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PENETRATIVE POWERS

of

DISINFECTANTS

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INTRODUCTION

Many authorities in the field of antiseptics and disinfectants have freely and justifiably criticized and readily altered the only accepted standard method of testing these compounds for their actual value. Since this method does not, obviously, give the user of commercial preparations more than a vague idea of their practical effectiveness in actual use, the attempt has been made to devise a method that will attain this end. It was decided that if any one common characteristic which all compounds must possess to be of practical value could be chosen and a feasible but relatively simple test devised to evaluate this characteristic a step forward would be made.

In view of the foregoing it seemed that the one characteristic which must be common to all compounds irrespective of their chemical constitution, is that of penetrative power, since in order to kill quickly they must be able to pass through the wall, or ectoplasm, of the organism and reach the life center. On first consideration it might appear a relatively simple matter to select or devise a test which would evaluate this characteristic, giving the proper evaluation to all compounds to which it was applied. The method so chosen is one to be described later as a speed test, and which in order to withstand critical analyses must have a substantial foundation that will reach beyond mere theoretical considerations. It was deemed advisable to first ascertain, as far as practically

possible, just how a few of the outstanding representatives of the various groups of compounds operated in bringing about destruction of organisms. Therefore, such methods were devised and employed to determine this particular phase but at the same time keeping in mind the specific item, penetrative power, which theoretical consideration demands must be present.

HISTORICAL REVIEW

The literature abounds in studies designed to systematize the investigation of the effect of killing agents upon organisms. Only the more important works will be reviewed in order to show the general trend. Robert Koch (32), 1881, started mercury on the road to questionable fame as an excellent bactericidal agent by the use of bacterial impregnated threads. In 1889-1891, Geppert (20) proved that the unneutralized sublimate carried by the thread in Koch's method was responsible for the exceptionally high values obtained with mercuric chloride. To eliminate this factor Kronig and Paul (33),1897, employed bacterial coated garnets as test objects and in their thorough study formulated tenets which have served as a foundation for subsequently devised methods. Rideal and Walker (39), 1903, started the trend toward the present day phenol coefficient method by devising a test tube method of examining chemicals for their killing action. Chick (10), 1908, did much to ferret out the probable mode of action of killing agents, and also introduced the use of added organic matter (Chick and Martin (11),1908) in the testing methods. In 1911, Anderson and McClintic (2) published a method designed to eliminate some of the objectional features of the Rideal-Walker method. This new method was known as the Hygienic Laboratory Method. Shippen (44) combined the best features of the Rideal-Walker and Hygienic Laboratory Method in a test which he put into practice about 1916 and which was published in 1927 by Reddish (37) as the "R-W Modified Method". Very little change

has been made in this test which is known today as the "Food and Drug Administration Phenol Coefficient Method".

In 1924 Conover and Laird (14) published a method utilizing petri-plates instead of seeding tubes. This was designated as a Direct Unit Phenol Coefficient but has received little recognition. Allen (1), 1929, proposed a method for examining antiseptics and disinfectants consisting of direct application of the test solution to the agar slant of a specially prepared culture. In 1933 Jensen and Jensen (23) proposed a modification of the method of Kronig and Paul (33) by using cover glasses instead of garnets.

Since that time more attention has been directed towards the toxicity factor of antiseptics and disinfectants in order to give a more practical aspect to the phenol coefficient. This is demonstrated by the work of Salle, McOmie and Shechmeister (40,41), 1937, employing embryonic chick heart, Nye (36), 1937, using leucocytes, and Samuels (42), 1938, using frog pharyngeal epithelium. All demonstrated that the majority of chemicals ordinarily employed as bacteriostatic and bactericidal agents in the presence of tissue were more toxic for tissue cells than for bacteria.

As illustrated by this brief review the bulk of the evolutionary work since the time of Koch has been directed toward introduction of new methods and refinement of the ones existing. Scant attention has been given to the underlying factor of penetration. To avoid a lengthy review only those works unquestionably dealing with penetration will be reported.

In 1897 Vincent (47) examined the ability of various chemicals to sterilize fecal matter pointing out that it was quite difficult to obtain penetration of the organic particles to kill the enclosed bacteria. Claudius (12), 1902, while searching for a method of sterilizing and preserving catgut, obtained penetration with iodine as demonstrated by the death of the organisms enclosed in the minute folds of the tissue. Kendall and Edwards (25), 1911, employed short uniform cylinders of seeded agar from which a small center core was removed at the end of the exposure period to demonstrate the penetrative ability of various substances. Seelig and Gould (43), 1911, used celloidin capsules and living animal skin to demonstrate the penetration of alcohol and iodine. In 1918 the committee on Standard Methods of Examining Disinfectants (13) in making their recommendations state that "A complete study of a disinfectant must also include its physical and chemical behavior in the medium in which it is employed, as for example, its diffusibility or powers of penetration, and especially its chemical permanence".

Carnot and Dumont (7), 1918, employed seeded agar plates containing a perforated glass cup centrally embedded to determine the penetrative powers of the compounds used in dressing war wounds. Chambers (8), 1922, worked with starfish eggs demonstrating the penetration of their walls by the acid and alkali groups of $NaHCO_3$ and NH_4Cl, respectively. Hirschfelder Malmgren and Creavy (21),1925, produced artificial edema in the dog and observed penetration of an intravenously injected dye from the blood vessels into the edema

fluid. Hirschfelder and Wright (22), 1930, in studying the action of dyes upon proteins, state that an antiseptic acts to "prevent growth of microorganisms by merely adhering to and altering the bacterial surface so as to prevent cell division; and it kills them either by altering the surface layer more intensely or by penetrating into the cell body and injuring its internal metabolism or by both processes simultaneously or successively".

In 1930 Knaysi (27-31) published a series of papers on disinfection including an extensive review dealing with the various conflicting theories as to the mode of action of killing agents. Knaysi (30) points out that penetration of the cell wall of bacteria is the one all important factor which has been consistently neglected. Knaysi and Gordon (29), 1930, demonstrated the penetration of yeast cells by iodine and mercuric chloride.

Karns (24), Karns, Cretcher, and Beal (25), 1932, studied various solutions of iodine using silk and guinea pig skin to demonstrate the mechanism of penetrative superiority of aqueous-potassium iodide solutions of iodine over alcoholic solution of this element. Biskind (5, 6), 1932, worked in conjunction with this group but employed frog skin to demonstrate that the aqueous forms of iodine were superior to the alcoholic forms from the standpoint of speed of penetration. Nyiri and Jannitti (35), 1932, studied the fate of iodine placed upon the unbroken skin of dogs and rabbits. They found that iodine penetrated the live skin in a period of 162 hours but only in the form of its compounds such as KI, and never as free iodine.

Mallmann and Chandler (34), 1933, found that of a group of recognized disinfectants only colloidal iodine was able to penetrate and render sterile the finely divided particles of avian fecal material.

Statement of the Problem

The tendency of the general consumer to employ one disinfectant or antiseptic for a variety of purposes rather than the specific purpose for which it is adaptable necessitated choosing the most severe of all the conditions which compounds must undergo in practical use. These conditions are: (1) Brief contact with the surface to be disinfected, usually under five minutes; (2) organic matter such as blood, serum, pus, dirt, in or beneath which the organisms are located; and (3) possible subsequent dilution.

Since disinfectants, unlike antiseptics and allied compounds of the bacteriostatic type, must kill rapidly, they are not allowed the privilege of rendering organisms innocuous by interfering with their enzymatic processes thereby leading to gradual extinction, but must reach and destroy the life center of the organism immediately. Therefore, assuming the conditions chosen to be sufficiently severe it was decided that the most important single feature which these compounds must possess (disinfectants primarily, antiseptics to a lesser degree, or not at all) is the ability to penetrate organisms.

It is difficult to prove just how a substance kills micro-organisms and because of their minute size it would be equally as hard to prove that the substance had penetrated the bacterial cell; hence, it was decided to employ methods of a grosser nature and by analogy apply the information obtained to bacteria thereby deriving the single test desired.

It would be well, before proceeding further, for the sake of clarity, to draw a line of distinction, even though arbitrary, between the commonly interchanged words antiseptic and disinfectant in order that a consistent meaning may be conveyed in this paper. The term antiseptic will be applied to compounds which render organisms innocuous, or incapable of exhibiting evidence of life, from the moment of contact, but only as long as the two are in contact. The term disinfectant will be used in the sense that the compound employed will kill the organisms during the time of contact implying that its subsequent removal will not result in the organisms exhibiting life. The terms disinfectant, germicide, bactericide, and parasiticide will be used synonymously; and the terms antiseptic, bacteriostat, and parasitistat will be used in like fashion. Further, to avoid confusing terminology, the term compound will be used throughout the work in a general sense to represent the substances used whether they are elements, or compounds.

Phenol Coefficients

To have information from an accepted standard method with which to compare, several phenol coefficients were run on each compound and the average taken as the basis for comparison with the results of other workers as well as for the experimental methods* to be described below. The method employed was that described in

* All media (nutrient broth and nutrient agar) employed throughout this work was prepared according to the specifications given in U.S.D.A. Circular No. 198.

the U.S.D.A. Circular No. 198, as "The F.D.A. Method, Staphylococcus aureus, 20°C ". To avoid inhibitory effects of the compound conveyed to the subculture a second subculture was made into plain broth (Shippen). At the same time that subcultures were made from the test solutions a subculture was also made into a broth containing a substance which stopped the action of the compound that was introduced.

The Staphylococcus aureus culture (originally obtained from Food and Drug Administration) used in the phenol coefficient tests as well as throughout the remainder of the work was taken from the stock collection of the Bacteriology Department of Michigan State College and answered all the requirements demanded by the F.D.A. Method. The Eberthella typhosa strain used in one part of the work was likewise obtained from the same source and met standard requirements for this species.

Colloidal Iodine* was selected as the chief representative of the halogen group while Lugol's solution and Tincture of Iodine (both prepared according to U.S.P.X) were included to demonstrate the important role different solvents play in disinfection. Merthiolate was chosen as representative of the newer type of mercurial compounds which are supposed to occupy a more or less intermediate position between the theoretically ideal disinfectant and the theoretically ideal antiseptic. Brilliant green was selected as representative of the dye compounds and as probably the nearest to

* Throughout this paper the term colloidal iodine will be used to refer specifically to Iodine Suspensoid Merck according to Dr. W. L. Chandler.

the theoretically ideal antiseptic or bacteriostatic type of compound. Phenol was employed as a reliable standard representative of the coal tar series. Chlorine, though another representative of the halogen group would seem superfluous, was, nevertheless, included because of the large variety of agents on the market which have this element as the active ingredient. Two types of such compounds were included, both commercial products, HTH as representative of the inorganic chlorine compounds, and azochloramid as a representative of the slower acting organic compounds.

The data derived (Table I) show without doubt that elemental iodine is, according to the phenol coefficient test, obviously superior to all the other compounds used. In addition there is no difference between the three forms, colloidal, Lugol's, or tincture, when used in the high dilution necessary for testing, the reason for which will be emphasized later. Chlorine in the hypochlorite form, HTH, is apparently effective when there are 150 p.p.m. available chlorine, yielding a phenol coefficient of 14.5. However, the stable type compound, azochloramid, in a saturated solution of one (1) part in a thousand could not be tested because it did not yield sufficient chlorine to kill in 15 minutes. It was necessary to extend the time of the test to 50 minutes before sterilization of the seeding was effected, therefore a coefficient is not given.

The necessity of the Shippen (44) modification is demonstrated in the results obtained with the two compounds, brilliant

TABLE I. - Determination of Phenol Coefficients. Food and Drug Administration Phenol Coefficient Method, S.aureus 20°C.

Compounds	Dilutions killing in 10 min. but not in 5 min.			Phenol Coefficient	
	Subcultures in			Plain Broth 2nd subculture	Sodium thiosulphate Broth
	Plain Broth		Sodium thiosulphate Broth		
	1st	2nd			
Iodine - Tincture	1:14,000	1:14,000	1:14,000	200	189
Iodine - Lugol's	1:14,000	1:14,000	1:14,000	200	189
Iodine - Colloidal	1:14,000	1:14,000	1:14,000	200	189
Merthiolate - Tincture*	1: 8,000	1: 1,500	1: 1,500	21.7	20.2
Merthiolate - Aqueous*	1: 8,000	1: 1,000	1: 1,000	14.5	13.5
Azochloramid	1: 1,000	1: 1,000	1: 1,000	Failed	Failed
HTH 15 - Aqueous	1: 1,000	1: 1,000	1: 1,000	14.5	13.5
Brilliant Green*	1:30,000	1: 1,900	1: 8,000	30.0	108.0
Potassium Iodide Solution	No action	No action	No action	-	-
Alcohol 50%-Acetone 10%	Undiluted	Undiluted	Undiluted	0.0285	0.027
Phenol	1:69	1:69	1:74	-	-

* These are the nearest to killing dilutions; stronger dilutions could not be tested owing to the amount of compound carried over from seeding pots.

green and Merthiolate. Brilliant green is a well known bacteriostatic compound and like practically all compounds of this class shows specificity, especially for Gram-positive organisms. It is not surprising to find that it apparently killed in a dilution as high as 1 to 30,000 for the particular stock dye that was employed in this work. However, with the double subculture procedure in use it was found that 1 to 1900 failed to kill in 15 minutes. The reason that lower dilutions gave negative subcultures, even when double subcultures were made, was due to the amount of compound carried over. This was indicated by the fact that the broth had a strong greenish tinge at a dilution of 1 to 1000 and the color was also perceptible at 1 to 1600. Failure of subsequent plantings into these tubes to produce growth provided conclusive evidence that there was an inhibitory amount of compound present even in the second subculture tubes. It is doubtful if the 1 to 1000 concentration killed within 15 minutes.

Merthiolate, in both aqueous and tincture (solvent: alcohol 50 percent, acetone 10 percent, water 40 percent) solutions, showed much the same picture as brilliant green. The 1 to 1000 aqueous solution failed to kill in 15 minutes whereas the 1 to 1000 stock solution of tincture did kill in 5 minutes. This was evidently due to the alcohol concentration since dilution up to 1 to 1500, lowering the alcohol concentration to 30 percent, failed to kill in 15 minutes. This information, of course, was obtained by using the Shippen (44) double subculture technique. Phenol coefficients of the

nearest estimated or strongest dilutions used were calculated and placed in the table.

The few items given in the above paragraphs illustrate some of the weaknesses of the test even when it is uncomplicated by additional factors such as added organic matter of various types or definite particle size as suggested by Garrod (19), 1935. These items illustrate why Reddish (38), 1937, has so strongly emphasized the fact that the test has been badly misused in expecting it to yield any, let alone equally as much, information for antiseptics. For, no matter what other arguments may be offered the paramount one is that the test has been designed for disinfectants only. In addition there is the potential possibility of misinterpretation depending upon the viewpoint of the investigator. Reddish (38) illustrates by giving the phenol coefficient of a certain percentage of carbolic acid which is straining the interpretation slightly since comparison is based on this compound and any strength compared to it should always be unity. In other words, comparing a yardstick with itself accomplishes very little. Another type of interpretation aptly illustrated by Dunn (15), 1937, is to give the phenol coefficient on the basis of solid substance or dry powder resulting in a very large figure which is definitely misleading if the compound is insoluble at high concentrations or so toxic that to be usable it must be diluted to such a weak concentration that the phenol coefficient of the usable dilution may be even less than unity.

Salle and Lazarus* et. al., (1935-37) in utilizing a tissue culture technique in conjunction with the regular phenol coefficient method devised a toxicity test for evaluating disinfectants which Salle, McOmie, Shechmeister and Foord (41), 1938, found needed revision in the form of added organic matter. The incorporation of horse serum, however, did not seriously alter the results but merely lowered the figures for all compounds. They showed by their first technique that Merthiolate, with a rather high toxicity index, had a phenol coefficient of 70. Yet when their test was revised adding the horse serum factor and taking into account the marked inhibitory properties of Merthiolate, they found that a 4 percent solution, the strongest that could be prepared, failed to kill S. aureus in 10 minutes. The well known dye, mercurochrome, which had by their first test a phenol coefficient of 0.6 (killing in a dilution of 1 to 40) was found by the modified (bacteriostatic determination) method to fail to kill the same organism in an 8 percent solution, the strongest solution they could prepare. On the other hand they found iodine (Lugol's solution) by their first technique to have a phenol coefficient of 308 or killing in a dilution of 1 to 20,000. By the revised technique the killing dilution was 1 to 3520, just a little less than a saturated water solution of iodine.

* Series of articles published in Proc. Soc. Exper. Biol. and Med. were condensed and published with complete description of test by Salle, McOmie and Shechmeister (40), 1937.

These facts are presented to illustrate how closely the findings in this work parallel those of these investigators.

Agar Cup Plate

The next method employed was the first step in demonstrating that penetrative power (assuming all other factors such as medicinal-organism contact, constant) was the chief factor responsible in the ultimate rapid destruction of organisms. This method is presented in U.S.D.A. circular 198. It has been credited to Dr. L. C. Himebaugh by Reddish but apparently was originated in 1918 by Carnot and Dumont (7) and known today as the agar cup plate method.

Preliminary trial runs indicated that the chief objections to this method are: (1) The phenomenon observed is evidently more that of diffusibility than penetration and (2) volatile compounds will give a false picture, even of diffusibility. The latter is accomplished by saturating the atmosphere immediately above the agar, redissolving in the moisture of the surface diffusing downward over the entire area at the same time the compound is diffusing outward from the center.

This last objection was easily eliminated by covering the agar with a layer of paraffin prior to cutting out the center portion with a cork borer. Repair of the center cup so produced was accomplished by filling with agar level with the paraffin and again

cutting out the center portion with a smaller cork borer leaving a wall or collar of agar. This formed a seal over the opening existing between the agar and the paraffin layer thereby preventing any of the compound subsequently placed in the cup from seeping out between the layers of capillary action. The bottom of the cup was repaired by a drop of agar to prevent capillary seepage between the agar and glass.

Employing 0.1 percent soluble starch and 0.1 percent potassium iodide as an indicator in the agar, data were obtained for iodine and chlorine (Table 2) which show that these volatile compounds gave fairly consistent readings, i.e., diffusing outward from the center of the plate a definite distance. On the other hand the data on uncovered agar plates (Table 2) appear to show that the iodine containing compounds (according to the starch-potassium iodide indicator) had, within the limits of reading time, 24 hours, diffused out to the periphery of the plate.

After noting some of these peculiarities of agar cup procedure a series of agar cup plates was set up using the test organisms, E. typhosa and S. aureus, for indicators rather than starch and potassium iodide. This change was necessary as the majority of the compounds listed were employed, and two, aqueous merthiolate and tincture of merthiolate, cannot be tested by color indicators. The plates were observed at 18 and 24 hours and readings made at 48 hours.

TABLE 2. - Determination of Penetration. Agar Cup Plate Method **

Temperature 25C

Compound	Concentration	Inhibition Zone width* in cm.			
		Nutrient Agar		Plain Agar	
		Uncovered	Paraffin Top	Paraffin Top	Uncovered
Iodine - Tincture	1:20	4.0	1.86	2.07	4.0
Iodine - Lugol's	1:20	4.0	1.5	2.45	4.0
Iodine - Colloidal	1:20	4.0	1.0	2.0	4.0
HTH 15	150 p.p.m.	-	-	0.1	0.4

* The average distance of all the plates used from the edge of the cup to the periphery of the plate was 4.0 cm. Hence, this figure denotes penetration of entire plate.

** Starch and potassium iodide used as indicators.

As was expected from the preliminary observations the 5 percent solutions of tincture of iodine and Lugol's iodine gave sterile plates in all seventeen trials (Table 3). The 5 percent suspension of colloidal iodine gave only two sterile plates out of the series, the remaining fifteen plates having well marked zones. The apparently larger zones given by colloidal iodine against E. typhosa was due to the two sterile plates occurring in this series, thereby raising the average. The 0.1 percent concentrations of Merthiolate, aqueous and tincture, gave identical results against E. typhosa and also S. aureus. These two solutions were apparently slightly more specific for the Gram-positive organism than the Gram-negative organism. HTH 15 in a concentration of 150 p.p.m. available chlorine gave the smallest inhibition zones obtained but was equally effective against both organisms. The more stable chlorine compound, azochloramid, in a saturated aqueous solution of 1 part in 1000 parts of water gave inhibition zones comparable with the Merthiolate solutions but showing slightly more activity against E. typhosa than S. aureus.

Since extent of penetration varies with the time, it was thought more definite information might be obtained about the relative penetrative power of the three iodine preparations, alcohol solution, potassium iodide solution, and aqueous iodine (colloidal suspension). Conversely the faults of the method would be more clearly shown. To observe relative penetrative power a third series

TABLE 3. - Determination of Penetration. Agar Cup Plate Method

Temperature 37°C

Compound	Concentration	Inhibition Zone, width* in cm. Indicators**	
		E. typhosa	S. aureus
Iodine - Tincture	1:20	4.0	4.0
Iodine - Lugol's	1:20	4.0	4.0
Iodine - Colloidal	1:20	2.9	2.3
Merthiolate - Tincture	1:1000	1.7	2.0
Merthiolate - Aqueous	1:1000	1.7	2.0
Azochloramid	1:1000	2.0	1.6
HTH 15	150 p.p.m.	0.7	0.8

* From the edge of the cup to the periphery of the plate the average distance for all the plates used was 4.0 cm., hence, complete penetration is denoted.

** Average of ten trials using E.typhosa, and seven trials using S.aureus.

of agar cup plates with paraffin tops were prepared and readings made at frequent intervals up to 38 hours to detect variation in progress of diffusion. From Table 4 and the companion graph (Fig.1) it appears evident that by this method Lugol's and tincture of iodine penetrate or diffuse much more rapidly than does colloidal iodine. This is explained by the fact that the particles which represent the reservoir in colloidal iodine settle down to the bottom of the cup so they do not exert the full vapor pressure force evenly throughout the suspension as does occur when the suspension is kept agitated during short time contact.

This last item, as will be further clarified by following tests, rather clearly demonstrates one of the definite objections to this method. The necessary lapse of time before reading the plates is so great that the worker has very little chance to place a correct interpretation upon his findings, which can be just as readily called diffusibility as penetrative power, since it is frequently necessary for 48 hours to lapse before reading the results. Reiterating, the most superficial reasoning would demand that, to be effective, compounds should have the ability to penetrate within a very short space of time. In a test of this sort where there is no way of knowing what might be taking place up to 24 hours (when the very first evidence of growth is detectable) about the most valuable information that can be obtained from it is whether or not a compound will yield any zone of inhibition. The size of the

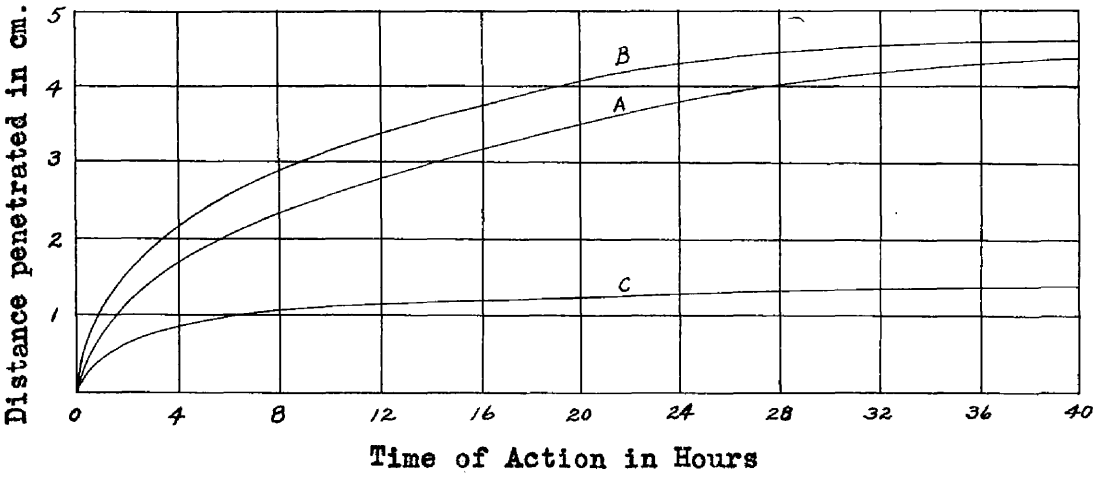
TABLE 4. - Determination of Penetration. Agar Cup Plate Method Using Paraffin Tops. *

Temperature 25°C

Time from filling, in hours.	Inhibition Zone, width in cm.		
	Tincture of Iodine	Lugol's Solution	Colloidal Iodine
00:30	0.25	0.4	0.1
00:60	0.37	0.6	0.2
1:30	0.45	0.7	0.25
2:00	0.6	0.85	0.33
2:40	0.7	0.95	0.38
4:40	0.92	1.11	0.46
10:00	1.3	1.6	0.55
13:00	1.45	1.76	0.58
24:00	1.95	2.15	0.65
38:00	2.2	2.3	0.7

* Nutrient agar containing 0.1 percent soluble starch.

Fig. 1. - Comparative Rates of Penetration of Tincture (A), Lugol's (B) and Colloidal Iodine (C) in Paraffin Covered Agar.



zone produced is definitely misleading as will be shown in the next section. For example, Merthiolate frequently gave plates that were entirely sterile as did some of the iodine preparations acting on uncovered plates. Yet when these two compounds were confined within an object that they had to penetrate in order to give any zone in the agar, the areas of the zones were surprisingly similar. One other minor objection to this method is that special porous plate tops must be employed to permit evaporation of moisture produced by bacterial metabolism. In addition considerable care must be exercised to prevent slopping of the compounds over the edges of the cup, particularly tinctures which have a tendency to "crawl". Those objections are readily eliminated by the technique employed in the next section.

Modified Agar Cup Plate.

As a result of the series cited, it was thought that an alteration might be made in the procedure which would show, without question, that penetration had been accomplished and that diffusibility had been entirely eliminated. The technique was as follows:

(1) A freshly collected length of adult chicken intestine with a fairly constant diameter and thickness was washed in Locke's solution,

(2) Cut in 4 cm. lengths, tied at one end with a loop of No. 40 thread,

(3) The section was filled with 1 ml. of compound and the open end tied with a loop of thread,

(4) The tied section was washed by dipping into a neutralizing solution and thence into two changes of distilled water,

(5) It was now placed in the center of a petri-dish,

(6) Agar seeded with S. aureus was poured in a thin layer into the petri-dish and allowed to set (this prevented floating),

(7) Additional seeded agar was poured to cover the tissue,

(8) The plates were incubated at 37°C. and,

(9) Readings were made at 24 and 48 hours, noting the size of the inhibitory zone obtained around the tubular section of tissue.

Employing this procedure the area of the zone need not bear the brunt of interpretation as to the penetrative power of the compound involved but rather serve as a crude yet definite indication that the compound had penetrated the tissue.

Table 5 shows the relative penetrative power of the compounds employed given in actual measurements obtained direct from the plates. It is to be borne in mind that while the elliptical zone sizes given in cm. (length and breadth measurements) represent the total area of inhibition minus the size of the tissue, the size of these areas are not to be interpreted in the sense that one compound is better than another simply because the zone is bigger. In fact, the tables show that the majority of the zones are of very nearly the same area. Several observations were made which are

TABLE 5. - Determination of Penetration. Adult Chicken Intestine Test.*

Compound	Concentration	No. of trials	Average size of zone in cm.	Average area of zone in $cm.^2$
Iodine - Tincture	1:20	22	3.65 x 2.57	9.28
Iodine - Lugol's	1:20	23	3.25 x 2.4	7.80
Iodine - Colloidal	1:20	23	2.35 x 1.44	3.38
Merthiolate - Tincture	1:1000	17	2.77 x 1.86	5.15
Merthiolate - Aqueous	1:1000	20	3.00 x 2.16	6.48
Azochloramid	1:1000	12	0.85 x 0.15	0.13
HTH 65	32,500 p.p.m.	2	3.4 x 2.3	7.82
Brilliant Green	1:20	2	3.9 x 2.8	10.92
Brilliant Green	1:100	1	3.8 x 1.9	7.22
Phenol	1:20	10	0.87 x 0.32	0.28

* 1 ml. of compound placed in 3 x 1 cm. length of intestine and embedded in S. aureus seeded agar. Clear zone produced by compound diffusing into the agar read at 48 hours.

considered worthy of inclusion here because of the bearing they have on the subsequent data. In addition these facts are more valuable in this interpretation than the actual measurements recorded.

The chlorine compound, HTH 15, employed in previous tests in a dilution containing 150 p.p.m. available chlorine (1 part powder - 1000 parts of water) had given such consistent negative results that a stronger solution of HTH 65 was prepared (5 percent solution of the powder) containing approximately 32,500 p.p.m. available chlorine which did give a zone of inhibition in this test but only after it had "eaten" its way through the tissue. The three forms of iodine employed while giving variable size zones, but within the limits of technical error, never failed to render sterile the tissue section in which they had been placed. On the other hand the two solutions of Merthiolate used while also giving a variable but frequently good sized zone in the seeded agar consistently failed to sterilize all parts of the tissue section.

This failure was noted at the ends of the section where the tie off was made indicating that the mechanically produced added thickness of the tissue at this point slowed the penetration of the compounds sufficiently to allow development of the organisms normally present on the washed intestines. These organisms, as a group, are predominately Gram-negative and less susceptible to these compounds than the S. aureus used as the indicator. The interpretation that

penetration was retarded is based upon the fact that subculture from these hazy zones at the ends of the tissue section gave no growth, nor would these subcultures support growth upon subsequent seeding. This was due to the amount of compound carried to the tube of broth in the small loop of fished agar, indicating that while the myriads of colonies had time to develop partially they were killed within the 48 hour time limit. This is a notable contrast to the sections containing iodine which invariably had a cleared zone all the way around the tissue that upon subculture was always negative and yet the subcultures would support growth upon subsequent seedings.

The slow acting chlorine compound, azochloramid, which in comparison with the more commonly known brands of chlorine compounds, is very stable in the presence of organic matter, gave a zone indicative of just bare penetration of the tissue as the zone rarely ever encompassed the whole tissue section. In other words only partial sterilization of the tissue section was obtained. This was also true of 5 percent phenol which was carried along in this series of experiments as the representative of the coal tar group of compounds. The tissue section filled with phenol was usually, but not always, surrounded by a visibly cleared zone of inhibition.

Brilliant green, when employed in a concentration of 1 to 1000 utterly failed to penetrate in a number of trials. Stronger concentrations of 1 to 100 and 1 to 20 were prepared and penetration was obtained. The zone sizes given in the tables for

these latter concentrations of the dye are the measurements of the area of dyed agar, and not a readable zone of inhibition, since the dye did not penetrate soon enough with the 1 to 100 concentration to prevent development of colonies within the zone covered by the dye in the 48 hour reading time. In both trials with the 5 percent concentration of the dye the penetration of the tissue was fairly rapid occurring shortly after pouring the agar, but not in sufficient concentration to give rapid diffusion into the agar so as to prevent the development of all colonies within the dyed area.

Penetration of Adult Chicken Ceca

The above findings led to the following experiment to determine the relative time necessary for these compounds to penetrate tissue of a slightly different type. The technique was as follows:

(1) Adult chicken ceca were obtained, washed in Locke's solution and filled with 10 ml. of compound,

(2) The open end tied with No. 40 thread,

(3) Washed by dipping in neutralizing agents and two changes of distilled water,

(4) Placed in a flask containing 100 ml. of a 24 hour broth culture of S. aureus,

(5) Shaken at intervals, and

(6) Subcultures made frequently to determine when the compound had penetrated the tissue as indicated by death of the bacteria in the surrounding medium.

To eliminate the possibility of variation in the structure of the ceca from bird to bird, pairs of ceca were obtained from two birds, one of each pair being used for colloidal iodine and the other of each pair being used for Lugol's iodine and tincture of iodine respectively.

The findings recorded in Table 6 are in reasonable accord with assumptions made in the preceding section. Lugol's solution proved to be the most rapid, in fact penetrating in just a few minutes whereas tincture of iodine required 39 minutes and colloidal iodine needed 5 hours to penetrate the tissue.

Both Merthiolate solutions penetrated this type of tissue in 6 1/2 hours while phenol required approximately 33 hours to sterilize the culture. All the other compounds, 1 to 1000 concentrations of HTH 15, azochloramid and brilliant green failed to sterilize the S. aureus culture or tissue in 44 days. At this time equal size portions of the negative cecal tissue were snipped from the terminal ends of the respective ceca, washed and embedded in S. aureus seeded agar to ascertain if there were any residual compound in the tissue. All compounds (but phenol), Lugol's, tincture, and colloidal iodine, aqueous and tincture of Merthiolate, showed inhibitory zones. The latter two zones being somewhat larger than those produced by the iodized tissue sections.

TABLE 6. - Penetration of Adult Chicken Ceca

Temperature 20°C.

Compound	Concentration	Time when 1st subculture became negative	Inhibition zone of plated tissue cm.
Iodine - Tincture	1:20	39 min.	0.05
Iodine - Lugol's	1:20	6 min.	0.1
Iodine - Colloidal	1:20	5 hrs.	0.1
Merthiolate - Tincture	1:1000	6½ hrs.	2.2
Merthiolate - Aqueous	1:1000	6½ hrs.	2.4
HTH 15	150 p.p.m.	44 days positive	-
Azochloramid	1:1000	44 days positive	-
Brilliant Green	1:1000	44 days positive	-
Phenol	1:20	33¾ hrs.	0

Penetration of Microscopic Living Units

Having determined that dead tissue could be readily penetrated by the compounds in use and having in a measure determined some of their limitations it was decided to conduct further tests upon living objects of sufficient size to make the end point visible with the aid of a microscope. Coccidia from fresh acute cases of fowl coccidiosis and freshly hatched long-tailed strongylid larvae of horses were chosen for these tests. These two organisms were selected because in both instances there exists a very definite membrane which must be penetrated before the life center is reached with ensuing death, namely the wall of the oocyst or the protective cuticle of the larvae.

Penetration of Coccidial Oocysts.

The selection of coccidial oocysts to meet the investigative purpose of this paper was based upon the evidence accumulated by Chandler (9), Becker (4), Fish (18) and others that the shell or wall of the oocyst is a membrane extremely difficult for compounds to penetrate. Chandler and Schalm * have demonstrated (with another objective in view, i.e., death of the organism) that a distinct difference exists between the three iodine preparations employed here, finding that, under constant controlled conditions, they kill the oocysts in the following order: (1) aqueous solution of iodine

* Unpublished data.

suspensoid, (2) Lugol's solution, and (3) tincture of iodine. It is well to bear in mind that thus far the tests made on agar cup plates and dead tissue have indicated that, if these tests are fair measures of penetrative power, that the above order should be (2), (3), (1).

The technique employed by Chandler and Schalm was modified slightly for this determination of penetrative power, which, as previously stated, varies only as the time of contact. In order to ascertain this end-point the following method was used:

(1) 0.25 ml. of a suspension of freshly collected, washed, coccidial oocysts was placed in 4.75 ml. of compound,

(2) Thoroughly shaken at intervals,

(3) At definite periods 0.5 ml. of the suspension was placed in 4.5 ml. of brilliant green-sodium thiosulphate and allowed to sporulate at room temperature. This procedure sufficed for all iodine and chlorine containing compounds,

(4) For the merthiolate preparations the sample of oocysts prior to placing for sporulation were first washed in saturated H_2S water then three washes of tap water by centrifugation,

(5) For phenol, brilliant green, and the acetone-alcohol mixture three washes with tap water sufficed,

(6) At the end of 48 hours the greater portion of supernatant liquid was drawn off and a drop of the sediment placed on a slide under a cover glass,

(7) Random fields were counted until a total of one hundred

oocysts had been observed, noting those that were sporulated, non-sporulated and fragmented,

(8) Control samples were sporulated at the same time,

(9) The total sporulation for the control samples which averaged 95 per hundred oocysts was taken as the 100 percent base upon which the percentages shown in the table were calculated for the test samples, and

(10) The recognized sporulation medium, 2 percent potassium dichromate was also used to show that the brilliant green-sodium thiosulphate medium produced maximum sporulation.

Of the rapid acting compounds (Table 7) it can be seen that colloidal iodine was the most rapid of all compounds in penetrating. Observation showed that it actually entered and stained the cytoplasm of the organism in less than 5 minutes. The time for Lugol's solution to enter and kill was over 15 minutes while 5 percent phenol needed approximately 4 hours and the 5 percent HTH65 solution (32,500 p.p.m. available chlorine) required better than 7 hours to give complete mortality.

From Table 8 it can be readily seen that the compounds which under other penetrative conditions (cf. agar plate methods) appeared possibly superior to colloidal iodine are in the light of these data actually inferior. Five percent tincture of iodine killed only 28 percent of the oocysts in 12 hours. Aqueous Merthiolate in the three concentrations 0.1, 1.0, and 4.0 percent was slightly better.

TABLE 7. - Penetration of Coccidial Oocysts. *

Compound	Concentration	Percent of Coccidia Killed													
		Minutes										Hours			
		1	2	3	4	5	15	30	45	60	90	4	7	10	48
Iodine - Lugol's	1:20	-	-	-	-	-	83	100	100	100					
Iodine - Lugol's	1:100	-	-	-	-	-	90	100	100	100					
Iodine - Colloidal	1:20	82	100	100	100	100									
Iodine - Colloidal	1:100	76	100	100	100	100									
Phenol	1:20	-	-	-	-	-	-	-	-	-	-	100	-	100	-
HTH 65	32,500 p.p.m.	-	-	-	-	-	-	-	-	-	-	-	47	100	100
HTH 65	6,500 p.p.m.	-	-	-	-	-	-	-	-	-	-	-	-	11	25

* Oocysts suspended in compounds; Aliquot portions removed at intervals and placed in brilliant green-sodium thiosulphate medium to sporulate. Test and sporulation conducted at room temperature 22°C. Read at 48 hours.

TABLE 8. - Penetration of Coccidial Oocysts. *

Compound	Concentration	Percent of Coccidia Killed				
		4 hr.	6 hr.	8 hr.	10 hr.	12 hr.
Iodine - Tincture	1:20	4	12	16	26	28
Merthiolate - Tincture	1:1000	0	0	1	9	26
Merthiolate - Aqueous	1:1000	6	7	8	17	69
Merthiolate - Aqueous	1:100	8	14	23	47	59
Merthiolate - Aqueous	1:25	5	7	10	19	71
HTH 65	650 p.p.m.	0	1	9	15	25
Azochloramid	1:1000	1	8	2	3	5
Alcohol 50% and Acetone 10%		8	3	5	11	13
Brilliant Green	1:1000	1	2	0	1	0
Controls: Potassium Dichromate	1:50	5**	6	5	5	6
Brilliant Green and $Na_2S_2O_3$		5**	6	6	5	4

* Oocysts suspended in compounds; Aliquot portions removed at intervals and placed in brilliant green-sodium thiosulphate medium to sporulate. Test and sporulation conducted at room temperature 22°C. Read at 48 hours.

** Actual number dead per hundred oocysts counted.

Sufficient of the compound passed through the wall of the oocysts in 12 hours to prevent subsequent cyclic division of the cytoplasm in 69, 59 and 71 percent of the oocysts, respectively. Apparently the 1.0 percent concentration of Merthiolate seems to be the optimum concentration for uniform action under the conditions of the test. The 0.1 percent tincture of Merthiolate shows the inhibiting effect of the solvent upon the chemical as only 26 percent of the oocysts were killed in the 12 hour period in contrast to the 69 percent mortality obtained with the same concentration of the aqueous solution. Part of the 26 percent mortality obtained by the tincture can be attributed to the solvent itself since the alcohol-acetone mixture alone produced 13 percent mortality in 12 hours. The saturated aqueous solution of azochloramid 1 to 1000 concentration proved to be a very poor killing agent, producing only a 5 percent mortality. On the other hand the inorganic compound HTH 65 in a concentration of 650 p.p.m. available chlorine killed 25 percent of the oocysts. The 1 to 1000 concentration of brilliant green failed to kill any of the oocysts.

In order to point out the role solvents may play in the penetrative power of a disinfectant the three iodine preparations were used with a much more resistant stock sample of oocysts so as to give a clearer picture with no border line distinctions. The results are given in Table 9. Here we have three decidedly different solutions of the same strength element, 5 percent available iodine. In colloidal iodine the only solvent present (and a very poor solvent, 1 to 3000 at

TABLE 9. - Penetration of Coccidial Oocysts. *

Compound	Concentration	Percent of Coccidia** Killed																
		Minutes											Hours					
		1	1½	2	3	4	5	10	15	20	30	45	1	2	3	4	24	48
Iodine - Tincture	1:20	-	-	-	-	-	0	-	-	-	0	-	0	-	-	-	0	
Iodine - Lugol's	1:20	-	-	-	-	-	0	0	-	28	30	30	80	90	100			
Iodine - Colloidal	1:20	0	0	25	25	-	50	100										

* Oocysts suspended in compounds; Aliquot portions removed at intervals and placed in brilliant green-sodium thiosulphate medium to sporulate. Test and sporulation conducted at room temperature 22°C. Read at 48 hours.

** Resistant sample used.

20°C.) is water, the suspending menstruum for the particulate iodine which forms the reservoir of instantaneously soluble iodine. This feature of instantaneous solubility constantly maintains the surrounding solution at a strength of 1 to 3000, higher or lower depending upon the temperature. In Lugol's compound the menstruum is again all water. However, all the iodine present is in actual solution, made possible by the strong solvent action of the potassium iodide present. The third compound, the well known tincture of iodine, has but a small amount of water and two tenacious solvents acting, namely potassium iodide and alcohol.

The results shown in Table 9 demonstrate that with all other factors identical (concentration, substrate, temperature) there is only one left which can be held responsible for the difference in penetrative power of the three solutions of iodine and since the solvents employed are the only variables present, it follows then that they must be responsible for the fact that colloidal iodine kills within ten minutes; Lugol's iodine requires three hours and tincture of iodine is not effective in 24 hours. This simple experiment also explains why identical results are obtained with these three solutions of iodine when they are used in dilute form (1 to 1000 or higher dilutions) as for example in the phenol coefficient method of testing where the solvent is essentially diluted out of existence, yet in practical use (concentrations of 1, 3 or 5 percent) yield such markedly different results. To add more information to this particular factor the following experiments were devised:

Penetration of Larval Cuticle

For this series freshly collected, hatched, long-tailed strongylid larvae of horses were employed. The technique consisted simply in placing a drop of the larval suspension containing not less than 100 larvae in an esmarch dish, adding 2 ml. of the test compound and observing under a low-power (20x) binocular microscope the changes that ensued. In some instances neutralizing agents were employed to stop the action of the compound after certain changes had occurred. This will be explained as each compound is discussed. One factor which recommends the choice of larvae for these tests is that by their constant, active movements in the solution the criticism of lack of continual agitation is obviated.

Here again as in the previous test we have a definite set-up which has an unquestionable end-point, namely irreversible demise of the organism. Further we have an analogous situation of a visible barrier, the cuticle or protecting sheath of the larvae, which must be penetrated by the compound before the life center of the organism is reached resulting in death.

From Table 10 it is obvious that, of the group of compounds studied, elemental iodine, irrespective of the original solvent present, proved obviously superior to every other compound employed in the rapidity with which the larvae were penetrated and killed. In addition it will be noted that no detectable difference existed between the iodine preparations when employed as previously stressed,

TABLE 10. - Penetration of Strongylid Larvae Cuticle.*

Compound	Concentration	Killing Time
Iodine - Tincture	1:1000	5 sec.
Iodine - Lugol's	1:1000	5 sec.
Iodine - Colloidal	1:1000	5 sec.
Merthiolate - Tincture	1:1000	3½ hr. 28 min.
Merthiolate - Aqueous	1:1000	62 hrs.
Merthiolate - Aqueous	1:100	62 hrs.
Merthiolate - Aqueous	1:25	62 hrs.
HTH 65	650 p.p.m.	75 min.
HTH 65	6,500 p.p.m.	15 min.
HTH 65	32,500 p.p.m.	3 min.
Azochloramid	1:1000	312 hrs.
Brilliant Green	1:1000	288 hrs.
Brilliant Green	1:100	144 hrs.
Brilliant Green	1:20	107 hrs.
Phenol	1:100	45 min.
Phenol	1:20	3½ min.
Alcohol 50% + Acetone 10%	undiluted	2¼ hr. 15 min.
Control - in Tap H_2O		alive 30 days

* Two ml. of compound added to one drop of larval suspension (approximately 100 larvae). Observed at intervals for visible changes or death of the larvae (20x binocular). Test conducted at room temperature 20°C.

in dilutions of 1 to 1000 or higher. All three compounds of iodine of 0.1 percent concentration killed within five seconds. These sharp end-points were obtained by neutralization with 0.1 N sodium thiosulphate, followed by three washes of aspirational sedimentation. The next quickest killing compound was aqueous HTH containing 32,500 p.p.m. available chlorine. While this compound required only three minutes to bring about death of the organism it was noted that in order to do so the organism was first dissolved. Briefly the process occurred in this way: The thin double layer of cuticle which formed the tail was the first part to disappear followed by a splitting or shredding of the posterior end of the organism simultaneously with appearance of bubble-like rupture of the cuticule along the whole length of the organism. Inactivity and death were almost immediately followed by dissolution. If the digesting action of the hypochlorite were stopped by neutralization with 0.1 N sodium thiosulphate at the instant the last organism ceased moving, followed by aspirational washing, none ever recovered and all had the appearance of floating shreds in the dish.

This picture was obtained by using the weaker concentrations of HTH 65 containing 6,500 p.p.m. and 650 p.p.m. available chlorine, respectively. The former concentration required only 15 minutes while the latter concentration needed 75 minutes to kill all the larvae. Maceration of the organisms followed in those samples not neutralized. The action of this hypochlorite is in accord with that reported by

Bashford (3), 1917), Taylor and Austin (46), 1918, Fiessinger and Clogne (17), 1918, who found that in attempted disinfection employing the classical Dakin's solution that digestion of the tissue is a paramount feature. Fiessinger and Clogne pointed out that in the treatment of war wounds by the Carrel continuous irrigation method that improvement was due more to the proteolytic action of the compound upon mortified tissue than to any sterilizing action.

Chandler (9), 1933, demonstrated that while hypochlorite solutions were very inefficient, free chlorine was quite active in killing coccidial oocysts. In the light of these observations it is well to recall that the HTH 65 solution containing 32,500 p.p.m. available chlorine required 7 hours to kill all the coccidial oocysts in the test sample (see Table 7). In connection with the mode of destruction of larvae given above it can now be pointed out that the prevention of cyclic division of the cytoplasm of the oocysts was accomplished by the hypochlorite digesting and entering the oocysts through the operculum followed by destruction of the life center in the cytoplasm. There is demonstrated then the imperviousness of the oocyst shell to ordinary chemical agents. At the same time it is shown that the mechanism of action of the hypochlorite type compound is not one of penetration and killing as with elemental iodine but instead death results from digestion or oxidation.

Phenol in a concentration of 1 to 100 brought about death in 45 minutes as an irreversible procedure without any external changes of the organism while the 1 to 20 concentration killed in three and one-half minutes. Attempts to revive the organisms by washing with ammonium sulphide ($(NH_4)_2S$) water followed by washing with plain tap water were futile indicating that the phenol had evidently penetrated sufficiently to bring about irreversible changes in the protoplasm.

For the two Merthiolate preparations, aqueous and tincture, the killing time was established by washing various lots of organisms at different intervals with water saturated with hydrogen sulphide, for 3 minutes, followed by not less than four washes of tap water. Control larvae kept in saturated hydrogen sulphide water for five minutes followed by not less than 4 washes with plain tap water showed no marked loss of activity or subsequent ill effects. It was found that whereas aqueous Merthiolate in 0.1, 1.0 or 4.0 percent concentrations would cause the organism to begin less active movements in 10 to 12 hours, irreversible death of all the organisms in any given test lot could not be brought about even by the strongest concentration used under 62 hours at 20°C. Apparently the tincture of this compound in 0.1 percent concentration was much better, killing the organisms under 3 1/4 hours, but when the menstruum, alcohol-acetone, was examined for its killing power and found to accomplish death in approximately the same time it was concluded

that the tincture was no better than the same concentration of aqueous Merthiolate.

Brilliant green in three concentrations of 0.1, 1.0 and 5.0 percent proved to be a very poor killing agent being unable to inactivate the more resistant organisms under 288, 144 and 107 hours, respectively. Likewise the stable chlorine compound, azochloramid, in a 0.1 percent concentration proved to be too stable to give up enough chlorine to inactivate the organisms in less than 312 hours. At the end of this period there was still available chlorine present as could be shown by titration. As already emphasized, readily available chlorine will bring about death of the larvae in a very short period if the concentration is high enough for rapid oxidation, but with azochloramid death is attained without microscopically visible oxidation as the organisms were all intact after death. After the above stated time period had elapsed washing once with 0.1 N sodium thiosulphate and several times with plain water failed to bring about recovery of any of the organisms.

To emphasize again the role solvents may play, several tests were made using the three iodine preparations in 5 percent concentrations and following the technique described above. It was noted (Table 11) that aqueous iodine (colloidal suspension) killed in less than 1 second and visibly stained the interior of the organism in less than 3 seconds, whereas the potassium iodide solution, Lugol's, required 15 seconds to kill and 30 seconds to stain the interior; the tincture needing 45 seconds and 5 minutes to accomplish the same

TABLE 11. - Penetration of Strongylid Larvae Cuticle by Selected Compound with Limited Time Periods. *

Compound	Concentration	Killing Time	Staining
Iodine - Tincture	1:20	45 seconds	5 minutes
Iodine - Lugol's	1:20	15 seconds	30 seconds
Iodine - Colloidal	1:20	1 second	3 seconds
			Recovery
Iodine - Tincture	** 1:1000	5 seconds	none
Iodine- Lugol's	1:1000	5 seconds	none
Iodine - Colloidal	1:1000	5 seconds	none
Iodine - Tincture	** 1:10,000	3 minutes	none
Iodine - Lugol's	1:10,000	3 minutes	none
Iodine - Colloidal	1:10,000	3 minutes	none

* Two ml. of compound added to one drop of larval suspension (approximately 100 larvae). Neutralized at the intervals given as killing time. Different trial lots were neutralized at various intervals to determine interior staining. Observations done with 20x binocular. Test conducted at room temperature 22°C.

** The 1 to 1000 and 1 to 10,000 dilution results are included to emphasize the role of solvent action.

results. Again showing conclusively that, with iodine at least, the weaker the solvent the more effective will be the iodine, or the greater its penetrative power.

An observation made throughout the work reported in this section on macroscopic living units which could be only hinted at by the data given in the tables is that the coccidial oocysts and strongylid larvae when acted upon by the slower compounds (Merthiolate, brilliant green, azochlorâmid, the higher dilutions of phenol and HTH) behaved very much like bacteria. Apparently each test sample contained a small percentage of susceptible and highly resistant organisms. Especially was this feature noticeable in working with the larvae. Approximately 25 percent of the organisms were inactivated in the first few hours or minutes of contact with the compounds and an additional 50-60 percent were inactivated by the time the test period had reached the half way mark for the particular compound in question. The remaining 15-25 percent of the organisms succumbed one by one during the last half of the test period until the time reached when they were recorded as dead or almost finished. This behavior is analogous to the death rates of bacteria under similar conditions of slow acting compounds as frequently reported in the literature.

Rabbit Skin Penetration

The next procedure employed to demonstrate the action of the compounds studied was based upon a technique devised by Etchells and Fabian (16), 1935, for determining toxicity of compounds placed upon the abdomen of a rabbit. Briefly the technique used was as follows:

(1) 25 ml. graduate cylinders were cut at the 5 ml. divisions,

(2) The edges ground to right angle smoothness using alundum and a smooth glass plate,

(3) The cup thus formed was attached to the shaved abdomen of a rabbit (prepared 24 hours prior to the test) by the aid of rubber bands terminating in hooks fashioned from paper clips,

(4) Collodion was then placed around the outside base of the cup and allowed to dry. The "setting" of the collodion and subsequent drying gradually drew the cup against the skin until sufficiently tight to prevent any leakage occurring,

(5) Several cups were placed in a row (usually two parallel rows equidistant either side of the midline were employed) and as each cup "set" sufficiently the rubber bands and hooks holding it were moved to a new position for holding another cup,

(6) Following placement of the last cup and removal of all bands and hooks the animal was ready for intravenous nembutal and intermittent ether anesthesia,

(7) Blunt needles were inserted subcutaneously beneath each cup for culture placement,

(8) Two ml. of compound were then placed in each cup,

(9) Zero time was taken as each cup was filled, the filling being done at intervals so as to allow for subculturing and final cup removed, so that each compound was in contact with the skin the same length of time,

(10) One-tenth ml. of a saline suspension of a 24 hour agar slant culture of S. aureus was then injected into the pocket beneath each cup,

(11) At intervals as shown in the tables subcultures were made by withdrawal of 0.01 ml. of the inoculum and,

(12) Seeding into broth tubes, incubated 48 hours at 37°C.

A study of Tables 12-16 show the culturing method to be a failure as apparently none of the compounds penetrated the live skin in sufficient concentration to sterilize the pockets since subcultures were all positive even after 11 hours. However, upon autopsy of rabbit No. 4 (Table 14) it was found that the iodines were macroscopically present in the muscular layer beneath their respective cups. These circular areas of muscle tissue beneath each cup were removed and plated in S. aureus seeded agar, incubated 48 hours and the zone of inhibition noted.

Because of certain technical difficulties encountered, including absorption of the inoculum, such that the time period could not be practically extended, the culturing phase of the technique was abandoned and attention directed toward determining in an unquestion-

TABLE 12. - The Effect of Selected Compounds on Live Rabbit Skin for 30 to 60 Minutes.

Compound	Concentration	Acting Time on Rabbit No.2		Acting Time on Rabbit No.1		Comment
		Time	Subculture	Time	Subculture	
Iodine - Lugol's	1:33 1/3	30 min.	+	60 min.	+	None of the
Iodine - Colloidal	1:40	30 min	+	60 min.	+	compounds
Merthiolate - Aqueous	1:1000	30 min	+	60 min.	+	penetrated
Brilliant Green	1:1000	30 min.	+	60 min.	+	the epidermis.

TABLE 13. - The Effect of Selected Compounds on Live Rabbit Skin for 5 and 5 1/2 hours.

Compound	Concentration	Acting Time On				Observations
		Rabbit No.1		Rabbit No.3		
		Time	Subculture	Time	Subculture	
Iodine - Tincture	1:20	5 hr.	+	5½ hr.	+	None of the Compounds penetrated.
Iodine - Lugol's	1:20	5 hr.	+	5½ hr.	+	
Iodine - Colloidal	1:20	5 hr.	+	5½ hr.	+	
Merthiolate - Aqueous	1:25	5 hr.	+	5½ hr.	+	
HTH 65	32,500 p.p.m.	5 hr.	+	5½ hr.	+	Caused severe Necrosis
Brilliant Green	1:20	5 hr.	+	5½ hr.	+	

TABLE 14. - Passage of Selected Compounds Through the Skin of a Dead Rabbit in 7 Hours.

Rabbit No.4

Compound	Concentration	Time Dead Skin	Subculture	Plated Muscle * zone of Inhibition in cm.	Comment
Iodine - Tincture	1:20	7 hr.	+	0.5	
Iodine - Lugol's	1:20	7 hr.	+	0.7	
Iodine - Colloidal	1:20	7 hr.	+	1.4	
Merthiolate - Aqueous	1:25	7 hr.	+	scant	Inhibition present Arc not measurable
HTH 65	32,500 p.p.m.	7 hr.	+	Not Tested	
Brilliant Green	1:20	7 hr.	+	0	Dye did not appear on under side of skin.

* The first layer of abdominal muscle was removed and plated in S. aureus seeded agar obtaining evidence of penetration to this depth of tissue. Zones read in 48 hours.

TABLE 15. - Penetration of Rabbit Skin by Selected Compounds Acting 5 1/2 Hours Before and an Additional 5 Hours After Death of the Rabbit.

Rabbit No.5

Compound	Concentration	Time Live Skin	Subculture	Time dead Skin	Plated Skin. * Zone of Inhibition in cm.		Comment
					Epidermis	Fascia	
Iodine - Tincture	1:20	5½ hrs.	+	5 hrs.	0.8	0	
Iodine - Lugol's	1:20	5½ hrs.	+	5 hrs.	1.6	0	
Iodine - Colloidal	1:20	5½ hrs.	+	5 hrs.	5.0	0.1	
Merthiolate - Aqueous	1:25	5½ hrs.	+	5 hrs.	7.6	1.4	Fascia zone indistinct.
HTH 65 **	32,500	5½ hrs.	+	5 hrs.	0.0	0	Severe Necrosis
Phenol **	1:20	5½ hrs.	+	5 hrs.	0.0	0	

* Demonstration of penetration by embedding skin and fascia in S. aureus seeded agar and reading inhibitory zone in 48 hours.

** Phenol and HTH 65 did not leave enough unbound compound to demonstrate inhibition when the epidermis was plated. Since HTH 65 showed macroscopic evidence of digestion of the epidermis but not completely through to the fascia no inhibitory zone was expected of the fascia.

TABLE 16. - Penetration of Rabbit Skin by Selected Compounds Acting $6\frac{1}{2}$ Hours Before and an Additional $4\frac{1}{2}$ Hours After Death of the Rabbit.

Rabbit No.6

Compound	Concentration	Time after Death	Subculture	Acting time on the Skin. Total *	Zone of Inhibition in cm.		Zone Subcultures		Skin** stretched over short tubes 1 ml. culture inside. Subcultures at Time in Hours			
					Epidermis	Areolar Tissue	Near Tissue	Edge of Growth	1:10	2:00	3:15	20
Iodine - Tincture	1:20	$4\frac{1}{2}$ hr.	+	11 hr.	1.8	0.1	-	+	+	+	+	
Iodine - Lugol's	1:20	$4\frac{1}{2}$ hr.	+	11 hr.	5.0	0.2	-	+	not done			
Iodine - Colloidal	1:20	$4\frac{1}{2}$ hr.	+	11 hr.	3.6	1.1	-	+	+	-	-	-
Merthiolate - Aqueous	1:25	$4\frac{1}{2}$ hr.	+	11 hr.	8.2	2.4	-	-	+	+	+	+

* At $6\frac{1}{2}$ hours, animal sacrificed, saline suspension of S. aureus injected beneath each cup and subcultured as noted.

** Unused epidermis removed from the left side of the animal, cut into squares and tied over one end of a short sterile tube. Tube with skin end downward floated in a narrow jar containing 50 c.c. of compound.

able fashion the length of time required for these compounds to penetrate both live and dead skin. The term "live skin" will be used in referring to the skin when it is on the intact, anesthesized animal with circulation unimpaired. The term "dead skin" will be employed in referring to the skin from the moment when the animal has been sacrificed and the general circulation stopped irrespective of whether or not the skin is used on the animal or removed immediately at death for test tube work.

In each test the animal was sacrificed as indicated by the termination of the experiments on live skin, Tables 14-19. The cups were emptied and the interior washed with a neutralizing agent and several changes of distilled water followed by swabbing dry, then cups were removed. The circular area of epidermis covered by each cup was removed as was the underlying areolar tissue and first layer of muscle, except where inflammatory reactions had occurred to such a degree that the fascia was more or less adherent to the skin. These bits of tissue averaging 1.3 cm. in diameter were placed upon a previously poured thin layer of S. aureus seeded agar, and covered with another layer of seeded agar. Incubation was carried out the usual 48 hours to allow a dense growth to clearly outline the area around the tissue where the compound had diffused into the agar, killing or inhibiting the colonies.

Inspection of Tables 14-19 show that the compounds to which definite killing action had been previously assigned were capable of

penetrating the skin, the areolar tissue, and into the muscle layers if certain conditions obtained. These conditions will be described in more detail later. Rabbit No. 4, Table 14, was sacrificed immediately after the suspension of S. aureus was placed in the pockets so that insight might be gained as to whether or not the failure to sterilize the inoculum through live skin was due to factors beyond reasonable control. This was evidently the case since positive subcultures were obtained even though the iodine compounds had macroscopically penetrated. Inspection of Table 14 shows that 4 percent aqueous Merthiolate and the three 5 percent iodine compounds penetrated the epidermis of the dead animal in some time under 7 hours since tissue plating demonstrated they were present in the first layer of abdominal muscle.

From Table 15 it can be seen that these same compounds were barely able to penetrate the dead skin in 5 hours, even though they had acted on the live skin 5 1/2 hours before the animal was sacrificed, a total of 10 1/2 hours action upon the epidermis. Incidentally, the stronger solution of aqueous HTH 65 used, (32,500 p.p.m. available chlorine) showed why it was virtually impossible of demonstration in the subcutaneous tissue by this technique. Since HTH penetrated by oxidation, causing necrosis and digesting the tissue as it went deeper, the compound itself was used up in the procedure. Therefore, insufficient residual compound was present in the tissue to be demonstrable when the epidermal layer was plated.

At the time the animal was sacrificed (5 1/2 hours) the skin was dissected loose from the muscle and the under side examined for macroscopic evidence of any of the iodine compounds. None were visible until 5 hours later, but edematous reactions in the areolar tissue were evident under all cups except 4 percent Merthiolate. Brilliant green was omitted because of the obvious length of time (by observation) necessary to penetrate the epidermis, while aqueous HTH and phenol were dropped (Tables 14 and 15) from further consideration because the amounts penetrating were apparently used up or bound by the epidermal cells so that no residual could be demonstrated by plating the washed epidermis.

Considering Table 16 next, it is readily seen that aqueous Merthiolate and the three iodine compounds penetrated the epidermis and the areolar tissue in 11 hours. In this test the compounds acted on the skin of a live animal the first 6 1/2 hours and on a dead animal the last 4 1/2 hours.

To distinquish between the compounds from the standpoint of speed, a section of the unused epidermal layer was removed at death, cut into sections an inch square and stretched over one end of a sterile two inch length of test tube. A ml. of a saline suspension of S. aureus was placed therein and the skin end of the tube then immersed beneath the surface of a compound. Subcultures from the inside at intervals proved colloidal iodine to be the most rapid, penetrating the skin in something less than two hours while over

three hours were required for tincture of iodine. The 4 percent aqueous solution of Merthiolate did not penetrate in sufficient amounts to sterilize the seeding in 20 hours. However, the compound had penetrated in sufficient quantity by this time such that the Shippen procedure, i.e., a double subculture, gave no growth in the first tube but excellent growth in the second broth tube.

The next two experiments (Tables 17 and 18) were designed to explode the myth of easy penetration of live skin. In these two tests a run was performed with the cups set up on one side of the mid-line of the abdomen with the animal alive. Duplicate cups were set-up on the other side of the mid-line and filled just as the animal was sacrificed. The first set of cups was then removed and the tissues plated. The second set of cups was treated in the same manner. The only variable introduced was time, the compounds being allowed to act upon the live skin longer than upon the dead skin.

Acting upon dead skin 7 3/4 hours (Table 17) all of the compounds employed (1 and 4 percent aqueous Merthiolate; 5 percent tincture, Lugol's and colloidal iodine) penetrated the areolar tissue in sufficient concentration to give definite zones on plating. Whereas, acting upon live skin almost an hour longer 4 percent aqueous Merthiolate was the only member of the group apparently able to penetrate the epidermis. It is evident from the areolar tissue zones and the relative size of the epidermal tissue zones produced by the 1 percent aqueous Merthiolate and the three 5 percent concentrations

TABLE 17. - Comparison of Penetrative Power of Selected Compounds Acting 8 1/2 Hours on the Skin of a Live Rabbit with 7 3/4 Hours Action on Skin of a Dead Rabbit.

Rabbit No.7

Compound	Concentration	Plated Skin* - Zone of Inhibition in cm.							
		Live Skin - Right Side				Dead Skin - Left Side			
		Time	Epidermis	Fascia	Muscle	Time	Epidermis	Fascia	Muscle
Iodine - Tincture	1:20	8½ hr.	0.05	0	0	7¾ hr.	1.5	0.1	0
Iodine - Lugol's	1:20	8½ hr.	0.1	0	0	7¾ hr.	6.2	1.7	0.2**
Iodine - Colloidal	1:20	8½ hr.	1.8	0	0	7¾ hr.	7.5***	1.4	0.3
Merthiolate - Aqueous	1:25	8½ hr.	7.9	2.0	0.5**	7¾ hr.	7.1	4.6	1.2
Merthiolate - Aqueous	1.100	8½ hr.	1.3**	0	0	7¾ hr.	3.9	1.5	0

* Demonstration of penetration by embedding tissue in S. aureus seeded agar and reading inhibitory zone in 48 hours.

** Indistinct zones. Estimation only.

*** Sterile plate.

TABLE 18. - Comparison of Penetrative Power of Selected Compounds Acting 6 Hours on the Skin of a Live Rabbit with 4 1/2 Hours Action on the Skin of a Dead Rabbit.

Rabbit No.8

Compound	Concentration	Plated Tissue* - Zone of Inhibition in cm.					
		Live Skin - Right Side			Dead Skin - Left Side		
		Time	Epidermis	Fascia	Time	Epidermis	Fascia
Iodine - Tincture	1:20	6 hr.	0	0	4½ hr.	0.2	0
Iodine - Lugol's	1:20	6 hr.	0.3	0	4½ hr.	0.4	0.3**
Iodine - Colloidal	1:20	6 hr.	1.3	0	4½ hr.	0.3	0.6
Merthiolate - Aqueous	1:25	6 hr.	8.0***	0	4½ hr.	1.2	0.7**
Merthiolate - Aqueous	1:100	6 hr.	1.5	0	4½ hr.	1.0	0

* Penetration demonstrated by embedding tissue in S. aureus seeded agar and reading inhibitory zones in 48 hours.

** Indistinct zone. Estimation only.

*** Sterile plate.

of iodine that the dead epidermal layer was much easier to penetrate than that of live skin. The aqueous forms of iodine, Lugol's and colloidal, show some superiority over the same strength of tincture of iodine by penetrating the first layer of abdominal muscle while the latter compound reached only as far as the areolar tissue within the allotted time period. The 4 percent aqueous concentration of Merthiolate showed similar superiority over the 1 percent concentration by penetrating the dead epidermis, areolar tissue and into the abdominal muscle while the weaker concentration failed to penetrate the muscle.

In the second experiment of this type (Table 18) the allotted time for penetration of the live and dead skin was shortened 2 1/2 and 3 1/4 hours respectively. This served to give a clearer distinction between the compounds. Acting upon live skin 6 hours none of the compounds penetrated the epidermis whereas acting upon dead skin 4 1/2 hours three of the group, Lugol's iodine, colloidal iodine and 4 percent Merthiolate penetrated the epidermis as demonstrated by their presence in the areolar tissue. It is noteworthy that with these shorter time periods smaller amounts of the compounds were present in the epidermal tissue as demonstrated by the relatively smaller inhibitory zones when compared with those obtained on dead skin in the previous experiment (Table 17).

From observations made upon the plated tissues given in Tables 14 to 18 it was noted that the zones given by the areolar

tissue under the 4 percent Merthiolate cups were barely evident. It is felt that the evidence of penetration given by these zones is false. It is possible that these zones were due to contamination of the areolar tissue by the Merthiolate when the epidermal layer was being cut from the skin. Even after most careful neutralization and thorough washing of the epidermal layer under the 4 percent Merthiolate cups there was considerable residual compound in this layer as demonstrated by the majority of plates being sterile or almost so. In view of this fact it was felt that if true penetration had occurred then the areolar tissue and muscle tissue layers would have enough compound to produce much more definite, distinct zones than were obtained. In this connection it is well to remember that the bacteriostatic properties of mercury are high and not easily inactivated by nutrient media, hence even a minute amount of the compound carried from epidermal layer to the subcutaneous layers by the instruments at time of dissection could easily account for the results.

Before proceeding to the final table of this series, it would be well again to call attention to the more obvious differences among the three iodine compounds employed. Whether acting on live or dead skin, colloidal iodine penetrated faster and deeper than did Lugol's iodine. The platings of surface skin, areolar and muscle tissue from colloidal iodine cups gave much larger inhibition zones than the Lugol's, indicating that more iodine was taken up by the tissue from the colloidal iodine. The same concentration of tincture

of iodine was an outstanding failure, in several instances not even penetrating the epidermal layer of tissue.

The data given in the final Table of this series (19) were obtained by the same procedure as used in Table 16, i.e., tying the skin over the open end of a short sterile test tube and placing a ml. of a saline suspension of S. aureus on the inside, replacing the cotton plug and inserting the skin end of the tube in the compound allowing it to float undisturbed, except to subculture at intervals.

Using the Shippen subculture procedure with Merthiolate and brilliant green it was possible in this lengthy test to demonstrate that the dye could penetrate dead skin but required 66 hours even to tint the saline suspension inside the tube and 99 hours to reach sufficient concentration to produce a negative subculture. The 4 percent concentration of aqueous Merthiolate required less than 37 hours to give a negative subculture but the 1 percent solution of Merthiolate needed something over 60 1/2 hours to give negative subcultures with the 0.1 percent tincture yielding negative subcultures between 47 and 60 hours.

The iodine group was again obviously superior. Colloidal iodine penetrated in 2 3/4 hours while Lugol's solution required 15 minutes longer to render the seeding sterile. Tincture of iodine was almost as slow as the strongest solution of aqueous Merthiolate, requiring 14 to 17 hours to penetrate. Here, too, is strikingly shown why, in practical use, tincture of iodine is of little value

TABLE 19. - Relative Penetrative Time of Selected Compounds Acting on Epidermal Tissue Stretched over One End of a Glass Tube.

Rabbit No.9

Compound	Concentration	Time in Hours *														
		1:10	1:30	2:00	2:30	2:45	3:00	9:00	12:00	14:00	17:00	37:20	47:30	60:30	81:00	99:00
Iodine - Tincture	1:20				+	+	+	+	+	+	-	-				
Iodine - Lugol's	1:20			+	+	+	-	-								
Iodine - Colloidal	1:20	+	+	+	+	-	-									
Merthiolate - Tincture	1:1000 ($\frac{1}{2}$			+				+	+	+	+	+	∓	-	-	-
				+				+	+	+	+	-	-	-	-	-
Merthiolate - Aqueous	1:100 ($\frac{1}{2}$						+	+	+	+	+	+	∓	∓	-	-
							+	+	+	+	+	+	-	-	-	-
Merthiolate - Aqueous	1:25 ($\frac{1}{2}$						+	+	+	+	+	-	-	-	-	-
							+	+	+	+	+	-	-	-	-	-
Brilliant Green	1:20 ($\frac{1}{2}$			+				+	+	+	+	+	+	+	∓**	∓
				+				+	+	+	+	+	+	+	+	-
Control	Ringers Solution ($\frac{1}{2}$															+
																+

* One ml. of a saline suspension of S. aureus was placed inside short tubes and the skin covered end immersed in the test fluid. Subcultures were made into plain broth at intervals to determine when the suspension had been rendered sterile.

** Dye tinted the saline suspension in 66 hrs.; color was strong at 81 hrs.

∓ Subculture negative at 24 hrs., positive at 48 hrs.

1,2 sub-culture performed to dilute any compound carried from the test suspension to the first subculture.

unless allowed to dry upon the skin releasing the iodine held in solution. The alcohol instead of acting as a penetrative assistant is in reality a retarding agent. Because of its great solvent power it allows very little of the iodine to penetrate the skin. As shown by the two experiments (Tables 17 and 18) where the compounds acted upon live skin 6 and 8 hours, barely enough iodine from the tincture penetrated to give a residual sufficient to record (Table 17) even a small zone when the live epidermal layer was plated.

The double subculture method employed with the organic mercurial and the dye (Table 19) illustrate a point worthy of emphasis. This point is used as a basis for interpretation of an observation of a constant phenomenon which was noted throughout the entire study. Wherever double subculturing technique (Shippen) was employed it was found that just at the breaking point, i.e., where sterilization occurred, the first subculture would show no growth in 24 hours yet slight but definite growth in 48 hours. The second subculture tube would show no growth even after several days incubation. The purpose of the second subculture is to dilute beyond inhibition any of the compound which might produce bacteriostasis in the first subculture, thereby render it negative and give a false presumptive of sterilization.

Apparently a condition obtains in the test suspension, wherein, although there is insufficient compound to cause inhibition when transfer is made to the primary subculture, nevertheless there

has been enough action to result in a slow reduction in the number of viable organisms. A degree of scarcity is finally reached resulting in a primary subculture which is negative at 24 hours but becomes positive in 48 hours. At the same time there are initially so few viable organisms present in the primary subculture the secondary subculture seeded therefrom failed to receive any viable organisms at all yielding a bacteriostatic paradox of obtaining a negative secondary subculture before obtaining a negative primary subculture.

The reason is that the compound had penetrated the skin in sufficient concentration to kill as was macroscopically evident by the presence of the dye. Due to the slow penetration of the organisms many hours longer were necessary to obtain positive evidence (negative subcultures) of killing. This will be demonstrated conclusively in the next section.

The entire phenomenon is illustrated by the subcultures of brilliant green in the last two columns of Table 19, items 81 hours and 99 hours respectively. The primary subculture at 81 hours being negative at 24 hours and becoming positive at 48 hours indicates two coincidental effects, (1) probable reduction in the number of vigorous growing organisms in the test suspension and (2) temporary inhibition in the primary subculture which was overcome within 40 hours. The secondary subculture in this instance showed excellent growth in 24 hours. The fact that there was a faint trace of dye in the primary

subculture tube and no visible evidence of dye in the secondary subculture tube indicates that the further dilution by double subculturing performed its office of eliminating the bacteriostatic action of the dye. The subcultures taken at the 99th hour of the test demonstrate the validity of the premise of slow reduction in the number of organisms. In this case a 48 hour positive primary subculture and a negative secondary subculture were obtained. However, at this time sufficient dye had not penetrated the skin to result in any color being transferred to the second subculture tube. This tube supported good growth upon subsequent seeding with S. aureus, proving that the negative result could not be due to bacteriostasis.

Through the various tests previously described data have accumulated which when correlated point to what has been rather definitely demonstrated in these skin tests, i.e., that in order for a compound having any antiseptic or germicidal characteristics to penetrate any living cell it must bring about definite physico-chemical changes. These changes, whatever their nature — coagulation, denaturation, chemical combination with parts of the protein molecule — are, one and all, synonymous with death. This point cannot be emphasized too heavily when speaking of penetration of individual cells, especially bacteria. In the more complex organisms of the multicellular variety such as the larvae used emphasis on this point is less important since the functions (protection, food getting and assimilation, reproduction) of the organism as a whole are

delegated to various groups of cells rather than all being contained in a single cell as in the case of the bacterium.

From the standpoint of practical surface disinfection, particularly skin disinfection, it is well to bear in mind the above premises when speaking of penetration of any epidermal surface. Penetration of the live skin as such, by any compound that is definitely germicidal or bacteriostatic is most improbable since as it goes deeper it must kill each stratum of cells in its path. Hence it is difficult for a compound to penetrate deeply into the skin and underlying tissue because as one layer of cells after another are penetrated and killed the adjacent tissue juices, lymph and capillary flow will carry away the compound. This condition makes it increasingly difficult for the compound to reach a concentration high enough to conquer the next barrier of cells. If comparison is made between the extremely rapid bactericidal agents, iodine and chlorine, on the one hand, and the slow acting almost strictly bacteriostatic compounds represented by the bland dye on the other hand, it becomes obvious that the penetrative powers of these compounds shown by the skin tests, particularly those shown in table 19 (three hours for the aqueous iodine; 3/4 to 2 1/2 days for Merthiolate and almost 3 days for brilliant green) parallel the known relative rapidity of these compounds when used in practice. Thus it becomes necessary to admit that any compound when placed upon any epidermal surface for the purpose of killing organisms must be deleterious to the surrounding tissue cells

in order to be of any value against the organisms present. The only saving grace is that when effective concentrations of some compounds are considered there are usable dilutions which will, in a specified time of contact, be much more destructive to organisms than to tissue cells. Admitting that all compounds having any right to bear the name antiseptic or disinfectant are toxic to tissue cells, our attention must then be turned to the matter of relative toxicity. That is, the compound which in a usable concentration combines the least toxicity to tissue cells with the greatest toxicity to bacteria (relative penetration) becomes the article of choice. That the short-acting aqueous solutions of iodine possess this feature in a manner superior to all other compounds tested here, is obvious. This view has been definitely substantiated by the work of Salle and Lazarus (40), Nye (36), Samuels (42) and others.

It is noticeable that little effort has been made to demonstrate borderline differences in this tissue work. While realizing that some of the niceties of technical precision and more precise analyses of results such as interface phenomena, surface charge, etc., have been omitted, it was felt that more concrete worth could be drawn from the work if the cruder approach yielded more definite, inescapable facts.

Penetration of Bacteria

Speed Test

Upon the basis of the information derived from the foregoing tests the following simple procedure was devised for determining the ability of compounds to penetrate bacteria.

The technique employed was as follows:

Materials: (1) 32 tubes of broth (each containing 9 ml. of broth) were arranged in pairs, numbered, and 16 sterile petri plates were labelled to correspond,

(2) 200 ml. of sterile nutrient agar was melted and cooled to 45°C,

(3) Ample, not less than 30, one ml. sterile tip delivery pipettes were procured,

(4) 499 ml. of the dilution of compound to be tested was placed in a sterile one liter Erlenmeyer flask,

(5) The flask was warmed to 37°C. and

(6) A saline suspension of a 24 hour agar slant culture of S. aureus was prepared making sufficiently turbid to contain not less than 10,000,000 organisms per ml.

Method: (1) culture control - 1 ml. of the seeding suspension was transferred serially through 6 broth tubes plating 1 ml. from the last tube,

(2) 1 ml. of the seeding suspension was placed in the test flask, mixed,

(3) At intervals of 15, 30, 45 and 60 seconds, 2, 3, 4, 5, 7, 9, 11, 13, 15, 20, 25 and 30 minutes 1 ml. was transferred from the flask to each of the tubes in the first line of broth tubes,

(4) After the 4th transfer (at 60 seconds) there was sufficient time, while waiting the next interval, to return to the 15 second subculture and transfer one ml. to the second broth tube and thence 1 ml. of this mixture to the corresponding petri plate,

(5) The succeeding time interval subcultures were treated likewise,

(6) At the first opportunity usually between the fourth and fifth minute subcultures the previously seeded plates were poured with 10 ml. of sterile nutrient agar,

(7) Second culture control - after the 30 minute subculture was completed, Step (1) was repeated to detect any increase in organisms.

(8) At the close of the test all broth tubes and petri-plates were placed for 48 hours incubation at 37°C. and,

(9) The findings were recorded, positive or negative tubes at 24 hours and 48 hours and plate counts at 48 hours.

Table 20 is presented as illustrative of the typical record obtained from the above procedure. For the sake of brevity this table is condensed with ten other tables to form Table 21 containing the pertinent facts. With the facts gleaned from the work described in

TABLE 20. - Penetration of Bacteria by Elemental Iodine Within Limited Time Intervals

Tincture of Iodine 1:1,000,000 Temperature 30°C

Subcultures *				Plate Count at 48 Hours	Total Number of Surviving Bacteria
Number	Exposure Time	1st	2nd		
Culture Control **	0			70	70,000,000
1	15 sec.	+	+	1240	62,000,000
2	30 sec.	+	+	224	11,200,000
3	45 sec.	+	+	123	6,150,000
4	60 sec.	+	+	22	1,100,000
5	2 min.	+	+	1	50,000
6	3 min.	+	+	0	0
7	4 min.	-	-	0	0
8	5 min.	-	-	0	0
9	7 min.	-	-	0	0
10	9 min.	-	-	0	0

* One ml. subcultures from flask of compound seeded with bacteria. Subculture made into 9 ml. broth, mixed, and subsequently subcultured. One ml. from each of the second subculture tubes plated to note decrease in number of organisms. Tubes and plates incubated at 37°C.; read at 48 hours.

** One ml. of saline seeding suspension of S. aureus transferred serially through 6 broth tubes plating 1 ml. to obtain number of bacteria employed in seeding the flask.

TABLE 21. - Penetration of Bacteria (S. aureus) by Selected Compounds within Limited Time Periods.

Compound	Concentration	Initial Seeding	Acting Time	Reduction in Organisms	
				Residual Number	Percent
Iodine	1:1,000,000	80,000,000	2 min.	0	100
Iodine	1: 100,000	70,000,000	30 sec.	0	100
Merthiolate	1: 10,000	70,000,000	20 min.	17,500,000	75
Merthiolate	1: 5,000	67,000,000	15 min.	3,000,000	95
Brilliant Green	1: 20,000	50,000,000	30 min.	50,000,000	0
Brilliant Green	1: 10,000	11,000,000	20 min.	2,500,000	77

the preceding sections it was decided to employ only iodine, Merthiolate and brilliant green for this test. It was felt that in the selection of these three compounds the entire range of rapid — moderate — extremely slow penetration would be best demonstrated by iodine, Merthiolate and brilliant green, respectively.

The data obtained (Table 20) are in harmony with the accumulated data of the preceding tests. Elemental iodine in a dilution of 1 to 1,000,000 penetrated and killed the bacteria in 2 minutes while a 1 to 100,000 dilution sterilized the seeding within 30 seconds. These results occurred consistently irrespective of whether tincture, Lugol's or colloidal iodine was the stock solution used.

Merthiolate in a dilution of 1 to 10,000 effected a 75 percent reduction of the initial seeding within 20 minutes with little additional effectiveness noted up to 30 minutes. When used in a dilution of 1 to 5,000 a 95 percent reduction in the initial seeding was obtained within 15 minutes but complete sterility did not result in 30 minutes.

Brilliant green employed in a dilution of 1 to 10,000 was as efficient as Merthiolate at the same concentration reducing the initial seeding by 77 percent within 20 minutes. However, when this concentration was cut in half (1 to 20,000) this compound failed to give any reduction in the initial seeding within 30 minutes. That the compound was definitely bacteriostatic was proved by the observation that of the two series of broth tubes used as dilution blanks in which the concentration of the compound was 1 to 200,000 and 1 to 2,000,000 respectively none of the tubes showed any evidence of

growth even after extending the incubation period from 48 hours to 96 hours.

It was found necessary to use four broth tubes instead of two as dilution blanks for each time interval when the stronger concentrations of Merthiolate (1 to 5,000) and brilliant green (1 to 10,000) were tested. When only two dilution blanks were employed sterile plates resulted.

These results explain some of the points emphasized in the preceding sections and support the contention held that in order for a given compound to be an effective germicide it must possess the power to penetrate bacteria rapidly.

It is to be noted that no organic matter was employed in this test. Organic matter was omitted because true penetration of the bacteria was desired without confusing side reactions to consider. For organic matter to be of any value in this particular test the organisms would have to be enclosed by the particles i.e., grown within the particles so that the compound would have to penetrate the organic coating before reaching the organism. To employ any particular organic matter even of uniform size as suggested by Garrod (19), 1935, would merely serve to introduce another variable into the test. Those compounds that penetrate rapidly would enter organic matter and bacteria alike, being removed from the solution in the procedure. Consequently, stronger solutions of such compounds are required to effect sterilization. Compounds which penetrate

bacteria slowly would also penetrate organic matter slowly. This virtue of slowness enable weak dilutions of slow penetrating compounds to do the same total work that requires, comparatively, much stronger dilutions of the rapid acting compounds to accomplish. This comparison of the slow penetrating compound with the rapid penetrating compound under conditions of organic matter testing would make the former stand out as the superior bactericidal agent which is the reverse of the true state of affairs as demonstrated by this work.

SUMMARY

The compounds employed in this work are summarized in Table 22 under each test used according to their relative effectiveness. In arranging this table, the factors of temperature, substrate, concentration, quantity, and time of action were all considered. The information embodied in this table may be condensed by arranging these compounds in the order of their penetrative ability:

1. Iodine - Colloidal
2. Iodine - Lugol's
3. Iodine - Tincture
4. Merthiolate - Aqueous
5. Merthiolate - Tincture
6. Phenol
7. H T H
8. Brilliant Green
9. Azochloramid

The phenol coefficients of a group of selected disinfectants and antiseptics were found.

The mechanism of the agar cup plate method was shown to demonstrate diffusibility rather than penetrative ability.

Modification of the agar cup plate method was accomplished and the penetrative powers of the iodines, Merthiolate, brilliant green, azochloramid and phenol unequivocally established.

TABLE 22. - Summary of Compounds Employed According to Their Relative Effectiveness in Each Test Used.

Order of effectiveness.	Phenol co-efficient	Agar cup plate	Modified agar cup plate - penetration of adult chicken intestine	Penetration of adult chicken ceca	Penetration of coccidial Oocysts	Penetration of strongylid larval cuticle	Penetration of rabbit skin	Penetration of bacteria
1	Iodine***	Iodine -Lugol's	Iodine -tincture	Iodine -Lugol's	Iodine -colloidal	Iodine -colloidal	Iodine -colloidal	Iodine***
2	Iodine***	Iodine -tincture	Iodine -Lugol's	Iodine -tincture	Iodine -Lugol's	Iodine -Lugol's	Iodine -Lugol's	Iodine***
3	Iodine***	Iodine -colloidal	Iodine -colloidal	Iodine -colloidal	Phenol	Iodine -tincture	Iodine -tincture	Iodine***
4	Brilliant green	Merthiolate -tincture	H T H 65	Merthiolate -aqueous	H T H 65	H T H 65	Merthiolate -aqueous	Merthiolate
5	Merthiolate -tincture	Merthiolate -aqueous	Brilliant green	Merthiolate -tincture	Merthiolate -aqueous	Phenol	Brilliant green	Brilliant green
6	Merthiolate -aqueous	Azochloramid	Merthiolate -aqueous	Phenol	Iodine-tincture	Merthiolate -aqueous	H T H 65**	-
7	H T H 15	H T H 15	Merthiolate -tincture	H T H 15 *	Merthiolate -tincture	Merthiolate -tincture	Phenol **	-
8	Azochloramid	-	Phenol	Brilliant green *	Azochloramid	Brilliant green	-	-
9	Phenol	-	Azochloramid	Azochloramid *	Brilliant green	Azochloramid	-	-

* Failed
** Not demonstrable by technique employed
*** Tincture, Lugol's, and Colloidal Iodine gave identical results.

The time was determined which is required for the compounds to penetrate adult chicken ceca.

Unquestionable penetration was accomplished in determining the time required to kill coccidial oocysts by certain concentrations of the iodines, phenol, H T H, Merthiolate, and azochloramid.

To show actual penetration strongylid larvae were exposed to various concentrations of the group of compounds, the death of the larvae indicating penetration of the cuticle and the time required.

To determine the penetrative powers of the various compounds acting upon skin a series of trials was made on the live and dead skin of rabbits. The results show that some of the compounds can penetrate dead skin; and can penetrate live skin only by destroying the cells as they go deeper.

In the experiments to determine the actual time required by selected compounds to penetrate the minute cells of bacteria and destroy them, it was found that the usual high dilutions of iodine killed within 30 seconds to 2 minutes. Merthiolate required 20 and 15 minutes to reduce the total number of viable cells by 75 and 95 percent respectively. Brilliant green would not destroy any of the bacteria within 30 minutes when used in a concentration of 1 to 20,000.

Practical methods have been shown to yield more usable information concerning the penetrative powers of disinfectants and antiseptics than the present accepted routine bacteriological tests.

CONCLUSIONS

Penetration of bacteria by an antiseptic or disinfectant is absolutely essential to their ultimate destruction.

Activity, or power to penetrate rapidly, is a prime requisite of all compounds designed to kill bacteria within a short time interval.

Penetrative power is indispensable to all compounds even when allowed an extended time interval of action.

Of the selected group of compounds studied, iodine is the only substance consistently penetrating under all the varied tests employed. These results are in harmony with its rapid, irreversible destruction of bacteria as compared with the slower reversible action of the organic mercurial and the dye.

Iodine in the true colloidal state possesses inherent penetrative powers definitely superior to the same element in true solution. This greater activity is probably due to an increased distribution coefficient in favor of the organic test object.

Chlorine penetrates only by oxidation, i.e., first destroying the cell surface.

All compounds which have the power to penetrate tissue cells or bacteria in the original unaltered state of the compound do so only by killing the cell penetrated.

The speed test employed upon bacteria is a true means of obtaining the penetrative power of disinfectants and antiseptics.

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