CIRCULAR No. 198 DECEMBER, 1931
UNITED STATES DEPARTMENT OF AGRICULTURE
WASHINGTON, D. C.

UNITED STATES FOOD AND DRUG ADMINISTRATION METHODS OF TESTING ANTISEPTICS AND DISINFECTANTS

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The methods herein set forth are prescribed for use, where applicable, in the examination of antiseptics and disinfectants in the enforcement of the insecticide act of 1910.

Arthur M. Hyde,
Secretary of Agriculture.

Washington, D. C., *October 15, 1931.*

INTRODUCTION

All antiseptics and disinfectants shipped or offered for shipment in interstate commerce or offered for import into or export from the United States are subject to the provisions of the Federal insecticide act, the Federal food and drugs act, or both. In the enforcement of these acts it is necessary to determine the accuracy of the bactericidal and antiseptic claims made for such products. A number of methods have been developed for determining bactericidal effectiveness, but all of them possess certain disadvantages. Of course it is impossible to devise tests which will apply in all cases, but during the past 20 years the Insecticide and Fungicide Board and the Food and Drug Administration have found certain methods to be particularly well adapted to their purposes.

Confusion has arisen from the fact that, in many cases, manufacturers have not used the same methods of testing their products, as a basis for preparing their labels, as those used by the administration. This possibility of misunderstanding would be obviated if the same methods were employed by all, and many manufacturers, recognizing this, have requested information as to methods employed by the Food

and Drug Administration. In view of this, it seemed desirable to publish them in a form which would make them generally available.

This circular, therefore, describes briefly the methods usually employed in the insecticide control laboratory for testing official samples of antiseptics and disinfectants. No attempt is made to review the literature of disinfectant testing in detail, but the most important papers relating to the methods here presented are cited.

DETERMINATION OF PHENOL COEFFICIENT[1]

There are in general use at the present time three methods of determining the phenol coefficient: the Hygienic Laboratory (H. L.) method (*11*)[2], that of Rideal-Walker (R-W) (*7*), and the method developed by this laboratory. It had been realized for a long time, especially among qualified workers in the field of phenol coefficient testing, that there were numerous handicaps and minor deficiencies to be encountered in the routine manipulation of both the H. L. and R-W methods. Lloyd P. Shippen, formerly bacteriologist of the Insecticide and Fungicide Board, after much experience in the testing of disinfectants, devised a method for obtaining phenol coefficients, utilizing as its basis the best features of the two older tests. Under pressure of a great volume of routine work this method was first put into practice more than 15 years ago and found to be so satisfactory that it has come to be used for testing the great majority of the germicides now received at the Food and Drug Administration.

George F. Reddish, successor to Doctor Shippen, later published this method under the name, "The R-W modified method" (*5*).

The procedure of Shippen has been little changed, but the standards for the resistance of the test organisms, *Eberthella typhi* (Schröter) Buchanan,[3] and *Staphylococcus aureus* Rosenbach, have been firmly established and provisions for the use of other organisms have been added. The method, as here published, is designated the "Food and Drug Administration phenol coefficient method" or, briefly, the "F. D. A. method."

The differences in the three methods are shown in Table 1.

There need be very little confusion arising from substituting the F. D. A. method as a test for products previously tested by either the R-W or the H. L. methods. The phenol coefficients of the large number of substances chemically related to phenol (the only type of disinfectants for which the H. L. method is accepted) (*11*) are, in most cases, practically the same, whether tested by the F. D. A.

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[1] For the benefit of those unfamiliar with testing disinfectants a brief statement of the principles of determining phenol coefficients is made. The phenol coefficient is a figure expressing the ratio of the killing efficiency of a disinfectant as compared with that of phenol tested under identical conditions. The sample to be tested is diluted and the dilutions arranged in a series of decreasing concentrations (increasing dilutions). To these a specified amount of the test organism in broth culture is added. At the end of fixed periods of time a small definite portion of the mixture of diluted disinfectant and test organism is transferred to a nutrient culture medium and incubated. No growth in the subculture indicates that the organism has been killed. The greatest dilution (weakest concentration) of the disinfectant killing in a definite time period is divided by the greatest dilution of phenol killing in the same time period. This ratio is the phenol coefficient. It should be noted that the phenol coefficient is not based on a comparison of different time intervals but on a comparison of different concentrations acting for specified time periods.

[2] Italic numbers in parentheses refer to Literature Cited, p. 20.

[3] Throughout this paper the term *Eberthella typhi* is used for *Bacillus typhosus*, in accordance with the nomenclature adopted by the committee on classification of the Society of American Bacteriologists (*1*).

or the H. L. method. Not only has continued use of the method in this laboratory shown this to be true (*2*), but collaborative experiments in five other laboratories (unpublished) confirm this fact. In comparing this method with the R-W method, similar results in general are obtained, although a somewhat lower coefficient usually results with coal-tar products having high coefficients. However, the higher results sometimes obtained by the R-W method may be misleading. R-W broth is not well adapted for the optimum growth of the test organism; hence negative subcultures frequently indicate that the organism has been killed, when in fact it may have been only rendered incapable of growing in this culture medium.

TABLE 1.—*Differences in media and manipulation of the three methods of determining phenol coefficient*

Item	F. D. A. method	R-W method	H. L. method
Composition of medium	Peptone[1], 10 gm Liebig's beef extract, 5 gm. Salt, 5 gm Water, 1,000 c. c Boil 20 minutes	Peptone[2], 20 gm Liebig's beef extract, 10 gm. Salt, 10 gm Water, 1,000 c. c Boil 30 minutes	Peptone [1], 10 gm. Liebig's beef extract, 3 gm. Salt, 5 gm. Water, 1,000 c. c. Boil 15 minutes.
Acidity of medium	pH 6.8	+1.5. No definite pH	Unadjusted but pH between 6.0 and 7.0.
Amount of culture medium in tube.	10 c. c	5 c. c	10 c. c.
Amount of culture added to diluted disinfectant.	0. 5 c. c. to 5.0 c. c	0.5 c. c. to 5.0 c. c	0.1 c. c. to 5.0 c. c.
Resistance of test culture to phenol (dilutions killing in 10 minutes but not in 5 minutes).	1–90	1–90 to 1–110	No limits stated.
Condition of tube in test	Plugged with cotton	Plugged with cotton	Open tubes.
Temperature of test	20° C	15–18° C	20° C.
Time intervals of the test	5, 10, and 15 minutes	2½, 5, 7½, and 10 minutes.	5, 7½, 10, 12½, and 15 minutes.
Amount of medication mixture transferred (size of loop).	4 mm. loop (of No. 23 B. and S. gage wire).	4 mm. loop (of No. 27 Imperial gage wire).	Spiral loop (four spirals wrapped around a No. 13 B. and S. gage wire. Made of No. 23 B. and S. gage wire).
Calculation of phenol coefficient.	Highest dilution not killing in 5 minutes but killing in 10 minutes divided by same for phenol.	Highest dilution not killing in 5 minutes but killing in 7½ minutes divided by same for phenol.	Mathematical mean of highest dilutions showing no growth in 5, 10, and 15 minutes divided by same for phenol.

[1] Armour's. Special batch set aside for disinfectant testing. [2] Allen and Hanbury's.

The curtailment in labor, time, and material through the use of the F. D. A. method renders it particularly valuable where a large number of samples are involved. The F. D. A. method is considerably superior to the R-W method in producing consistent results (*4, 12*). The medium employed is better adapted to bacterial growth, and the technic is not restricted to the use of one test organism (*Eberthella typhi*) as is the case in the R-W and H. L. methods. Moreover, the stock cultures of *E. typhi* and *Staphylococcus aureus*, the organisms principally used in germicidal testing, remain sufficiently constant in their resistance to phenol, when grown on an adjusted medium, to necessitate but one phenol control, though two controls are used frequently as an additional check. This allows the use of nine dilutions of the unknown with 30-second intervals between transfers, or 14 when 20-second intervals are used. With a little practice, 20-second intervals allow sufficient time.

The F. D. A. method will be used by this laboratory in determining the dilutions at which miscible coal-tar disinfectants, and many other products to which the method is applicable, should be used for disinfecting purposes. As heretofore, this dilution should be at least equal in strength to a 5 per cent solution of phenol when tested against *Eberthella typhi* (20 times the phenol coefficient figure) and should be based on a phenol coefficient not higher than that obtained by the F. D. A. method.

FOOD AND DRUG ADMINISTRATION METHOD

APPARATUS AND TECHNIC

TEST ORGANISM AND CULTURE MEDIUM

The test organism is a 22–26-hour culture of *Eberthella typhi* (Hopkins strain) incubated and grown in nutrient broth at 37° C. The broth contains the following ingredients: 5 gm. of Liebig's beef extract, 5 gm. of chemically pure sodium chloride, and 10 gm. of Armour's peptone (for disinfectant testing) in 1,000 c. c. of distilled water. The mixture is boiled for 20 minutes, made up to original weight (or volume) with distilled water, and adjusted with NaOH to pH 6.8 using the colorimetric method (*3, p. 405–421*). It is then filtered through paper, tubed (10 c. c. to each tube), and the tubes plugged with cotton and sterilized at 15 pounds pressure for 40 minutes. The test culture is transferred daily in this medium for not more than one month. At the end of each month, a fresh transfer is made from the stock culture. The stock culture is carried on agar slants of the same composition as the broth medium plus 1½ per cent Bacto-Agar (Difco), adjusted to pH 7.2 to 7.4. This medium is also filtered, tubed, plugged with cotton, sterilized, and slanted. The stock culture is transferred once a month, and the test organism is taken from the month-old stock culture. When the test organism has not been transferred daily, it is advisable to make four or five consecutive daily transfers in broth before using it for testing purposes, to be reasonably sure of its conforming to the phenol resistance requirements. When only one transfer has been skipped the following transfer from the 48-hour culture is usually satisfactory for use after 24 hours. Transfers are made with the platinum loop used in the test. Only cultures giving readings within the following limits are considered satisfactory:

	5 minutes	10 minutes	15 minutes
Phenol:			
1–90	+	+	0
1–100	+	+	+
Or			
1–90	0	0	0
1–100	+	+	0

The following reading is that most usually obtained and is the most convenient:

	5 minutes	10 minutes	15 minutes
Phenol:			
1–90	+	0	0
1–100	+	+	+

PHENOL

The phenol used must meet the requirements of the United States Pharmacopoeia, and in addition the congealing point must not be below 40° C. A 5 per cent solution may be used as a stock solution if kept in a relatively cool place in well-stoppered amber-colored bottles protected from the light. This 5 per cent solution should be standardized with decinormal bromine (described under "phenol" (*10, p. 283*)), or with sodium bromide and bromate solution (*9, pp. 404–405*).

APPARATUS

Besides a number of accurately graduated pipettes, 100-c. c. glass-stoppered graduates or volumetric flasks are almost essential for the making of correct dilutions. All pipettes and graduates should be standardized. The test tubes for containing the dilutions should be large enough to permit transfers being made without touching the sides with the transfer needle. Lipped pyrex (to withstand constant flaming) test tubes 25 by 150 mm. serve very well as these seeding or medication tubes. A water bath for holding the dilutions at the desired temperature must be provided. To maintain the temperature practically constant during the period of the test, the bath should be made so as to contain a relatively large volume per surface area, and should be insulated. The lid is made with well-spaced holes admitting the 25-mm. tube, but not the lip. The most convenient form of subculture tubes (tubes containing medium for incubating the tested organisms, as well as for growing the test culture) are ordinary nonlipped bacteriological test tubes 20 by 150 mm. The racks for holding the subculture tubes may be any convenient style. Blocks of wood with a series of holes bored in them are quite satisfactory. Dimensions depend somewhat on the size of the incubator, but the holes should be well spaced to insure quick selection and easy manipulation during the test. It is an added convenience to have the holes large enough to admit the medication tubes while dilutions are being made. The transfers are made with a 4-mm. (inside diameter) single loop of number 23 B. & S. gage platinum wire, 1½ to 3 inches long, set in a suitable holder such as an aluminum or glass rod approximately 0.5 cm. in diameter. (Fig. 1, A.)

PROCEDURE

One per cent stock dilutions of the substance to be tested (or any other convenient dilution of the disinfectant, depending on the strength) are made up, usually in the glass-stoppered cylinders or volumetric flasks from which the individual dilutions are then prepared. For rapid routine work, the final dilutions may be made directly in the medication tubes. In this case all excess over 5 c.c. must be removed. For more precise work and when high dilutions

are required or volatile substances are dealt with, it is preferable to make up all of the dilutions in volumetric flasks and then transfer 5 c. c. of the final dilution to the medication tubes. These tubes containing 5 c. c. of each dilution (including the phenol control) are placed in the water bath at 20° C. for five minutes until the temperature of the bath is reached. Even slight variations in temperature may affect the results. The dilutions should cover the range of the

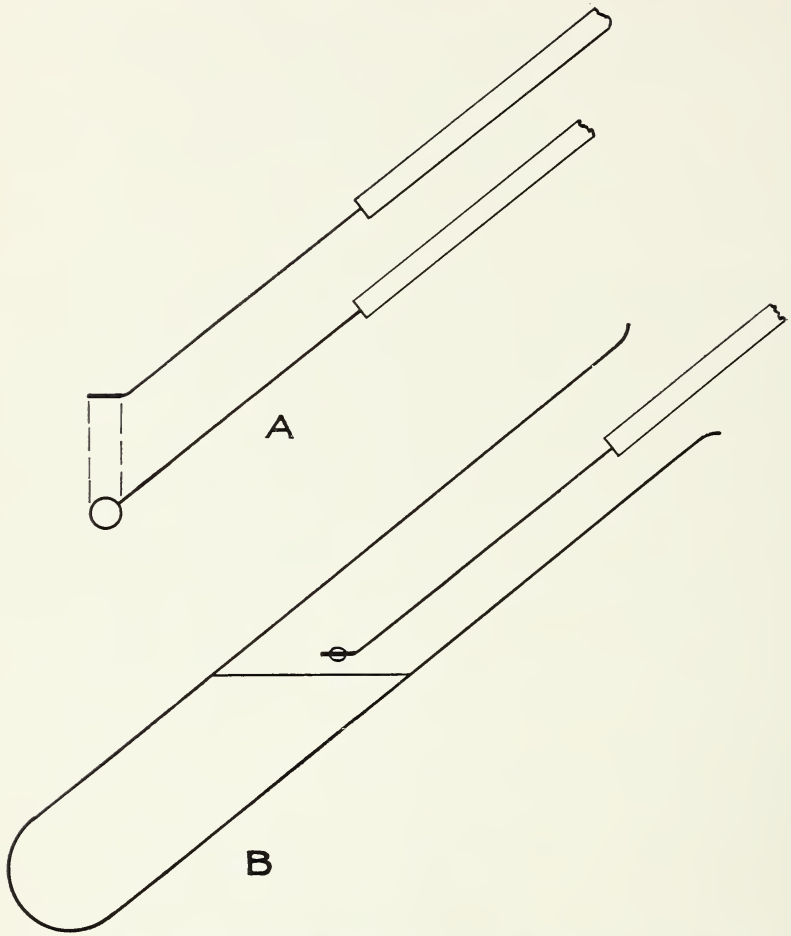

FIGURE 1.—A, Type of needle used in the F. D. A. test: B, the relationship of loop to the surface of the liquid on transfer

killing limits of the disinfectant within 5 and 15-minute periods and should at the same time be spaced sufficiently close together to insure the desired accuracy. Five-tenths of a cubic centimeter of the test culture is then added to each of the dilutions at a time interval corresponding to the interval at which the transfers are to be made. Thus by the time 10 tubes have been seeded at 30-second intervals, four and one-half minutes will have elapsed and a 30-second interval intervenes before the transference to the subcultures is commenced. The culture is added from a graduated pipette hold-

ing sufficient culture to seed all the tubes in any one set. The pipette may be loosely plugged with cotton at the mouth end before sterilizing, as a precautionary measure. Unfiltered culture is used, but it should be thoroughly shaken 15 minutes before use and allowed to settle. The temperature of the culture should be practically that of the water bath before being added.

In inoculating the medication tubes they should be held in a slanting position, after removal from the bath, and the culture run in without the tip of the pipette touching the disinfectant. The tip may be allowed to rest against the side of the tube just above the surface of the liquid. The tubes are agitated gently but thoroughly after the addition of the culture to insure even distribution of the bacteria. Five minutes from the time of seeding the first medication tube, transfer 1 loopful of the mixture of culture and diluted disinfectant from the medication tube to the corresponding subculture tube. To facilitate transfer of uniform drops of the medication mixture, the loop is bent to form a slight angle with the stem and the medication tube is held at an angle of 60°. In other words, as the loop is withdrawn, its plane should be parallel with the surface of the liquid. (Fig. 1, B.) At the end of 30 seconds, a loopful is transferred from the second medication tube to the second subculture tube and the process continued for each successive dilution. Five minutes from the time of making the first transfer, a second set of transfers is begun for the 10-minute period and finally repeated for the 15-minute period. Before each transfer the loop is heated to red heat in the Bunsen flame and the mouth of every tube is flamed. Sterilization of the loop is effected immediately after making the previous transfer (before replugging the tubes) to allow time for sufficient cooling. Time does not permit flaming the tubes after making the transfer. For this reason, care in transferring and seeding is necessary. Due caution is observed to prevent either the seeding pipette or the transfer needle from touching the sides or mouth of the medication tube; neither should cotton threads be found adhering to the sides or mouth of these. After completion of the transferring, the subculture tubes are incubated at 37° C. for 48 hours and results read. Macroscopic examination usually suffices for this, but occasionally agglutination with antityphoid serum will aid in reading doubtful results. A 3-day incubation period or agar streak or microscopic examination may be resorted to in determining feeble growth, especially when organisms other than *Eberthella typhi* are used.

There are certain types of germicidal agents, such as many of the mercury compounds, which give very high results by phenol coefficient tests (*8*). Due to the high inhibitory value of such substances in preventing growth in the subcultures these figures are frequently misleading. For germicides used in the disinfection of such objects as surgical instruments, this is of particular importance and must be taken into account. Failure to appreciate this characteristic of certain compounds is much more likely to lead to error when *Staphylococcus aureus* is used rather than *Eberthella typhi* as the test organism. That false values may not be obtained for products of this type, or for any other disinfectant giving suspiciously high results, the subcultures should contain very large amounts of medium (not less than 200 c. c.) or they should be retransferred by carrying at

least 4 loopfuls from the first subculture to a second tube of broth, as recommended by Shippen (*8*).

Other groups of disinfectants in common use, for which the phenol coefficient method of testing is not well adapted, are those compounds containing chlorine as the active agent as well as oxidizing agents in general. These are affected so materially by the presence of organic matter that a phenol coefficient statement may grossly misrepresent their value under practical conditions of use and is very apt to be misleading to the consumer when placed on the label.

CALCULATION OF THE PHENOL COEFFICIENT

The results of the test are expressed in terms of the phenol coefficient. This represents the germicidal value of the diluted disinfectant as compared with the diluted phenol control. It is a figure obtained by dividing the numerical value of the greatest dilution (the denominator of the fraction expressing the dilution) of the disinfectant capable of killing *Eberthella typhi* in 10 minutes but not in 5 minutes, by the greatest dilution of phenol showing the same results; that is, by the phenol control. Thus, if the results were as follows:

	5 minutes	10 minutes	15 minutes
Disinfectant (X):			
1–300	0	0	0
1–325	+	0	0
1–350	+	0	0
1–375	+	+	0
1–400	+	+	+
Phenol:			
1–90	+	0	0
1–100	+	+	+

The phenol coefficient would be $\frac{350}{90} = 3.89$.

If none of the dilutions shows growth in 5 minutes and killing in 10 minutes, the hypothetical dilution may be estimated in certain cases. This may be done only when any three consecutive dilutions show the following results:

The first, no growth in 5 minutes; the second, growth in 10 minutes but not in 15 minutes; and the third, growth in 15 minutes; for example:

If the results were as follows:

	5 minutes	10 minutes	15 minutes
Disinfectant (X):			
1–300	0	0	0
1–350	+	+	0
1–400	+	+	+
Phenol:			
1–90	0	0	0
1–100	+	+	0

the estimated phenol coefficient would be $\frac{325}{95} = 3.42$.

To avoid giving an impression of fictitious accuracy, the phenol coefficient is calculated to the nearest 0.1 unless the coefficient is less than 1.0. Thus, in the examples cited above, the phenol coefficients would be reported as 3.9 and 3.4 instead of 3.89 and 3.42.

In the preceding description, *Eberthella typhi* has been mentioned as the test organism. Wherever any expression of phenol coefficient occurs in literature, on labels, etc., it is assumed to mean the *E. typhi* phenol coefficient, unless otherwise stated. It is, however, the distinct intention of this department not to limit the test to the use of one organism. In fact, the test has been found adaptable to the use of a wide variety of bacterial species in the determination of phenol coefficients. In cases where some of the more strictly parasitic bacteria are used, modifications in media are necessitated, and, of course, a change in the phenol dilutions. The writers are not in a position at this time to prescribe the limits of resistance for many of the organisms that might be used. Therefore discussion of the exact technic is here omitted, with the exception of that for *Staphylococcus aureus*. Suggestions for the use of certain representative types may, however, be found in a paper by Reddish (5). When any test organism other than *E. typhi* is used it should be distinctly designated when stating the phenol coefficient.

S. aureus has been found to be an extremely useful organism for testing disinfectants and antiseptics and has been used for this purpose for a number of years. When substituted in the above test the technic remains exactly the same. The phenol dilutions, however, must be changed. The resistance of any strain of *S. aureus* used in this test must come within the following limits: At 20° C. it must survive a 1–60 dilution of phenol for 5 minutes and a 1–70 dilution for 15 minutes. The following is the minimal resistance that would be acceptable:

	5 minutes	10 minutes	15 minutes
Phenol:			
1–60	+	0	0
1–70	+	+	+

In the bacteriological examination of disinfectants, the *Eberthella typhi* and the *S. aureus* phenol coefficients give, in general, sufficient information to render tests with other organisms unnecessary, except in special instances. The commonly accepted criterion that disinfectants for general use be employed at a dilution equivalent to the germicidal efficiency of 5 per cent phenol against *E. typhi* (that is, 20 times the *E. typhi* phenol coefficient) allows a reasonable margin of safety for the destruction of infective agents likely to be the object of general disinfection about premises with the possible exception of *Mycobacterium tuberculosis*. *S. aureus*, due to its ubiquity, resistance and every-ready tendency to cause infection, should always be employed in testing those substances recommended for personal use or as applications for wounds. If the disinfectant is recommended for use externally the temperature of test should be 20° C., but where such substances are recommended for use in the body

cavities, such as for mouth washes, gargles, douches, etc., this test should be conducted at 37°. In such case the test should be designated "The F. D. A. method (special) *S. aureus*, 37° C." At body temperature the *S. aureus* should show the following resistance to phenol:

	5 minutes	10 minutes	15 minutes
1–80	+	0	0
1–90	+	+	+

Or

	5 minutes	10 minutes	15 minutes
1–80	+	0	0
1–90	+	+	0

The previous description of this method (*5*) differed from this only in allowing a slightly wider latitude in the resistance of the test organism against phenol.

OTHER TESTS FOR GERMICIDES AND ANTISEPTICS[4]

The limitations of the phenol coefficient make it necessary in some cases to judge the germicidal preparation by other tests or by additional tests. This is particularly true of preparations that are not completely soluble or miscible in water. It is also true of certain preparations designated as antiseptics.

Soluble antiseptics or antiseptics completely miscible with water can be tested, of course, by the procedure already described as the F. D. A. *Staphylococcus aureus* phenol coefficient method. In the testing of these substances, however, the phenol coefficient is not obtained necessarily, the phenol figure being used merely as a check of the resistance of the test organism. The information desired is the concentration which will kill in five minutes.

In an effort to simulate practical conditions, it is frequently advisable to conduct the tests in the presence of blood serum. Sterile horse serum in a concentration of 10 per cent is ordinarily used, both in the germicidal and inhibitory tests. Special claims and uses of a product, however, frequently indicate the desirability of a higher concentration of this organic enrichment.

The following methods designed for the testing of insoluble and immiscible products are in use in this laboratory at the present time. Some of them have been used for years and have been described previously (*6*). Laboratory tests, of course, can not duplicate the exact

[4] According to current usage the word "antiseptic" has two meanings; to kill bacteria or to prevent their growth, depending upon the use of the product. Products such as salves, ointments, and dressings that remain in contact with the body for long periods of time, may be designated properly as antiseptics if they inhibit the growth of bacteria. On the other hand, mouth washes, douches, gargles, and preparations of like nature are in contact with the body for but brief periods of time and exert negligible inhibitory action. These may be described properly as antiseptics only if they will destroy bacteria under the conditions of use; that is, in the dilutions recommended and in a period of time comparable to that in which they would have an opportunity to act when used as directed.

conditions found in practice. The procedures here outlined, however, are as close an approach to practical conditions as is feasible in routine laboratory tests, and reveal the obviously useless preparations. It should be noted that inhibitory tests are considered along with other facts in interpreting whether or not the substance will be of value in practical use. It must be remembered that not only bacteriological but physiological and pharmacological facts frequently must be taken into consideration in judging many substances.

THE WET FILTER-PAPER METHOD

The wet filter-paper method is a germicidal test rather than a test of inhibitory properties. It is used when the substance to be tested is not soluble or completely miscible with water, or for substances that are to be used in high concentration, such as soaps, tooth pastes, suppositories, dyes, dusting powders, salves, and ointments. If the substance is to be used in the body cavities the test is carried out at 37° C.; if not, the test is carried out at 20°, or at room temperature, and the temperature is recorded.

No. 2 Whatman filter paper is cut into pieces about 0.5 cm. square, and sterilized in a plugged test tube at temperatures below 170° C. to prevent charring. A suitable number of the paper squares are then impregnated with *Staphylococcus aureus*, or other test organisms, by immersion in a 24-hour broth culture of the organism. The culture must have the standard resistance required for phenol coefficient testing. The wet inoculated squares are then placed in the liquid or solid substance to be tested in such a way as to be completely covered and in intimate contact. At the end of 5 minutes, 10 minutes, 15 minutes, or 1 hour, or any other desired length of time, the wet papers are removed with a sterilized, stiff, platinum wire bent at a sharp angle to form a hook and placed in 10 c. c. of sterile broth. After as much of the disinfectant as possible has been removed (in the case of sticky substances, the needle must be used to aid in freeing the squares of adherent germicide) the squares are retransferred to a fresh tube of sterile broth (10 c. c.) and the tubes incubated at 37° for 48 hours, when they are observed for evidence of growth.

It will be noted that in this test resubcultures are always required, since the first tube of broth to which the filter-paper squares have been added frequently contains sufficient antiseptic to exhibit inhibition of growth. Both tubes of broth are usually incubated.

THE DRY FILTER-PAPER METHOD

The dry filter-paper method is used in tests of fumigants and of oils that are to be used where moisture is absent. It is similar to the wet filter-paper test, squares of paper being used that have been impregnated as described under the test above, except the squares are dried for two days in a sterile Petri dish in the 37° C. incubator. This test can be used successfully only with organisms capable of resisting the drying. *Eberthella typhi* will not withstand the drying. In the writers' work *Staphylococcus aureus* is the usual test organism. The inoculated dried paper squares may be used at any time after drying up to 30 days, but the resistance of the organism at no time should fall so low that it is incapable of withstanding a 1–80 dilution

of phenol for five minutes at 20°. It should be noted that control tests with nonmedicated squares should always be carried out to test the viability of the test organism. As in the wet filter-paper method, resubcultures are always necessary.

THE AGAR-PLATE METHOD

The agar-plate method is a test for inhibitory properties and is used for substances remaining in contact with the body in the absence of serous body fluids. Examples of substances which may be tested by this method are salves, dusting powders, creams, plasters, pads, adhesive tape, catgut, and suppositories. The test organism

FIGURE 2.—Agar-plate method showing good antiseptic inhibitory properties and good diffusion (dark zone)

ordinarily used is *Staphylococcus aureus*, but for special purposes the test may be used with any organism capable of growing on agar. The agar is of the same composition as that previously described for carrying stock cultures of the test organism.

Fifteen to twenty cubic centimeters of agar is melted and cooled to 42°–45° C. To this is added 0.1 c. c. of a 24-hour broth culture of the test organism. The inoculated agar is then poured into a sterile Petri plate and allowed to harden. As soon as the agar has hardened, the test substance is placed in intimate contact with the sur-

face of the agar. If a salve, it is first warmed just sufficiently to soften it and thus secure a complete peripheral contact. As a control, warmed sterile petrolatum may be placed on another portion of the plate. The plates are incubated 24–48 hours under unglazed porcelain tops at 37° C. and then are examined for evidence of inhibition. If the preparation is antiseptic or inhibitory, a zone of clear agar will be noted around the place where the substance has been in contact and the width of the zone will indicate the diffusibility of the inhibitory (antiseptic) agent. If there is no inhibition, growth of the test organism will be observed adjacent to and even under the test substance. (Figs. 2, 3, 4.)

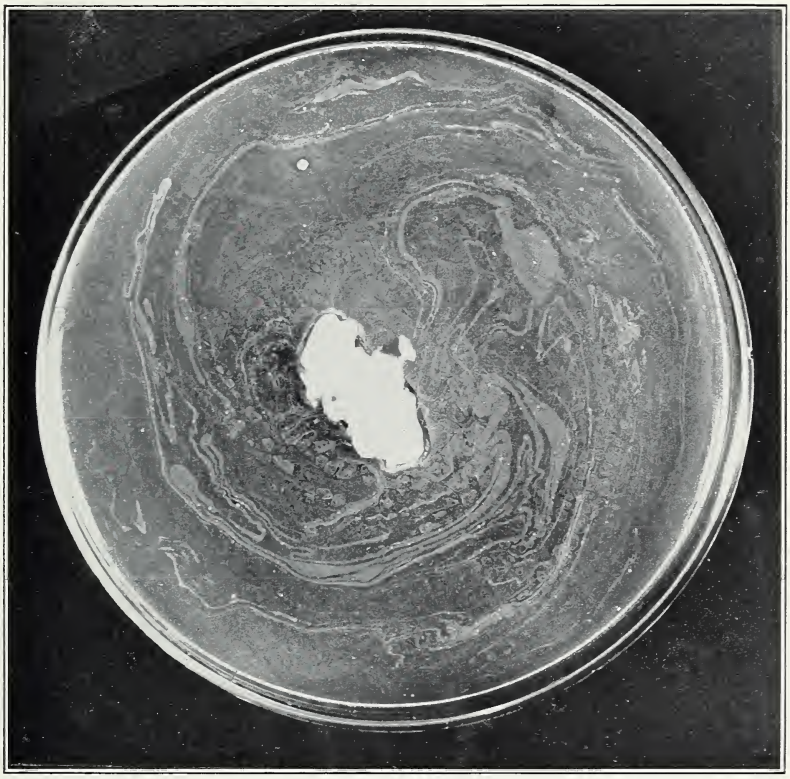

FIGURE 3.—Agar-plate method showing no antiseptic properties, or evidence of diffusion

THE SERUM AGAR-PLATE METHOD

Preparations recommended for use on open wounds, cuts, etc. will be effective only if they exhibit activity in the presence of serous fluids. In testing such preparations the agar-plate method is modified by the addition of 10 per cent sterile horse serum to the agar.

THE AGAR CUP-PLATE METHOD [5]

The agar cup-plate method is merely a variation of the agar-plate method. It is to be used on products liquid at the temperature of the test. The agar or serum agar is inoculated as in the agar-plate method. Before the agar cools, a depression or cup is made in the medium by standing a sterile flat-bottomed glass tube, 1.5 cm. in diameter, in the liquefied agar. On hardening, the glass tube is removed by slightly twisting and pulling at the same time. Insertion of a sterile wire down the side of the tube for the introduction of air will eliminate much of the cracking of the agar. Another method of preparing the agar-cup plate is to allow the medium to

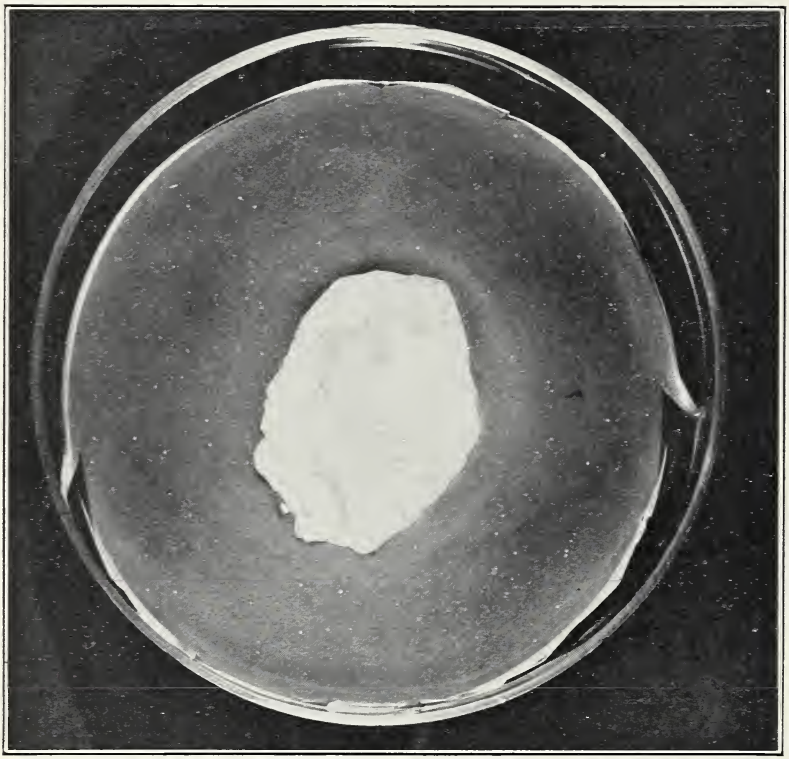

FIGURE 4.—Agar-plate method showing no antiseptic properties but good diffusion; note stimulation zone. (The culture medium has contracted because of drying)

harden and then cut out a disk in the agar, by means of a cork borer, 1.5 cm. in diameter. One or two drops of melted agar are placed in the cup to seal cracks or crevices. After the agar cup plate is prepared, 6 drops of the liquid to be tested are placed in the cup and the plate incubated under an unglazed porcelain top for 24 to 48 hours. If there is a clear zone about the cup, the substance under test has inhibitory properties. Here, as well as in the agar-plate test, the agar in the clear zone may be tested for growth by subculture in

[5] The authors are indebted to L. C. Himebaugh for this method.

broth to indicate whether the action is germicidal or merely inhibitory. (Figs. 5, 6, 7.)

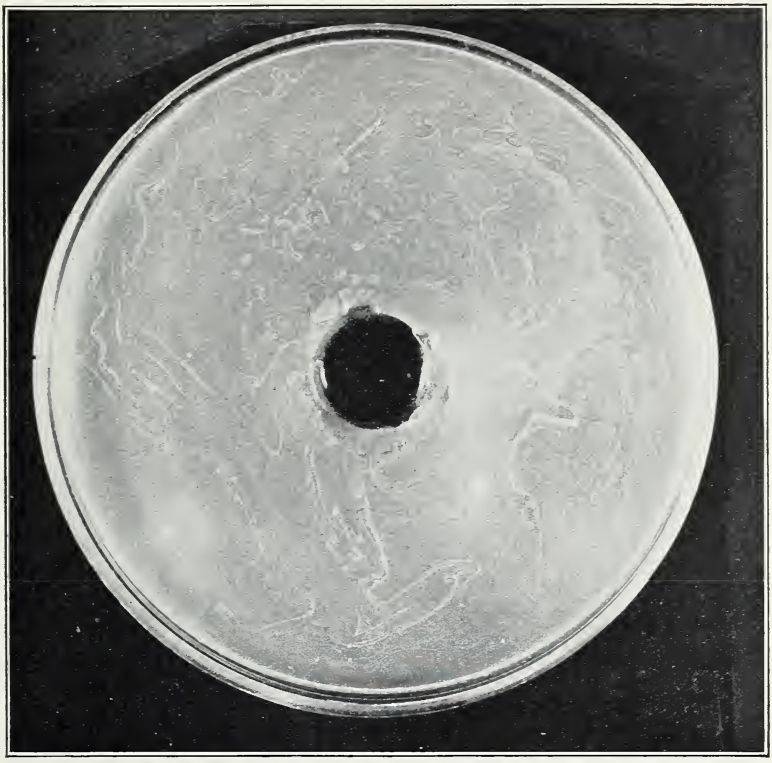

FIGURE 5.—Agar cup-plate method; no antiseptic properties; colonies grow to edge of cup

TESTS IN THE PRESENCE OF ORGANIC MATTER

In general, the tests outlined above will take care of the bulk of the preparations coming to this laboratory. However, special tests may be required to determine the value of products recommended for certain purposes. For instance, recommendations on the label may make advisable the use of various additions of organic matter, such as increased amounts of peptone or the addition of gelatin, blood, ascitic fluid, saliva, urine, or feces, depending upon the information desired.

TENTATIVE GUIDE TO METHOD APPLICABLE IN A GIVEN CASE

Substance	Method applicable
Soluble disinfectants:	
A. Preparations for general use [6]	Phenol coefficient—*E. typhi* at 20° C.
B. Preparations for antiseptic use	Phenol coefficient technic—*S. aureus* 20° C. or 37° C. as indicated.
C. Preparations for surgical instruments.	Phenol coefficient technic—*S. aureus* 20° C.

[6] For oxidizing compounds and compounds depending on active chlorine, see p. 8. For compounds suspected of high inhibitory properties, such as mercury preparations, see p. 7.

Substance	Method applicable
Insoluble and concentrated disinfectants:	
A. Preparations for general use (oils, tarry substances, powders, lime, etc.).	Wet filter paper—*E. typhi* and *S. aureus* Room temperature.
B. Preparations for use on dry surfaces (spraying oils, sweeping compounds, powders, lime).	Dried filter paper—*S. aureus*. Room temperature.

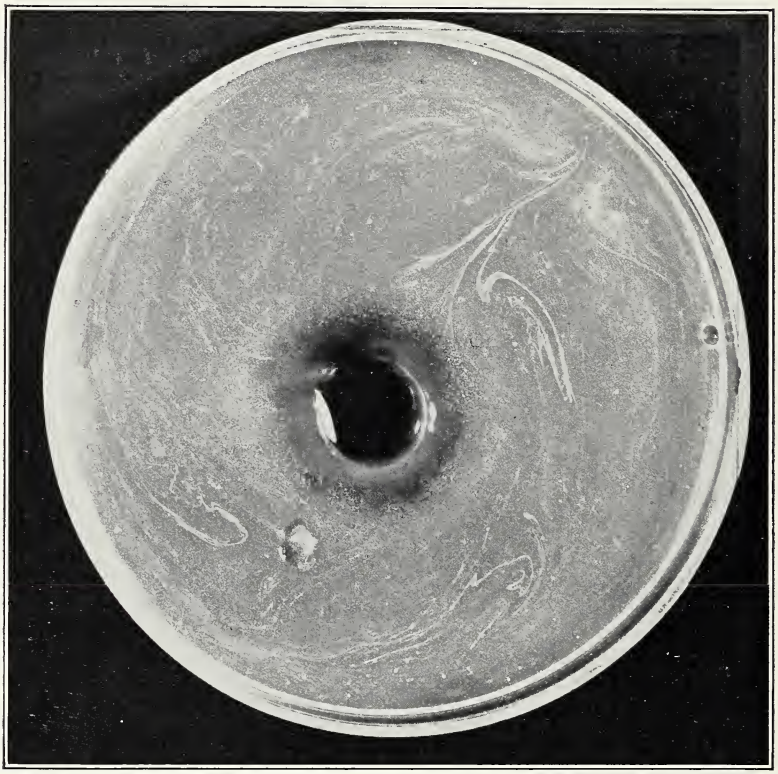

FIGURE 6.—Agar cup-plate method showing moderate antiseptic properties. Inhibition only fair; absolute inhibition confined to small area on one side of cup. Partial inhibition exhibited over wider area. An outside ring of stimulation may be noted

C. Preparations for fumigation (filter paper exposed to gas in confined space).	Wet filter paper—*E. typhi* and *S. aureus;* room temperature. Dried filter paper —*S. aureus;* room temperature. (Extension of time may be indicated.)
Soluble and liquid antiseptics:	
A. Preparations to be applied for a short time (washes, mouth washes, gargles, douches, etc.)	Phenol coefficient technic—*S. aureus* 20° C. or 37° C. as indicated.
B. Preparations for use on open wounds, etc. (washes).	Phenol coefficient technic—*S. aureus* 20° C. or 37° C. as indicated. (Tested in presence of 10 per cent serum.)
C. Preparations remaining on site of application (dyes, wet dressings, rubbing preparations, etc.).	Agar cup-plate.

Substance	Method applicable
Soluble and liquid antiseptics—Continued.	
D. Preparations for use on open wounds, etc. (dyes, wet dressings, etc.).	Serum agar cup-plate.
E. Preparations remaining on site of application but claiming germicidal properties.	Wet filter paper—*S. aureus* 37° C. Extension of time may be indicated.

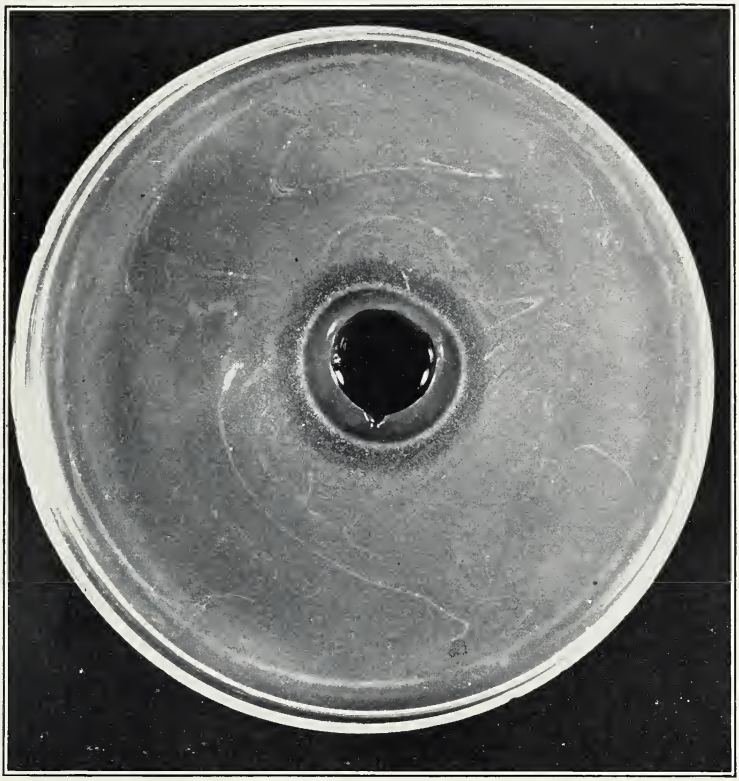

FIGURE 7.—Agar cup-plate method showing antiseptic properties. Definite inhibition zone surrounded by a stimulation zone and a secondary zone of partial inhibition

Solid soluble antiseptics:	
A. Lozenges, tablets, etc.	Wet filter paper—*S. aureus* 20° C. or 37° C. as indicated. Note: Saturated aqueous solution or in dilution indicated.
Insoluble and concentrated antiseptics:	
A. Preparations remaining on site of application (dusting powders, ointments, salves, suppositories, plasters, dressings, etc.)	Agar plate.
B. Preparations remaining on site of application but claiming germicidal properties.	Wet filter paper—*S. aureus* 37° C. Extension of time may be indicated.
C. Preparations for use on open wounds, etc.	Serum agar plate.
Antiseptic materials, appliances, etc.:	
A. Bandages, dressings, catgut, etc.	Serum agar plate.
B. Tape, pads, etc.	Agar plate.

Substance	Method applicable
Solid and semisolid antiseptics:	
A. Preparations used for a short time (soaps, soap powders, tooth pastes, tooth powders, etc.).	Wet filter paper—*S. aureus* 20° C. or 37° C. as indicated. (Note: Undiluted and diluted with equal parts water or diluted with sufficient water to form a thick paste or heavy emulsion.)
Disinfectants and antiseptics for use in the absence of organic matter:	
A. Preparations for drinking water.[7]	Phenol coefficient technic—*E. typhi* 20° C. (Note: 0.1 c. c. of culture to 10 c. c. of diluted preparation.)
Intestinal antiseptics:[8]	

FIGURES USEFUL IN MAKING DILUTIONS IN TESTING DISINFECTANTS (11)

(5 c. c. of disinfectant + 95 c. c. of distilled water = solution A)

Dilution	Solution A	Distilled water	Solution A	Distilled water	Solution A	Distilled water
	C. c.	*C. c.*	*C. c.*	*C. c.*	*C. c.*	*C. c.*
1:20	= 20	+ 0	or 10	+ 0	or 4	+ 0
1:25	= 20	+ 5	or 10	+ 2½	or 4	+ 1
1:30	= 20	+ 10	or 10	+ 5	or 4	+ 2
1:35	= 20	+ 15	or 10	+ 7½	or 4	+ 3
1:40	= 20	+ 20	or 10	+ 10	or 4	+ 4
1:45	= 20	+ 25	or 10	+ 12½	or 4	+ 5
1:50	= 20	+ 30	or 10	+ 15	or 4	+ 6
1:55	= 20	+ 35	or 10	+ 17½	or 4	+ 7
1:60	= 20	+ 40	or 10	+ 20	or 4	+ 8
1:65	= 20	+ 45	or 10	+ 22½	or 4	+ 9
1:70	= 20	+ 50	or 10	+ 25	or 4	+ 10
1:80	= 20	+ 60	or 10	+ 30	or 4	+ 12
1:90	= 20	+ 70	or 10	+ 35	or 4	+ 14
1:100	= 20	+ 80	or 10	+ 40	or 4	+ 16
1:110	= 20	+ 90	or 10	+ 45	or 4	+ 18
1:120	= 20	+ 100	or 10	+ 50	or 4	+ 20
1:130	= 20	+ 110	or 10	+ 55	or 4	+ 22
1:140	= 20	+ 120	or 10	+ 60	or 4	+ 24
1:150	= 20	+ 130	or 10	+ 65	or 4	+ 26
1:160	= 20	+ 140	or 10	+ 70	or 4	+ 28
1:170	= 20	+ 150	or 10	+ 75	or 4	+ 30
1:180	= 20	+ 160	or 10	+ 80	or 4	+ 32
1:200	= 20	+ 180	or 10	+ 90	or 4	+ 36
1:200	= 20	+ 180	or 4	+ 36	or 2	+ 18
1:225	= 20	+ 205	or 4	+ 41	or 2	+ 20½
1:250	= 20	+ 230	or 4	+ 46	or 2	+ 23
1:275	= 20	+ 255	or 4	+ 51	or 2	+ 25½
1:300	= 20	+ 280	or 4	+ 56	or 2	+ 28
1:325	= 20	+ 305	or 4	+ 61	or 2	+ 30½
1:350	= 20	+ 330	or 4	+ 66	or 2	+ 33
1:375	= 20	+ 355	or 4	+ 71	or 2	+ 35½
1:400	= 20	+ 380	or 4	+ 76	or 2	+ 38
1:450	= 20	+ 430	or 4	+ 86	or 2	+ 43
1:500	= 20	+ 480	or 4	+ 96	or 2	+ 48

[7] In the cases where chlorine compounds are used for this purpose the effectiveness is usually judged on the basis of "available" chlorine content.

[8] Intestinal antiseptics do not readily lend themselves to laboratory tests. When medical opinion concerning the physiological and therapeutic properties of such preparations is to be confirmed by the results of bacteriological tests, the products may be considered to be germicidal only when tested in the presence of liberal amounts of organic matter, such as saliva, feces, etc.

(1 c. c. of disinfectant + 99 c. c. of distilled water = solution B)

Dilution	Solution B		Distilled water		Solution B		Distilled water		Solution B		Distilled water
	C. c.		C. c.		C. c.		C. c.		C. c.		C. c.
1:100	= 100	+	0	or	10	+	0				
1:110	= 100	+	10	or	10	+	1				
1:120	= 100	+	20	or	10	+	2				
1:130	= 100	+	30	or	10	+	3				
1:140	= 100	+	40	or	10	+	4				
1:150	= 100	+	50	or	10	+	5				
1:160	= 100	+	60	or	10	+	6				
1:180	= 100	+	80	or	10	+	8				
1:200	= 100	+	100	or	10	+	10	or	4	+	4
1:225	= 100	+	125	or	10	+	12½	or	4	+	5
1:250	= 100	+	150	or	10	+	15	or	4	+	6
1:275	= 100	+	175	or	10	+	17½	or	4	+	7
1:300	= 100	+	200	or	10	+	20	or	4	+	8
1:325	= 100	+	225	or	10	+	22½	or	4	+	9
1:350	= 100	+	250	or	10	+	25	or	4	+	10
1:375	= 100	+	275	or	10	+	27½	or	4	+	11
1:400	= 100	+	300	or	10	+	30	or	4	+	12
1:400	= 10	+	30	or	4	+	12	or	2	+	6
1:450	= 10	+	35	or	4	+	14	or	2	+	7
1:500	= 10	+	40	or	4	+	16	or	2	+	8
1:550	= 10	+	45	or	4	+	18	or	2	+	9
1:600	= 10	+	50	or	4	+	20	or	2	+	10
1:650	= 10	+	55	or	4	+	22	or	2	+	11
1:700	= 10	+	60	or	4	+	24	or	2	+	12
1:750	= 10	+	65	or	4	+	26	or	2	+	13
1:800	= 10	+	70	or	4	+	28	or	2	+	14
1:850	= 10	+	75	or	4	+	30	or	2	+	15
1:900	= 10	+	80	or	4	+	32	or	2	+	16
1:900	= 5	+	40	or	4	+	32	or	2	+	16
1:1, 000	= 5	+	45	or	4	+	36	or	2	+	18
1:1, 100	= 5	+	50	or	4	+	40	or	2	+	20
1:1, 200	= 5	+	55	or	4	+	44	or	2	+	22
1:1, 300	= 5	+	60	or	4	+	48	or	2	+	24
1:1, 400	= 5	+	65	or	4	+	52	or	2	+	26
1:1, 500	= 5	+	70	or	4	+	56	or	2	+	28
1:1, 600	= 5	+	75	or	4	+	60	or	2	+	30
1:1, 700	= 5	+	80	or	4	+	64	or	2	+	32
1:1, 800	= 5	+	85	or	4	+	68	or	2	+	34
1:2, 000	= 5	+	95	or	4	+	76	or	2	+	38
1:2, 200	= 5	+	105	or	4	+	84	or	2	+	42
1:2, 400	= 5	+	115	or	4	+	92	or	2	+	46
1:2, 600	= 5	+	125	or	4	+	100	or	2	+	50
1:2, 800	= 5	+	135	or	4	+	108	or	2	+	54
1:3, 000	= 5	+	145	or	4	+	116	or	2	+	58
1:3, 200	= 5	+	155	or	4	+	124	or	2	+	62

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U. S. GOVERNMENT PRINTING OFFICE: 1931

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