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Automated System for Analytical Microbiology II: Construction of System and Evaluation of Antibiotics and Vitamins

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Abstract ☐ An automated system for photometric microbiological analysis was designed, constructed, and evaluated. The system consists of two completely automated modules and a water bath. One module pipets accurately measured volumes of sample and dilutes them with nutrient broth into assay tubes. The other module measures and records the subsequent turbidities of the suspensions in the assay tubes. Measurements are made on a rapidly flowing stream to eliminate flow birefringence problems. Data can be acquired on printed paper tape, punched tape, or punched cards or can be attached to an on-line computer. Antibiotics and vitamins are assayed by this system with at least a fivefold reduction in analytical error and a twofold increase in the productivity of laboratory personnel relative to manual assays. Examples of data from assays for penicillin G, erythromycin, vitamins B_{12}, and nicotinic acid are given.

Keyphrases ☐ Microbiological assay—automated ☐ Automated system—photometric microbiological analysis ☐ Antibiotics, vitamins—automated analysis procedures ☐ Turbidimetric analysis, microbiological—automated procedures

In a previous paper, basic theoretical and practical considerations for the design of an automated system for turbidimetric microbiological analysis were discussed (1). This paper reports on the construction of a system meeting these design requirements. Applications of this system to the analysis of several antibiotics and vitamins are reported, and some typical results are presented.

EXPERIMENTAL

Diluter Module—Figure 1 shows a photograph of the diluter module. Both this and the reader module employ a specially designed tube transport mechanism. Empty test tubes are placed in carriers shown in Fig. 2. Each carrier holds an 8 × 10 tube array. These carriers are stainless steel and have a oneway handle so that they can be inserted into the transport mechanism in only one orientation. Two of these carriers can be loaded into the tube transport mechanism at one time. The carriers are moved by a rake mechanism which advances the carriers as required under either manual or automatic control.

Associated with the diluter module is a control panel, shown at the upper right of the module. This control panel contains all the controls necessary for operation of the module. Mounted on the left upper portion of the module is an automatic sampler and turntable which can hold up to 40 tubes for automatic sampling. The struc-

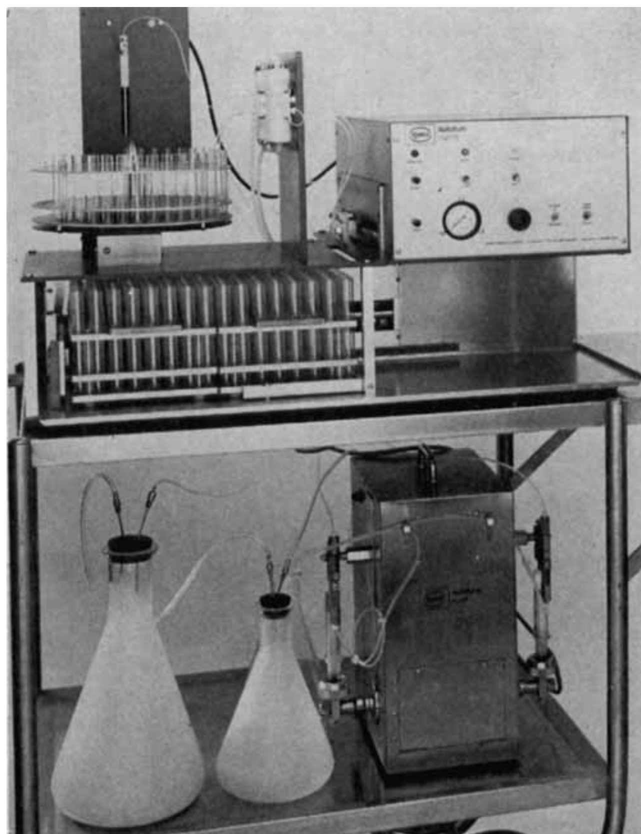

Figure 1—*Diluter module.*

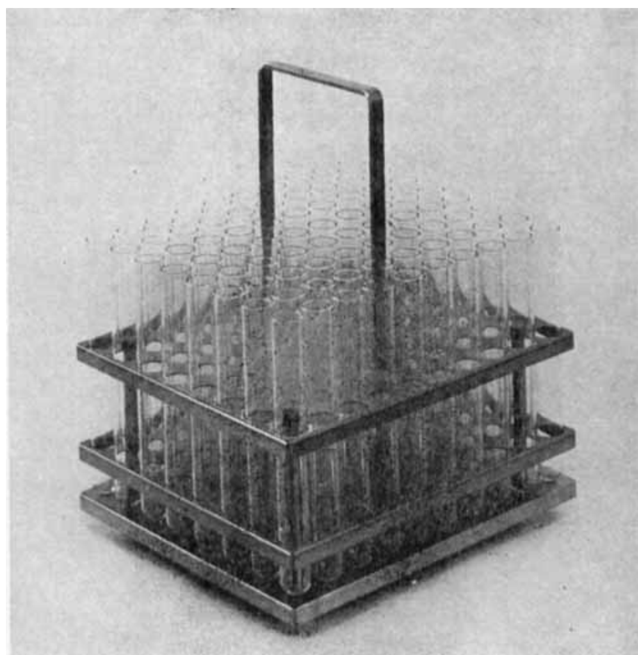

Figure 2—*Tube carrier.*

ture between the sampler and the control section is the valve system for metering the samples from the turntable. Below the diluter module is a delivery device for providing the necessary amount of diluent for each sample.

In practice, the operator places a container of nutrient broth, which may or may not be inoculated with test organism, in front of the diluent unit and drops the Teflon lines leading to the delivery unit into the nutrient broth. A wash trough (Fig. 3) is placed in one carrier, and the carrier is placed into the diluter unit. The diluter is automatically purged, and the washings are collected in the waste trough. Meanwhile, the operator places a turntable loaded with sample tubes on the sampler. One or two carriers of empty tubes are then placed into the diluter. The diluter starts processing the samples on the turntable as soon as the empty tubes are in position, and the operator presses the "start" switch.

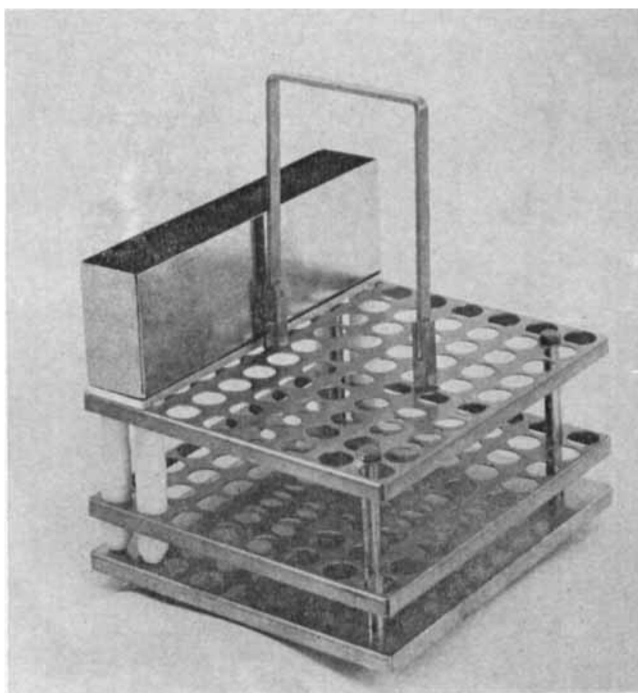

Figure 3—*Wash trough inserted in carrier.*

From this point until the last row of assay tubes in the carrier is filled, the diluter operates without attention, and the operator is free to do other tasks. Four dilutions are made from each sample in the turntable, and each dilution is performed in 5 sec. Thus, a complete load of 40 sample tubes, providing 160 dilutions, requires less than 14 min. of dilution time. This time interval is so short that, under most conditions, no significant growth of inoculum or interaction between organism and drug substance occurs during this period.

The entire load resides in two carriers or assay units. Since these two units are handled separately in subsequent steps, they should be considered as individuals; for maximum precision, each carrier must contain its own standard curve. Thus, each assay unit (carrier) requires less than 7 min. for dilution; since each unit contains its own standard curve, many such units can be processed from one batch of inoculated broth. The carriers are manually transferred to a specially designed water bath for incubation.

Cleanup of the diluter is also very easy and rapid. The rake is reversed, and another carrier with two waste troughs is inserted. The lines in the nutrient broth container are lifted out of the container, and then the lines are transferred to a container of hot water. The diluter is again started and run for less than 2 min. For longer sterilization, a "purge" switch can be used to cycle the diluent delivery unit continuously for as long as desired, or one or more extra wash troughs can be inserted initially. The hot water container is then removed and substituted by one of cool water or another of nutrient broth and the diluter is again turned on for a short time, depending on how many wash troughs are inserted. The diluter is now either cleaned or primed for the next samples, depending on whether the cooling liquid was water or inoculated nutrient medium. In either case, about 5 min. is required for cleanup or changeover from one nutrient broth to another. Some workers prefer to replace the hot water sterilization with another agent such as dilute formalin[1].

The dilution is performed in a unique manner. Two 8-port diluting valves are used, and both are connected by a T arrangement to a sample probe and a common waste receptacle. Each valve has two Teflon sample loops attached to it. In these laboratories, each valve is fitted with a 0.1- and a 0.15-ml. loop. These loops are easily removed and can be replaced with loops of other volumes in a matter of seconds. The two loops operate 180° out of phase with each other, so that while one is being filled, the other is being emptied of its contents. The loops are filled by vacuum and are emptied by discharging a predetermined quantity of nutrient broth through them.

Dilution is performed by a repetitive series of sequential events. A sample probe fitted with a small diameter line is lowered into a sample tube on the sampler turntable. Vacuum is applied through sample loop 1 to the probe, flushing out the previous contents of the sample loop and filling it with the new sample. The vacuum is then shut off, and the sample loop remains filled with the new sample. The 8-port valve is now moved pneumatically to its alternate position to connect the sample loop between the delivery line and the diluent pump. A metered amount of nutrient broth is then passed through the sample loop, washing out its contents into an empty test tube in the carrier located below the delivery line. While this action is occurring, the second sample loop is being filled with another portion of the same sample in the same manner as just described. The diluent valve is now returned to its original position, and the sample in loop 2 is washed into another test tube by a second portion of nutrient broth delivered in the same manner as before. During this time, sample loop 1 is filled with the contents of the next sample in the turntable.

Since two diluent valves operate in synchrony, four dilutions are automatically made from each sample. The extent of dilution is determined by the internal volume of each of the four loops attached to the diluent valves and the volume of broth delivered. These alternate actions are performed until all tubes in the carriers are filled. Since, in effect, the metering valves operate 180° out of phase with the sampler, only two dilutions of the last sample in the tray are delivered as diluted samples. To obtain four dilutions from each sample and to get complete samples in each assay rack, positions 20 and 40—or the last of any intermediate series in the sampler tray—should be buffer or tubes of sample dilution fluid. In practice, this turns out to be an advantage, since the first and

[1] This is possible since all materials in contact with the nutrient broth are Teflon, Kel-F, or stainless steel.

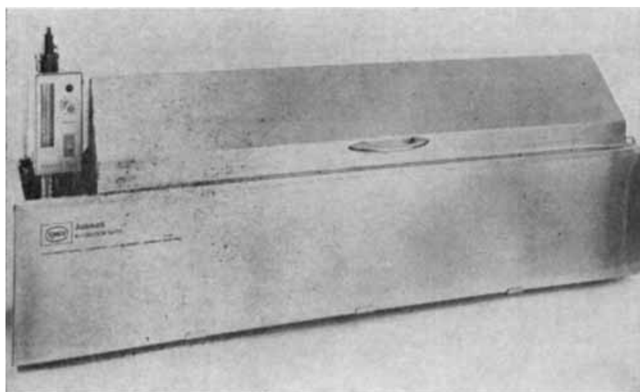

Figure 4—*Incubation bath.*

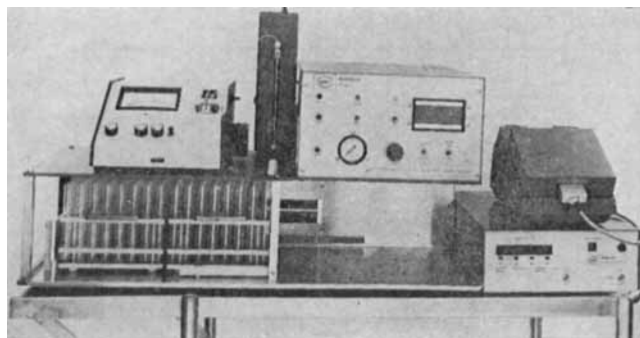

Figure 5—*Reader module.*

last two positions in each carrier contain no drug substance and any drift that might occur can thus be easily monitored.

The amount of sample removed from the sample tube is determined by the length of the withdrawal cycle, the diameter of the tubing, and the vacuum. Changes in the amount of sample withdrawn can be made by changing the vacuum by means of the vacuum control valve located on the control panel. This is especially useful if nonaqueous solutions, such as methanol or acetone, are to be sampled.

The dilution system used is a closed tubing system. Regardless of loop size, the volume of diluted sample remains the same because the contents of the sample loops are replaced with an equal volume of diluent during each cycle. For this reason, loop sizes can be changed without affecting the final volume of diluted sample; only the dilution ratio is changed. All the critical interconnecting tubing was carefully selected to be of minimum volume so that sample interaction would be essentially eliminated. The metering valves are of the zero dead-volume type and do not contribute any interaction. The only tubing that could be responsible for interaction between samples is either thoroughly washed with the next sample or remains filled with the diluent common to all samples. When the system is used properly, the only carryover from one sample to another is the minute amount on the outside of the sampler probe. This can be illustrated by use of concentrated dye solutions followed by water samples. Dye solutions up to 10 times the usual concentrations can be used before any indication of interaction is seen.

All solutions in this system flow in one direction, and there are no dead sample cavities or cross-connections. No part of the sample probe or tubing connected to it ever contacts the solutions containing the test organism. This arrangement of connections and flow pattern makes cleaning easy. Flushing the sample probe with a tube or two of sterile water while the medium lines are being sterilized by pumping hot water through them, as if performing a dilution, gives adequate cleaning. Depending upon the sequence of test organisms, media, and antibiotics, sterilization may not be necessary.

Incubation Module—After dilution, the assay carriers are moved manually to a specially designed water bath (Fig. 4). This bath holds four carriers, *i.e.*, 320 tubes equivalent to 76 samples and standards. The bath provides rapid water circulation and precise temperature control. The importance of proper incubation becomes evident when operational errors normally found in manual methods are eliminated. For example, some antibiotic assays can be influenced by a temperature nonuniformity of 0.05°. Most antibiotic assays are not that sensitive, but temperatures should be controlled so that the maximum difference between any two tubes in a test would be less than 0.1°.

After the optimum period of incubation for each test, the assay carriers are removed from the incubation bath and further growth of the test organism is stopped. A convenient method of temporarily stopping growth in antibiotic assays involves immersion of the carrier into an 80° water bath for 1–2 min., followed by subsequent cooling to about room temperature. With vitamin assays, cooling in a chilled water bath is adequate. Some assays may require shaking of the tubes before measuring turbidity. For those assays, a device is being designed to shake an entire carrier of tubes at one time.

Reader Module—The readout is accomplished automatically. Figure 5 shows the reader module. The basic tube transport mechanism is the same as for the diluter. A spectrophotometer is placed on the upper left portion of the transport mechanism. Its output is monitored by a digital voltmeter located on the control panel. The output of the spectrophotometer is adjusted so that the output voltage is 1.000 v. representing 100% T (transmittance). A reading such as 0.648 v. then represents 64.8% T. A probe assembly is substituted for the delivery-line-carriage assembly of the diluter module.

After the carrier of incubated tubes is placed in the reader and the "start" button pushed, the probe assembly advances to the position of the first tube, and the probe lowers into the tube directly below it. A solenoid valve in the vacuum line opens, and the suspension of bacteria in the tube is drawn rapidly through the flowcell of the spectrophotometer. The spectrophotometer continuously monitors the flowing stream. After about 6 sec. of flow, the reading on the voltmeter is automatically stored. The voltmeter reading can be read manually or the value can be recorded automatically by one of several means.

After the reading of the spectrophotometer is made, the flow is stopped, the probe is raised from the tube, and the probe assembly advances to the next position. If other tubes are to be read, the same cycle is repeated for each tube until the test is completed or until the "stop" switch is depressed. The inside tube of each row in the carrier is sensed; if any of these positions are vacant, the rake advances the carrier to the next occupied position or clears the carrier.

Each reading cycle takes 10 sec., and each carrier is read in about 13.5 min. With automated data recording, an operator can leave

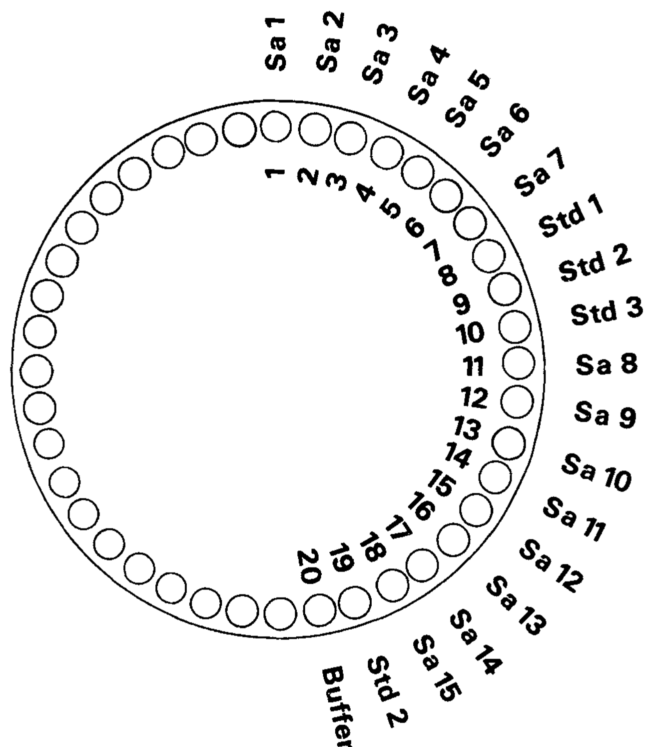

Figure 6—*Sampler turntable arrangement.*

Table I—Dilution Pattern

Tube	Row 8	7	6	5	4	3	2	1	Start
1	Sa 15	Sa 12	Sa 10	Std 3	Std 1	Sa 5	Sa 3	Buffer	From previous run
2	Sa 15	Sa 12	Sa 10	Std 3	Std 1	Sa 5	Sa 3	Buffer	
3	Sa 15	Sa 13	Sa 10	Sa 8	Std 1	Sa 6	Sa 3	Sa 1	Loop 1
4	Sa 15	Sa 13	Sa 10	Sa 8	Std 1	Sa 6	Sa 3	Sa 1	Loop 2
5	Std 2	Sa 13	Sa 11	Sa 8	Std 2	Sa 6	Sa 4	Sa 1	Loop 3
6	Std 2	Sa 13	Sa 11	Sa 8	Std 2	Sa 6	Sa 4	Sa 1	Loop 4
7	Std 2	Sa 14	Sa 11	Sa 9	Std 2	Sa 7	Sa 4	Sa 2	
8	Std 2	Sa 14	Sa 11	Sa 9	Std 2	Sa 7	Sa 4	Sa 2	
9	Buffer	Sa 14	Sa 12	Sa 9	Std 3	Sa 7	Sa 5	Sa 2	
10	Buffer	Sa 14	Sa 12	Sa 9	Std 3	Sa 7	Sa 5	Sa 2	

delivery line movement (↓ down the tubes); assay rack movement (→)

the reader unattended for periods up to about 27 min. It should be emphasized that the reader monitors a fast-flowing stream. The amount of sample withdrawn is regulated by the vacuum control valve on the control panel.

Automated Readout—All readers used in these laboratories have some form of automated data acquisition attached to them. The simplest system uses a digital converter to accept the BCD (Binary Coded Decimal) output from the digital voltmeter and to drive a paper tape printer. Printed on the tape is a test code, three digits of automatic sequence data, and four digits of data representing percent T to the nearest 0.1% T.

Several systems also contain a coupler for an IBM 526 summary card punch to punch simultaneously cards for off-line computer calculation of the data. One system drives a paper tape punch rather than a card punch. Most of these systems will eventually be interfaced directly to an on-line computer to provide computed data as soon as each carrier has been read. The interface is being designed and should be operational in the near future.

ANALYSIS PROCEDURE

The assay design is different for antibiotics and vitamins, and each will be treated separately. In each, the sample is automatically assayed at two concentrations because more than one concentration is required to detect aberrant samples. For example, in the assay of antibiotics, if the two potencies obtained from the two concentrations of sample differ from the mean by more than 5%, a problem should be suspected. If the two potencies differ from the mean by 10% or more, standard and sample differ in some significant manner, and the problem should be investigated. The capability to detect such problems was provided by the use of two sizes of measuring loops in each system.

Antibiotic Assay—A statistical study of this system showed that the potency of a sample was independent of the dilution at which it was assayed. Therefore, any dilution that provides a concentration of active material falling between the two ends of the standard curve is suitable. No special effort need be taken to dilute the sample exactly to an estimated predetermined concentration. The most accurate method of sample preparation should be used, even though the resulting concentration is estimated to be a number such as 5.67 mcg./ml. Standards and samples should be diluted by means of volumetric flasks and transfer pipets or by an *identical* schedule using automatic diluters. The diluting medium must be the same for samples and standards if highest accuracy is to be obtained.

A wide range of total growth (zero tube or the tubes without antibiotic) will give satisfactory assays. The range depends upon characteristics of the test bacterium, composition of medium, temperature of incubation, and time of incubation.

The standard curve should be selected so that the smallest concentration gives not more than 90% of the growth obtained in the zero tube while the high end of the curve gives not less than 20% of the growth of the zero tube. The range may be from 2- to 10-fold, depending upon the assay system. As an example, the range for the erythromycin standard curve can be 2, 4, 6, and 8 mcg./ml. in the sample tubes, resulting in concentrations of 0.02–0.12 mcg./ml. in the assay tubes. For samples whose content is likely to be unpredictable, this long curve is used and the sample is diluted to an estimated concentration of about 5 mcg./ml. If the product potency can be accurately estimated, a standard curve of 3, 4, and 5 mcg./ml. is used and the sample is diluted to an estimated 4 mcg./ml. concentration. Spacing of the concentrations of standards is on a linear scale, not a logarithmic one as for a diffusion assay.

Also needed is a tube of the solution used for diluting the samples, usually a buffer, water, or nonaqueous solvent. The samples and standards are placed in the sample turntable in the desired order. One possible arrangement is shown in Fig. 6 where the contents of one-half of a sample turntable are shown.

Standards can also be placed at the beginning or at the end of each test or can be randomly distributed throughout the array. The single duplicate standard is used as a check sample. It can be replaced with a secondary standard of known concentration. The last position must be a buffer or zero tube. The pattern shown in Fig. 6 would produce a diluted array as shown in Table I.

A single-strength nutrient medium is prepared and is inoculated with test organism just before dilution. Because of the speed of dilu-

Table II—Reproducibility Using Dye Solutions, Readings in Percent T

	0.1-ml. Loops: Loop 1	Loop 2	0.15-ml. Loops: Loop 3	Loop 4
	99.9	100.0	100.0	99.9
	47.9	47.9	33.2	33.2
	47.8	47.9	33.2	33.3
	47.8	47.8	33.2	33.3
	99.8	99.9	100.0	99.9
	99.9	99.9	99.9	100.0
	47.9	48.0	33.3	33.2
	47.8	47.9	33.2	33.3
	47.8	47.8	33.2	33.3
	47.8	48.0	33.2	33.3
Average	47.83	47.90	33.21	33.27
Range	0.1	0.2	0.1	0.1
CV, percent	0.102	0.170	0.113	0.146
Absorbance	0.321	0.320	0.479	0.478
Average absorbance of series of manual dilutions	0.319		0.479	
Ratio of 0.15/0.1-ml. loops 0.4785/0.3205 = 1.49				

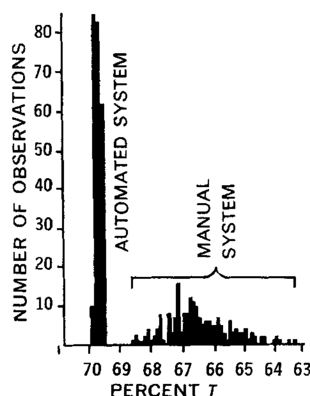

Figure 7—*Reproducibility of readings obtained by reading individual tubes of a pooled suspension of* Lactobacillus leichmannii.

Table III—Penicillin G Assay—Automated System

Known Potency of Sample, units/ml.	Day 1	Day 2	Day 3	Day 4	Day 5	Average	Range
1.50	1.49	1.50	1.50	1.49		1.50	1.49–1.50
2.00	2.03	2.03	2.04	2.01	2.00	2.02	2.00–2.04
2.50		2.49	2.52	2.55	2.54	2.52	2.49–2.55
		2.50	2.53	2.53	2.51		
3.00	2.97	3.04	2.99	2.97	3.05	3.00	2.97–3.05
3.33		3.33	3.34	3.37	3.43	3,37	3.33–3.43
4.00	4.04	4.05	4.00	3.99	4.04	4.03	3.99–4.05

tion, nutrient medium at room temperature is usually satisfactory if dilutions are performed as soon after inoculation as possible. In certain cases, the medium might have to be chilled or left at room temperature for some time after inoculating to obtain optimum results. Stirring of the medium is not always necessary and, in fact, may be harmful to the accuracy of the assay if significant heating of the medium is produced by the stirring device.

After incubation, the suspensions of killed organisms are measured on the reader module. When paired dilutions are used, the pairs can be observed for a quick check on the quality of the data. In general, if manual calculations are used, the results from each pair are averaged and separate standard curves are plotted for the 0.1- and the 0.15-ml. loops. Unknowns are referenced to the appropriate curves, and two assay values are obtained for each sample in the sample turntable. These two values, if they match the slope criterion, are averaged for the reported answer.

In most antibiotic assays, reduction in growth rate of the test organism by the test drug causes the test response. Therefore, the bacterial population at any time is proportional to the initial population (inoculum). An error in the inoculum causes an equal error in bacterial concentration at the termination of incubation. The volume of broth delivered to the assay tubes and, consequently, the concentration of inoculum differ by 0.5%, with the inoculum in the assay tubes receiving 0.15 ml. of sample being the smaller. This seemingly small difference in inoculum can cause an appreciable difference between the two dose–response lines. The difference in quantity of inoculum prevents the responses from the two sizes of samples from being portions of one dose–response line. The two sets of assay tubes are, in effect, different assays and must be treated as such; otherwise, accuracy of the system will be degraded. The influence of inoculum size is greater in certain assays than in others.

The millivolt output of the spectrophotometer should be plotted against concentration of antibiotic in the sample tube, using an inverse semilogarithmic graph (2) when manual interpolation is to be done. If a computer is used, millivolts are transformed to absorbance and log *A versus C* is plotted. This equation may also be used in manual computations, although it is less convenient than the inverse semilogarithmic graph. A point-to-point curve is constructed, and the results are interpolated from the curve. If a computer is used for calculation, it is preferable to calculate each loop against itself, resulting in four standard curves and four responses for each sample. The necessity for precise calibration of the loops and exact adjustment of the diluent unit is eliminated when each loop is compared only to itself. Rounding-off errors in the averaging of two measurements are also avoided.

Table V—Vitamin B_{12} Assay

Sample	Theory	Automated Assay	Manual
Secondary standard			
Day 1	107.1	107 mg./ml.	107
2		106 mg.	112
3		108 mg.	111
4		106 mg.	120
5		106 mg.	
6		107 mg.	
7		108 mg.	
8		108 mg.	
Lot A	42.5	45.0 mg./ml.	43.9
		41.8 mg.	50.2
Lot B	2.15	2.00 mg./ml.	2.22
		2.03 mg.	2.31
Lot C	28.0	28.0 mg./ml.	43.7
		28.4 mg.	29.8
		28.2 mg.	19.6
		28.0 mg.	27.7

Vitamin Assay—Total growth (concentration of microorganisms, not percent *T*) in a vitamin assay is proportional to the amount of vitamin put into the tube up to a limit set by the composition of medium and subsequent pH. There is a linear range with a span adequate for practical assays. The upper limit of the standard curve should be set at some point below the upper end of the straight-line portion of the curve. If points at 0 and at one-half of the high level standard are included for both standard and sample, the slope ratio method of calculating potency may be used as in common zero-five-point assays (3). Two separate assays would be obtained, just as in the antibiotic assay, because each size loop is treated separately. In this design, the standard would be at concentrations of 0, 1, and 2 and the samples at a concentration ratio of 1:2. Each sample would be assayed at two sample levels. This design is precise but may be more precise than is needed. This ideal response of concentration of bacteria is the one to use when investigating assay procedures and the quality of dose–response lines.

A more practical approach may be to use a multipoint standard curve and a single concentration of sample. A representation is then selected that gives a curve as nearly straight as possible. Such a curve may be obtained by first transforming millivolts into absorbance and plotting *A versus C*. The two different levels of sample, automatically produced, are different enough to detect abnormal samples.

Any desired design can be used, but the last tube must be a sample blank. Two tests can be loaded into the diluter at once. Single-strength medium without inoculum is used. After the tubes are diluted, they are capped and sterilized by autoclaving. The tubes are cooled prior to inoculation. Inoculation must be carried out very carefully since many vitamin tests are now run, at least partially, as growth-rate assays. The use of one drop of a suspension of bacteria per tube does not provide a sufficiently reproducible inoculum for each tube in the assay. A more satisfactory method is to dilute the inoculum by a factor of 10 and inoculate each tube with 0.5 ml. of this suspension. A precise repetitive pipeting device should be used to add a constant volume of inoculum to each tube.

Table IV—Precision Study on Erythromycin Using the Automated System

Assay Date	Sample Set	Test A, mcg./ml.				Test B, mcg./ml.			
		Std. 3	2.5	3.75	5.0	Std. 3	2.5	3.75	5.0
Day 1	1	3.02	2.25	3.35	4.42	2.94	2.21	3.33	4.38
	2	2.99	2.22	3.33	4.50	3.06	2.25	3.40	4.52
Day 2	3	2.99	2.20	3.41	4.46	3.01	2.26	3.42	4.46
	4	2.92	2.19	3.34	4.44	2.90	2.22	3.35	4.36
Day 3	5	2.99	2.26	3.40	4.45	2.99	2.25	3.32	4.51
	6	2.94	2.26	3.32	4.39	2.95	2.24	3.32	4.40
Day 4	7	3.01	2.24	3.34	4.39	2.92	2.25	3.38	4.42
	8	2.98	2.23	3.26	4.37	3.00	2.26	3.32	4.46
Day 5	9	2.96	2.22	3.35	4.44	2.99	2.25	3.32	4.47
	10	2.98	2.14	3.24	4.38	3.03	2.29	3.32	4.46
Average		2.98	2.22	3.33	4.42	2.98	2.25	3.35	4.44

Table VI—Nicotinic Acid

Sample		3-Day Acidimetric	1-Day Turbidimetric	Theory
Secondary standard		116.0	99.6	100
		95.2	100	
		99.5	98.8	
		97.3	101.6	
	$\bar{x}$	101.9	100.0	
Sample A		27.5	29.4	28
		30.3	27.7	
	$\bar{x}$	28.9	28.5	
Sample B		37.2[a]	27.9	28
		24.4	29.6	
	$\bar{x}$	24.4	28.8	
Sample C		26.1	29.1	28
		36.2[a]	28.1	
	$\bar{x}$	26.1	28.6	
Sample D		17.4	19.6	20
		14.2	20.1	
	$\bar{x}$	15.8	19.8	

[a] Invalid assays.

Incubation of the inoculated tubes is carried out in the normal manner. Termination of growth can be accomplished by chilling in a cold water bath before reading. Each tube must be shaken to resuspend the organisms before measuring turbidity with the reader module.

RESULTS

Dilution Error—Considerable effort was expended in designing a dilution system that would be rapid and precise and have a minimum of sample interactions. The system was evaluated using dye solutions to test these design goals. Table II shows the total error involved in the dilution and readout process using dye solutions. FD&C Blue No. 1 was used as the dye, and its percent *T* was read at 620 nm. The dye samples were interspersed with water, and all the readings are shown in Table II. Good precision is obtained and no significant interaction is observed between dye samples and water.

The loops can be cut to match exactly, if desired, and their ratios can also be exact, if necessary. Such matching requires three or four successive calibrations, with the loop length being trimmed each time. If a computer is used for calculating the results on each individual loop, only one calibration is necessary since the loops do not have to be closely matched. Loops have maintained their calibration for over 3.5 years. This is to be expected since the loops do not contact any moving parts and have walls heavy enough to prevent any change in internal volume. Since they are of small diameter [*i.e.*, 0.08 cm. (0.030 in.) i.d.] and are flushed with 10 ml. of liquid in about 5 sec., the high flow rate of liquid in every cycle thoroughly flushes the loops and prevents any accumulation of debris which could affect internal volume. The loops, being made of Teflon, provide a smooth inner surface which is not subject to coating by foreign materials. Microorganisms have no opportunity to grow on the walls of the tubing.

Table VII—Table of Coefficients of Variation[a], Production Control Department

	Manual	Initial Automated	Recent Automated
Erythromycin standard check	13.1	5.76	1.88
Erythromycin secondary standard	12.4	4.66	1.58
Tylosin standard check	12.7	4.40	1.37
Tylosin secondary standard	8.71	6.07	2.00
Vitamin B_{12} secondary standard	4.63	1.50	1.41
Calcium pantothenate standard	16.0[b]	4.50	3.20
Calcium pantothenate secondary standard	16.0[b]	6.00	3.42
Cephalexin	9.34[c]		1.79
Neomycin	19.9[c]		1.76

[a] Coefficient of variation is defined to mean 1 *SD* (calculated on single assays) divided by the average obtained on these assays. [b] This value is typical but obtained by another method. [c] These results represent plate assays.

Fast-Flow Readout—Figure 7 illustrates the practical application of the use of fast flow systems. A pooled suspension of *Lactobacillus leichmannii* was prepared and subdivided into 400 tubes. Two hundred tubes were read on the existing semiautomated system. This system consisted of a Lumetron with a conventional flow-through cell, a Leeds and Northrup type H potentiometer, a timer, a shaft position encoder, and a translator to drive an IBM 526 card punch. The operator emptied the suspension into the flowcell and, after a 13-sec. automatically timed interval, the result was punched on an IBM card. The results are seen on the left of Fig. 7 and range from 63.4 to 68.8% *T*, with one reading at 74.5% *T*. This variation is not due to drift since the results varied in a random manner. The readings of the other 200 tubes were made on the automated fast flow system and are shown at the right of Fig. 7. These readings range from 69.6 to 70.0% *T*. This same comparison has been conducted in these laboratories several times with essentially the same results.

Analysis of Antibiotics—Two illustrations of the use of the equipment for antibiotics will be presented. A large number of other examples could be given, but these results are typical of those obtained with many other antibiotics.

The data presented in Table III are a summary of 5 days' results on a series of dilutions of a standard penicillin solution. The first column is the known value and, reading across, the values obtained on each of the 5 days. Each value represents the result calculated from four individual diluted tubes or one sample in the sampler turntable. Each of these results is obtainable in 20 sec. of dilution time and 40 sec. of readout time. Standards were run at 1, 2, 4, and 6 units/ml., and the ratio of the number of sample tubes to standard tubes was 5 to 1. It can be seen from the range that all the results except one were accurate to ±2% of the true value, and the averages show no bias.

A more comprehensive precision study is shown in Table IV. This study was made on erythromycin base. The only components common to all the assays were: the stock standard solution, the operator, the slant of the test organism, the lot of assay broth, and the assay system. Ten weighings were made of slightly more than 100 mg. of the sample of erythromycin base into 100-ml. volumetric flasks. The base was dissolved in acetone, and the solution was diluted to give 1.000 mg. of solids/ml. Two freshly prepared dilutions were assayed each day in two tests. Each solution and its standard were diluted to assay level using volumetric pipets and flasks. Different flasks and pipets were used for the different tests. Two inocula were prepared. Two lots of media were prepared from the same lot of B.B.L.[2] Antibiotic Assay Broth No. 3. Both sets of samples and standards were assayed in each of the two daily assays. The standards were diluted to 2, 3, 4, and 5 mcg./ml. The samples were diluted to 2.5, 3.75, and 5 mcg. of solids/ml. Dilutions to assay levels were made in pH 8 phosphate buffer. Thus, each assay was linked to the other by no more events than if it had been performed on a different day. Each assay contained two independently prepared standards and samples.

The data were analyzed as a $2 \times 5 \times 3$ factorial experiment with one covariate. The factors were days, dilutions, and "tests," with the standard measurement being the covariate. The dependent variable was observed in micrograms per milliliter. However, in addition to the analysis carried out on this variable, the observations were expressed in percent recovery and analyzed. This was necessary in order to see if the percent recovery was affected by dilution. For each "treatment," two replications were observed. There was no evidence to indicate a statistical difference in either days or dilutions or the interactions: "test" by day, "test" by dilution, day by dilution, and "test" by day by dilution.

From the results of this study, it appears that there is 95%

[2] Baltimore Biological Laboratories.

certainty that approximately 95% of the individual assays will lie within ±2.7% of the recovery percentage. This figure is derived from the calculated standard deviation and takes into consideration that S is only an estimate; S has been multiplied by a factor found in standard tolerance factor tables for normal distribution to obtain the quoted confidence interval (4). Also, it appears with 95% certainty that the true potency is within ±1.35% of the mean of five assays when the response variate is in terms of percent recovery. The statements are predicated on the assumption that there is a relative sample standard deviation of 1.05%. Of couse, 1.05% is an estimate (calculated from these data) and is itself subject to variation.

Analysis of Vitamins—Again, two of many possible examples will be given to illustrate the use of the automated system for vitamin analyses. Results on vitamin B_{12} are shown in Table V. This table shows the results of automated analysis *versus* manual analysis run on some of the same days. The results on a secondary standard run on 8 days have a range of ±1% of the average value, and the average value is the same as the average obtained on multiple chemical assays. Each of the automated assay values represents the average of the results of two tubes in the sampler turntable, one a high sample and the other a low sample. Manual results show a much greater variation.

The results obtained on nicotinic acid are shown in Table VI. Here, a 3-day acidimetric assay was replaced by a 1-day turbidimetric assay. Even though the assay time was reduced by 2 days and involved much less hand labor, the results by the automated method were far superior to those obtained by the manual method. In the manual method, an unusually large number of invalid assays was customarily obtained, but the automated method rarely produced an invalid assay using the same criteria of invalidity.

DISCUSSION

The system just described is a fourth-generation system, with all previous systems still being used. All the systems employ the same basic principles, but each successive generation improves convenience, is faster, and eliminates former minor maintenance problems. If all systems are included, cumulative experience with the basic concept totals over 11 years. In this period, it has been evident that the equipment can provide precise analytical results if the method is properly designed and if operators are well trained and observant.

In any automated system, the operator is still a big factor in realization of the maximum potential of the system. Table VII illustrates this point very clearly. This is a table of coefficients of variation obtained on known standards and secondary standards which are routinely run to check for validity of the assay. The first column represents the results of the last manual assays before conversion to the automated equipment. The second column represents the results of the automated assay after about 3 months' experience with the equipment. The last column shows the results of a more recent set of 25 consecutive values obtained on the automated equipment. Even with relatively little experience, the automated method had a markedly lower coefficient of variation than the manual method. The really significant comparison, however, is between the last two columns which compare the more recent work with prior automated results obtained over a year earlier. This clearly demonstrates the improvement possible with the automated equipment, minor optimization of the equipment, and the use of well-trained operators. It also illustrates the precision obtainable in a routine production control operation. During this time, there were no major changes in the equipment, no changes in methods or operators, and no changes in calculation of results. It should be emphasized that these calculations represent all the results obtained over each period of the study for all the methods, and strict validity criteria would undoubtedly reduce the coefficient of variation stated.

A further item of interest is that there are no early automated results for cephalexin and neomycin since these were not run by this method until recently, yet the present precision is consistent with other tests. This would indicate that good precision is not a matter of the operator learning how to run a specific test over a long period, but that training in the general technique carries over into new uses of the equipment.

The results shown in Table VII are only representative of the many assays to which the equipment has been applied; the selection was made only to present a wide spectrum of uses. The turbidimetric assay has been used as a replacement for plate assays in cases in which the unique properties of agar diffusion are not required. Higher precision and significant cost savings are realized when automated turbidimetric methods replace manual turbidimetric and diffusion methods.

In these laboratories, this equipment has been well received by the operators, their supervisors, and management. The operator is still very much involved in the assay. The significant difference is that the operator has been relieved from the routine, boring operations, yet he has an opportunity to interact with the assay and take pride in the results of the assay. Supervision has been satisfied since very few assays are lost because of failure to meet validity criteria. In addition, each operator has become much more productive and is processing 2–4 times as many samples as previously possible. Management can observe the much higher level of precision obtainable and has a much higher degree of confidence in the results produced by the laboratory. Fewer assays need be run on each sample, and yet the confidence interval can be substantially reduced. Running fewer assays generally can result in faster turn-around time and better service can be offered. Space requirements using the automated equipment are much smaller than that for the previous manual assay, and total sample capacity is still very high. If greater capacity is required in any one location, it can be obtained by extending the effective workday to beyond 8 hr. to take better advantage of the equipment.

SUMMARY

In summary, a highly versatile system for turbidimetric microbiological analysis has been described. It is a relatively simple, rugged, and reliable system with few moving parts. It is capable of analyzing a wide variety of antibiotics, vitamins, and other substances that can produce a microbiologial response. The equipment is compact so that it can be installed in a number of areas where needed, thus eliminating the necessity of a large central analytical service area and its attendant problems of sample transportation and delay. Starting up the equipment and its subsequent cleanup are easily and effectively accomplished because of the small interconnecting lines and ease of operation. The operation of the system is such that a well-trained laboratory technician can operate it with minimum support, much like the situation found in conventional automated chemical analyzers. Experience with the system shows it can provide rapid and reproducible results at a lower cost per assay than conventional manual methods. As a result of this experience, this equipment is now being used in several installations and is furnishing analytical information that assures management of a higher level of quality assurance than previously obtainable by manual methods.

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