Prototype of a Fully Automated Device for Determination of Bacterial Antibiotic Susceptibility in the Clinical Laboratory[1]

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A completely automated system for the performance of antibiotic susceptibility tests in the clinical laboratory is described. With a modicum of personnel involvement, data on 40 specimens tested against 13 antibiotics are obtained every hour after an initial 3-hr period. The step by step explanation of the functioning of this prototype system, based on a thoroughly tested manual model, is presented. The system compares well with the standard diffusion test and has a potential for application to other endeavors of the clinical microbiology laboratory with a comparable saving in time and labor.

The overriding responsibility of clinical microbiology is the presumptive and rapid recognition of a significant microorganism in a clinical specimen and the report of its presence, along with its antibiotic susceptibility profile, to the clinician while this information is still of consequence to the diagnosis and treatment of the patient (7, 8, 11). Although other divisions of laboratory medicine have successfully employed modern advances in automation, clinical microbiology has been content to use the methods of 100 or more years ago. Now that the clinician has finally acknowledged the need for specific guidance of antibiotic therapy, as predicted by Ericsson (3) and Barber and Garrod (1), this need cannot be met with the modern instruments and modalities at the disposal of other laboratory scientists. This consideration, coupled with the suggestion that some standardized method be used for the performance of the antibiotic susceptibility test (1, 2, 4, 5, 12), made it seem natural to use this particular test to acquaint clinical microbiologists with the advantages of automation.

Automated monitoring procedures for manufacturing processes involving microorganisms have been used for a number of years. They usually involve only a single genetically stable assay microorganism and most frequently a single product, such as an antibiotic agent. In contrast, procedures in the clinical field must provide information on the reaction of different bacteria to a

dozen or more drugs. Thus, the convenient indicators used in the automation of industrial processes or quality control, such as carbon dioxide production and pH or E_h changes, could not be applied universally to all of the fast-growing bacteria subject to screening for antibiotic susceptibility in the clinical laboratory. It was this important requirement, namely, universal applicability of the monitoring method, which led to several attempts to find a rapid and accurate indicator of microbial proliferation and its inhibition. To this end, natural and radioactive isotopes of phosphorus, glucose utilization, adenosine triphosphate and other compounds were examined. These approaches were accompanied by variations in microbial responses, difficulties in reagent standardization, lack of reproducibility, and expense which made their suitability as universal indicators questionable. However, a modified particle-counting system was quite satisfactory.

To investigate this modality, a manual progenitor of the fully automated system was devised. This system, reported earlier (6, 9), outlined steps required for the fully automated system. Briefly, the methods consisted of preparing an inoculum of clinically significant organisms isolated on enrichment or selective media in the clinical laboratory. The inoculum consisted of 5 to 10 colonies which were suspended in 2 ml of optically clean Eugonbroth. Their turbidity was adjusted with a barium sulfate standard (2). A 1:1,000 dilution of the inoculum was prepared in the same medium,

distributed in 2-ml portions, and incubated at 37 C for a 30-min equilibration; this was followed by the addition of antibiotics in the form of discs or appropriate dilutions of antibiotic solution. Two control tubes without antimicrobial agents accompanied each particular test run for each bacterium. Bacteria in one of the tubes were killed with Formalin at time zero, i.e., after the 30-min preincubation and at the time of the addition of antimicrobial agents. At the end of 150 min, Formalin was added to all of the remaining tubes. The second tube without antibiotic thus served as a growth control. After a 10-min hold, to insure killing, the contents of the tubes were decanted into a carrier system which was assayed automatically with the particle counter. All antimicrobial susceptibility examinations were accompanied by the performance of agar diffusion tests according to the instructions of Bauer et al. (2).

This report describes the prototype apparatus for a fully automated system capable of performing antibiotic susceptibility testing on clinically significant, fast-growing bacteria and applicable in the clinical microbiology laboratory.

MATERIALS AND METHODS

Bacteria. Microorganisms which were isolated as described (8) from pathological specimens, and which would normally have been tested for antibiotic susceptibility profiles, were included in the study. In addition, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were tested with each battery of microorganisms undergoing examination.

Inoculum broth. The earlier study had shown that Eugonbroth (BBL) contained the least number of optically discernible particles after autoclaving. Eugonbroth was therefore chosen as the medium for use in the automated antibiotic susceptibility tests. For the preparation of the inoculum, one-quarter strength Eugonbroth (OCIB), filtered through 0.45-μm membrane filters (Millipore Corp.) before autoclaving, was used.

Medium. For the automated procedure, full strength Eugonbroth (OCB), Millipore-filtered as described prior to autoclaving, was contained in a large plastic bag from which it was delivered automatically to the dispensing mechanism for the individual test.

Formalin. A 25% aqueous solution of Formalin was stored within the machine and delivered to the various stations as required for killing the microorganisms.

Agar diffusion tests. Each and every bacterium tested by the automated procedure was also examined by the standardized Kirby-Bauer method (2). The procedure was followed to the letter. Only high-content discs, certified by the Food and Drug Administration, were employed. Mueller-Hinton agar plates filled to the proper depth, supplied through the courtesy of Bioquest, Division of Becton, Dickinson & Co., were used. After 18 hr of incubation at 35 C, the zones of inhibition were measured with calipers, recorded in

TABLE 1. *Discs used for the automated determination of antibiotic susceptibility*

Disc	Content	Final concn/ml
Penicillin	2 units	1 unit
Methicillin	10 μg	5 μg
Ampicillin	10 μg	5 μg
Cephalothin	10 μg	5 μg
Tetracycline	20 μg	10 μg
Erythromycin	10 μg	5 μg
Oleandomycin	10 μg	5 μg
Chloramphenicol	10 μg	5 μg
Lincomycin	2 μg	1 μg
Kanamycin	20 μg	10 μg
Colistin	10 μg	5 μg
Gentamicin	50 μg	25 μg
Carbenicillin	100 μg	50 μg

millimeters, and interpreted according to the guide published by the originators of the method.

Antibiotic discs for the automated system. The antibiotic discs in strengths listed in Table 1 were delivered to each proper individual carrier for the determination of antibiotic susceptibility. As reported earlier (6, 9), the delivery of these antibiotic discs served merely to carry the antimicrobial agents into the broth. It is necessary to reiterate the published findings that the liberation of antibiotic agents from the carrier paper occurs in all instances in 1 min or less (6, 9).

Additional system specifications. All excess material aspirated in the process of counting goes into a collection unit. The cups are practically dry at the end of the determination. It is important to appreciate that the entire system is maintained at 37 C, and 100% of the air is circulated through a HEPA filter. Thus, 10% fresh air and 90% recirculated air constitutes each laminar flow cycle. The 10% of air exhausted carries with it the Formalin fumes.

RESULTS

The sequential steps carried out by the fully automated device are demonstrated in Fig. 1. Of course, fully automated is not meant to imply that no human intervention is required. At station 1 an individual will have to load trays into the machine. The supply of sterile Eugonbroth has to be stored in the machine at various time intervals depending on the requirements of the individual laboratory; routinely, one plastic soft container holds broth for testing 100 samples. Unknown and control samples are introduced at station 3a; antibiotic disc supplies are maintained at station 5. At this station, as well as at station 7, Formalin delivery from a general reservoir is required. The reservoir provided can serve for several days' examinations.

Sample preparation is in keeping with the conventional method. Approximately 10 colonies of an isolated organism recognized on the basis of
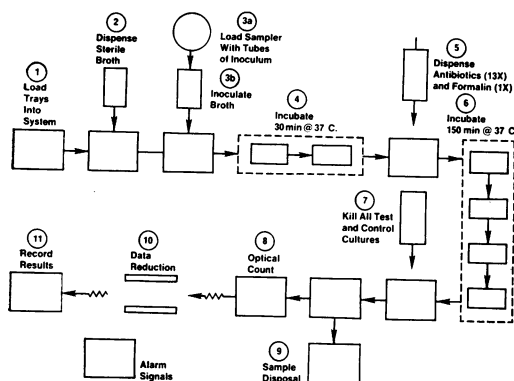
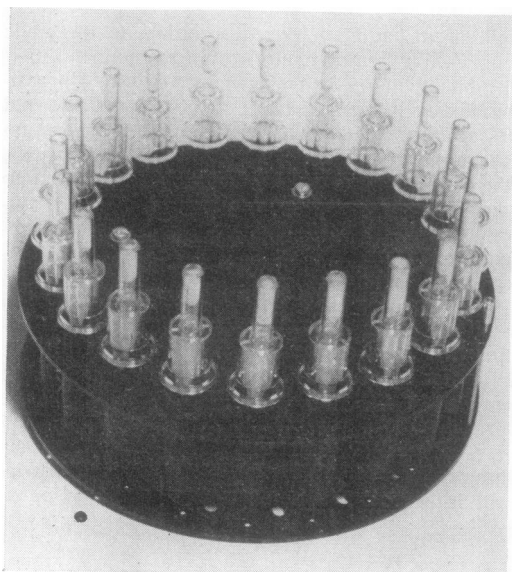FIG. 1. *Schematic representation of the automated device.*

FIG. 2. *Inoculum tubes with specially designed pipette closure, added after nephelometric standardization, in carrier tray used for insertion into the system.*

colonial morphology are suspended in a tube containing OCIB. It is more convenient to use tubes with stainless-steel or plastic closures. The inoculum is then standardized in a nephelometer, an instrument supplied but separate from the fully automated device. The nephelometer is so calibrated that a specified reading on the scale corresponds to approximately 10^5 bacteria per ml. After nephelometric standardization, the tubes are closed with a special disposable pipette containing closures and are inserted into the carrier as demonstrated in Fig. 2. This carrier holding 20 tubes of inoculum preparation broth is then introduced into the fully automated system at station

3a (Fig. 1). This introduction does not interfere with the maintenance of the temperature and air-flow environment of the device.

Light metal scaffold trays bearing plastic disposable cups or vessels are introduced into the system at station 1 (Fig. 1). Each tray consists of 10 rows, and each row contains 15 individual disposable cups. Each cup has a volume of 4 ml. Each row of 15 vessels represents a single test run. Thus, each tray is suitable for the performance of 10 antibiotic susceptibility determinations by the automated system. Trays are advanced automatically. This advance is predetermined, synchronized with the entire system, and controlled by a resetting clock mechanism. At station 2 (Fig. 1), prewarmed OCB is dispensed into each of the 15 cups sequentially. As can be seen in Fig. 3, a metal delivery needle attached to the dispensing system advances to each cup and delivers 2 ml of broth into each cup. At the end of the row and after a programmed hold, the tray advances one stop and the delivery needle fills the following row of vessels in the opposite direction. When a tray reaches station 3 (Fig. 1), an inoculum-dispensing arm
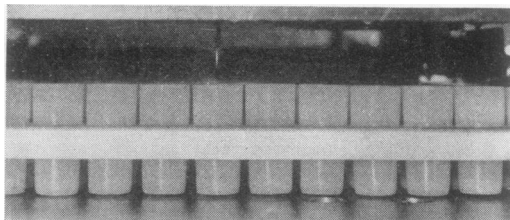

FIG. 3. *Close-up view of broth dispensing. Note needlelike tip of dispensing mechanism centered over single cup.*
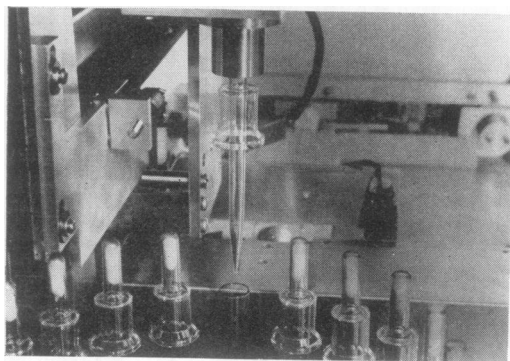

FIG. 4. *Inoculum pipette pick up. The inoculum aspirator arm descended, aspirated inoculum, and, while retaining the inoculated broth, has descended and is now ready to proceed toward the cups seen in the background.*
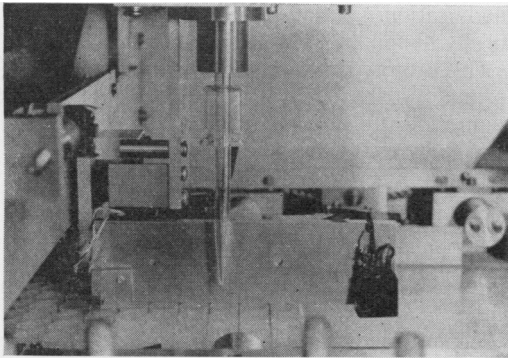
FIG. 5. *Inoculation. Pipette carrier has descended and has engaged the drop counter, represented by the metal bar carrying the solid-state light source on the left side and the optical detector (black box) on the right side.*
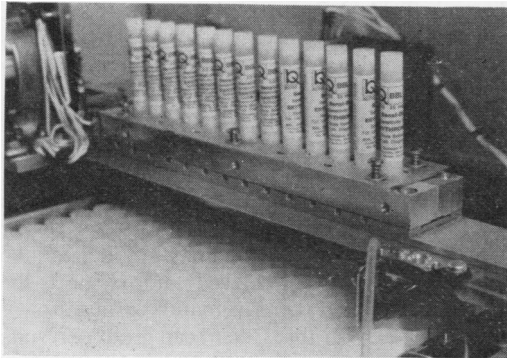

FIG. 6. *Antibiotic disc dispensing. The 13 drug carriers are obvious. The tubing and partly hidden dispensing needle over the first cup in each row adds Formalin and establishes the T_0 number. The second cup in each row is below the metal block supporting the disc carrier. No drug is added to this second cup which constitutes the T_{150} growth control.*

descends over the proper pipette, covering the inoculated broth in its special compartment. As is demonstrated in Fig. 4, the device then aspirates a sample of the inoculated broth and lifts the pipette; it then descends within 2 mm of the cup tops in its particular row and delivers a drop, approximately 0.1 ml in volume, into each vessel, as shown in Fig. 5. Inoculation is controlled by an optical device, shown as a small black box in the lower right of Fig. 5, in front of the lower portion of the pipette carrier. This device does not permit the advance of the pipettor to the next cup until a drop has been delivered into each cup. At the conclusion of the inoculation of the row, the inoculating device reverses direction, lifts the pipettes into its original tube, and disengages. Now,

the rotary inoculum tray advances one stop, permitting the inoculating device to center on the next tube to serve as inoculum. This event does not occur until the tray within the system has advanced one row. After inoculation, the trays enter the preincubation phase. This 30-min preincubation permits the bacteria to equilibrate and overcome the initial lag phase. At the conclusion of the 30-min period, the tray has arrived at the antibiotic-dispensing station (station 5, Fig. 1). Here, the first cup in each row receives 0.5 ml of 25% Formalin. The second cup has no addition, and the remaining 13 cups each receive an antibiotic disc as shown in Fig. 6. Once the antibiotic has been delivered to the last row in a tray, the tray enters an elevator (Fig. 7), which descends slowly to the sampling station, a process which encompasses 150 min. As each tray completes this incubation, Formalin is added to the 14 cups in each row. After a short hold, the contents of each cup are aspirated through the optical counting system (Fig. 8).

The cell counting device is represented diagrammatically in Fig. 9. The optical system consists of a reverse dark-field with Koehler-type illumination. A field stop in the form of a very precisely defined slit in the electro-formed material is placed at the lamp condensing lens. This field stop is imaged by a small-aperture objective lens with a half-field angle of approximately 3° into the image plane within the flow cell. The collector lens on the other side of the flow cell contains the dark stop disc designed to collect the near forward angle scattered light in the region of about 5 to 15° half angle. Because of this exclusion, a large portion of diffracted light is collected by the system, compensating for the relatively small light-collecting angle of the objective lens on the illumination side. The position field stop and small-aper-
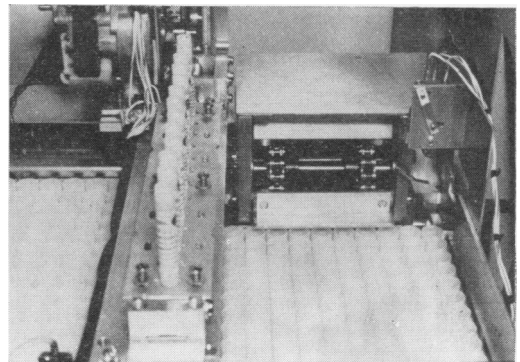

FIG. 7. *Incubation arrangement. After addition of antibiotics, trays progress onto the supports, one side of which is seen in front of chains. Trays descend at a rate which brings them to final station in 150 min.*
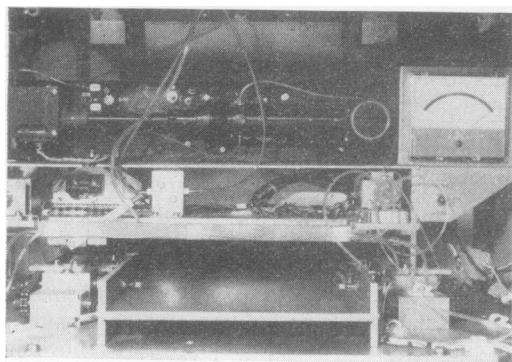
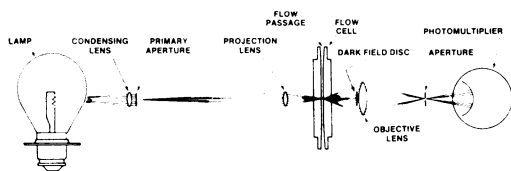FIG. 8. Sampling station including optical system.

FIG. 9. Diagrammatic view of optical system.

ture objective lens permit the imaging of a precisely defined illuminating sensing volume within the flow cell. Furthermore, the small subtended angle of the objective lens results in a relatively large depth of focus. The position of particles anywhere within the flowing stream does not affect to any appreciable extent the signal pulse height amplitude. The narrow illuminating angle greatly reduces stray scattered light within the system; in other words, noise is reduced to a minimum. The most important achievement of this

modification is a very precisely defined and uniformly illuminated sensing volume (10).

This prototype system reports its findings in the form of a strip chart which presents growth ratios as an indicator of susceptibility. These ratios are derived by use of the following formula:

$$F = \frac{X_{150} - T_0}{T_{150} - T_0}$$

where X_{150} is the cell count in the presence of an antibiotic after 150 min of incubation, T_{150} is the cell count in the untreated growth control after 150 min of incubation, and T_0 is the cell count in the vessel to which Formalin was added at the end of the 30-min preincubation.

Some typical results obtained when the same inoculum was used in the fully automated system and in the Kirby-Bauer agar diffusion test are shown in Table 2. By use of the automated system, with antibiotics in concentrations as yet not finalized, it seemed possible to judge susceptibility by fixing the ratio of susceptibility at 0.15 or less. Ratios ranging between 0.15 and 0.25 were in the indeterminate or equivocal zone of susceptibility, and values larger than 0.25 invariably were resistant organisms. The identical evaluation of ratios was obtained with the manual progenitor of the fully automated system (9). The data indicate the extent of agreement achieved and support the conclusion reached with the manual progenitor of this prototype that this system can produce results comparable to those obtained with agar diffusion. The instances of disagreement, e.g., the response of Proteus mirabilis to cephalothin and of Klebsiella pneumoniae to kanamycin, are slight; equiv-

TABLE 2. Comparison of results obtained by automation and agar diffusion

Drug	Escherichia coli			Staphyloccoccus aureus			Proteus mirabilis			Klebsiella pneumoniae		
	ADT zone[a]	AS ratio[b]	Interpretation[c]	ADT zone	AS ratio	Interpretation	ADT zone	AS ratio	Interpretation	ADT zone	AS ratio	Interpretation
Ampicillin	17.5	0.095	S	30	0.11	S	22.5	0.095	S	6.0	0.68	R
Cephalothin	19.0	0.05	S	32	0.095	S	17.0	0.095	E/S	19.0	0.09	S
Chloramphenicol...	24.0	0.095	S	21.5	0.085	S	14.0	0.50	E/R	21.0	0.04	S
Colistin	14.0	0.03	S	6.0	0.78	R	6.0	1.00	R	12.0	0.02	S
Erythromycin	10.0	0.855	R	23.0	0.06	S	6.0	1.00	R	10.0	0.89	R
Kanamycin	20.0	0.085	S	21.0	0.06	S	21.0	0.07	S	17.0	0.06	E/S
Lincomycin	6.0	0.94	R	19.0	0.05	S	6.0	1.00	R	6.0	0.96	R
Methicillin	6.0	0.93	R	20.5	0.07	S	6.0	1.00	R	6.0	0.97	R
Oleandomycin	6.0	0.97	R	21.0	0.02	S	6.0	1.00	R	6.0	0.94	R
Penicillin	6.0	0.96	R	33.0	0.01	S	17.5	0.99	E/R	6.0	0.99	R
Tetracycline	20.0	0.085	S	22.0	0.07	S	9.0	0.99	R	19.0	0.05	S
Carbenicillin	21.5	0.02	E/S	32.0	0.01	NA	25.0	0.05	S	12.0	0.32	NA
Gentamicin	18.0	0.035	S	20.0	0.03	S	20.0	0.02	S	17.0	0.01	S

[a] Size (in millimeters) of the zone of inhibition obtained in the agar diffusion test (ADT).

[b] Ratio obtained with the automated system (AS).

[c] S = susceptible; R = resistant; E = equivocal; NA = official interpretation not available.

ocal results were obtained by the Kirby-Bauer method whereas the automated system results indicated susceptibility. A similar discrepancy was observed in the *E. coli* response to carbenicillin. *P. mirabilis* responded with equivocal zones to chloramphenicol and penicillin whereas the automated results suggest resistance. In all of these instances, the agar diffusion response places the results at the limits of the equivocal zone measurement interpretations.

DISCUSSION

This prototype of a fully automated system for the determination of antibiotic susceptibility in the clinical laboratory meets all the specifications by which automation is defined. The apparatus reduces the time required for the performance of the antibiotic susceptibility test from 24 hr to 3; results on 40 bacterial specimens per hour are generated after an initial 3-hr incubation period; accuracy and reproducibility are excellent; and a modicum of human involvement is required. Perhaps the most important aspect of this procedure, which is difficult to translate into laboratory terms, is the speed with which information is provided to the clinician. Although it was stated at the outset of this paper that it is the function of clinical microbiology to do precisely this, the clinician has frequently met this claim with disbelief if not disdain. The use of a fully automated system for the determination of antibiotic susceptibility will enable the laboratory to provide this meaningful information to the clinician practically at the same time that it reports on the isolation of a significant bacterium, the identity of which need not have been established at that moment.

It may be reasoned that an automated system ought to be able to provide pertinent microbiological information sooner. At the present time, no system is available which can perform such a task on a sound microbiological theoretical basis. There is no indicator for the action of an antimicrobial agent on bacteria which does not involve multiplication of these organisms. The 150-min incubation is based on the theoretical consideration that at least three doublings of the population should take place within this time period. Actually, antibiotic susceptibility tests, whether employing diffusion or automation, are performed with bacteria that have comparatively short generation times, the so-called fast-growing bacteria. These organisms in their exponential growth phase divide at approximately 30-min intervals. However, there is no guarantee of synchrony at the onset of multiplication. Thus, the 150-min period might have resulted in eight multiplications were all of the organisms dividing simultaneously. Nevertheless, the increases observed both with the manual progenitor and with the fully automated system indicate that at least three doublings do occur. In many instances, as many as four or five doublings in the number of bacteria present at time zero have been observed. It is the consideration of antibiotic action on proliferating organisms which demands that the organisms be given this opportunity. The action of antibiotic agents cannot be estimated in the face of unnaturally high concentrations of antibiotics which may result in cytological changes or cell death. The basis for this consideration is obvious. The test as performed in the clinical microbiology laboratory must have some resemblance to conditions which the clinician may encounter. Concentrations of antibiotic in environments, whether test tubes or the microenvironment of a pathological lesion, cannot be compared if the concentrations of antibiotics employed are unrealistic. This is not to suggest that one can compare with impunity conditions in vivo with those in the laboratory. To be sure, all clinical microbiologists are very much aware that the laboratory estimates the relationship only between the drug and the microorganism, and of necessity ignores completely the very significant contribution made by various host factors and pharmacological peculiarities of the drugs which govern in vivo conditions. Nevertheless, at the present time, the only real guide to the interaction between drug and bacterium is the ability of the drug in comparatively low concentrations to interfere with the proliferation of the bacterium. The fully automated system described here is able to assess this reaction at a faster rate than was possible heretofore, with a reproducibility difficult to achieve with conventional tests. Agreement in results between laboratories using the manual progenitor has been extremely good. The fully automated system with its reduced chances for human intervention and error brings with it the promise of real standardization of the antibiotic susceptibility test in clinical laboratories.

The discs used in the automated procedure do not serve the function usually associated with them in the performance of the agar diffusion modality. Here the discs are carriers of specified amounts of antibiotic. Within 1 min of their addition, the drug has diffused into the broth, and the organisms at time zero encounter a uniformly distributed antibiotic in a specified concentration. This is in contrast with the critical concentration of antibiotic encountered by a critical population of bacteria in a critical time period which establishes the zones of inhibition during agar diffusion testing. It is also noteworthy that the concentrations of antibiotic employed in the study with the manual progenitor and the automated system

have not been agreed upon finally. Although several thousand clinical specimens have been exposed to analysis, especially by the manual system, decision has been delayed to allow an exhaustive search for all possible variations. As stated earlier, these tests are being carried out in conjunction with concomitant screening by the standardized Kirby-Bauer method. Just as zone sizes for each antibiotic may differ from bacterial species to bacterial species, so will it be necessary to adjust the interpretation of the ratios obtained with the fully automated system. These adjustments may involve changing the concentration of antibiotic in certain discs slightly or adjusting the classification of ratios obtained according to the bacterial species and the drug used. It must also be remembered that the usual inoculum preparation for the automated system incorporates 5 to 10 colonies, usually 10. Therefore, an overt expression of heterogeneity in a particular bacterial population from a clinical specimen is enhanced. There exists, in addition, the possibility that the established differences between dilution and diffusion assays of antibiotic susceptibility may become manifest occasionally and lead to negligible differences of interpretation in results obtained by both the standard diffusion and the automated methods. Complete appreciation of these differences must await the previously mentioned exhaustive study.

It is very important to recall that this is a prototype system for the performance of antibiotic susceptibility tests in a clinical laboratory. As with all prototypes, many modifications will be incorporated in future models. Thus, instead of a strip chart displaying the ratios representing antibiotic susceptibility, digital printout of the ratios will be made available. Easier access to parts of the automated machinery will be incorporated. It will be possible also to control the length of incubation. Although we do not intend to shorten the incubation or the preincubation periods, it may be desirable to test microorganisms with longer generation times by doubling or tripling the 150-min incubation. It may also be possible to limit the number of cups per row to be inoculated. This change or choice will provide the user with the opportunity to expand the versatility of the machine. The application of automation to clinical microbiology is limited only by the ingenuity of its users; for example, the automated system might be used to screen urine specimens quantitatively for the presence of viable bacteria per unit volume, to use simple identification schemes of fast-growing bacteria, and to determine minimal inhibitory concentrations.

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