Use of a Fiber Optic Probe for Spectral Measurements and the Continuous Recording of the Turbidity of Growing Microbial Cultures

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Measurement of turbidity is a common and convenient means for estimating the size of a bacterial population. These measurements were the subject of a thorough review by Kavanagh (9). The first systematic use of photoelectric colorimeters for the turbidimetric estimation of microbial populations was by Alper and Sterne (1) and by Longworth (13). These workers recognized that turbidimetric measurements required the construction of calibration curves for the particular instruments used. Hershey (8) clearly differentiated between the increase in mass and the increase in numbers of organisms in a culture as parameters of growth. He further showed that the turbidity increase was related to increased mass and that turbidity measurements were independent of changes in cell size that occurred during different phases of growth.

Two classes of instruments have been designed for the continuous estimation of turbidity of a growing culture: (i) the culture vessel is placed in the photometer or (ii) the culture itself is pumped through a photometer cell. Representative of the first class is the Bonet-Maury apparatus used by Coulthar and Hutchison (4). This is a cumbersome apparatus introducing individual tubes, each containing a growing culture, into a photometer. Forrest and Stephen (6) also described a device for the continuous recording of the turbidity of a suspension of growing microorganisms with a nephelometer. Although a simpler device, it has limited use because the growth of a single small culture is monitored within the photometer housing. Representative of the second class are continuous cultures controlled by pumping the culture through a photometer (5) or by mounting the growth chamber in the light path of a photometer (15). Blachere and Jamart (3) used a flow-cell photometer in which the interior optical surfaces are periodically swept clean by the piston, which also moves the culture. This photometric device has an additional analogue circuit which converts the photometer reading to dry mass of the organism.

A device for the continuous recording of bacterial turbidity which would have a rapid response time and does not require sample removal or pumping through a large dead space would be a highly useful instrument for the examination of the growth of microbial populations. In this paper, we describe the design and application of a fiber optic probe as part of a system for continuous recording of the turbidity of growing microbial suspensions. This probe was designed by Gilford Instrument Co. and was built by American Optical Co. We have used it to continuously follow the growth of Saccharomyces cerevisiae, Escherichia coli, and Streptococcus mutans.
MATERIALS AND METHODS

Microbiological. Three organisms were used in these studies: a laboratory strain of E. coli, a variety of S. mutans (SL-1) from the culture collection of the National Institute of Dental Research, and S. cerevisiae ATCC 7921. E. coli and S. mutans were grown at 37 °C, and S. cerevisiae was grown at 30 °C. The organisms were grown on one of three media: Brain Heart Infusion medium (Difco), Penassay Broth (Difco) adjusted to pH 6.8, and a medium devised for the anaerobic growth of yeasts (2).

Determinations. Numbers of bacteria or yeast were determined by use of a Coulter model B particle counter equipped with a high-gain, low-noise preamplifier. The numbers of bacteria were determined by use of the 30-μm aperture and the numbers of yeast with the 100-μm aperture.

Determination of dry weight of organisms. The dry weight of microorganisms in suspension was determined by filtering a sample on Metricel type VM-6 membrane filters (2.5-cm diameter, 0.45-μm pore size; Gelman Instrument Co., Ann Arbor, Mich.). The filters were numbered with a ball-point pen, dried in vacuo over phosphorus pentoxide at 50 °C, and reweighed repeatedly to constant weight on a Cahn "gram" balance by use of the 25-mg scale. To determine the limits of error in weighing, 10 successive weighings of a single filter disc which had been equilibrated to a dryness were recorded, and the error between weighings was determined. The standard deviation of these successive weighings was 7 μg. The organisms retained on the filter disc were washed twice with approximately 2 ml of distilled water per washing. The discs were placed in petri dishes and were again dried and weighed.

The components of the growth unit are shown in Fig. 1. The basic growth unit is a Chemapce vibromixing fermentor with a glass container of 1-liter capacity, jacketed and thermostatted with a circulating fluid by use of a Haake circulator. Agitation of the cul-
ture was set to achieve mixing of the medium without excessive foaming or bubble formation in the bulk phase of the medium. Rubber stoppers in all ports of the fermentor were sealed with a heat-shrinkable polyester product (Scotchtight; 3M Co.) that shrinks during autoclaving.

**Maintenance of constant pH.** The culture was maintained at a constant pH by the use of a combination glass and calomel electrode (Ingold type 465, sterilizable), a Radiometer PHM no. 26c pH meter, a TIT 11B titrator, and a type ABU 1B autoburette. The output of the burette with standardized titrant maintaining the set pH of 6.8 was fed directly into the fermentor. The volume of standardized base added during any interval could be determined by the displacement of the recorder pen. The atmosphere in the growth chamber was flowing, filter-sterilized nitrogen plus 5% carbon dioxide saturated with water vapor, unless otherwise specified.

**Description of the probe.** The fiber optic probe used in these studies was a prototype supplied to us by Gilford Instrument Co., Oberlin, Ohio. Figure 2 is a photograph of the probe and the optical assembly which fits into the sample compartment of a Gilford 300N spectrophotometer. Figure 3 shows a block diagram of the optical path of the Gilford 300N spectrophotometer with the fiber optic probe inserted. Light of a selected wavelength is conducted from the monochromator of the spectrophotometer to the end

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**Fig. 2. Optics which fit in sample compartment of spectrophotometer and the end of the probe.**

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**Fig. 3. Diagram of the spectrophotometer with and without the probe in the optical path.**
of the probe by means of a glass-fiber bundle. There it is transmitted across the opening and reflected back by the mirror at the other side of the probe. The light reflected from the mirror goes into adjacent fiber optic bundles that conduct the light into the photomultiplier of the spectrophotometer. Organisms in the opening of the probe may deviate light from its original path, and the remaining light reaching the phototube will be read as a numerical value which we will call "apparent absorbancy." The fiber optic bundles coming from the monochromator and returning to the photomultiplier are concentrically wrapped with one another and are covered by an inert plastic. Figure 2 shows the assembly removed from the sample compartment. The normal cuvette-holding assembly of a Gilford 300N spectrophotometer is removed, and in its place is inserted the mirror system which is attached to the fiber optic bundles leading to and from the probe.

The output of the Gilford 300N spectrophotometer, representing the changing turbidity of the growing culture, is recorded continuously by use of a Leeds and Northrup Speedomax W multipoint recorder. The span of the recorder was adjusted so that its full range would record absorbancy of 2 optical density units.

Sterilization of the fiber optic probe. The probe was sterilized prior to use with gaseous ethylene oxide in the following manner. An ethylene oxide mixture in the volatile liquid carrier was introduced into the bottom of a 100-ml graduated cylinder. The probe end, along with its accessory rubber cap used to fit the probe into the growth chamber, was suspended above the liquid in the bottom of the graduated cylinder. Cotton was packed around the fiber optic bundles leading out of the graduated cylinder, and aluminum foil was used to cover the cotton stopper. The probe remained in the graduated cylinder for at least 1 hr after the liquid had evaporated from the bottom of the cylinder.

RESULTS

Instrument characteristics. To evaluate the light loss due to probe insertion in the Gilford 300N spectrophotometer and to determine whether it is uniform at all wavelengths, the "spectral response" curve was evaluated (Fig. 4). The instrument was set to an arbitrary null at the emission maximum of the tungsten lamp, 550 nm. Absorbance values were measured as wavelength was varied away from the maximum; thus, the absorbance values are a combined measure of the output of the lamp, the effect of materials in the light path (i.e., wavelength shifters, absorbers, etc.), and the response of the phototube to varying wavelengths. To plot "probe-in" and "probe-out" values on the same figure, it was necessary to subtract approximately 2.6 absorbance units from the "probe-in" values, which is the approximate loss of light with the probe in place. The results also indicate a slight shift in the absorption minimum of the system. This is explainable by the increased voltage applied to the tungsten lamp necessary to zero the instrument; the lamp emission shifts toward higher wavelengths as the voltage is increased. The only significant difference between the two curves is the increased absorption observed at lower wavelengths with the probe in place. For example, the difference between probe-in and probe-out in excess of light attenuation due to reduced aperture is 0.05 absorbance unit at 550 nm, 0.3 absorbance unit at 500 nm, and 0.5 absorbance unit at 450 nm. Below 400 nm, the probe does not conduct sufficient light to allow the instrument to be useful; at all higher wavelengths, the probe appears only to lower the available light with no other effect on the operation of the system.

The absorption maxima measured with the two instruments were identical when an absorption spectrum of a solution of riboflavin taken with a Gilford 300N spectrophotometer equipped with the fiber optic probe was compared with the same absorption spectrum taken with a Gilford 2400 spectrophotometer. In addition, measurements of the relation between absorbancy and concentration were made with the same ribo-
flavine solutions by use of a Gilford 2400 spectrophotometer and a Gilford 300N spectrophotometer equipped with the fiber optic probe. Both of these instruments were set at 450 nm, which is the absorption maximum for the flavine solutions. The relation was linear through absorbancy values up to 2.0. There is a difference in slope between the two straight lines which can be accounted for by the difference in path length of the fiber optic probe and the 1-cm path length of the cuvette used in the Gilford 2400 spectrophotometer. The effective path length for the opening of the fiber optic probe is 8.4 mm, twice the actual opening in the probe, because light is reflected by a mirror in the end of the probe. The actual opening is 4.2 mm.

**Effect of ambient light.** There was a significant effect of ambient light when the probe was used for turbidity measurements. Measurements of apparent absorbancy values of turbid suspensions of organisms were significantly lower with the room lights on than with the room lights off. This is demonstrated by the following measurements. *S. cerevisiae* cells grown in the usual manner were centrifuged, washed with water, and made up to a 200-ml volume with distilled water. Twofold serial dilutions of the original suspension were made in distilled water and dispensed into Erlenmeyer flasks. Two successive sets of readings at 550 nm were taken by dipping the probe into each flask. One reading was made with the room lights on (normal overhead fluorescent light fixtures), and a second with the room lights off. The apparent absorbancy of the original yeast suspension was 1.84 with the room lights off, whereas the apparent absorbancy of the same suspension was 0.92 with the room lights on. The effect of ambient light was much less pronounced when measurements were made with less turbid suspensions. There was no difference in the apparent absorbancy measurements made with the room lights on or off for dilutions of this yeast suspension of 1:32 or greater. Because of this ambient light effect, measurements with the fiber optic probe were normally made in a darkened room.

**Calibration procedures.** The relation between the apparent absorbancy and the dry weight of microorganisms was determined in the following manner. The organisms were grown to the stationary phase, centrifuged, and resuspended in a smaller volume of the same medium. Suitable dilutions of the organisms were made in medium and kept in an ice bath during the course of subsequent determinations. The dilution of each of the suspensions was determined by counting the number of particles per unit volume with a Coulter counter and taking the ratio of the particle count in each suspension to that of the undiluted suspension. The dry weight of a sample of the undiluted suspension was determined as described in Materials and Methods. The mass per unit volume of each suspension was calculated from the dry weight of the original suspension and the dilution was calculated from the particle counts. Additional turbidity measurements on the same suspension of microorganisms were made with a Gilford 2400 spectrophotometer and a Gilford 300N spectrophotometer with a conventional 1-cm cell. Two independent readings of the apparent absorbancy for each concentration of microorganisms were made to calibrate the probe. A polynomial relating apparent absorbancy to dry weight was fitted by means of a linear least squares computer program deemed satisfactory according to the criteria of Wampler (19). The data were successively fit to polynomials from the second degree to the fifth degree, and the best fit was chosen on the basis of the lowest values of standard deviations of the observed and computed data and the lowest value of the residual. By these criteria, the relationship was fit best by a polynomial of the third degree for all three organisms. A computer program was written to transform the apparent absorbancy readings of the suspensions into milligrams (dry weight) of organisms per milliliter of suspension and into the logarithm of this value.

Figure 5 shows the calibration curves of the fiber optic probe for the three different organisms drawn by a Calcomp plotter. The calibration curves for the two bacterial species are almost coincident with one another, whereas the calibration curve for the yeast is substantially different. Presumably, the optical properties of an organism of the size of the yeast are different from those of a particle of the size of the bacteria. Similar calibration curves were determined with the same organisms by use of Gilford 300N and Gilford 2400 spectrophotometers in a conventional manner. For all three organisms, the relationship between apparent absorbancy and dry weight determined with each of these instruments was very similar to the values obtained with the fiber optic probe.

Figure 6 demonstrates that turbidimetric data obtained with the probe are comparable to turbidimetric data taken in the conventional manner throughout the course of the growth cycle of the culture. The medium in the fermentor was inoculated with an exponentially growing culture of *S. mutans*, and at the time intervals shown in Fig. 6 the room lights were turned off and the apparent absorbancy of the culture was
read on a Gilford 300N spectrophotometer equipped with the fiber optic probe. A sample was then removed from the culture, put into a 1-cm cuvette, and read at 550 nm in a Gilford 2400 spectrophotometer. The time interval between readings of the two instruments was about 1 min. The apparent absorbancy readings taken with the probe and the corresponding measurements made in the conventional manner were converted to milligrams (dry weight) per milliliter of culture by use of the appropriate constants for each instrument derived from the calibration procedures. There was no substantial difference in the results obtained by these methods. The insert in Fig. 6 shows a plot of the raw apparent absorbancy values obtained with the probe versus the corresponding value measured with a Gilford 2400 spectrophotometer in the conventional manner. A "least squares" straight line was fitted to the values taken throughout the course of growth of the culture. The standard deviation of the data from the fitted line is 0.036.

The use of the probe to collect data continuously during batch-culture growth of *S. mutans* on Brain Heart Infusion medium is shown in Fig. 7. There are two traces on this graph. One shows the values of apparent absorbancy from the fiber optic probe; the second represents the addition of alkali to maintain the culture at a constant pH of 6.8.

To recover the data from the original records and put these data in a useful form for further processing, the charts were digitized semiautomatically with a Calma digitizer. One hundred points per inch were recovered from these data, representing a resolution of less than 1 min on the time scale of the growth of the culture. The appropriate computer programs were written to convert the arbitrary units of the digitizer to apparent absorbancy units and then to the logarithm of the dry weight of organisms per unit volume corresponding to those absorbancy units. The transformation of apparent absorbancy units to dry weight was based on the cubic equations obtained in calibrating the fiber optic probe. These data were then used for the data sets for a Calcomp plotting routine.
It is apparent from the raw data shown in Fig. 7 that as the culture ceased producing acid there was a concomitant peak in the turbidity curve where growth apparently ceased. This was followed by a noticeable reduction in turbidity of the culture. This may be similar to the lytic phenomenon reported by Hadjipetrou and Stouthamer (7) when glucose was depleted from an exponentially growing culture of Bacillus subtilis. Figure 8 shows a plot of the turbidity data after digitization and conversion to the logarithm of the dry mass against time. The irregularities seen at the lower end of the curve are due to the nature of a logarithmic plot, and represent a very small fraction of a similar displacement at the upper end of the curve. With the exception of the transient spike, the growth curve is conventional with a lag phase, a phase of exponential growth, and a phase in which the organism is declining from exponential growth before growth stops, presumably owing to a limitation of glucose. Since this particular strain has been shown to be a homofermentative lactic acid bacterium (17), the amount of acid produced is a measure of the amount of glucose which has been fermented. If a plot is made of the logarithm of the milliequivalents of KOH added versus time for the exponential phase of growth, the slope of the curve obtained agrees within 5% with that of the corresponding portion of the curve for turbidity. If the same organism is grown in a static air atmosphere instead of the nitrogen-carbon dioxide atmosphere used in the previous experiment, the general features of this curve are similar, except that the slope of the exponential phase is less and the terminal spike present at the end of the growth phase is more pronounced. Another example of transient changes which can be detected by continuous monitoring is shown in Fig. 9, which illustrates the growth of E. coli on Penassay Broth. The medium in the fermentor was inoculated with an exponentially growing culture and sampled at the time shown on the graph by opening a sampling port and withdrawing a sample with a pipette. The growth
rate appeared to be altered at each sampling time, and there was an apparent transient decrease in cell mass after the sample taken at 2.8 hr. No similar effects were seen when either *S. mutans* or *S. cerevisiae* was sampled in an identical manner. Thus, an alteration in the culture condition associated with sampling indicates an unexpected change which could be detected by continuous monitoring.

Figure 10 shows a growth curve for the yeast. In this case, a conventional growth curve was seen, with a lag phase, a phase of exponential growth, and a phase of decline from exponential growth. In this particular experiment, the particles were counted at selected time intervals. A plot of the logarithm of cell numbers against time during the exponential phase of growth of this particular culture gave a slope which differed by no more than 5% from the slope obtained from a plot of the log of the dry mass against time.

**DISCUSSION**

With the use of the fiber optic probe, the optical path of the spectrophotometer is extended beyond the physical confines of the sample chamber while the essential features of a spectrophotometer are retained. A spectrophotometer that can be equipped with a fiber optic probe should prove to be useful for conventional spectral measurements, in addition to its use as a device for the continuous measurement of turbidity. Conventional measurements can be made with this system in places to which there is limited access or in samples which it is not possible to transfer to a cuvette. Measurements which were made with the riboflavine solution show that Beer's law was followed to an absorbancy value of 2, the limit of the rated linearity of the spectrophotometer itself. The absorption spectrum of riboflavine proved to be identical to one taken with a spectrophotometer used in the conventional manner. If there had been any shifting of the wavelength during passage of light through the probe, it would have been seen as an apparent shift in the absorption maximum. The sensitivity of the measurements in both nonturbid solutions and turbid suspensions would be reduced if ambient light were allowed to pass to the photomultiplier along with the signal from the monochromator. The measurement in a nonturbid solution would...
be affected by "noise" imposed by the ambient light; the situation should be more complex for the measurement of turbid suspensions because additional ambient light would be scattered toward the photomultiplier by the particles in suspension.

Spectral measurements made with solutions are different from measurements made with suspensions of particles, and the numerical value read on the spectrophotometer has a different meaning in each case. When light of a particular wavelength is attenuated by the change in electronic energy of a molecule in solution, we use the term absorbancy or optical density in which we imply an adherence to Beer's and Lambert's laws. When light of a particular wavelength is attenuated by scattering due to particles in suspension, the value we read on this same instrument should not be thought of as conforming to these laws of absorption spectroscopy. There may be particular cases of segments on a curve where the use of a linear relationship between the value for absorbancy and the mass or numbers of the organisms is justified; however, these conditions should be very carefully specified in the treatment of turbidity measurements. It is wise for the investigator to construct a calibration curve relating the optical properties of the particular instrument he is using to the mass or numbers of organisms, and the interpretations of these data should be restricted to the limits of these calibration curves.

The optical properties of particles in suspension, as these properties relate to our measurements and to the properties of this probe, may be summarized as follows: the formulation for the scattering properties of particles of this size is Rayleigh-Gans treatment; the light scattered by particles of this size is more a function of the mass of the particles than of their number; and light is scattered in a forward direction with the Rayleigh-Gans relationship holding for scattering angles up to 30° in the direction of propagation of the light (10-12, 16, 18). Although the relationship between the amount of light transmitted through a turbid suspension and the dry weight of the suspension per unit volume is represented by cubic polynomial equations, the coefficients relating these data for the yeast are much different from the coefficients for the two bacteria. According to Rayleigh-Gans theory, there is an effect of volume of the particle on the scattering function which may account for this difference. In the construction of the probe used in these studies, the total path length of the opening is folded by the use of a mirror at the end of the probe. Light is scattered in both directions going across the opening of the probe. In these particular studies, the use of calibration curves has allowed us to ignore the effect of backscatter of light. In the design of an improved device, these factors should be considered, and the light should cross the opening in one direction. This modification would allow one to predict the effect of a change in the opening of the probe with a greater degree of confidence than is possible with an instrument with a folded path. Other possible design changes would allow sterilization of the probe in the culture vessel and a means of mechanically separating from the light path of the probe bubbles which will interfere when the rates of agitation are higher than those used in these studies.

The major advantage of a device of the type described in this paper for studies of microbial growth is the ability of the investigator to collect large amounts of data in short periods of time. This will allow a much more detailed investigation of transient changes in growth rate imposed by sudden changes in the physical or chemical environment. These changes in growth rate may be associated with profound changes in the macromolecular composition of the cells, as has been demonstrated by many workers and summarized by Maaløe and Kjeldgaard (14). For transient events, data processing itself could become the limiting factor, so it would be highly desirable to have the data acquired in a digital form eliminating the transcription of points and allowing direct computer processing.

LITERATURE CITED
1. Alper, T., and M. Sterne. 1933. The measurement of the opacity of bacterial cultures with a photo-electric cell. J. Hyg. 33:497-509.
2. Bauchop, T., and S. R. Elsden. 1960. The growth of micro-organisms in relations to their energy supply. J. Gen. Microbiol. 23:457-469.
3. Blachere, H., and G. Jamart. 1969. A flow cell photometer for bacterial growth monitoring. Biotechnol. Bioeng. 11:1005-1010.
4. Caultas, M. K., and D. J. Hutchinson. 1962. Metabolism of resistant mutants of Streptococcus faecalis. IV. Use of a biophotometer in growth-curve studies. J. Bacteriol. 84:393-401.
5. Eisler, W. J., Jr., and R. B. Webb. 1968. Electronically controlled continuous culture device. Appl. Microbiol. 16:1375-1380.
6. Forrest, W. W., and V. A. Stephen. 1965. A simple recording nephelometer for studies of bacterial growth. J. Sci. Instrum. 42:664-665.
7. Hadjipetrou, L. P., and Stouthamer. 1963. Autolysis of Bacillus subtilis by glucose depleton. Antonie van Leeuwenhoek. J. Microbiol. Serol. 20:256-260.
8. Hershey, A. D. 1939. Factors limiting bacterial growth. IV. The age of the parent culture and the rate of growth of transplant of Escherichia coli. J. Bacteriol. 37:285-299.
9. Kavanagh, F. 1963. Elements of photometric assaying. p. 141-217. In F. Kavanagh (ed.), Analytical microbiology. Academic Press Inc., New York.
10. Koch, A. L. 1961. Some calculations on the turbidity of mitochondria and bacteria. Biochim. Biophys. Acta 51:429-441.
11. Koch, A. L. 1968. Theory of the angular dependence of light scattered by bacteria and similar-sized biological objects. J. Theoret. Biol. 18:133–156.
12. Koch, A. L., and E. Ehrenfeld. 1968. The size and shape of bacteria by light scattering measurements. Biochem. Biophys. Acta 165:262–273.
13. Longworth, L. G. 1936. The estimation of bacterial populations with the aid of a photoelectric densitometer. J. Bacteriol. 32:307–328.
14. Maaløe, O., and N. Kjeldgaard. 1966. Control of macromolecular synthesis. W. A. Benjamin, Inc., New York.
15. Moss, F. J., and F. Bush. 1967. Working design for a 5-liter controlled continuous culture apparatus. Biotechnol. Bioeng. 9:585–602.
16. Powell, E. L., and P. J. Stoward. 1962. A photometric method for following changes in length of bacteria. J. Gen. Microbiol. 27:480–500.
17. Tanzer, J. M., M. I. Krichevsky, and P. H. Keyes. 1969. The metabolic fate of glucose catabolized by a washed stationary phase caries conducive streptococcus. Caries Res. 3:167–177.
18. Van de Hulst, H. C. 1957. Light scattering by small particles. John Wiley & Sons, Inc., New York.
19. Wampler, R. H. 1969. An evaluation of linear least squares computer programs. J. Res. Nat. Bur. Stand. Sect. B 73:59–90.