THE BACTERIOLOGICAL STANDARDIZATION OF DISINFECTANTS.

SOME FURTHER SUGGESTIONS.

H. C. HAMILTON and TATSUZO OHNO.
From the Research Laboratory of Parke Davis & Co., Detroit, Mich.

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When any substance which finds application directly or indirectly in therapeutic measures cannot readily be standardized by any chemical method, other methods are usually attempted by which to determine its value. The medicinal substances which fail to respond to any chemical assay method are standardized by various means, of which perhaps the most important is the biological assay.

An example of valuable products whose values cannot, in most cases, be determined by chemical assay is that of the coal-tar disinfectants. While it is established that the value of the coal-tar oils as disinfectants resides largely if not entirely in those constituents similar to phenol, the crude product contains these phenols in such an endless variety that the chemical assay of any oil can give only a hint as to the actual value of that oil as a germicide. We resort, therefore, to a bacteriologic method and attempt to decide its value by testing it as a germicide.

It is almost impossible to make a laboratory test of a disinfectant which will duplicate the practical use of that product even in one particular case. How much more difficult it would be to duplicate every use to which such a product may be applied, is very evident. The infinite variety of conditions under which disinfection is practised opens wide the field for discussion as to the minor points which it is desirable to consider in outlining a method for their standardization.

This is but natural. One investigator, realizing the difference in resistance of different organisms, chooses an exceptionally resistant one, as *B. pyocyaneus*. Another, who is vitally interested in the disinfection of excreta, suggests *B. typhosus*. Another, wishing to avoid the danger lurking in a culture of *B. typhosus*, chooses *B. coli communis*.

Again, one investigator considers that a disinfectant should be valued on its prompt action and suggests that the dilution to be compared with standard should be that which kills the organism in a time between one and five minutes. Another suggests a half-hour as the maximum time, on the logical supposition that the disinfectant will be acting for at least

that period. So, too, opinions vary as to the temperature at which disinfectants should be tested and as to the medium in which the organism should be grown. While these points are not unimportant, any method which attempts to incorporate all of the possible suggestions would necessarily be too cumbersome for practical use.

Looking over the field of discussion, it is evident that one may eliminate as less important all methods but two; namely, that proposed by Doctor Samuel Rideal and T. Ainslie Walker and that proposed by Doctors John F. Anderson and Thomas B. McClintic, known, respectively, as the Rideal-Walker and Hygienic Laboratory Methods. The others may be eliminated from any specific reference because so many of their valuable features have been incorporated in these two methods.

The authors of this paper published a method (AMERICAN JOURNAL OF PUBLIC HEALTH, May, 1912), which has been in practical use for the standardization of commercial disinfectants for a period of fourteen years. In its essential details, the Rideal-Walker Method resembles it so closely that for all practical purposes they are the same.

In a later paper (AMERICAN JOURNAL OF PUBLIC HEALTH, June, 1913), the authors suggested some changes in following the Hygienic Laboratory Method, changes which appear to simplify what is really a complicated process. In that paper reference was made to the possiblity of evolving from the wealth of material at hand a simple practical method for standardizing disinfectants.

At this time we wish to present some data pointing to the marked variation in the results of germicidal assays by the Hygienic Laboratory Method. This data consists of: first, reports of tests of two disinfectants which had been submitted to three bacteriologic laboratories; second, the reported coefficients on several well-known disinfectants by various investigators; third, results obtained by using, as the test organism, two different strains of *B. typhosus* grown in the same culture medium; fourth, results of a long series of tests on two disinfectants under a variety of conditions, including the use of test organisms (*B. typhosus*, Hopkins) obtained from different sources and from the same source at different times; also the growing of the organism on or in different media.

As a conclusion to these illustrations, we wish to suggest certain steps which might be adopted to advantage as a means of obtaining uniform results in standardizing disinfectants.

When the Hygienic Laboratory Method was made public by the appearance of *Bulletin No. 82*, the authors of this paper immediately set about becoming familiar with the technique, with the idea that the method would soon become official. Such variable results were obtained, however, that it was decided to submit two samples to three prominent bacteriologists.

The remarks of these bacteriologists quoted in the letters below are sufficient to indicate that the method is one which leaves much to be desired. From letter dated January 28, 1914, signed by Dr. Herbert D. Pease, we excerpt the following:

"I believe now that it is, or will very soon be possible to obtain results with the Hygienic Laboratory Phenol Coefficiency Method which would be within 10 per cent. or 15 per cent. of each other. What I stated in my letter held good at that time, but I do not think it holds true in all respects at the present time. We are making a critical analysis of our methods at the present time and I expect to have them very greatly improved in the near future.

"Some of the points that I brought out in my letter of February 19 are still expressive of my opinion. The last sentence of the first paragraph, 'The variations in results are always greater the higher the coefficiencies, and are smaller the lower the coefficiencies.' I think even the percentage variation is slightly greater with the higher coefficiencies than with the lower.

"I do not believe we have yet learned all there is to know about the Hygienic Laboratory Method. It is quite possible that we can improve it, although I do not see very clearly at the present time just wherein any changes would produce such a result. I believe we should all keep working at the matter as far as we possibly can—I intend to try to do my part."

Hygienic Laboratory Phenol Coefficient No. 578273 $\left\{ \frac{\frac{275}{80} = 343}{\frac{400}{110} = 363} \right\} = 3.53.$

Hygienic Laboratory Phenol Coefficient No. 581507 $\left\{ \frac{\frac{275}{80} = 3.43}{\frac{375}{100} = 357} \right\} = 3.59.$

(signed) H. D. PEASE.

The letter of Dr. Joseph McFarland we quote in full as follows:

PHILADELPHIA, January 14, 1913.

We have at last completed the tests of Kreso 1 (Rx 578275) and Kreso 2 (Rx 581507). It was a long and tedious piece of work, fraught with many difficulties, and complicated by the rapidity of transplantation necessitated by the method worked out by Anderson.

The culture employed for the tests was the "Hopkins Typhoid Bacillus" obtained from Dr. Anderson. The carbolic acid used was Merck's "Absolute." We at first worked with a stock solution made by weighing out both the carbolic acid and the water, but subsequently abandoned this for a new stock solution made by measuring the water and weighing the carbolic acid. As you may note by an examination of the protocols, it is the latter solution that coincides with the solution used by Anderson.

You will see that the results obtained at the different tests were not uniform. To secure, as nearly as possible the precise strength of the Kreso solution, we made many tests of both and averaged them. We think that their averages give the strength as nearly as it can be determined, Kreso—1=4.58, Kreso—2=5.18.

We hope that these results are in agreement with your own.

Very truly yours,

(Signed) JOSEPH MCFARLAND.

Dr. Webster's letter including report is as follows:

We enclose you herewith report for examinations of Kreso Nos. 581507 and 578273. The phenol coefficient of this disinfectant was determined according to the method of *Bulletin No. 82*, Hygienic Laboratory, Public Health and Marine Hospital Service of the United States.

The delay in getting the report to you was occasioned by the fact that it was necessary to run three different series of tests before we could be sure of the test typhoid organism.

Trusting that this report may be satisfactory to you and thanking you for your favor, we remain.

Very truly yours,

CHICAGO LABORATORY.

(Signed) R. W. WEBSTER, per L.

Kreso No. 578273 $\frac{\frac{400}{80}+\frac{500}{110}}{2}=\frac{5+4.54}{2}=4.77.$

Kreso No. 581507 $\frac{\frac{300}{80}+\frac{450}{120}}{2}=\frac{3.75+3.75}{2}=3.75.$

TABLE I.

REPORTS SUMMARIZED.

Tested by.	Disinfectant.		Date of Tests.
	578273.	581507.	
	Coefficient.		
Dr. Herbert D. Pease	3.53	3.59	
Dr. Joseph McFarland	4.58	5.18	Oct., 1912.
Dr. R. W. Webster	4.77	3.75	
Tatsuzo Ohno	5.45	4.95	
H. C. Hamilton	4.75	4.3	
Average of the five results	4.61	4.35	
	Results on different dates.		
Ohno	7.1	7.9	July
	4.88	4.53	Oct.
	5.67	5.	Oct.
Hamilton	6.	5.	June
	4.75	3.6	Oct.
	5.	4.1	Oct.

That the Hygienic Laboratory Method often gives different results in the hands of different workers, is also evident from the following results compiled from different sources:

TABLE II.

Disinfectant.	Authority.	Result.
F	Hamilton	3.9
	Ohno	4.
	Hygienic Laboratory Bull. 82	6.06
	Label	6.
G	Hamilton	9.2
	Ohno	9.4
	Hygienic Laboratory Bull. 82	15.
	Label	15-16
H	Hamilton	9.
	Ohno	10.
	Pearson	22½
	Hygienic Laboratory	16.6
	Texas State Board of Health	18.
	Dr. Prescott	12.2
	Walker	22

(Ameriean Medicine, May, 1912)

The above instances are sufficient to make one doubt the correctness of any of the tests. It should be noted that the authors' results were in every case obtained by testing the same sample and by using the Hopkins' organism, coming either directly or indirectly from the Hygienic Laboratory, and grown for one week in bouillon according to the method described in *Bulletin No. 82* of the Hygienic Laboratory.

These instances show, too, that phenol alone cannot be depended upon as a safe control. If it were a reliable standard, the coefficient would not vary, since different conditions would affect standard and sample equally. Cultures of *B. typhosus* seem to acquire an increased resistance towards the coal-tar disinfectants, while remaining unchanged toward phenol. If not, why should we obtain results like in Table III.

It is possible that the character of the emulsifying agent in this case (gelatine) may have influenced the results obtained.

These tests were made about a week to ten days after receiving a fresh agar culture of the test organism from the Hygienic Laboratory.

The Hygienic Laboratory Method has invariably given a lower value to a coal-tar disinfectant than one obtainable by the other methods cited. The question, therefore, arose, what feature of the tests is responsible for this lower value. By planting culture No. 0190 in the medium used in the Hygienic Laboratory Method, it was at once found that the

test organisms are decidedly different in resistance, the Hopkins' strain being considerably stronger toward the coal-tar disinfectants than the strain previously employed (No. 0190. See Table IV). The Hopkins' strain, however, as is shown in Table I, had at one time not much greater resistance than culture No. 0190.

TABLE III.

GERMICIDAL ASSAY—HYGIENIC LABORATORY METHOD.

Test of Disinfectant, K.

Sample.							Phenol.						
Dilutions.													
						April 10, 1912.							
1600	−	−	−	−	−	−	90	−	−	−	−	−	−
1800	−	−	−	−	−	−	100	+	−	−	−	−	−
2000	+	−	−	−	−	−	110	+	+	−	−	−	−
2200	+	+	+	−	−	−	120	+	+	+	+	−	−
2400	+	+	+	+	+	+	130	+	+	+	+	+	+
						April 11, 1912.							
1900	−	−	−	−	−	−	90	−	−	−	−	−	−
2000	+	−	−	−	−	−	100	+	−	−	−	−	−
2100	+	+	−	−	−	−	110	+	−	+	−	−	−
2200	+	+	+	−	−	−	120	+	+	+	+	−	−
2300	+	+	+	+	+	−	130	+	+	+	+	+	+
Coefficient 20.													
						Sept. 9, 1912.							
1200	+	+	+	+	+	+	90	−	−	−	−	−	−
1300	+						100	+	−	−	−	−	−
1400	+	all					110	+	+	+	+	−	−
1500	+	growth					120	+	+	+	+	+	−
1600	+						130	+	+	+	+	+	+
						Sept. 10, 1912.							
800	−	−	−	−	−	−	90	−	−	−	−	−	−
900	+	−	−	−	−	−	100	+	−	−	−	−	−
1000	+	+	+	−	−	−	110	+	+	+	−	−	−
1100	+	+	+	+	+	−	120	+	+	+	+	−	−
1200	+	+	+	+	+	+	130	+	+	+	+	+	+
Coefficient 9.													

To determine whether the method of growing the organism has any appreciable effect on its resistance, a long series of tests was devised and carried out by the authors working independently. The experiment included the test of three disinfectants on the Hopkins' organism, three different cultures, lettered a, b, and c, being obtained at different times and grown in three ways, namely;

x continuously on bouillon,
y " " agar,
z alternately on agar and bouillon.

TABLE IV.

HYGIENIC LABORATORY METHOD.

Min.	Phenol.				D.				E.			
					Hopkins' Culture.							
2½	−	+			−	+			−	+		
	90	100	110	120	14	15	18	19	8	9	11	12*
15			−	+			−	+			−	+
				Coefficient	A	15.9				B	9.5	
					Culture No. 0190.							
2½	−	+			−	+			−	+		
15	100	110	120	130	16	17	22	23	10	11	13	14*
			−	+			−	+			−	+
				Coefficient	A	17.2				B	104.	

* Dilutions of D and E are in hundreds.

The first (x) was transplanted daily from bouillon to bouillon. The second (y) was transplanted weekly from agar to agar, a bouillon culture being made every other week and transplanted to bouillon daily. The third (z) was transplanted from bouillon to agar, where it grew one week, then transplanted to bouillon from this medium, transplants being made daily for one week, then to agar again for one week.

The results of seventeen tests covering a period of eight and one-half months is summarized in the following tables:

TABLE V.

Average for each culture grown under each of the three different conditions:

	B.		C.
ax	4.56	ax	9.38
ay	3.45	ay	9.30
az	4.24	az	8.91
bx	4.37	bx	9.12
by	4.28	by	9.43
bz	4.28	bz	9.24
cx	4.75	cx	9.24
cy	3.82	cy	8.81
cz	4.21	cz	9.03

Averages when culture is grown and transplanted differently:

x	4.58	x	9.25
y	4.11	y	9.18
z	4.24	z	9.4

Averages with the different cultures:

a	4.38	a	9.20
b	4.31	b	9.25
c	4.26	c	9.03

The averages shown in these tables are remarkably close, considering what variable results this method of testing has given on other occasions. But when one observes the extremes, the variable results obtained are more apparent.

The following tables show the lowest and highest coefficients obtained with each culture and each way of growing the culture, and shows also the difference between the extremes and the percentage this difference is, of the lowest coefficient.

TABLE VI.

DISINFECTANT B—EXTREME COEFFICIENTS.

	Lowest.	Highest.	Difference.	Per Cent.
ax	4.	5.5	1.5	37½
ay	3.68	4.9	1.22	33
az	3.48	4.92	1.44	41
bx	3.88	5.5	1.62	42
by	3.8	4.72	.92	24
bz	3.48	4.77	1.29	37
cx	4.1	5.7	1.6	40
cy	3.	4.77	1.77	59
cz	3.6	5.	1.4	41

DISINFECTANT C—EXTREME COEFFICIENTS.

	Lowest.	Highest.	Difference.	Per Cent.
ax	7.5	10.45	2.95	40
ay	7.6	10.4	2.8	37
az	7.6	9.54	1.9	25
bx	7.7	10.	2.3	30
by	7.4	10.7	3.3	44
bz	7.5	10.4	2.9	40
cx	7.6	10.3	2.7	35
cy	7.8	9.5	1.7	22
cz	7.7	10.	2.3	30

The plates illustrating this experiment consist of the curves obtained by plotting the averages of the results. The abscissas are the numbers of tests, the ordinates are the dilutions of the disinfectants.

Solid lines are results with cultures grown in bouillon continuously (x). Broken lines are results when the cultures were grown on agar continuously (y). Dotted lines, when the cultures were grown alternately on bouillon and agar (z). Cultures y and z are identical in first test; y, only, is recorded.

A, B, and C are the three disinfectants, being phenol; a, b, c, the three different cultures used. The black lines show results obtained by Hamilton, the red, those by Ohno.

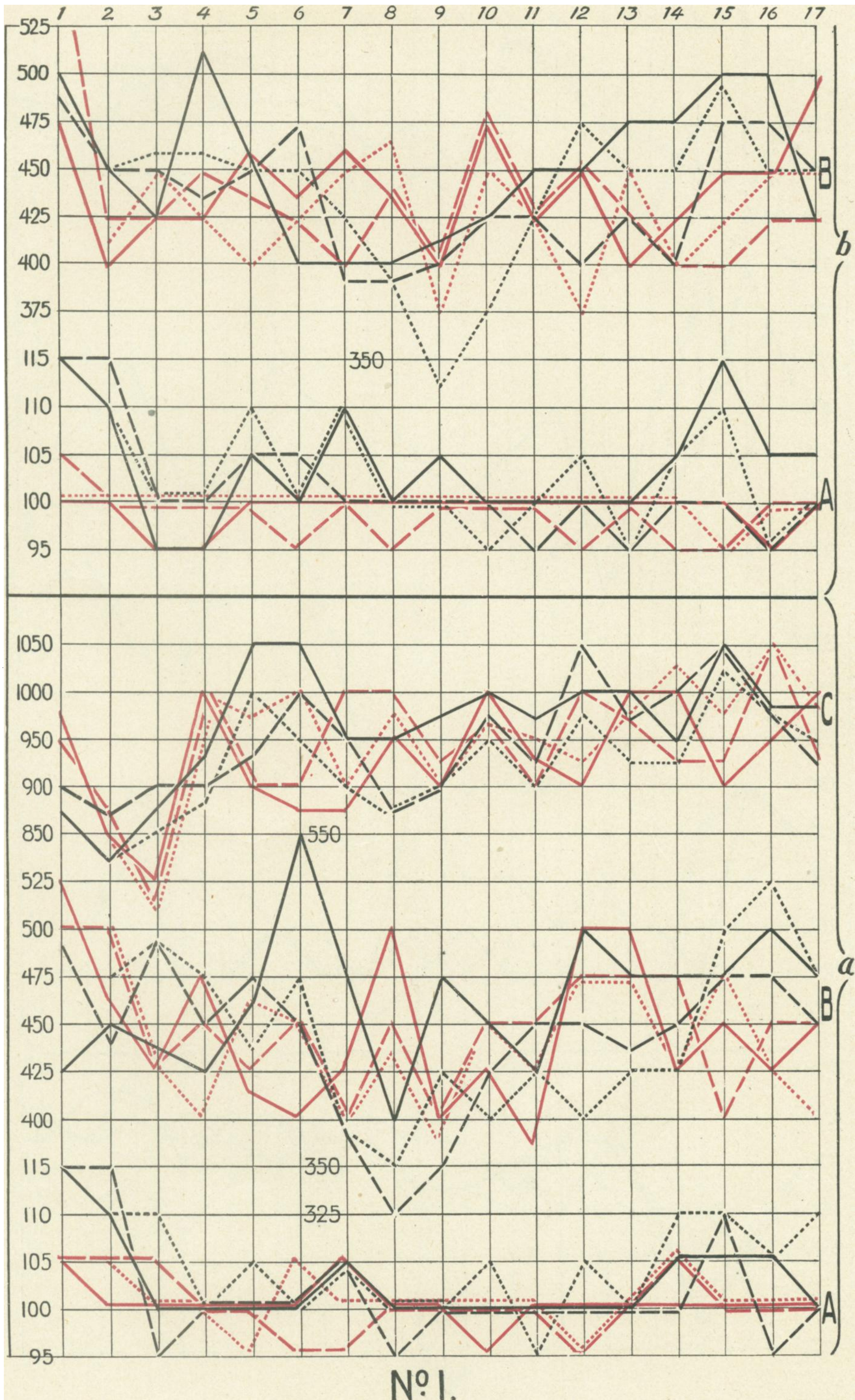
The location of the points determining the curve was obtained by using the average of the highest dilutions killing at 2½ and 15 minutes. For example, in Plate 1, the first test of carbolic acid (A), with the culture first obtained from Washington (a), and grown continuously in bouillon (x) by Hamilton (black solid lines), gave an average dilution 115 this being half the sum of the two efficient dilutions, *i.e.*, that allowing no growth in 2½ minutes and that allowing no growth at 15 minutes.

A straight black line separates the results obtained with (a) from those obtained with (b); an irregular line separating (b) from (c) begins between numbers 95 and 1050, on Plate 2.

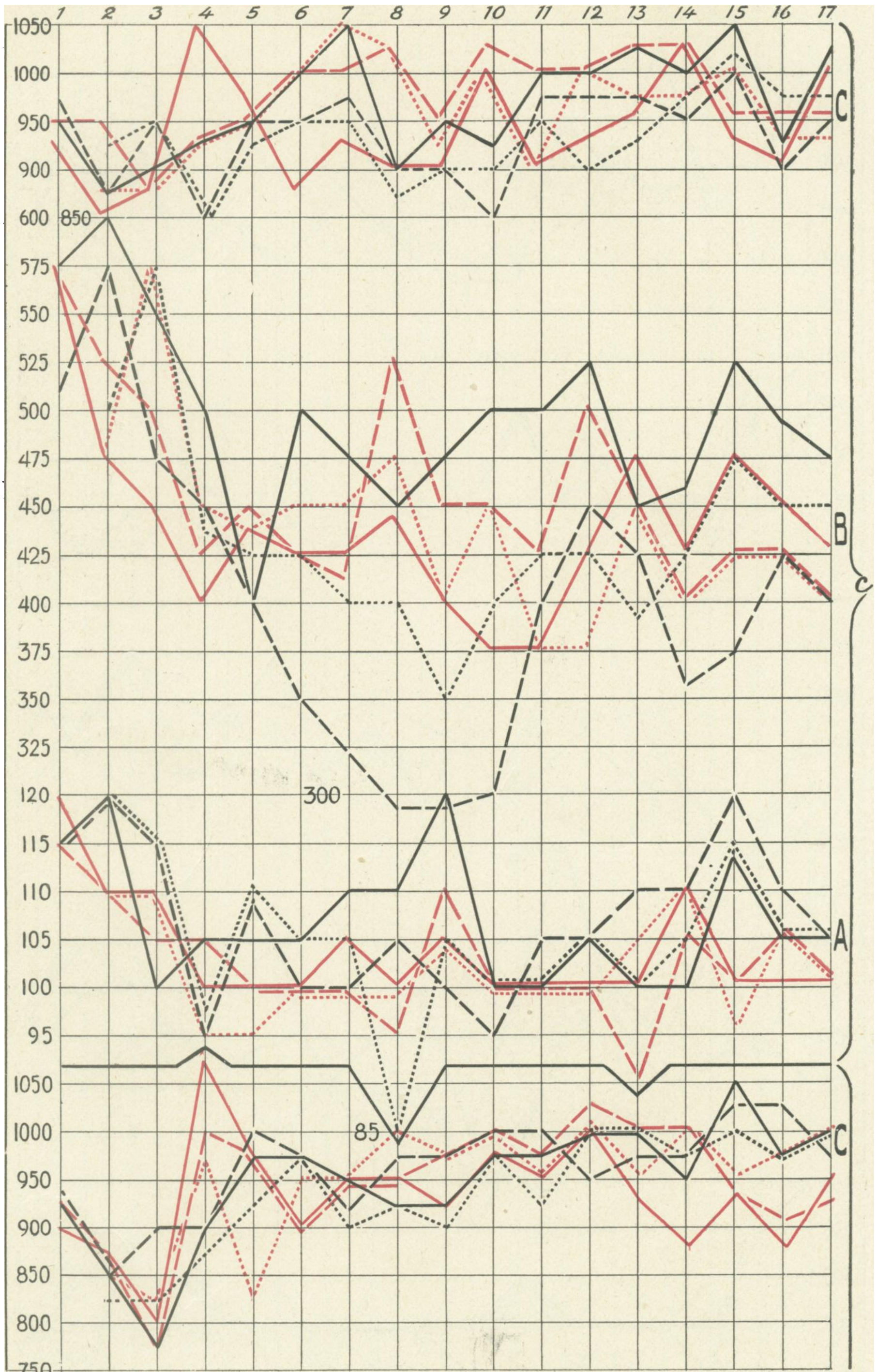
The zigzag character of the curves shows the very variable results obtained, while the fact that the red and black lines correspond so rarely shows that the medium used and the temperature of the incubator were not influencing factors since these were identical for both. One might conclude that culture (b), the second obtained from Washington and grown alternately on agar and in bouillon, gives the least variable results. The difference between this and the others, however, is only slight and unimportant.

We are not prepared at this time to explain many of the results shown in the above tables and curves. We may conclude, however, that certain unknown conditions very profoundly affect the resistance of the test organism. In fact, it seems almost unquestionable that the different and varying resistance of the test organism is responsible for more of the variable results than is the technique of testing.

We have shown that the Hopkins culture is more resistant than culture No. 0190 in either medium. The temperature at which the test is carried out is of secondary importance, but some certain temperature should be adopted for the sake of uniformity. The amount of the culture medium used should not be less than 5 cc., but it is only in the case where an amount of the disinfectant carried over in a loopful might be antiseptic in 5 cc. that any larger quantity is necessary. The amount of a healthy broth culture of the organism which should be inoculated into the 5 cc. of disinfectant is unimportant within reasonable limits. Results obtained



No. I.



Nº 2.

by using 0.1, 0.2, 0.4 cc. did not vary more than the variation due to the personal factor.

The point at which the comparison with standard is made seems unimportant and so little is gained by comparison at two points that one may be omitted to advantage. An average in the extremes of time rather than averaging the results at two times seems much more logical.

We wish to suggest, therefore, the following points which, as stated in the introduction, might be used to advantage in attempting to standardize disinfectants.

The Test Organism. There are some objections urged against the use of *B. typhosus* but no other organism seems better adapted to the purpose. The Hopkins strain suggested by the Hygienic Laboratory is perhaps as satisfactory as any other, although one which is more sensitive to the disinfectants with a coefficient of 5 or over is more accurate since it shows finer shades of differences between samples. Culture No. 0190 has been in use many years, and while occasionally the bouillon culture has been noticed to change in its resistance, the agar culture seems to be exceptionally uniform.

Method of Growing. To obtain the greatest degree of uniformity in the vitality of the culture apparently requires very little attention other than that which is so essential in bacteriologic technique; namely, pure cultures, sterile apparatus, uniform temperature and medium in which to grow and an occasional comparison of the bouillon culture with a fresh culture from the agar.

The Culture Medium. A medium containing more nutriment than that adopted by the Hygienic Laboratory seems to give more uniform results.

Proportion of Culture to Disinfectant. An average amount of culture for inoculating, such as 0.2 cc. per tube of disinfectant, is sufficient for obtaining good subcultures, is easily measured, and is not an excess.

Dilutions of the Disinfectant. The dilutions of the disinfectant to be tested are logically those which are approximately proportioned to the dilutions of the standard. If phenol is the standard and the dilutions increase by addition of 10, a disinfectant with a coefficient of 2 may have its dilutions increase by 20, if its coefficient is 5, by 50, if 10, by 100.

Loops for Transferring Subcultures. These should be of No. 23 U. S. gauge platinum wire, the loop being 4 mm. inside diameter. The means by which they are sterilized can be left to the ingenuity of the individual worker. See American Journal of Public Health, Vol. 3, No. 6.

The Temperature During the Test. Any convenient room temperature such as 20° to 22°C. should be adopted and maintained by any con-

venient method during the test. That suggested in the article previously cited (AMERICAN JOURNAL PUBLIC HEALTH, Vol. 3, No. 6) is very satisfactory.

Seeding Tubes. While one more surely guards against contaminating the subcultures by using the narrow seeding tubes recommended in both the Parke, Davis & Co. and the Rideal-Walker Methods, the wide tubes suggested by the Lancet Commission and adopted in the Hygienic Laboratory Method are more convenient and in a laboratory where contaminating influences are at a minimum, the use of wide seeding tubes is recommended.

Time of Contact between Organism and Disinfectant. An average time of 5 minutes after which all organisms should be dead seems a logical time limit for the reaction to take place, and since it is convenient to use a difference of 2½ minutes between times of subculturing the inoculated dilutions of the disinfectant, the logical way is to accept for comparison between sample and standard, those dilutions of each which fail to kill the organism in 5 minutes but which contain no live organisms in the 7½-minute subculture. All other dilutions and times of subculturing are non-essential under these circumstances and to eliminate them shortens and simplifies the process very materially. When subcultures are taken at only the two times; namely, after 5 minutes and 7½ minutes' contact with the disinfectant, one person can inoculate five dilutions in 2½ minutes, allowing one-half minute for each inoculation. Then, after a wait of 2½ minutes, one proceeds with the subculturing, planting one from each seeding tube in succession and immediately taking a second subculture from each. The first test of a disinfectant whose coefficient is not known can be made with so wide a range of dilutions that its character can be determined; then the second test can be made in comparison with the appropriate standard. Ten dilutions should be sufficient in the second test to cover the necessary range both for the sample and the standard. With an assistant to shake the seeding tube after it is inoculated and to aid in subculturing, 15 seconds is sufficient time for each operation and ten tubes can be inoculated and two subcultures taken from each in 10 minutes.

The Standard. It has been noticed repeatedly that changes in the resistance of the test organism toward coal-tar disinfectants, having coefficients of 5 or over, are not accompanied by a corresponding change in its resistance toward phenol. The critical dilution of the latter appears not to fluctuate nearly so much as that of the coal-tar disinfectants with different strains of the culture and at different times of testing. It seems advisable, therefore, to compare disinfectants with standards of similar origin and approximately the same coefficient.

TABLE VII

ILLUSTRATION OF PROPOSED METHOD.

Hopkins' Culture,
Liebig's Extract Medium,
Temperature—20°C.
Amount of Culture—0.2 Cc.

Minutes	Phenol		Disinfectant A*				Disinfectant B.*			
7½	−	+	−	−	+	+	−	−	+	+
	110	120	22	24	26	28	18	19	20	21
5	+	+	−	+	+	+	−	+	+	+

Coefficient A $\frac{2400}{110} = 22-$
Coefficient B $\frac{1900}{110} = 17+$

Steps in the process and time consumed for each.
Inoculating 2½ minutes.
Wait 2½ minutes.
First Subculture at 5 minutes.
Second Subculture at 7½ minutes.

* Dilutions of A and B are in hundreds.

If we have succeeded in proving the importance of the test organism and of the standard and the minor importance of many details in obtaining uniform results when testing disinfectants, and if our suggestions by which the process may be materially shortened and simplified receive consideration, the object of this paper will have been attained.