or high-energy physics, for example. The potential reward, however, in new insights into the age-old questions of the universe, challenges the imagination, and I believe that it more than justifies the money and effort required.

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Capillary-Tube Scanner for Mechanized Microbiology

A photoelectric scanner measures growth in agar-filled capillaries and gives a new approach to microbiology.

Robert L. Bowman, Philip Blume, Gerald G. Vurek

We are reporting an instrumental method for counting viable bacteria and determining their antibiotic susceptibilities. The instrument counts the light-scattering pulses from developing colonies in a capillary tube filled with agar containing various nutrients. New or larger scattering pulses are produced by growth of organisms but not by inert scattering points. Antibiotics added to the agar inhibit growth or fail to modify it according to the susceptibility of the organisms. The system will reduce the burden of colony-counting and accelerate the determination of antibiotic sensitivities. The time saved in determining antibiotic sensitivity of organisms isolated from clinical specimens permits rational antibiotic therapy to be instituted earlier. Multiple parallel tests are feasible through the use of cheap disposable capillaries that require small amounts of both sample and reagent, and little laboratory space. The hazard of contamination is low because the samples are held in gelled agar inside sealed capillaries.

We have used methods of manual manipulation to permit easy variation of the techniques, but the extension to automated procedures can be accomplished easily. The sample is suspended in gelled agar, as in conventional pour plates, so that problems arising from motile organisms are reduced, and the growth dynamics of each colony derived from a viable organism in the original sample can be studied. The volume occupied by the organisms is so small that, at least for the first few hours of incubation, the capillary represents a large reservoir for nutrients and sink for metabolites. Mixed populations grow independently in dilute suspensions. Although a trained microbiologist can count and obtain partial identification of microcolonies soon after a pour plate is prepared (*1*), the man-hours involved are so great that this procedure is not used routinely. Petri-dish scanners and counters have been made, but they are expensive, bulky, and complex (*2*). The compact, inexpensive capillary system and the information made available by repeated scans and by analysis of the pulse-height distribution can provide a useful and powerful tool for research as well as for clinical testing.

Instrumentation and Method

Figure 1 is a schematic representation of the equipment we are developing. Light from a linear tungsten filament lamp (*3*) is focused by a microscope objective (3.2×) into the center of the filled capillary. Another objective (3.5×) collects light scattered from material within the capillary and directs it through a stop to a photomultiplier (*4*). The axes of the illuminating beam, the capillary, and the observation line are coplanar. The photoelectric signal is recorded graphically or registered on a counter whenever the amplitude exceeds a predetermined threshold. A synchronous motor-driven carriage translates the capillary through the light beam, and the recorded charts or accumulated counts of successive scans of a capillary can be compared directly.

The details of the equipment used for many of the studies to be described below are shown in Fig. 2; the photomultiplier housing is at the left, the drive motor and capillary carriage are in the central foreground, and the illuminating optics are at the right. Six separate capillary holders were used so that we could follow the development of growth within several capillaries without disturbing the orientation of the tubes in their holders. The holders were designed to be replaced precisely on the carriage with semikinematic mounts. During use, a cover (removed for the photograph) encloses the carriage region to reduce the effect of room light. An earlier apparatus (*5*) used the light scattered at a right angle from a laser-source to indicate the presence of microcolonies, but that unit was put aside when we found the coplanar forward-scattered light signal measured with the new apparatus was adequate. In addition to the unit shown in Fig. 2, we are using equipment that has essentially the same configuration except that the capillary carrier holds several tubes that can be scanned successively (*6*).

The chance that colonies will not be counted because of coincidence loss depends on the concentration of colonies and the volume of the region formed by the intersection of the illuminating beam and the observing "beam." We selected the size of our viewing stop so that coincidence losses

Dr. Bowman is chief, Dr. Blume is staff scientist, and Dr. Vurek is senior investigator at the Laboratory of Technical Development, National Heart Institute, Bethesda, Maryland 20014. Dr. Blume's present address is: Department of Laboratory Medicine, University of Minnesota, Minneapolis 55455.

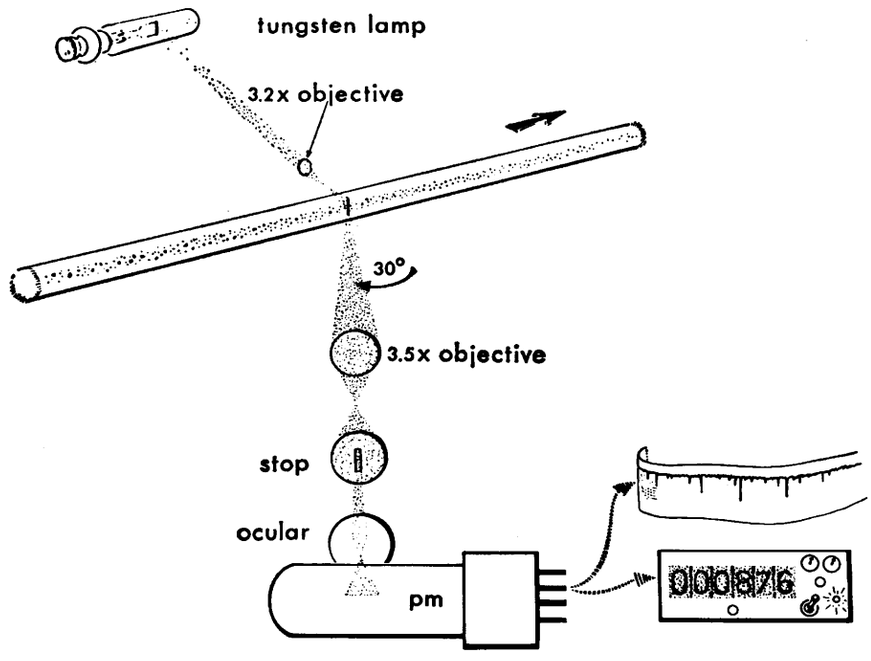

Fig. 1. Schematic illustration of the capillary scanner, showing light source, observation optics, and photomultiplier (*pm*).

would be small for concentrations below 10^5 organisms per milliliter. Urine specimens containing more than 10^5 viable bacteria per milliliter are generally considered to be from patients with an active infection of the urinary tract.

The gel structure of the agar with added nutrients scatters a significant amount of light into the viewing system. This background scatter signal obscures the signal from individual organisms, so that they must divide several times before they can be detected. The threshold of our counter was set to be about four times the background signal. We tried many types of suspending media, including other kinds of agar, agarose, cellulose derivatives, gums, pectin, and gelatin, but none was significantly better, in terms of low background, than the purified agar used in the experiments to be described.

We rinsed 2-ml ampoules with filtered (pore size of filter, 0.45 μm) demineralized water before filling them with 0.5 ml of agar. Trypticase-soy broth was the nutrient added for most of the experiments, but deoxycholate, veal infusion, Meuller-Hinton, thioglycollate, and brain-heart infusion media were also used for some tests. These nutrients were mixed with 2 percent (weight/volume) purified agar (*7*), filtered through a steam-jacketed, pressure-operated membrane filter (pore size, 1,2 μm) unit (*8*), and distributed into the rinsed ampoules. The ampoules were then autoclaved, sealed, and stored at 4°C until they were used.

The ampoules were heated in a bath of boiling water for a few minutes to melt the agar and were then placed in a temperature-regulated aluminum heater block at 50°C. When we were ready to prepare a sample, we opened an ampoule, added the suspension of bacteria and antibiotics (if any) with disposable syringes and needles (bringing the final volume to approximately 1 ml), mixed the contents by swirling, and filled the desired number of capillary tubes (*9*) from the ampoule. The melted agar was drawn into the tube either by gentle suction or by capillary action. Less than 150 μl was needed to fill each tube. After the tube was filled, the upper end was sealed with red sealing wax or with plastic plugs (*10*). The lower end, which had been dipped into the agar, was considered contaminated and was disinfected with Amphyl (*11*); it was then sealed with wax or a plug. After we had prepared a group of tubes, we cleaned the outside with detergent and demineralized water, to remove dust and finger marks, and placed them in holders for the initial scan.

We scanned the tubes and made graphic records or recorded the number of superthreshold counts. After the initial scan the tubes, in their carrier, were placed in an incubator at 37°C until time for the next scan. For most of the studies described here, further scans were made after 2 hours' incubation and hourly thereafter until the end of the work day. Occasionally, scanning was done at half-hour intervals and sometimes after overnight incubation.

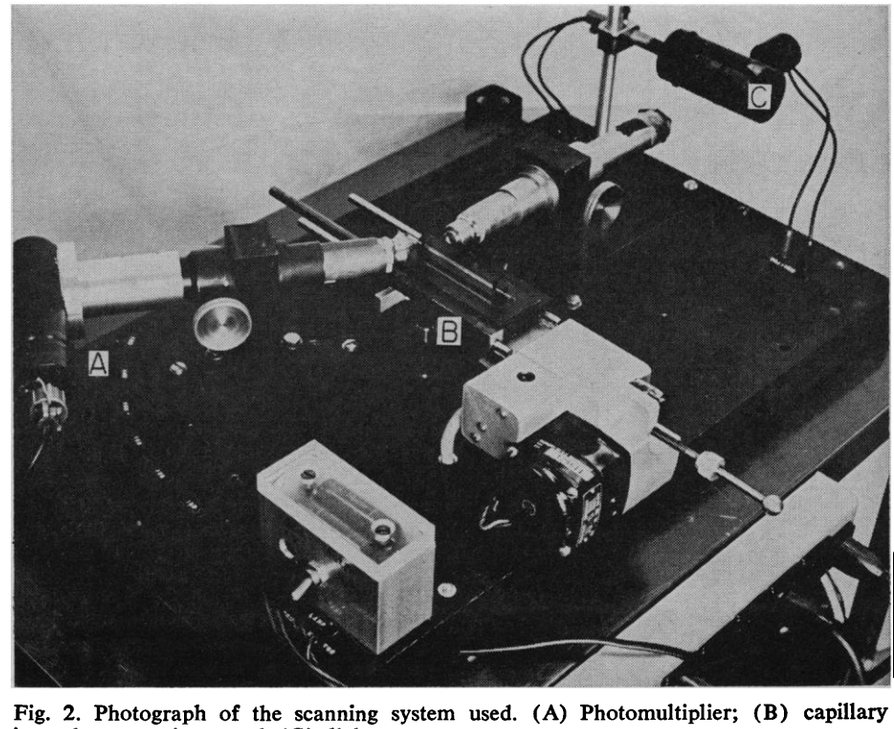

Fig. 2. Photograph of the scanning system used. (A) Photomultiplier; (B) capillary in rack on carriage; and (C) light source.

Results

We made serial dilutions of a culture of *Escherichia coli* and compared the net count obtained with the scanner with the number of colonies observed in agar pour plates made from the same dilutions. Figure 3 shows the net counts obtained after 5 hours and the "true" concentration as determined from counting the pour plates after incubation overnight. Each point is the mean of counts obtained from three capillaries. For our apparatus, the factor of proportionality between counts per tube and the concentration of colonies was about 500 for concentrations of organisms below 10^5 per milliliter and increased rapidly above that count. The factor of dilution of the sample must be included; for our work this was usually two (0.5 ml of sample plus 0.5 ml of agar).

The net count levels off at high concentration of colonies because the system is not capable of resolving closely spaced colonies. Two or more colonies in the volume formed by the image of the source (50 μm wide) and the demagnified stop will count as one. Because the edges of the observation volume are less well illuminated and viewed, colonies growing in those regions must grow larger than colonies in the center in order to be counted. This is the main reason that the count increases with time for preparations that initially were uniform. We have observed that the count for a fast-growing organism, such as *E. coli*, will stabilize in about 5 hours, while slow-growers may take 12 hours or more. This effect is not as pronounced at high concentrations, because two small overlapping colonies will scatter enough light to register a count sooner than a single colony can grow to a countable size. In general, 5 hours is enough time to make a quantitative estimate of the number of viable bacteria, and statements concerning the relative degree of growth can be made within 2 hours.

The size that a growing colony must reach in order to be counted determines the sensitivity and response time of the capillary system. The smaller the size that can be detected, the fewer divisions the bacteria must undergo and the sooner an indication of growth can be demonstrated. We made an estimate of the minimum size of a detectable colony by preparing a set of tubes containing about 10^5, 10^4, and 10^3 *E. coli* per milliliter and then scanning them at half-hour intervals. When growth was indicated on the graphic record, the tubes were brought to room temperature, and a portion of their contents was spread on a slide. The agar was stained and size of the colonies was measured with a filar micrometer. In addition, we estimated the number of organisms in each colony that was measured. Table 1 summarizes the results of this experiment. The early detection time observed at high concentrations of colonies is probably due to overlap, but detection time at lower concentrations is quite good. Colonies 8 μm in diameter are detectable (this was confirmed by observation of countable signals from dilute preparations of guinea pig red blood cells), and the organisms need go through only four to six division cycles to be counted.

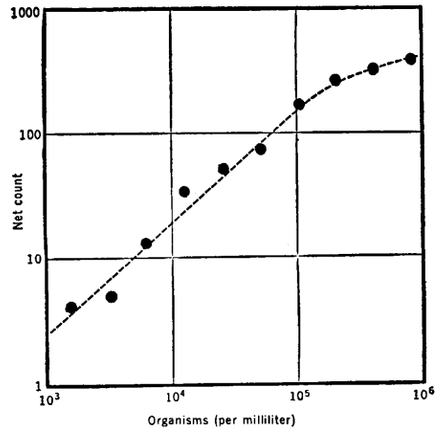

Fig. 3. Graph showing relation between net counts per tube (mean of three tubes per point) and number of *Escherichia coli* per milliliter in each tube. Stop size: 0.2 mm in diameter.

The organisms continue to grow in the tube until nutrients are exhausted or they are stopped by the accumulation of metabolic wastes. Figure 4 shows the appearance of a tube containing a relatively low concentration of colonies. The colonies reached a size of about 80 μm in diameter. At higher concentrations, growth stops quickly and the limiting size is much smaller than those in this figure. The colonies never appeared to be larger than about 400 μm, even in the most dilute preparations.

We tested the system for use with not only facultative organisms but with aerobes and anaerobes as well. Preparations of *E. coli*, group D *Streptococcus*, *Proteus mirabilis*, *Klebsiella sp.*, *Staphylococcus aureus*, *Staphylococcus epi-*

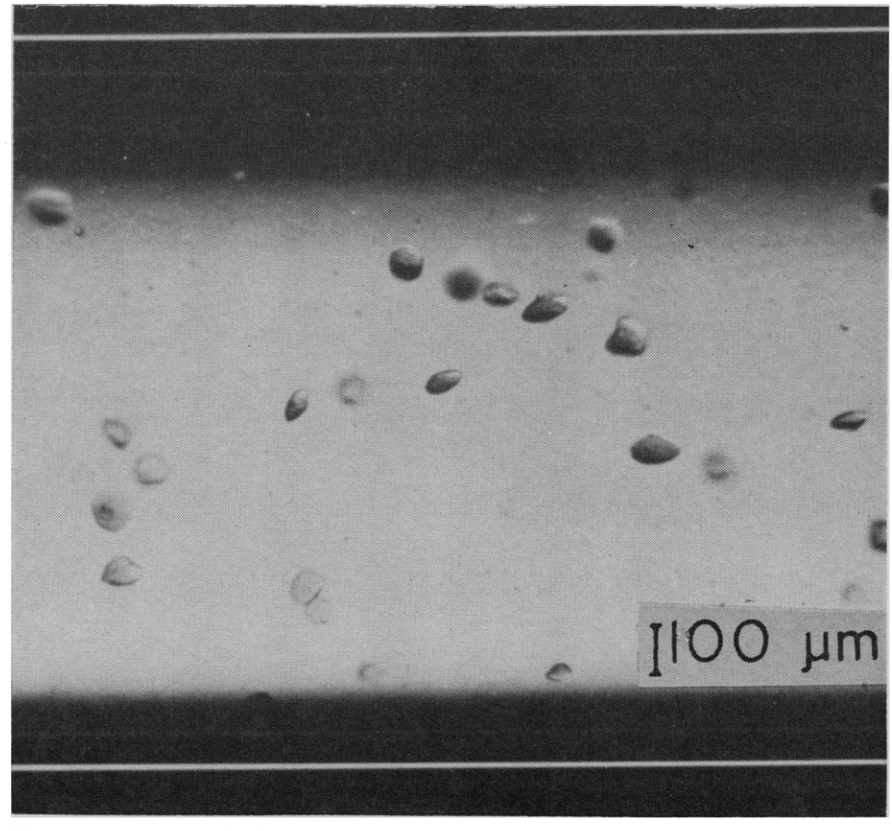

Fig. 4. Appearance of microcolonies in capillary with an outside diameter of 1.7 mm.

dermidis, Pseudomonas aeruginosa, and *Clostridium perfringens* were made in trypticase-soy agar, and growth curves were prepared. Figure 5 shows the results of part of the tests. *Klebsiella* grew as well as *E. coli,* but *C. perfringens* did not grow at all. We tried several other media for growing clostridia and were able to get good growth in veal-infusion, thioglycollate, and brain-heart infusion media. Deoxygenation of the saline suspension before it was added to the agar also seemed to facilitate the growth of clostridia. Improved growth of *Pseudomonas* was obtained with suspensions from freshly prepared broth cultures.

We then tested the system for its suitability in the examination of urine specimens. Many of these samples contained particulate material which was counted initially; we deducted these initial counts from the final count in order to estimate the number of viable organisms in the original sample. Figure 6 shows the graphic record of a sample of urine containing more than 10^5 organisms per milliliter. Note the increase in large scattering pulses displayed after only 2 hours of incubation. We have made and are continuing to make counts of samples obtained from patients at the Clinical Center of the National Institutes of Health. (It is not possible to make more than a qualitative comparison between the scanner results and the pour plates prepared by the Infectious Diseases Section of the Clinical Center, because they do not routinely make quantitative counts of their pour plates.) At this writing, we have tested 236 samples; of these, 174 had fewer than 10^5 organisms per milliliter as measured by both methods; 11 had more than 10^5/ml estimated by the pour-plate method, but less than 10^5/ml by the scanner (false negative); 35 had fewer than 10^5/ml by the pour-plate method, but more than 10^5/ml by the scanner (false positive); 16 samples had more than 10^5/ml estimated from both pour plates and the scanner. Our estimates were based on new counts accumulated in 4 to 5 hours. We are continuing to obtain samples to determine the reasons for the relatively high fraction of "false" reports obtained by the scanner.

We have been evaluating the capillary techniques as a rapid method for testing antibiotic sensitivity. We prepared overnight broth cultures of *E. coli, Klebsiella,* group D *Streptococcus,* *Staphylococcus aureus,* and *S. epidermidis,* and aqueous dilutions of the following (unstandardized) antibiotics: free chloramphenicol (Parke-Davis), Na-colistimethate, kanamycin sulfate, cephalothin penicillin G (Lilly), neomycin sulfate, polymixin B sulfate, and tetracycline HCl. The antibiotics were serially diluted with sterile saline from 50 to 0.39 μg/ml in steps of one-half. To each ampoule of nutrient agar we added 0.1 ml of an antibiotic dilution and 0.4 ml of a 1 : 1000 dilution of an overnight broth culture of an organism. After the antibiotics and cultures were mixed, the capillaries were filled, sealed, and scanned. Counts were made after 2 hours, and at hourly intervals thereafter. The concentrations of antibiotics that inhibited the growth of organisms in the capillaries were compared with results obtained by tube-dilution tests from an unrelated experiment conducted by the Infectious Diseases Section. Of the 25 comparisons, 14 differed by a factor of two or less. Figure 7 shows the growth curves obtained in the test of *Klebsiella* against Na-colistimethate.

Table 1. Size distribution of *Escherichia coli* microcolonies grown in trypticase-soy agar in capillaries.

Conc. (No. organisms per milliliter)	Detection time (hr)	No. of colonies measured	Diameter of colonies (μm)		No. of organisms per colony
			Mean	Range	
10^5	2	25	5	3–8	8–30
10^4	3	17	8	3–12	10–25
10^3	3½	8	12	7–18	40–60

Discussion

Only a small fraction of the information available from the scanning procedures has been used to obtain these results. Adjustable or multiple apertures in the viewing optics would permit accurate counting of more dilute or more concentrated samples. The presence of aggregates of viable bacteria, or bacteria associated with larger cells, can be determined with a multichannel pulse-height analyzer. The equipment described here discriminates against large light-scattering particles if they are countable on the first scan, and, although they may grow, they do not make a contribution to the net count. More thought and effort will be required to simplify handling of the sample and to improve the presentation of the results to make them more convenient for the clinician.

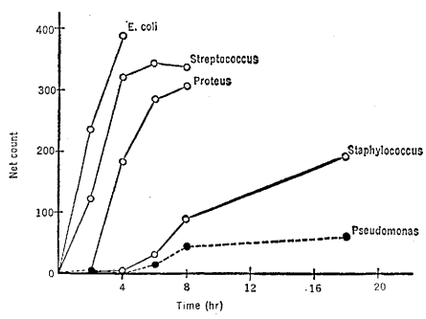

Fig. 5. Growth curves of aerobic and facultative organisms obtained with the scanner.

Preparing separate ampoules for each antibiotic and each dilution is tedious and time-consuming, and we have been exploring ways to precoat capillaries with a uniform, standardized amount of antibiotic. This would simplify testing for sensitivity, not only because fewer ampoules would have to be prepared, but also because the preloaded capillaries could provide the precision of standard tube-dilution technique with the convenience and economy of the disk method. We have tried several ways to precoat the capillaries, and the most promising technique seems to be to extrude a filament of antibiotic in 1 percent agar inside the capillary and to dry the filament with a stream of dry nitrogen. The uniformity of the coating was tested by using thiazine red R dye instead of antibiotic and then measuring the light absorption along the length of the capillary. The variation of the absorption was less than ± 15 percent of the mean for 18 capillaries prepared in three separate groups. By adjusting the diameter of the agar filament and the concentration of the antibiotic, we can distribute the desired amount within the capillary with good precision. A linear array of several concentrations can be put in the same capillary. Tests with capillaries coated with tetracycline run in parallel with tetracycline added to ampoules, with *E. coli* as the test organism, gave identical

results for minimal inhibitory concentrations on three separate occasions. Storage of dried tetracycline-coated tubes for 3 days at room temperature and humidity does not seem to affect the potency of the antibiotic. The dried agar does not inhibit diffusion of dye into the inoculum, nor does there seem to be any additional background scatter.

Identification of the organisms is also a problem we have been investigating. We have extruded the agar filament from the capillary with a little air pressure, stained it with the Gram stain (allowing longer times to permit diffusion into the bulk of the agar), and observed the stained microcolonies. Preparations of *Bacillus globigii* (pigmented variety of *B. subtilis*) and *Serratia marcescens* were stained with specific fluorescein-labeled antibody, and the labeled colonies were easily identified. By applying gentle suction to a capillary, we have put the stained agar back into the tube and scanned it again. It is possible to count the number of stained colonies by either their fluorescence or their absorption of light, by the use of appropriate filters, and, for absorption, by rotating the viewing optics to measure the directly transmitted light rather than scattered light. In addition to staining procedures, chemical indicators and selective media can be used, and the formation of gas in the capillaries is easily observed.

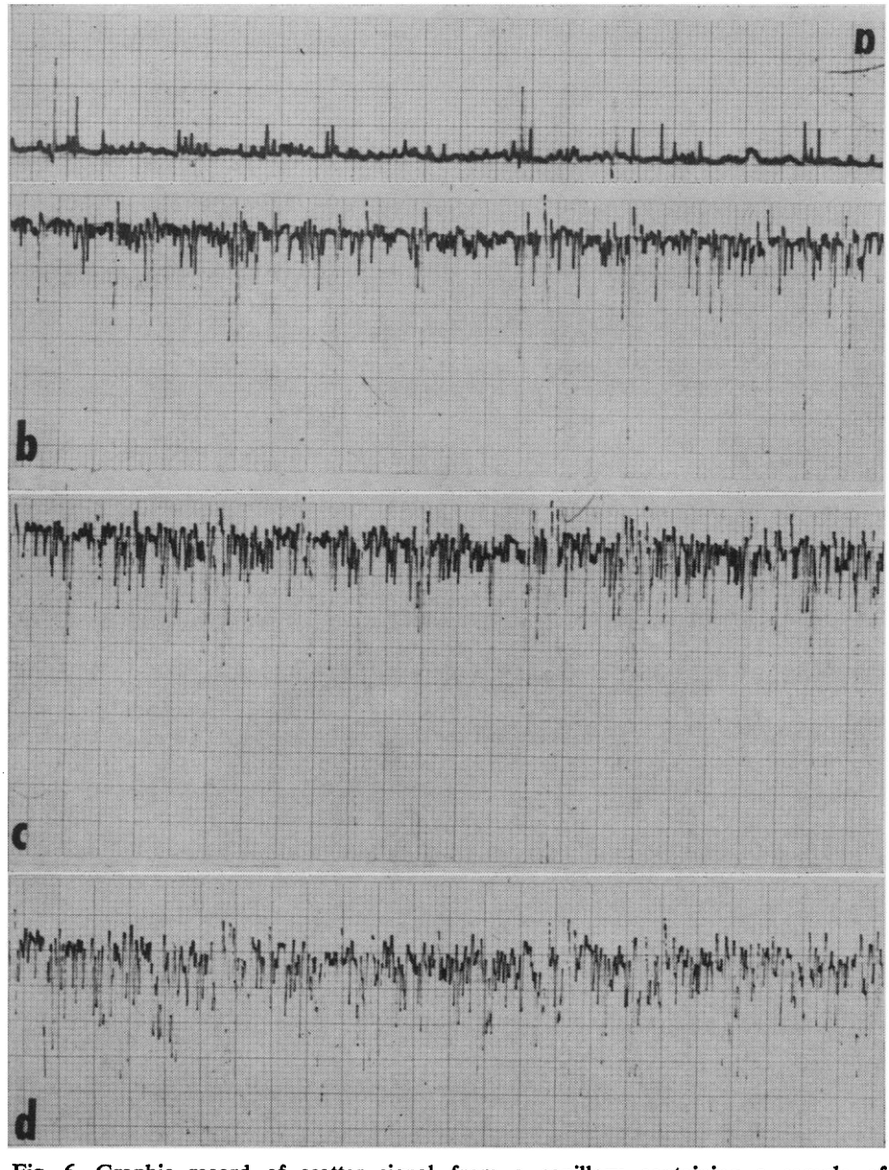

Fig. 6. Graphic record of scatter signal from a capillary containing a sample of urine. The top record shows the scan prior to incubation, and the lower records show scans after 2, 4, and 6 hours of incubation. Record scale: one major horizontal division equals 1 second; (a) initial scan; (b) after 2 hours; (c) after 3 hours; and (d) after 4 hours.

What we measure is the change in the optical properties of particulate material. We have made exploratory efforts to adapt the capillary-scanning technique to studies of the formation of tissue culture clones, to the assay of phages, and to the hemolytic-plaque technique. We were able to grow clones of mouse fibrosarcoma (P388) suspended in a plasma clot within the sealed capillaries and observed the development of these clones from single cells in less than a week. Unfortunately, the cells had settled to the wall of the tubes before the clot had set and we were not able to use the scanner to quantify the growth of the clones. However, this should be possible with some modification of both the instrument and the preparation of the sample. The environment of the capillary did not inhibit growth of the cultured cells. The plaque technique for the assay of phages (*12*) seemed ideal for the scanner, but we have not been able to obtain confluent growth of organisms within the capillary. By treating the phages as an "antibiotic" we added various concentrations of phage particles to a preparation of *E. coli* and were able to estimate the concentration of phage required to inhibit the appearance of countable colonies. Microscopic inspection of the capillaries showed numerous small "holes," presumably the pockets left by infected and lysed microcolonies. The released phage particles could not diffuse effectively through the agar to infect some of the adjacent colonies, which continued to grow to their maximum size.

The hemolytic-plaque (*13*) technique permits counting the number of antibody-producing cells obtained from spleens by combining the antibody produced with a sheep red cell–immune hemolytic system. Each antibody-producing cell can be seen by the clear zone of hemolysis in the background of red blood cells in a petri dish. We arranged our scanner to make transmission measurements and to accommodate a capillary with an outside diameter of 0.5 mm and an inside diameter of 0.3 mm.

Samples of a mixture of spleen cells from mice (immunized with sheep red blood cells), sheep red blood cells,

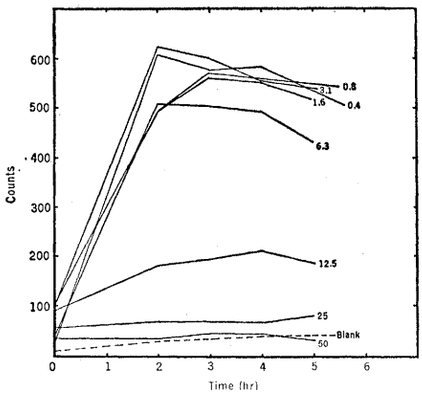

Fig. 7. Growth curves of *Klebsiella* sp. suspended in agar containing Na-Colistimethate. Number at the right of each line represents ten times the concentration of antibiotic in each capillary. The blank tube contained no organisms.

guinea pig complement, and agar were drawn into these capillaries, which were incubated and scanned. These preliminary experiments showed excellent correlation between the number of plaques observed as regions of increased light-transmission in the capillaries and the number of plaques counted on standard petri-dish preparations from the same spleen-cell mixture. In addition, the widths of the cleared zones, as indicated on the graphic record, were related to the size of plaques.

There are many other applications of the capillary technique. The information about the growth rate of individual colonies is available, and with appropriate techniques for pulse-height analysis one should be able to follow the kinetics of growth of a large number of separate colonies. Our single-channel analysis discards much of this pulse-height information, and we can be misled into designating as debris an initially countable particle when, in fact, it may be an aggregation of viable organisms which was not included in the census of viable organisms because the particle was included in the "blank." The change in pulse-height distribution with time would provide information about such occurrences. In addition, we have grown colonies in porous glass capillaries inside the solid-wall glass capillaries, so that it should be possible to change the nutrient of the cells and follow the kinetics of growth as they accommodate to the new environment. Since the individual colonies are held in a matrix which prevents cell motility but permits small molecule diffusion, this technique may provide a tool for studying the adaptability of organisms to all sorts of environmental stress, and provide a way to determine the relative importances of mutation and inherent adaptability.

Summary

Growing microcolonies of bacteria can be detected by their light-scattering property. We have described a system in which growing bacteria in glass capillaries filled with nutrient agar are counted as the capillaries move through a narrow beam of light. Increased counts after incubation indicate the presence of viable organisms in the original sample; the equipment can detect colonies when they grow to a diameter of 8 μm. Aerobic, anaerobic, and facultative organisms can grow in the capillary environment. The sensitivities of several organisms to antibiotics were determined by adding antibiotics to the agar before the capillaries were filled.

We chose to show the utility of the capillary-tube scanning method for counting bacteria and for assay of antibiotics because we felt that these procedures were most in need of automation, but during the development of the method it became apparent that several other biological systems could be studied by scanning cells or particulates in a semisolid gel and rescanning to demonstrate swelling, growth, or lysis. The methods are not presented as complete solutions but as demonstrations that acceptable clinical and research methods, that utilize the basic principles outlined, can be developed.

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