudsen (7), by Greenberg (8), and by Brewer (9). Knudsen demonstrated that the probability of accepting lots having a given percentage of contaminated containers is directly related to sample size rather than batch size. Brewer considered the problems associated with sampling for sterility testing and pointed out the mathematical limitations of the procedures. The relationships of the probabilities of acceptance of batches of varying assumed degrees of contamination to sample size were tabulated. Probability values for eight sample sizes were given. Greenberg stated that from a controller's viewpoint it is essential that sterility tests be performed, but he made it clear that regardless of the size of the sample and the techniques used, the tests would detect only grossly contaminated lots. To compensate for this, he advocated that pharmaceutical and biological manufacturing be strictly controlled.

The difficulty of establishing procedures that would be accepted internationally is evident in the WHO Report of General Requirements for the Sterility of Biological Substances (10). The WHO Study Group considered various rules for sampling finished containers which had been proposed or adopted in different countries. The Study Group agreed that it was not possible to decide which of these rules for sampling should be preferred and that the Requirements should

permit the adoption of any rule, based on the principles of sampling statistics, found to be satisfactory by the national control authorities of individual countries. For sampling among final containers, one of its members (10) proposed the testing of a sample equal to four-tenths of the square root of the number of articles in the batch. Mathews (11) cast doubt on the validity of this proposal, since it appeared that the best control would be achieved (in batches contaminated 2–4%) when the sample size was proportional to the number of articles in the batch. However, he agreed with Knudsen (7) that proportional sampling would lead to very poor control over small batches and would be impracticable for extremely large batches due to overwhelming interference by accidental contaminations. Proom (12) showed that the size of the sample is not limited in practice by statistical consideration of the risk of accepting contaminated batches. The size of the sample is limited by empirical considerations of the risk of rejecting sterile batches because of accidental contamination during the testing process. Another report (13) stated that it is difficult to fix a value for a certain fixed risk (r) of extraneous contamination during the sterility test. However, a value of 1% for r was regarded as a reasonable possibility.

At the London Round Table Conference on Sterility Testing in 1963 (14) the participants concluded that the control authority should satisfy itself that the manufacturer has taken all necessary precautions to ensure the production of a sterile product, since sterility tests employing reasonable sampling detect only gross contamination. They recognized that the lowest contamination rates which can be detected with at least 95% probability are 28, 15, and 7% when testing 10, 20, and 40 samples, respectively, and that accidental contamination will weaken the test further. Most of the speakers wanted the number of final containers tested to be independent of the batch size. The majority concluded that experience of manufacturing laboratories showed that testing between 10 and 20 vials from a final lot has regularly given no untoward reactions associated with lack of sterility. Therefore, this number of vials was regarded as sufficient for the test on a final lot.

The USP and NF require a representative sample of 10 units to be examined from products sterilized by steam under pressure, and a representative sample of 20 units for all other products. The *Public Health Service Regulations* require 20 final containers from each filling of each lot, selected to represent all stages of filling from the bulk container. The *Antibiotic Regulations* require 20 immediate containers collected at approximately equal intervals from each filling operation. A filling operation is defined as that period of time not longer than 24 consecutive hours during which a homogeneous quantity of a drug is being filled continuously into market-size containers and during which no changes are made in equipment used for filling.

CULTURE MEDIA

From the time of the first sterility tests until the present, the test results have been influenced by the types and sensitivity of the culture media. Since no single medium will support the growth of all bacteria, molds, and yeasts, more than one medium must be used. The question of which to use has been the subject of many conferences, study groups, and published reports. However, Pittman (15) concluded that none of these have supplied adequate support for the selection of any medium in preference to others in use in various control laboratories. She pointed out a possible shortcoming of published work in that the growth-promoting properties of various media were determined by using microorganisms considered to be potential contaminants, rather than by using organisms actually isolated from contaminated products. She advocated that emphasis be given to the recovery of organisms subjected to insult by preservatives.

Many media are being used for sterility testing, and the formulas for these appear in the pharmacopeias of many countries. In the report of a WHO Study Group (10) formulas were listed for nine media for culturing bacteria and six for culturing fungi. The Group could not recommend any one medium in preference to another because of lack of comparative data.

In 1949 Brewer (16) introduced the use of sodium thioglycollate to provide aerobic and anaerobic conditions in one medium. After extensive studies of the medium for the sterility test, Pittman (17) agreed that it provided both aerobic and anaerobic conditions in one test tube. In addition, it neutralized the bacteriostatic action of mercurial preservatives. Sabouraud liquid medium has been widely used for the detection of fungi since 1950. Since the advent of these media, few changes have been made.

Benković and Higy-Mandić (18) presented comparative studies of the growth of yeasts and bacteria in various common media. Although fluid thioglycollate had become firmly established by 1956 for the cultivation of both anaerobic and aerobic bacteria some investigators preferred the use of sodium hydrosulfite suggested by Bonnel (19) as an oxidation-reduction potential regulator. Jeskova (20) found that of five media tested against 272 strains of 20 species, fluid thioglycollate and the Clausen modified hydrosulfite media gave the best results. Mathews (11) reviewed the controversy centered around the use of sodium hydrosulfite as an oxidation-reduction potential regulator in lieu of thioglycollate. The hydrosulfite medium, the thioglycollate medium, and a corn steep liquor with both thioglycollate and hydrosulfite were discussed. Mossel and Beerens (21) studied the inhibitory properties of four different types of thioglycollate media. Using wet spores of fourteen strains of *Clostridium*, he found sodium thioglycollate to be toxic *per se* to almost all strains tested. The degree of toxicity was influenced by other components of the medium. He recommended that cysteine hydrochloride be used as the redox potential reducing compound instead of sodium thioglycollate.

Chauhan and Walters (22) used common air-borne saprophytic fungi to demonstrate that the *British Pharmacopoeia* test for sterility was unable to recover fungal contamination. The inclusion of a specific test for fungi comparable to that described in the USP was recommended for the BP

Two recent papers (23, 24) present data to show that thioglycollate medium does not support growth of

Bacillus subtilis spores when they are entrapped or held so that the organisms cannot be released into an environment of high oxygen tension.

Although liquid Sabouraud medium has been used successfully to recover molds and yeasts from small inocula, many investigators oppose the use of a selective medium in sterility testing. Since the purpose of performing the test is to detect as many microorganisms as possible, it is undesirable to use a medium such as fluid Sabouraud that was designed to inhibit certain bacteria. For this reason, it is believed that the 18th revision of the USP will replace Sabouraud medium with a soybean-casein digest medium which has been shown to support the growth of many bacteria as well as fungi.

TIME AND TEMPERATURE OF INCUBATION

Originally the sterility test medium was incubated at 37° for pathogenic bacteria, and at about 25° for psychrophilic bacteria and fungi. Since common airborne saprophytic bacteria represent a greater potential source of contamination of pharmaceutical products than the more fastidious pathogens, in 1950 the compendia reduced the temperature of incubation from 37° to 32–35°. The stimulus for the 1955 change to 30–32° for fluid thioglycollate was brought about by a dramatic incident (15). The Division of Biologics Standards of the National Institutes of Health discovered a failure to detect the presence of a pseudomonad contaminant in plasma. The contaminated plasma caused severe shock when administered to patients. It was later discovered that the contaminant grew at room temperature but was killed at 35°. As a result of this finding, in 1955 the temperature for the incubation of fluid thioglycollate was lowered from 32–35° to 30–32° in the Federal Regulations and in the official compendia.

Pittman and Feeley (25) showed that although the yeasts and fungi they studied were cultivated easily at 22° (compared with other temperatures within the range of 4–35°), the number of strains recovered in fluid thioglycollate medium incubated first at 22° for 3 days and then at 30° for seven days was only slightly less than in the best combination of medium and temperature.

Mathews (11) has reported that the method of incubating first at a low temperature and then at a higher one has been used in some laboratories for years. He also postulates that it is plausible that starting incubation in any medium at a low temperature may encourage elution, from the surface of any microorganism present, of any antiseptic which might be adsorbed. Thus, the preliminary low temperature incubation would mitigate the harmful effect to be expected if the organism were to be exposed to the action of adsorbed antiseptic at a higher temperature.

The use of a single medium, thioglycollate, incubated for 3 days at 21 ± 1° and 7 days at 31 ± 1° for the sterility testing of antibiotics was explored and rejected (26). Brewer and Keller (27) presented data which support the existence of slow-growing organisms that could not be detected until the 21st day of incubation. These findings are in keeping with those of other workers who have pointed out the slow growth of organisms subjected to less than the lethal radiation dosages.

The 18th revision of the USP will likely require no less than 14 days of incubation for fluid thioglycollate and for soybean-casein digest broth, except for preparations tested by membrane filtration. The incubation time for preparations tested by filtration sterility test will probably be no less than 7 days. The fluid thioglycollate will be incubated at 30–35° and the soybean-casein digest medium will be incubated at 20–25° for pharmaceutical preparations tested by either of the two prescribed sterility tests. If the nature of the product or the sterilization procedure used is conducive to producing the "slow-grower" phenomenon, additional incubation time for these preparations may be required.

BIOLOGICAL INDICATORS

In recent years biological indicators have been employed in addition to sterility tests to demonstrate the adequacy of some sterilization procedures. A single species of viable microorganisms of known resistance to the sterilization process being employed may be added directly to representative units of the batch being sterilized. If this is not feasible, the culture is added to disks or strips of paper or metal, or glass beads which are incorporated in or on the product. The biological indicator (BI) is removed after the sterilization cycle, transferred to culture media, and incubated at the appropriate temperature to determine whether the microorganisms of the BI have been destroyed. The effective use of BI's for monitoring a sterilization process requires a knowledge of the product being studied and of the probable types and numbers of the microbial population in the product prior to sterilization. Brewer and Phillips (28), in a paper which elaborates on the proper use of BI's, discussed the selection of the indicator to be used with various sterilization procedures, the preparation and calibration of the indicator carrier system, and the placement of the BI in the sterilization system. Bruch (29) emphasized that BI's should not be used as biological thermocouples. They are not suited to measuring physical processes which can be monitored by other types of indicators. For example, if the measurement of time and temperature is a necessary part of the sterilization cycle, it is better to use physical monitoring techniques. The BI's should be employed to demonstrate that the cycle used is capable of killing the largest population of resistant organisms that can be expected to contaminate any batch of the product to be sterilized. Bruch believes that the use of BI's is a necessary adjunct to gaseous and radiation sterilization since all the variables in the process cannot be properly monitored by physical instrumentation.

A variety of microorganisms have been used for BI's to verify the sterilization procedures used for pharmaceuticals. Spores of *Bacillus subtilis* var. *niger* are widely used as a wet and dry heat sterilization control. *Bacillus pumilus* is used in Europe and America for radiation sterilization control. Christensen *et al.* (30) has suggested *Streptococcus faecium* as a BI for radiation sterilization, but warned that the radiation resistance of any microbiological species shows great differences in resistance between various strains of the same species. The use of *Bacillus stearothermophilus* spores has been approved by the National Institutes of Health as a sterilizer control

for use in licensed establishments which produce biologicals. This organism meets the rigid requirements of the PHS *Regulations*, Title 42, Part 73 (4) because it does not produce pyrogens or toxins, is not pathogenic for man, and does not grow at or below 37° within a 2-week period. In addition, these Regulations give detailed instructions concerning the handling of microbial spores and prohibit their transfer to culture media in areas used for manufacture of products.

In the last few years manufacturers of pharmaceuticals have found that BI's, in addition to sterility tests, are of great value in establishing and monitoring their sterilization procedures. Due to the increased acceptance, a number of biological spore indicators are commercially available.

In order to give official recognition to the use of BI's as meaningful adjuncts to the sterility tests, it is expected that the XVIII revision of the USP will include guidelines for their proper use.

ENVIRONMENTAL CONDITIONS

It is axiomatic that if the results of sterility tests are to be reliable, they must be performed in a sterile environment. For this reason Federal Regulations and the compendia state that the tests should be performed in an area as free from microbial contamination as is possible to achieve. It is anticipated that the chapter on Sterility Tests for USP XVIII will state that ideally, the sterility test area should comply with Class 100 conditions as described in Federal Standard No. 209A, entitled "Clean Room and Work Station Requirements, Controlled Environment," and NASA Standard for "Clean Rooms and Work Stations for Microbially Controlled Environment," as described in NHB5340.2, Aug. 1967.

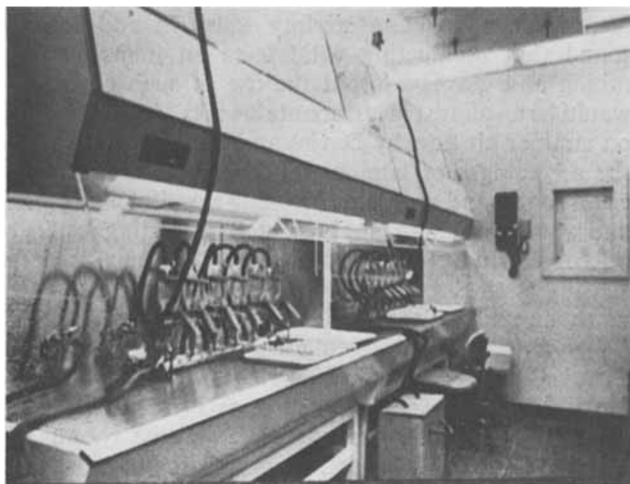
For many years sterility tests were performed in conventional clean rooms, equipped with germicidal lamps and filtered air under positive pressure. All surfaces of the rooms were washed daily with germicides. Personnel who performed the tests donned sterile gowns, caps, masks, shoes, and gloves. Nevertheless it was apparent that all these precautions were inadequate. Airborne microbial contamination was a constant problem which could cause false-positive results. One difficulty was that employees working in these areas were constantly shedding particles containing microorganisms (31). Whitfield (32), working for the aerospace industry, was assigned to study and improve the conventional clean rooms which were then in use. He noted that in the conventional clean room the filtered air, which is forced through wall or ceiling ducts, creates swirls and eddies in the airstream which, in turn, trap particles and microorganisms within the room. From this observation there evolved in 1961 the concept of laminar air flow—a bank of filtered air moving through a work area at just the proper speed to sweep contamination with it and to create a minimum of turbulence and a minimum effect on workers. Laminar flow is defined by Federal Standard 209a as "air flow in which the entire body of air within a confined area moves with uniform velocity along parallel lines, with a minimum of eddies" (33). Brewer (34) reported on the use of laminar flow hoods in sterility testing. He concluded from his experience that the use of laminar flow equipment affords a practical means for conducting sterility tests. In addition, he stated that conducting valid tests on items such as tubing and gloves without the use of such equipment would be an almost insurmountable task. A review paper on laminar air flow by Davies and Lamy (35) described the advantages of laminar air flow over conventional clean rooms and suggested its use in hospital pharmacies and wards. Manning (36) presented interesting results of microbial studies in a laminar air flow unit used for sterility testing. Over 200 tests were performed in this unit without a contamination problem. One publication (37) described the successful use of a laminar flow unit to aseptically fill and close 460 syringes. Parisi and Borick (38) performed mock sterility tests in a laminar flow sterile room and in a conventional sterile room. The tests employed all of the necessary testing motions with the exception of placing a sample into the medium. The tests performed in the laminar flow sterile room yielded 0.5% positives whereas 1.5% positives were obtained in the conventional sterile room. He concluded that laminar flow is significantly better for providing a microbiologically clean area for sterility testing procedures than a conventional sterile room with filtered air. Bowman (39) evaluated the use of vertical laminar flow hoods for the sterility testing of antibiotics and insulin. An aerosol study employing the tracer organism, *Serratia marcescens*, proved the ability of these hoods to remove airborne contamination. Three 1.8-m. (6-ft.) vertical laminar flow hoods installed in clean rooms (see Fig. 1) have been used satisfactorily for approximately 2 years for performing sterility tests on antibiotic and insulin preparations. The air entering each clean room is filtered through HEPA[1] filters located in the ceiling. There is an air change in each room every three minutes.

In order to obtain quantitative information on the control of the air in clean rooms, laminar flow rooms, and the work areas of the laminar flow hood, a variety of volumetric samplers have been designed and evaluated. After six years of fundamental research, Luckiesh *et al.* (40) developed two highly efficient electrostatic air samplers. Kuehne and Decker (41) conducted studies on several factors affecting the efficiency of air sampling when vegetative cells of microorganisms were collected for extended periods of time. He found that slit samplers afford a time-concentration relationship, permit air sampling for longer periods of time at a good collection efficiency, and require a minimum of labor, personnel, and equipment to take the samples. *Public Health Monograph* No. 60 (42) gives an excellent discussion of commercially available instruments for sampling airborne bacteria. It also describes techniques for numerical determination of the microflora of air.

METHODS FOR STERILITY TESTING

There are two basic recognized methods for performing sterility tests of pharmaceuticals. One is the direct method, which allows the test sample to be inoculated directly into the appropriate culture medium. The other is the bacterial membrane filter method, in which the sample is solubilized in a non-toxic diluting fluid which

[1] High Efficiency Particulate Air Filters.



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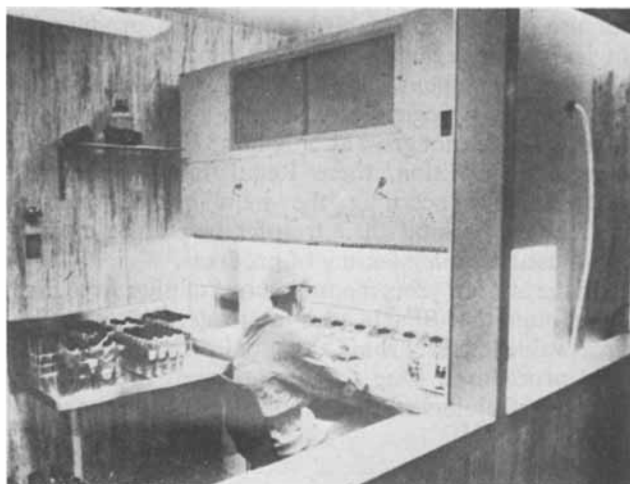

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Figure 1—*Laminar air flow hoods used in the sterility testing of antibiotics and insulin.* A, *Two 1.8-m. (6-ft.) vertical laminar flow hoods;* B, *One 1.8-m. (6-ft.) vertical laminar flow hood;* C, *One 1.2-m. (4-ft.) vertical laminar flow hood used for aseptically transferring sterile enzymes to sterile broth. Reprinted from* Bull. Parenteral Drug Assoc., *22, 57(1968).*

is then filtered through a bacterial-retentive membrane usually composed of cellulose esters. The membrane is washed to remove any inhibitory substances contained in the sample, and is then transferred aseptically to an appropriate medium. This filtration method was first introduced by Holdowsky (43) to separate microorganisms from the antimicrobial effects of antibiotics in order to obtain reliable sterility tests of antibiotic drugs. Research on the application of the membrane filtration technique to the sterility testing of antibiotic drugs by Bowman (44) produced practical methods for solubilizing and filtering a number of antibiotic preparations. The *Antibiotic Regulations* (45) were amended in 1964 to incorporate the filtration procedures, which greatly increased the sensitivity of the antibiotic sterility tests. The direct type of sterility test formerly used, when applied to all antibiotics except penicillin, detected only those organisms highly resistant to the inhibitory action of the particular antibiotic. In the case of penicillin, the enzyme penicillinase was added to the medium to inactivate the antibiotic.

Several reports (46–52) confirmed the soundness of the membrane filtration approach for sterility testing of antibacterial substances. The filtration techniques have also been successfully applied to testing the sterility of oils and ointments (53–55).

The 1963 *British Pharmacopoeia* introduced the membrane filtration test and required its use for antibiotics other than penicillin. It is anticipated that the eighteenth revision of the USP will allow a filtration sterility test which will be especially valuable for products that contain bacteriostatic or fungistatic preservatives. Alternatively, these products may be tested by the direct method provided these substances are diluted beyond an

inhibitory level. The details of the performance of the sterility test are given in all official compendia and Federal Regulations. They include the media to be used, the time and temperature of incubation, and directions on how to interpret the test results. All regulations governing sterility testing allow for the contingency of accidental contamination introduced in the performance of the test, and therefore provide for one or more retests.

Whatever testing system is employed, it is essential that the technique be continuously and adequately controlled. The USP and NF require that control tubes of each medium be incubated at the time of the test to assure sterility of the entire batch of medium. They further require that each lot of medium be tested for its growth-promoting qualities, using two or more strains of microorganisms that are exacting in their nutritive requirements. Sykes (56) presented an excellent review of the information made available by the Standardization Subcommittee of the Society for General Microbiology on methods of manufacturing bacterial culture media. Farber and Seligmann (57) recommended the use of small inocula of *Bacteroidis vulgatus* ATCC 8482 to test the anaerobic growth-promoting qualities of fluid thioglycollate medium.

DISCUSSION

The state of the art of sterility testing is changing rapidly and such changes are reflected in revisions and amendments to official requirements for sterility tests. There is still controversy over whether it is better to test for the efficiency of the sterilization process or to test the sterilized product. Most control authorities insist that the sterilization process be proven and that the sterile products also be tested. Our objective is to seek the maximum information from a balanced amount of testing, and to provide pharmaceuticals that are free from microbial contamination.

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RESEARCH ARTICLES

Surface Pressure Relaxation and Hysteresis in Stearic Acid Monolayers at the Air-Water Interface

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Abstract □ Time-dependent changes in the surface pressure of stearic acid monolayers were examined using an automated Wilhelmy-type film balance. Different surface pressure-area isotherms were obtained for two different rates of compression. Pressure relaxation from preselected surface pressures was examined as a function of time. The results indicated two types of relaxation which, along with the compression rate effect, may be rationalized on the basis of changes in molecular orientation and redistribution, together with expulsion from the monolayer at areas below the limiting area per molecule. Marked hysteresis effects were also noted when stearic acid monolayers were subjected to compression-expansion cycles. The effect of repeated cycling and the minimum area of compression on hysteresis were investigated. The onset and extent of hysteresis may also be explained on the basis of expulsion and reentry and orientation and redistribution of molecules at the interface.

Keyphrases □ Stearic acid monomolecular films—effect of compression rate □ Surface pressure relaxation—stearic acid monolayers □ Hysteresis—effect of minimum area of compression □ Cycling, requested, effect—hysteresis □ Air-water interface—Surface pressure

In recent years, the study of monomolecular films of biologic materials spread at the air-liquid interface has become increasingly significant in pharmaceutical and medical research. Serving as simulated biologic interfaces, these systems have been used to examine the interactions of such medicinal agents as the phenothiazines, local anesthetics, bactericides, and antibiotics with various cell membrane constituents. The evaluation of such studies is based primarily on the surface pressure-area per molecule (π-A) relationships exhibited by the system and the manner in which these change with time.

There are many factors affecting the shape and type of surface pressure-area diagram obtained for monolayers. Influencing factors such as pH, temperature, and the ion content of the supporting media have received fairly extensive investigation and are well documented in several texts (1–4). The time-dependency of surface pressure has been examined under conditions of slow discontinuous compression (5–7) and more rapid continuous compression (8–11). With the exception of the work of Rabinovitch *et al.* (11), the treatment of the time effects in these papers is limited. Examination of the literature shows that monomolecular film studies have been undertaken using a wide range of compression rates and methods of compression. This arises, presumably, from the lack of appreciation of the time-dependent properties of monomolecular films. Accordingly, investigations were undertaken to examine the effects of compression rate on the surface pressure-area isotherms of stearic acid monolayers and to study surface pressure relaxation, *i.e.*, the decrease in surface pressure with time when compression is stopped and the film held at constant area.

The property of surface hysteresis was also examined since it appeared there might be a link between the time-dependency of surface pressure and this property. Surface hysteresis or the significant separation seen between compression and expansion surface pressure-area isotherms has been examined in the case of the biological surfactants obtained from lung tissue, the so-called pulmonary or lung surfactants (12–15); however, the ability of simpler compounds such as stearic acid to exhibit similar behavior has been almost ignored. Ries and Walker (16) have stated, without presenting any evidence, that compression-expansion isotherms for stearic acid show hysteresis.

As a result of studies on the monolayer properties of biologic amphiphiles currently under investigation in this laboratory, the authors wish to report their observations on (*a*) the effect of compression rate on the π-A isotherm; (*b*) pressure relaxation following compression to preselected film pressures; and (*c*) surface hysteresis as affected by repeated cycling and minimum area of compression.