# Automated System for Analytical Microbiology I: Theory and Design Considerations

N. R. KUZEL and F. W. KAVANAGH

**Abstract** □ Quantitative microbiological assays use either of two responses, growth inhibition or growth promotion. Antibiotics and germicides are assayed by growth inhibition, and vitamins and amino acids by growth promotion. Previous automation efforts in these types of assays are critically reviewed. Design considerations are presented for a new automated system which meets all essential requirements of microbiological turbidimetric analysis. Optimum requirements are discussed separately for dilution, incubation, and measurement. The automated system duplicates all essential characteristics of well-known manual procedures. Dilution and measurement are performed separately and simultaneously, and incubation is made independent of both functions so that incubation time can be optimized for each analysis. This arrangement furnishes the flexibility, versatility, and precision so necessary in present-day automated analytical microbiology.

**Keyphrases** □ Microbiological assay, automated—theory □ Design consideration—automated microbiological assay □ Automated analysis, microbiological—theory, design

The increasing use of turbidimetric microbiological analysis has emphasized the need for a precise, versatile, automated system. Such a system should meet the needs of a large variety of users and the stringent requirements of a living biological system. The theory in this field is very complex and sometimes not well understood. Effective utilization of these theoretical considerations in the design of a practical and versatile automated system has required simplification of theory and its reduction to essentials. The present paper summarizes this theory, relates this work to other efforts in the field, and presents the design considerations that led to the construction of an automated system. In the accompanying paper (1), this system is described in detail, and a laboratory evaluation is presented of the systems used for analysis of certain antibiotics and vitamins.

## BASIC THEORY

Quantitative photometric microbiological assays take advantage of two responses, growth inhibition or growth promotion. Growth-inhibiting substances and microbes interact to decrease the mass of the latter. The two parameters of bacterial growth affected by antibacterial agents are growth rate and lag time. Most antibiotics which have been studied in sufficient detail to discover the dynamics of their action reduce growth rate. A few substances increase lag time. A cell with a lag time of 5 hr. or more is dead for analytical purposes. Each population of organisms apparently makes one of two responses to inhibitors of growth. Either the entire population makes the same response to the same degree or it does not. The first type of response is characteristic of antibiotics that reduce growth rate. The second response is elicited by such substances as cetyl trimethylammonium bromide, which seem to affect only a portion of the population (2).

Requirements for a growth-inhibition assay are: (*a*) a substance capable of inhibiting growth rate or increasing lag time, (*b*) a test organism sufficiently susceptible to the test substance, and (*c*) a liquid medium nutritionally sufficient so as not be be altered significantly by substances accompanying the test substance in the assay solution or by metabolic products of the growing test organism.

Growth-promoting substances and microbes interact to increase the mass of the latter. The test substance must be required by the microbe, and the microbe must not be able to synthesize the test substance, at least at the rate needed. The substances most often determined by growth-promotion methods are the B vitamins and amino acids. Other organic compounds and certain minerals may also be measured.

Requirements for a growth-promotion assay are: (*a*) a substance essential for growth of the test organism, (*b*) the test organism, and (*c*) a liquid medium deficient only in the substance being measured. The preferred assays are those for which the deficiency is absolute, although assays for substances that affect only growth rate can be done. Kinetics of vitamin assays are simple and well known; the organism grows in the presence of the test substance and does not grow in its absence. Time of incubation and composition of the medium are such that the amount of the test substance in the assay tube is the only limiting factor. Under these conditions, the total cell mass is proportional to the amount of test substance in the tube.

If all the above-mentioned criteria are met for either growth-inhibition or growth-promotion assays, the one additional requirement is that the test substance be the only variable introduced into the assay. This means that all dilutions are performed precisely, that no variable environmental factors are introduced, and that the resultant growth can be measured with sufficient precision so as not to degrade the final result. In conventional manual performance of these assays, each of these factors can be directly influenced by the analyst; each sample or standard is handled individually and uniquely by the analyst. Not only do all the operations require considerable time if performed precisely, but the only assurance that any one sample is directly related to its standard is based on the confidence resulting from the analyst's previous performance. Thus, the results of the analysis are extremely operator dependent. The means usually used to overcome these deficiencies are a high degree of replication of the assay and the use of rather elaborate statistics to evaluate the results. These required replications of procedures cause a high cost of analysis and an undesired degree of error in estimations of sample concentration.

It is to avoid this high cost and/or concomitant error that automation of this assay has been attempted. Automated analysis, when performed properly, should assure that the dilution and measurement have been performed in a reproducible manner. Environmental changes must still be monitored by an appropriate number of standards run in the same manner as the samples. The greater the time difference between processing of sample and standard, the greater is the opportunity for some change to occur. Ideally, to avoid environmental influence, the sample and its reference standard should be analyzed at exactly the same time and under exactly the same conditions, or all conditions should be held so constant that differences in time have no influence on the end result. The extent to which various automated systems approach this ideal governs their usefulness as suitable assay systems.

## DISCUSSION

**Manual Analysis**—Practical quantitative photometric microbiological assays were introduced by McMahan (3) in 1944 as an improvement over both the serial dilution tube assay and the diffusion assay. Very little was published on additional methods or theory until the publication of Treffers (4) in 1956 on the antibiotic dose–response line. Kavanagh (5, 6) continued the development begun by Treffers of the dose–response line and, in addition, discussed in detail the theory and present practice of photometric assaying.

**Continuous-Flow Automation**—Continuous-flow methods for automation of microbiological turbidimetric analysis have been reported in the literature for many years. A review of these early methods was published by Gerke and Ferrari (7). A more recent review was published by Kuzel *et al.* in 1969 (8). Gerke *et al.* (9) first described the use of automated continuous-flow bioassays for turbidimetric analysis. Haney *et al.* (10) introduced some improvements on this method. Further details on this method were presented by Gerke *et al.* (11). Shaw and Duncombe (12) used a biostat to provide continuously grown inoculum. Continuous dilution rather than intermittent sampling for turbidimetric analysis was introduced by Platt *et al.* (13), based on earlier work by Pagano *et al.* (14). A variation of the continuous-flow technique was described by Berg *et al.* (15). These authors prepared their dilutions by continuous-flow methods into discrete vessels and then incubated and read out the dilutions as discrete samples.

Very recently, two papers on continuous-flow turbidimetric analysis were published by Jones and Palmer (16) and Grimshaw and Jones (17). These authors state that three technicians, using two continuous-flow systems, can run 16 samples per day at a precision of ±3%. This experience appears to be indicative of the problem with application of continuous-flow methods to microbiological assays. These methods generally depend on precise and reproducible long-term control of all environmental conditions, including a continuous, dynamic supply of test organisms. Deviations from absolute control are seen as drift and are compensated for by very frequent analysis of standards and randomization of replications. The necessity of precise, long-term, environmental control also requires a relatively long period of equilibration of the system before useful data can be obtained. Incubation times are restricted by the back-pressures built up in the incubation coils and by aggregation of viable organisms which collect in various recesses of the system. Since incubation times are restricted to 30–60 min., these methods [with one exception, as described by Davis *et al.* (18)] cannot be used for analysis of vitamins. For these and other reasons, continuous-flow turbidimetric methods—despite years of experience—have had only scattered instances of routine applications.

**Discrete Analysis Automation**—An alternate approach to automation of turbidimetric microbiological assays has been by batch or discrete analysis methods. These, in some instances, have been somewhat more successful. McMahan (19) reported on an elaborate system for such analysis. The system automatically dilutes samples and standards simultaneously into a moving rack, adds the appropriate nutrient broth, controls the movement of the diluted tubes through a water bath, and then automatically transfers the contents of the tubes into a colorimeter for readout. All steps are controlled by punched cards, which are decoded at each work station. The analysis rate was reported to be about 50 samples/hr., each sample consisting of eight observations, four on the sample and four on a concurrent standard.

Gualandi and Morisi (20) described an automated discrete analyzer for both chemical and microbiological analyses. Samples were diluted, incubated, and read out automatically in a temperature-controlled air incubator. Capacity was limited to 18 samples per run. Each run required about 3.5–4 hr. for antibiotics and about 5.5–6.5 hr. for vitamins, so sample capacity varied from 18 to a maximum of 36 samples per working day. Simoncini *et al.* (21) reported on the use of this equipment in their laboratory.

Burns and Hansen (22) described a totally automated system for turbidimetric analysis. Their system accepted unfiltered fermentation broths, filtered them, diluted the filtrate, added inoculum, incubated the samples in an air incubator, measured the turbidity, and calculated the results by means of an on-line computer. In a later publication, Burns *et al.* (23) described a modified system which was faster (120 observations an hour) and used a batch dialysis scheme for effecting sample purification. Davis *et al.* (18) reported on the use of a commercial apparatus designed for chemical analysis, for turbidimetric microbiological analysis of folate activity. Millbank *et al.* (24) further elaborated on the use of this equipment.

Most of these systems involve straight-line or full automation by means of a series of integrated, timed, discrete steps. Such systems cannot be used for long incubation times such as are required for analysis of vitamins, unless the unit is loaded with samples, partially processed, shut off for a number of hours, and then restarted for the remainder of the processing. This ties up the entire apparatus for the duration of incubation and also limits the number of samples that can be processed to the capacity of the incubator associated with the apparatus.

## DESIGN CONSIDERATIONS

**General**—Since manual turbidimetric analysis has been used by so many workers in the field and since the theory behind the use of this methodology is complex or sometimes unknown, it appears desirable to take advantage of these years of experience by staying with the same technology. This would dictate the use of the same test organisms and the same nutrient broth. It would also require measurement of the same microbiological response according to the same assay design. This parallelism of methodology should make the task of conversion from manual to automated methods rapid and simple. Official methods could then be accommodated with no significant changes, and acceptance of the automated results by regulatory agencies should be expedited.

The automated system should have a large sample-handling capacity because of the large number of assays normally performed in most microbiological laboratories. An analysis rate of at least one sample per minute would be desirable. The equipment should be convenient to use and easy to clean after use. There should be essentially no interaction between adjacent samples so that an unexpected high concentration of one sample would not affect succeeding samples. This elimination of interaction would also allow more than one kind of sample to be run without intermediate cleanup; that is, more than one antibiotic could be run in a group of samples, provided the test organism and nutrient broth are the same for the group of samples. Changeover from one test organism or nutrient broth to another should be easily performed since several changes need be made each day in many laboratories.

The system should be able to operate unattended for sufficiently long times so that an operator could perform other productive tasks such as preparing samples, setting up the next test, and cleaning the laboratory area. The analyzer should be small enough to fit in a conventional laboratory facility and should not require an unusual laboratory environment. Obviously, it should be mechanically simple and reliable for routine use.

A system that could perform analyses on both vitamins and antibiotics on the same day has some additional requirements. Vitamin assays normally require a least 16 hr. for incubation, while antibiotic assays require 3–5 hr. If samples can be diluted in a discrete module, incubated independently in one or more incubators, and then read out in a third module, dovetailed operations can be considered. The diluter module can dilute antibiotics in the morning and vitamins in the afternoon. The incubator can be used for antibiotics during the earlier part of the working day and for vitamins during the remainder of the day and overnight. The reader can be used for the readout of the previously incubated vitamin tests in the morning and for the antibiotic tests in the afternoon.

A careful study of the logistics of manual analysis revealed that two steps, the dilution step and the readout step, were the most difficult, time consuming, and inaccurate; the incubation step was not difficult or time consuming. Conversely, the incubation phase had been, in past automation attempts, the most difficult and costly to automate and had placed the greatest restrictions on the versatility of previous systems. Separation of the incubation process has other advantages. It gives the analyst freedom to select optimum incubation times and temperatures independent of the rest of the system. It allows the rest of the system to operate at the full speed of each module without consideration of incubation requirements. The incubation step can be divided among several incubators to provide different temperatures. These incubators can be stacked to conserve space. Temperature control of smaller units is easier to accomplish than is control of much larger units. Diluted tubes in appropriate carriers can be placed manually into and removed from the incubators, obviating the need for mechanical devices dynamically controlling the transport of tubes through the incubator.

All the above-mentioned requirements dictated the need for separate dilution, incubation, and readout modules in a system designed to duplicate essentially the basic steps required for conventional manual analysis. Consideration now had to be given to the optimum design for each of these modules.

**Diluter Module**—Many requirements were placed on this module. It had to make dilutions rapidly. If dilutions could be made so rapidly that no appreciable change in the concentration of the assay

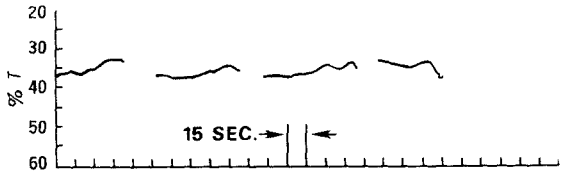


**Figure 1**—*Representative curves obtained by repetitive scans of a static suspension of* Lactobacillus leichmannii.

organism would occur during dilution, fewer standards would have to be run. (A change in concentration of assay organisms between samples and their corresponding standards causes a drift of test results.) Previous workers attempted to control short-term drift by keeping the inoculated nutrient medium chilled. Keeping the nutrient broth uniformly chilled has certain operational disadvantages. If the dilution time for any set of samples and their standard could be reduced to 10 min. or less, chilling should not be necessary for most assay organisms.

Samples should be routinely evaluated at more than one concentration so that parallelism of the slopes of response can be compared for the sample and its standard. This multiple dilution should be performed automatically for each sample. A module that automatically performed four dilutions might be a satisfactory arrangement. At least two different dilutions would be needed, and these could be performed in duplicate. The same equipment should also be easily convertible to produce up to four different dilutions, if necessary.

The dilution step should be performed with sufficient precision to eliminate this as a source of error in the test. The required precision was estimated to be better than $\pm 0.5\%$. The diluter must be constructed of materials that will withstand the corrosive action of the various salts contained in nutrient broths. All lines that contact the assay organisms should have a smooth lumen and no recesses where the organisms could be trapped. This would prevent a cluster of organisms from being accumulated and then subsequently released in a localized high concentration. Rapid sterilization of the equipment should be possible without requiring disassembly of the diluter. Hot water or normal sterilizing fluids must be accommodated. The diluter should also be able to dilute samples in solvents such as methanol, ethanol, and acetone. The diluter mechanism should be rugged, reliable, and easy to maintain. It should be easy to calibrate and retain its calibration for long periods of time.

**Incubation Module**—A water bath is required that provides a very uniform temperature, precisely controlled, throughout the entire bath. This is best accomplished with a precision temperature controller and very rapid and unobstructed water flow. The bath should be easily accessible and capable of being stacked to conserve space. It should be constructed of stainless steel and easy to clean. It was believed that the incubation process had been a long neglected variable in turbidimetric analysis; precise, uniform temperature control of the contents of the assay tubes would minimize one more important variable.

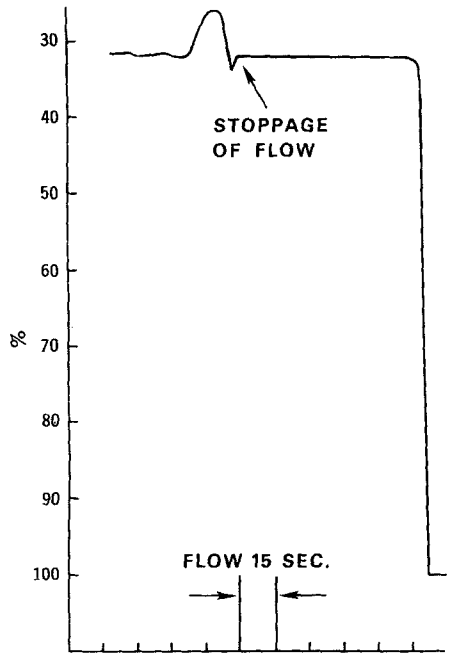


**Figure 2**—*Recording of the photometric response obtained on a fast-flowing stream of* Lactobacillus leichmannii.

**Reader Module**—The major design features of this module should include ruggedness, reliability, and maximum convenience. No appreciable interaction between the readings of adjacent samples should occur. The readings should be performed as rapidly as possible to be consistent with the speed of the diluter and meet the sample load requirements. It should perform the measurement of turbidity with such precision that it does not become a significant source of error in the assay. This would require the readout of fast-flowing streams. Previous experience with fast-flowing streams indicated their utility in solving the problems of flow birefringence.

Flow birefringence is a phenomenon exhibited by elongated particles, such as rod-shaped organisms, in which the particles align themselves with slow-moving currents in a container of liquid. When this container is a flowcell and the motion is imparted by the filling of the cell, many minutes are required before currents cease to exist. Additional impetus can be given to the currents if any appreciable heat is absorbed by the contents of the cell from the incident beam of the spectrophotometer. Since the slowly changing alignment of the elongated particles presents a variable cross section of particles to the beam, a variable reading is obtained. With certain flowcells and with flow rates of about 1 ml./sec. or more, these effects are not evident and stable readings are obtained even with long rod-shaped organisms such as *Lactobacillus leichmannii*. Figure 1 shows the effects of flow birefringence normally encountered in photometric analysis. Each recording is a new filling of the flowcell. There is a highly variable response at each filling with very little predictability of the exact result. Figure 2 shows the same suspension in a flowing stream; it is evident that a stable reading can be obtained in only a few seconds and the reading remains stable until the flow is stopped.

When flow is stopped, serious deviation from steady state is again observed.

## CONCLUSION

The design considerations discussed in this paper were evaluated in terms of all previously reported approaches to automation of turbidimetric microbiological analysis. All previously reported systems did not meet the design goals in one or more major requirements. This led the authors to design and evaluate a prototype of a new automated system. This was constructed, and several improvements were subsequently made on later models of this system. Information on the latest system are presented in the accompanying paper (1), and a discussion is given on how closely the design goals stated here have been achieved.

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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-

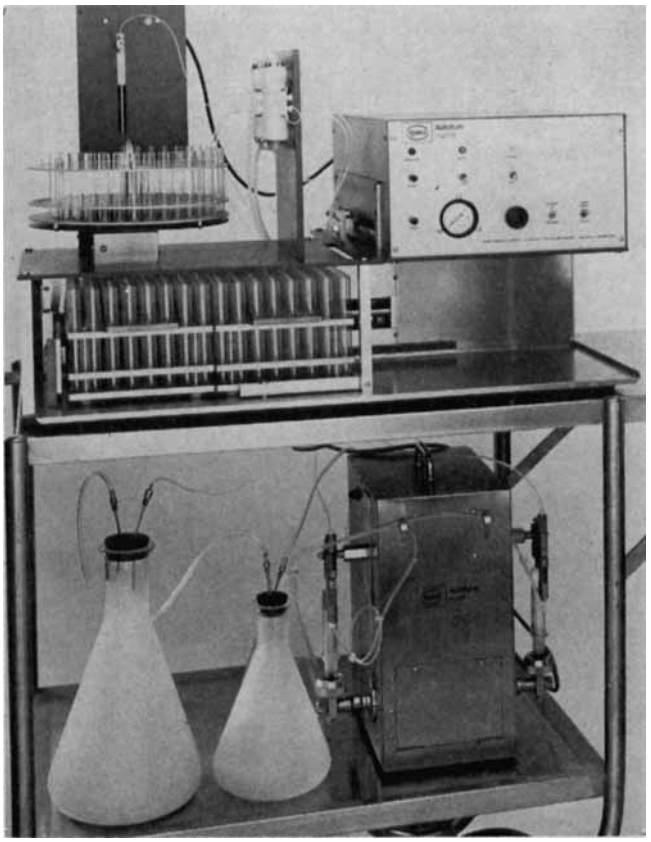

**Figure 1**—*Diluter module.*