Microbiological Diffusion Assay I: Operations Studied with Cooper Equation

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Abstract □ The Cooper equation relating zone diameter to certain parameters was used to study the influence of variations in technique upon the assay of antibiotics. Variations in prediffusion time, incubation temperature, density of inoculation, composition of medium, and thickness of agar affected zone size. From 80 to 90% of certain effects could be removed from the assay by applying the Food and Drug Administration's correction to the petri dish single-dose assay. The two-dose petri dish design was the least sensitive to variations in parameters. Large plate assay was sensitive to variations in prediffusion times but not to the other parameters. Since these variations can be minimized, large plate methods were less susceptible to operational details than the one-dose petri dish method. Suggestions are given for minimizing errors.

Keyphrases □ Antibiotics—microbiological diffusion assay, influence of variations in prediffusion time, incubation temperature, density of inoculation, composition of medium, and thickness of agar studied using Cooper equation □ Microbiology—diffusion assay of antibiotics, influence of variations in technique studied using Cooper equation □ Diffusion assay—microbiological assay of antibiotics, effects of prediffusion time, incubation temperature, density of inoculation, composition of medium, and thickness of agar on zone size

The theoretical basis of the diffusion assay was established primarily by Cooper and his students beginning with the paper by Cooper and Woodman (1). Other researchers (2–5) published equations more complex but no more useful than that of Cooper. For detailed discussion of the several equations and contributions by the other authors, see the review by Cooper (6) which is the general reference for this paper.

The Cooper equation will be used to show the size of error in an assay caused by variations in the several parameters of the equation. In addition to application to plate assays in the usual sense, the equation will also be applied to tests of sensitivity of bacterial isolates by the disk technique. The assumption will be made that the Cooper equation is applicable to disk, cylinder–plate, and cup (holes in the agar) methods as well as to linear diffusion in tubes (superposition method) for which it was derived. The lessons learned from this study of the Cooper equation as applied to the design of equipment and operating procedures of petri dish assaying will be published later.

Diffusion or plate assays have been the most important method for measuring concentrations of antibiotics for the last 35 years. Most official methods around the world are plate assays. It is the principal method used to assay millions of samples each year. Two petri dish designs, cup–plate methods employing large plates, and the disk plate methods had assumed essentially their present forms by 1945. Several parameters affecting the quality of these assays had been discovered by then. However, there was no unifying theory to guide development of operating procedures. That came later when Cooper and his students published their work which, however, was never applied.

EXPERIMENTAL

Several forms of the Cooper equations will be used. Zone sizes usually will be reported as X^2. The equations will be written using natural logarithms to permit easier computation using small scientific pocket calculators.

For the first part of this study, the simplifying assumption is made that isothermal conditions prevail; this is not true in an actual assay. The Cooper equation is:

$$X^2 = 4D(T_0 + h)(\ln m_0 - \ln m') \qquad \text{(Eq. 1)}$$

where X is the distance diffused from the solution–agar boundary, D is the diffusion coefficient of the antibiotic, T_0 is the critical time, m_0 is concentration of antibiotic in the cup, m' is the critical concentration of antibiotic, and h is the prediffusion time, *i.e.*, time of incubation of plates (after addition of antibiotic) at a temperature too low to permit growth of the test organism (D_h must be for the lower temperature for the time h). Critical time is the time required for a constant number of generations of the test organism to develop from a standard inoculum (7). Since the number of generations is the same for all growth conditions, anything that affects growth rate also affects the critical time period. Lag time may be thought of as a prediffusion time occurring at incubation temperature and is included in T_0. Therefore, the equation may be rewritten as:

$$X^2 = 4DT_0 \ln (m_0/m') + 4D_h \ln (m_0/m') \qquad \text{(Eq. 2)}$$

Prediffusion Time—Calculations will now be made to show the effect of prediffusion upon zone size of a petri dish assay. The situation in assays when done without special precaution may be to fill the standard cylinders 1 or even 2 hr before the last sample is filled into cylinders. The standard and first samples will have a prediffusion time of as much as 2 hr, but the last samples will have a prediffusion time of only a few minutes.

In this example, the constants in Eq. 2 obtained in the study of Cooper and Gillespie (8) were: D_{25} = 0.83 mm²/hr, D_{37} = 1.1 mm²/hr, T_0 = 8 hr, and m' = 1 μg/ml. Therefore, by substituting in Eq. 2, it becomes:

$$X^2 = 35.2 \ln (m_0) + 3.32h \ln (m_0) \qquad \text{(Eq. 3)}$$

In a Food and Drug Administration (FDA) one-dose design, the reference zone on the sample plate is used to correct the sample zone to a standard condition. For this example, the standard curve is taken as 32, 40, 50, 62.5, and 78.1 μg/ml as in an FDA design with the 50-μg/ml standard as the reference concentration. The standard curve is put on first in the assay, followed by the sample plates for the next hour. The examples are the last three sets of samples to show how a prediffusion time of the standard of 1 hr at room temperature affects the measured potency. The first sample is the reference of 50 μg/ml, the second sample is one at 70 μg/ml,

Table I—Influence of Prediffusion Time of 1 hr upon Zone Size in a Streptomycin Assay Using *Staphylococcus aureus*

Concentration, μg/ml	Zone Size, X^2, mm²		Increase in Zone Size, %
	Prediffusion Time		
	0	1 hr	
8	73.2	80.1	9.4
16	97.6	106.8	9.4
32	122.0	133.5	9.4
64	146.4	160.2	9.4
128	170.8	186.9	9.4

and the third one is at 40 μg/ml. Results are listed in Tables I and II.

Temperature—Temperature affects zone size through several mechanisms. The first to be considered is the effect upon the diffusion coefficient, D, and the critical time, T_0. The value of D increases with an increase in temperature whereas T_0 decreases with an increase in temperature. Since T_0 decreases more rapidly than D increases, the product DT_0 decreases with an increase in the temperature of incubation. For a streptomycin assay using *Staphylococcus aureus*, values of DT_0 were computed at 36 and 37° from the data of Cooper and Gillespie (8); DT_0 was 9.40 at 36° and 8.8 at 37°. These values were used to compute the influence of temperature upon zone size and, consequently, upon potency of the samples. Results are listed in Table III.

Thickness of Agar—Thickness of agar is not an element of the Cooper equation, which was derived for linear diffusion in tubes. Of the five equations listed by Cooper (6, Table II), only that of Humphrey and Lightbown (4) has a factor for agar thickness. They show that X^2 (where X is distance diffused) is related to logarithm of agar thickness as was found by the experimental work already mentioned. Since thickness occurs in the equation as the logarithm, zone size is not very sensitive to variations in thickness for layers thicker than 1 mm.

The data of Humphrey and Lightbown (4) for inhibition of *Sarcina lutea* by penicillin were used in their equation to investigate the effect of thickness of the agar layer upon zone size. Distance diffused and zone diameters were computed for agar thickness of 4 mm for the standard plates and of 3 mm for the sample plates. The samples were the two extreme standards. Results are given in Table IV for potencies before and after applying the FDA correction.

Biological Factors—To this point, the influences of three physical factors (prediffusion time, thickness of agar, and incubation temperature) upon zone size have been illustrated. Now the biological factors of lag time, L, generation time, G, inoculum size, N_0, and critical concentration, m', will be considered. To this end, a different form of the equation is used (7):

$$X^2 = [4D \ln (m_0/m')][L + 1.44269G \ln (N'/N_0)] \quad \text{(Eq. 4)}$$

where N' is the critical population that forms the zone edges only at the end of the lag period or after cell division. Prediffusion time will be considered to be zero. The lag, L, is a property of the organism, medium, and treatment of the culture. In the example here at 37°, L = 0.75 hr, D = 1.1, m' = 0.75 μg/ml, G = 0.466 hr, and $N' = 3 \times 10^7$. Rewriting Eq. 4 to incorporate the constants yields:

$$X^2 = [4.4 \ln (m_0/0.75)][0.75 + 0.672 \ln (3 \times 10^7/N_0)] \quad \text{(Eq. 5)}$$

To find the error caused by differences in the size of the inoculum, N_0, a standard curve was constructed using 10×10^3 as the inoculum.

Values of X^2 were computed for m_0 = 50 for different sizes of inoculum, and the apparent potencies were obtained by interpolating from the standard curve (Table V). The proportional change (error) in the apparent potency of a sample was less than the proportional change in the size of the inoculum because of the logarithmic relationship.

DISCUSSION

The Cooper equation has been used to investigate the influence of variations in prediffusion time, incubation temperature, concentration of test organism, growth rate, thickness of agar upon zone size, and, from these, potency of a sample. The most significant factors in influencing petri dish assays are prediffusion time and concentration of bacteria. Both are usually poorly controlled.

Prediffusion Time—Prediffusion of standards causes an increase in the size of the inhibition zone and, consequently, a negative bias in the uncorrected potencies of the sample. As shown in Table I, a 1-hr prediffusion time of standards and not of sample causes a substantial difference in zone size. This difference corresponds to overestimates of potency ranging from 22% at the 8-μg/ml level to about 60% at the 128-μg/ml level in uncorrected assays. A difference in prediffusion time of 4 min caused an error of about 2% in uncorrected potency at 50 μg/ml.

Two classes of errors are caused by differences in prediffusion times in petri dish assays. One is caused by differences of prediffusion times occurring among the containers within a plate; no correction for these errors is practical. The other is caused by differences in prediffusion times between plates; the FDA correction is applicable to these.

Differences in prediffusion times within the sets of standard plates can be a source of unrecognized and uncorrectable error. The usual practice in petri dish assays is to fill all cylinders holding a particular concentration of standard and then to fill the set containing the next concentration. Filling the cylinders may require considerable time and during this time the drug diffuses from the cylinders. Thus, prediffusion time is different for each concentration of standard on the set of standard plates. A time as short as 5 min between filling of successive concentrations of standards into the cylinders on a plate can cause a measurable error. The error is maximum if the order of filling sets of cylinders is such that the highest concentration is filled first and minimum if it is filled last. The FDA correction is not applicable to errors caused by within-plate differences in prediffusion times. The errors can be reduced to insignificance by working rapidly. The 18 cylinders in a three-dish set can be filled in about 1 min, which is a time too short to cause appreciable errors.

The FDA procedure (9) corrects the sample zones on a plate for the deviation of the reference zones on the sample plate from the reference zones of the standards. This correction can be expected to be only a first approximation because the basic assumption is made that any environmental or operational cause of change in zone size does not affect the slope of the dose–response line. Consideration of the Cooper equations reveals this assumption to be incorrect (10). Nonetheless, the FDA correction is a good first approximation as inspection of Table II reveals. Under the worst conditions (the highest potency), the FDA correction reduced by 90% the error caused by prediffusion.

Table II—Effect of Prediffusion and FDA Correction upon Accuracy of a Streptomycin Assay

Concentration of Standard, μg/ml	X^2, mm²	Not Corrected		FDA Correction			
		Potency	Error, %	Reference Zone	Corrected Sample	Potency	Error, %
32	133.5						
40	142.1						
50	150.7						
62.5	159.3						
78.1	167.8						
Sample 1	137.7	35.68	−28.6	137.7	150.7	50.0	0
Sample 2	149.5	48.47	−30.7	137.7	162.5	67.94	−2.9
Sample 3	129.8	29.1	−27.25	137.7	142.8	40.78	+1.95

Table III—Effect of Temperature upon Zone Size by Influencing Values of D and T_0 in the Cooper Equations

Concentration, μg/ml	X^2, mm²		37° Standard			
	Temperature				FDA Correction Applied	
	37°	36°	Potency at 36°	Error, %	Potency	Error, %
32	130.3	122.0	—	—	—	—
40	138.7	129.8	51.6	+29	39.38	−1.55
50	147.1	137.7	65.2	+30.4	50	0
62.5	155.5	145.6	83	+32.8	63.47	+1.55
78.1	163.8	153.4	—	—	80.34	+2.88

The FDA correction is effective only when the reference standard and sample are filled into cylinders of a plate at the same time. This means that the prediffusion times of the solutions in the six cylinders (or disks) on a plate must be identical which, of course, is not true in the usual cup or cylinder assay. It can be achieved in a disk method. The disks containing the samples are placed on glass plate and all are transferred to the agar at the same time. Both petri dish and large plate designs were described (11).

The principal factor affecting large plate assays is prediffusion time, which is different for each cup. However, if the filling of the cups proceeds at a constant rate, as Simpson (12) insisted it must to achieve the highest precision, the total prediffusion times are the same for all test solutions in a balanced Latin square or quasi-Latin square design and assure lack of bias from this source only if each row of cups is filled in sequence always starting from the same side of the array. Prediffusion causes error in completely random designs and no correction is possible. Such designs generally are considered to be of low precision. The other operations that can have such large effect in petri dish assays have minimal effect in large plate methods. This is one of their advantages.

Temperature—Results in Table III are given for both the uncorrected potencies and for potencies after FDA correction. Even a difference in incubation temperature of only 1° between standards and samples caused large error (~30%). The FDA correction reduced the error to less than 3%, depending upon the potency of the sample. This example shows the importance of having uniform incubation conditions for assays. Temperature differences of only 0.1° from one part of the plate to another would cause appreciable error in large plate assays unless the gradient was linear and the design was a balanced Latin square.

The usual practice was to place the large plate on a shelf in the incubator as soon as the last hole was filled or the last disk was put in place. The plates were not stacked in the incubator. Since Latin square designs usually were employed, temperature gradients (they should have been small) would not affect the assays significantly unless there was a strong nonlinear component from one part of the plate to another.

The situation with petri dish assays was quite different in an unfavorable manner. Usually the dishes were stacked in layers of three or five high. Quite often they were handled in wooden boxes. The numbers were so large that an assay could occupy a considerable volume in the incubator and, thus, be subjected to large temperature gradients. Each plate within a stack had a different temperature history and, consequently, different zone sizes for the same sample (13).

The general rule is that lowering the incubation temperature increases the zone size. In sensitivity testing by the Bauer–Kirby technique, substantial departure from the standard temperature of 35° could cause misclassification of a culture.

The Cooper equation represents the complete dose–response line. Usually only a small portion of the line is ever presented. To illustrate the effect of changing the parameters upon the line, computed dose–response lines for the streptomycin–*S. aureus* system are given at two incubation temperatures for a sensitive strain and at one temperature for a resistant strain. The data were taken from Cooper *et al.* (14). The assigned diameter of the cups was 8 mm. The results of the computations are given in Fig. 1 as zone diameter (not square of the distance diffused) *versus* concentration.

The dose–response lines show the influence of incubation temperature and sensitivity, m', of the test organism upon zone size. The line obtained at 30° has the steeper slope because of the larger DT_0. The difference between the two lines for the sensitive strain is caused solely by the difference in slope, DT_0, because m' is independent of temperature. The difference between the lines at 40° is caused by the different values of m'; DT_0 was the same for the two strains.

If the square of distance diffused, X^2, were plotted instead of zone diameter, the lines would be straight. The two lines for the sensitive strain would have different slopes because of the different values of DT_0. The two lines at 40° would be parallel because the slope is determined by physicochemical factors that are independent of sensitivity of the test organism. Decreasing susceptibility of the organism moves the line toward the right in Fig. 1. Note that the lines are not straight.

Thickness of Agar—As shown by the results in Table IV, the influence of variation in thickness upon potency is much smaller than the percentage change in thickness. Therefore, nonuniform thickness of the agar should be of minor significance because total thickness is easily controlled with the required precision.

A plot of zone diameter *versus* log penicillin concentration gave slightly curved lines that were not parallel. The FDA correction applied to such is not perfect (Table IV). The residual errors are caused by use of inappropriate, although customary, dose–response lines. The uncorrected answers had a large error (~33%) in agreement with the observations of Hayes (15). These results show the utility of the FDA correction for reducing the effect of this operational defect.

In large plate assays, the agar should be of uniform thickness because compensation and correction are not possible. However, a small gradient of thickness from one side to another probably would be compensated for in a balanced Latin square or quasi-Latin square design.

A source of error more serious than variations in total thickness of the agar is variations in thickness of the inoculated layer of two-layer plates. Usually the lower layer is 20 ml of uninoculated agar and the top layer is 4–6 ml of inoculated agar. Four milliliters of agar forms a layer 0.67 mm thick in a 90-mm dish. Fortunately for the analyst, the top layer unlike the lower layer does not assume a surface determined solely by gravity. The agar in the thin top layer begins to harden as soon as applied to the base layer and, consequently, thickness is determined by forces in addition to gravitational. A small variation in thickness of the inoculated layer can

Table IV—Influence of Agar Thickness upon Potency in a *Sarcina lutea* Penicillin Assay[a]

Concentration, Units/ml	Standard d = 4 mm	Sample d = 3 mm	Potency		Error, %	
			Uncorrected	FDA Correction	Uncorrected	FDA Correction
0.1	16.38	16.97	0.1330	0.1046	+33	+4.6
0.2	17.76	18.29	—	—	—	—
0.4	19.01	19.50	0.534	0.400	+33.5	0
0.8	20.17	20.63	—	—	—	—
1.6	21.24	21.68	2.135	1.537	+33.4	−6.2

[a] Reference concentration is 0.4 unit/ml.

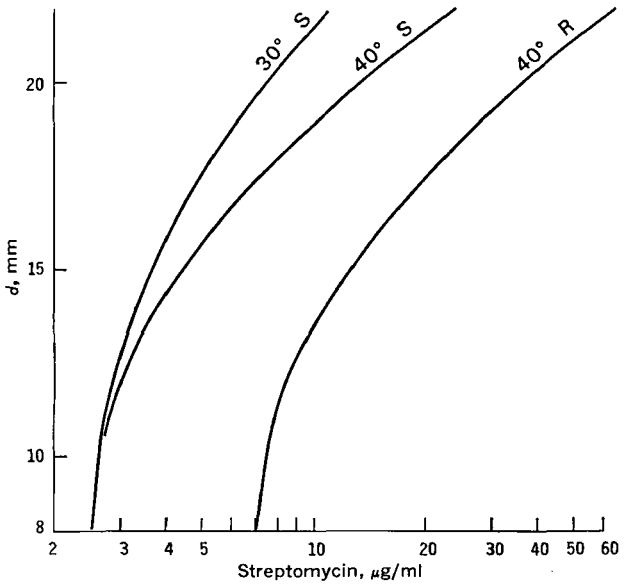

Figure 1—*Diameters of zones of inhibition by streptomycin of* Staphylococcus aureus *at 30 and 40°. The sensitive strain is S* (m′ = *2.5 μg/ml), and the more resistant strain is R* (m′ = *7 μg/ml). The diameters,* d, *were obtained from 2X + 8, where 8 was the inside diameter of the cup.*

cause a significant error because thinning of the layer, in effect, also reduces the concentration of the test organism. Errors caused by uneven top layer would not be corrected by application of the FDA procedure. Such error can only be avoided.

Biological Factors—The generation time, G, is a function of temperature, composition of medium, and inherent properties of the test organism. Therefore, composition of medium should be the same in all bottles of a multibottle assay. Changing the value of G changes the slope of the dose–response line. Therefore, the FDA correction would be incomplete. An increase in G of only 0.1 min would increase the uncorrected potency at the 50-μg/ml concentration of Table V by 1%. Vitamins, amino acids, minerals, and other growth substances present in a sample and not in the standards may increase the growth rate (decrease in value of G) of the test organism and, thereby, reduce the size of the zone. This kind of interference is well known to those who must assay low potency samples of natural origin such as those encountered in residue testing.

The critical concentration, m', of the antibiotic was independent of incubation temperature but not of medium composition for inhibition of *S. aureus* by streptomycin (6). Salts increase values of m' for inhibition by streptomycin, thus accounting for the well-known influence of salts upon streptomycin assays. The value of m' was greater for a resistant strain of *S. aureus*.

Changes in the value of m' will change the zone size but not the slope of the dose–response line. In the previous example, an increase of m' of 0.05–0.80 μg/ml caused a decrease in zone size equivalent to a decrease in potency of 6.25%.

A common practice is to use petri plates prepared from several bottles of agar. Each bottle is inoculated with *approximately* the same volume of inoculum. Results in Table V indicate that the inoculum must be identical if substantial error is to be avoided. However, if the assay is large plate or is one to which the FDA correction can be applied, the error caused by variation in inoculum can be reduced greatly. One advantage of the large plate method is immediately obvious; variation in inoculum cannot occur (if the inoculum is properly mixed with the agar) because the plate is poured from only one bottle.

Table V—Influence of Concentration, N_0 in the Cooper Equation, of Inoculum upon Computed Zone Size

Inoculum, $N_0 \times 10^{-3}$	X^2, mm²		Sample of 78.1 μg/ml			
	m_0		Uncorrected		FDA Correction	
	50	78.1	Potency	Error, %	Potency	Error, %
8	116.85	129.26	88	+12.5	78.96	+1.1
10	114.08	126.19	78.1	0	78.1	0
12	111.82	123.69	71.5	−8.45	77.41	−0.8

A 10-fold change in quantity of inoculum could cause a 3-mm change in zone diameter. The plates with the smaller inoculum would have the larger zones. This much change in zone size in a sensitivity test could move an organism from susceptible to resistant or vice versa as Linton (7) suggested. The example was for streptomycin inhibiting *S. aureus*. Other antibiotic–organism systems may be more sensitive to the size of the inoculum.

The first diffusion assays were done with agar inoculated on the surface. Surface inoculation was certain to produce large plate-to-plate and, perhaps, even within-plate variations in surface density of organisms. The importance of uniform inoculation (Table V) was not recognized at first. Surface inoculation was abandoned early by analysts (16). Ericsson (17), however, was still using surface inoculation in 1960 and it is used in sensitivity testing. All precise diffusion assays are now done with bulk-inoculated agar following the technique introduced by Foster and Woodruff (16).

In certain kinds of assays, of which testing for residual antibiotic in animal tissues is an example, the assays are operating at the limit of sensitivity. The day-to-day variation in responses may be large as in the examples of Kline and Rathmacher (18). Maximum sensitivity is sought in these assays; consequently, the assays are very sensitive to operating conditions. Every factor in Eq. 4 must be carefully controlled to produce consistent daily sensitivity. Here, the problem is not so much accuracy as it is the difficulty in producing a detectable zone reliably over several days.

Testing of clinical isolates of bacteria for susceptibility to antibiotics usually is done by procedures not suitable for quantitative work. The method of inoculating is certain to cause considerable plate-to-plate variation. However, this variation usually is not important because categorization of the organism usually is based upon the results from only one plate. Correlation of zone sizes with minimum inhibitory concentration (MIC) but, more importantly, with clinical efficacy is what is significant when using the test in hospitals. The results of such tests are only a guide to therapy and not an indication of the certainty of successful treatment. Nonetheless, attention to test details can increase reliability of the method.

CONCLUSIONS

The influence of operations upon four types of assays have been considered. They are the original single-dose petri dish assay, the single-dose petri dish assay incorporating the FDA correction, the two-dose petri dish assay of FDA, and the large plate assay. The single-dose assay is the most susceptible to variations in operations and the two-dose method is the least. Errors could be large in the single-dose method without the FDA correction. A two-dose assay is particularly attractive because each dish is a complete assay that can be quickly set up. The Latin square large plate design can be precise if filling is at a constant rate. A disk method can avoid the prediffusion problem. This study of the design of diffusion assays shows the advantages of the large plate method over the usual petri dish ones.

Nonuniform incubation temperature, agar thickness, and inoculum were potent sources of error. Uncorrectable errors were caused by differences in prediffusion times between reference standards and samples in all petri dish assays. Certain designs of tests and procedures will minimize errors caused by the inevitable variations in operations. These errors cannot be eliminated from petri dish assays but they can be reduced to insignificance. Procedures known to reduce errors are: *(a)* incubating the plates in one layer in an incubator free from temperature gradients, *(b)* preparing all plates used in a test from one bottle of inoculated agar, *(c)* pouring the agar into plates sitting on a truly level surface, and *(d)* filling all cups on a plate in 1 min or less. These conclusions concerning procedures needed to produce accurate assays were obtained from a quantitative study of operation using the Cooper equation.

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Automated System for Analytical Microbiology IV: Accuracy of Measurements

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Abstract □ The automated parts of a system designed for performing microbiological turbidimetric assays were tested for accuracy and precision of photometric and volumetric measurements. The system was tested with potassium ferricyanide solutions. The photometric measurements were accumulated and processed by an on-line computer. They had an average standard deviation of about 0.020. The diluter apparently could contribute an additional small variance to the measurements. The answers had a slight positive bias. Uncorrected multiple reflections in the cell can cause significant errors. The use of a multipoint standard curve makes it unnecessary to correct for multiple reflections in the cell in high precision measurements. Digitizing errors were very small.

Keyphrases □ Automated system—analytical microbiology, studies on precision and accuracy of photometric and volumetric measurements □ Microbiology, automated analytical—determination of accuracy of photometric and volumetric measurements □ Reproducibility—automated system for analytical microbiology, precision and accuracy of photometric and volumetric measurements evaluated

A system[1] for performing turbidimetric microbiological assays was described in detail previously (1, 2). Application of computer technology to the system was shown to improve precision and accuracy by reducing computational errors (3).

The system consists of a diluter, an incubation bath, and a reader. The diluter prepares dilutions of 66-fold and 100-fold in pairs and delivers the diluted sample to an array of test tubes in a carrier. The reader causes solution from the test tubes to flow through a fixed cell in a spectrophotometer and records percentage transmittance of the flowing solutions. The accuracy and precision of the measurements performed by the system are the results of the accuracy and precision of the measurements made by the two modules. The errors and variances were small because the philosophy governing the design was to reduce the electromechanical variations to such an extent that they would not contribute a significant variance to the microbiological assays. The goal was a maximum variation of 0.1% in dilutions or transmittances. Experience with routine measurements of dye solutions indicated probable achievement of the goals. It was also recognized that the accuracies and precisions reported were not necessarily the best values because of the limited resolution of the 3-digit voltmeter used with the spectrophotometer.

Improvements accompanying installation of an on-line computer made an evaluation of the system's accuracy and precision worthwhile. The system was tested by processing colored solutions. No assay, not even a high precision one, was suitable because of the introduction of variances external to the system being tested by the microbiological portion of an assay.

EXPERIMENTAL

The purpose was to test the accuracy and precision of the volumetric and photometric measurements of the system. Test solutions were prepared from solutions of potassium ferricyanide dissolved in pH 7 phosphate buffer. These solutions were used because they were shown (4) to follow Beer's law with requisite accuracy (1 part in 10,000). Measurements of transmittance were made at 415 nm by one of two spectrophotometers[2].

Two classes of test solutions were prepared. One was used to test photometric accuracy and the other to test the diluter as well as the photometer. The first class was prepared by diluting a careful-

[1] The AUTOTURB System was obtained from the Elanco Division of Eli Lilly and Co.

[2] Coleman-Hitachi model 101 with Hewlett-Packard 6203B dc power supply for the lamp or a modified Turner model 330 as modified by Arthur H. Thomas Co.