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The Rapid Estimation by Light Scattering of the Course of Growth of PPLO in Liquid Media

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THE RAPID ESTIMATION BY LIGHT SCATTERING OF THE COURSE OF GROWTH OF PPLO IN LIQUID MEDIA

by

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INTRODUCTION

Mycoplasma are intermediate in size between bacteria and viruses. As such they are the smallest free-living form now known. They vary in diameter from 0.1u to greater than 10u. Their lack of cell wall as evidenced by resistance to cell wall inhibitors and by electron micrograph studies in which only a membranous outer structure is seen give the reason for such morphological characteristics as pleomorphism, pliability, and fragility.

We became interested in this organism because Mycoplasma may offer a unique system for the study of microbial physiology and genetics. Their small size and free-living nature indicate a less complex model of a free-living system. In the area of polysome studies, the fragility of the organism and its less complex nature is advantageous for the study of cell-free protein synthesis and for the study of the natural distribution of ribosome populations. Susceptibility to osmotic shock makes the Mycoplasma an ideal system to work with since most other organisms require extremely harsh methods to liberate ribosomes, which results in a distortion of normal polysome patterns.

To those interested in membrane structure and function, the external covering of Mycoplasma is analogous to the cytoplasmic membrane of many organisms. The absence of contaminating cell wall material presents a clean model for micro-anatomical and structural analysis of its multi-functional and vital subcellular structure. Likewise, the easily attainable yet pure membranes permit studies on the chemical composition, permeability and terminal respiration unhampered by contaminating wall or cytoplasmic constituents. The

problem of latency, relapse of infection, hyperimmune phenomena and interference with host regulatory mechanisms may be studied readily with these organisms. Mycoplasma may also offer a less complex system for the study of regulatory systems.

The advantages that obviously accrue from the reduction in size also present some operational problems. The lack of complexity results in requirements for a very rich growth medium that must also take into account such factors as osmotic stability as well as complex growth requirements. To accomplish this, optically dense medium is usually required.

This, coupled with a very small increase in weight per generation, does not allow the application of the standard bacterial methods of quantitation. A decade ago, Smith (16) wrote "accurate and reliable methods for the quantitative measurement of the growth of Mycoplasma have never been developed. Studies on members of this group of organisms have advanced in recent years to the stage at which further developments will be impeded by the lack of such methods." In the ten years which have elapsed little change in this aspect of the field has taken place.

During this period numerous methods of quantitating growth have been used; unfortunately, these are time consuming, tedious, and do not lend themselves to routine laboratory work. The standard rapid technique for observing the development of a bacterial culture is by periodic measurement of the turbidity of that culture. Turbidity or absorbancy is proportional to cell number and cell mass. Curve determined by diluting and plating of the organism and subsequently, correlating viable cell count with absorbance allow

one to define cell numbers on the basis of a simple absorbance reading. Unfortunately, absorbance is not a helpful index for the growth of Mycoplasma because absorbance is absent during most of the development of the culture and develops only late during the log phase of the culture, usually within one or two generations preceding the onset of the stationary phase (2).

The most accurate technique (2, 16) for the determination of a growth curve or the growth rate constant of Mycoplasma is by a plot of plate counts of samples taken during the development of the culture. The actual computations are then make a few days later after the colonies have developed to the point at which they can be counted under a dissecting microscope.

Smith (16) suggested that sensitivity of the turbidimetric method could be increased by a ten-fold concentration of cells via centrifugation. However, further studies have shown that (a) as many as 10^8 (colony forming units) ml remain in the supernatant fluid after centrifugation at 20,000 x g; (b) under these severe conditions for Mycoplasma, large numbers of cells may be ruptured and finally, (c) this technique is not applicable to routine laboratory work.

The method of most probable numbers has been used successfully to estimate the growth of M. hominis (7) and M. gallisepticium (3). This technique is applicable to less fastidious species which are capable of growth starting from minute inocula. It does not, however, give precise enumeration of the organism and requires large numbers of tubes to reduce experimental error.

A number of other methods have been employed in an attempt to obtain a meaningful growth curve. Measurement of mean colony diameter on plates under standardized conditions is useful for some species (15). It is essential to

standardize the agar concentration in the medium and to minimize overcrowding. Acid production also has proven useful as an assay of growth for fermentative species (2). Growth of all species can be quantitatively assayed by measurement of dry weight (2, 14), DNA or protein nitrogen (2, 10). Caution is necessary when measuring dry weights since the cells must be washed in a salt solution to prevent loss of cellular material, yet transfer of organisms to the weighing vessel must be done with water to eliminate error due to the weight of salt. Dry weight measurements or protein nitrogen content do not correlate even in the same species grown under different conditions. Thus, the ratios of dry weight to protein nitrogen may vary from 10 to 17 (15) values far above the usual 6.25 for protein nitrogen. More recently, Anderson (1) has used the electron microscope for counting and also to study the size distribution of M. laidlawii, but this is not practical on a routine basis. Most of the methods mentioned above are tedious, time consuming and do not lend themselves to routine laboratory use.

An obvious solution to this problem is the use of the principle of light scattering. The amount of light scattered by a suspension of particles or microbes is proportional to the particle or cell number.

We have introduced the technique of light scattering to reduce the time and tedium previously required to determine the usual growth parameters of Mycoplasma. It also allows one to monitor continuously the development of the growing Mycoplasma culture.

MATERIALS AND METHODS

Organism, isolation and purification. Mycoplasma laidlawii, an isolate from sewage, obtained from Dr. H. Neimark (State University of New York, College of Medicine, Brooklyn, New York) was used in these studies. To ensure the purity of the culture, four successive single colony transfers have been made starting with a typical single colony. The culture derived from an isolated colony was inoculated into a two liter flash containing a liter of medium I enriched with 5% bovine serum. The flask was incubated at 37 C on a rotary shaker for 36 hours. To obtain uniform inocula, 3 ml of this culture were transferred aseptically into sterile vials and frozen at -30 C. These were defrosted as required, a few minutes prior to an experiment.

Growth media and buffer. Medium I (PPLO broth): 0.5% trypticase soy broth (BBL), 0.5% bacto-peptone (Difco), 0.2% yeast extract (BBL) and 0.5% NaCl. Medium II (13): 2.0% trypticase soy broth (BBL), 0.5% Tris·HCl and 0.5% NaCl. Media were adjusted to pH 8.0 with NaOH. Sterile bovine serum to a concentration of 1-10% was added as specified in the text. B buffer contains 0.15 M NaCl, 0.05 M Tris·HCl and 0.01 M B-mercaptoethanol adjusted to pH 7.4. For plate counts, medium I supplemented with 10% bovine serum was solidified with 1.5% agar. Plates were incubated at room temperature for three days as a sterility check as well as to remove excess surface moisture. Dilutions were made in medium I. The plates were sealed with tape after spotting with Mycoplasma to prevent dessication and incubated for 2-3 days at 37 C before counting under 10 X magnification with a dissecting microscope.

Fluorescence and light scattering measurements. A 36 hour culture of M. laidlawii containing 10^9 CFU/ml was harvested and the cells washed once in medium I, twice in B buffer, resuspended in 10 ml B buffer and finally diluted to a concentration of 10^9 organisms/ml. This suspension was examined in the Aminco-Bowman Spectrophotofluorometer for fluorescence and light scattering. The initial recording was obtained with an excitation wavelength of 300 mu; the emission wavelength was scanned from 300 mu to 700 mu. The excitation wavelength was then successively increased by 20 mu until 700 mu was reached, a total of some 20 scans. This procedure was repeated for (a) uninoculated medium II and (b) a 36 hour culture of M. laidlawii grown in medium II.

Growth curve determination. Three ml of freshly defrosted stock culture were inoculated into 1 liter of test medium. The contents of the flask were mixed thoroughly and a 0.5 ml sample was withdrawn for the zero time CFU assay and light scattering determination. The flask was incubated at 37 C on a rotary shaker set at 90 oscillations/min. Samples were examined at 3 hour intervals for the first 24 hours and at 7 hour intervals for the next 48 hours. Growth curves were determined in the following three media: medium I, medium I + 0.5% bovine serum and medium II + 1% serum, by light scattering and viable count.

Viable count determinations. The technique we used for determining viable counts was essentially that described by Butler and Knight (2). One-half ml samples were serially diluted in 4.5 ml of medium I and 0.01 ml of each dilution was spotted in sextuplicate onto dry agar plates. The plates

were incubated at 37 C for 2 days. Those plates containing 50 to 200 colonies per spot were counted.

Growth curve determinations by light scattering. Since maximum light scattering was noted at 460 mu, the extent of light scattering for the succeeding experiments were measured at 460 mu. The fluorometer was blanked to zero on the 0.3 full scale with uninoculated medium in the sample chamber by adjusting the slit width. Of five aliquote of each sample, one was: (1) plated for determination of CFU/ml, (2) diluted in medium I, (3) diluted in medium I + 2% glutaraldehyde, (4) diluted in medium I + 2% formaldehyde, (5) diluted in medium I + 10% formaldehyde. Aliquots 2 through 5 were examined in the fluorometer using 460 mu for both excitation and emission wavelengths.

Particle count. M. laidlawii was inoculated into medium I enriched with 1% bovine serum. The complete medium had been sterilized by filtration through an 0.45 u diameter membrane filter prior to inoculation. A filter sterilized, uninoculated batch served as a sterility check. Samples were withdrawn during the growth of the culture and added to equal volumes of Sorenson's phosphate buffer or B buffer. Both buffers contained 2% glutaraldehyde. Particles were counted in a Coulter multichannel particle counter with a 4 u diameter aperture, in the laboratory of Dr. Kubitschek of the Argonne National Laboratory, Argonne, Illinois.

Growth curve determinations with formaldehyde. In performing a number of experiments simultaneously, it is often desireable to simply collect and store samples until after the experiment is concluded and then to complete growth

determinations at a more convenient time. This requires that growth be stopped immediately. We accomplished this by adding a constant amount of formaldehyde ranging from 1-10% to the sample and the blank. The concentration of formaldehyde required to stop growth is not only dependent on the numbers of organisms, but also on the medium used.

RESULTS

Figure 1 shows a typical growth curve of Mycoplasma laidlawii growing on a medium containing 2% serum, measured as colony forming units. Included in this figure is a measurement of the absorbancy of the culture. Absorbancy does not increase during most of the development of the culture and becomes apparent only late in the development of the culture at a cell concentration approximately 10^9 Mycoplasma per ml, which in this case is approximately two generation times before the onset of the stationary phase. Very little change in absorbance at 450 mu or 600 mu is noticeable during the development of the culture. An increase of approximately 0.08 absorbance units heralds the passage of 8 cell doublings.

Even after plate counts show that the culture has entered the stationary phase, the absorbancy readings continue to increase, indicating that at some period in the stationary phase, the Mycoplasma begin to form large bodies or become optically denser. Thus, not only is the sensitivity of this spectrophotometric method poor because of small increases in absorbancy, but the resulting curve is not an accurate reflection of the late stages of growth.

Consequently, the usefulness of the light scattering technique for Mycoplasma and the wavelength which gives maximum light scattering was examined in an exponentially growing 24 hour culture of M. laidlawii. In Figure 2 the curve drawn for the data shows the relative intensity of the emission using the corresponding excitation wavelength. The maximum emission for any given wavelength occurred at the same excitation wavelength. The maximum emission for all sets examined occurred at 460 mu. The fact that maximum transmitancy occurs

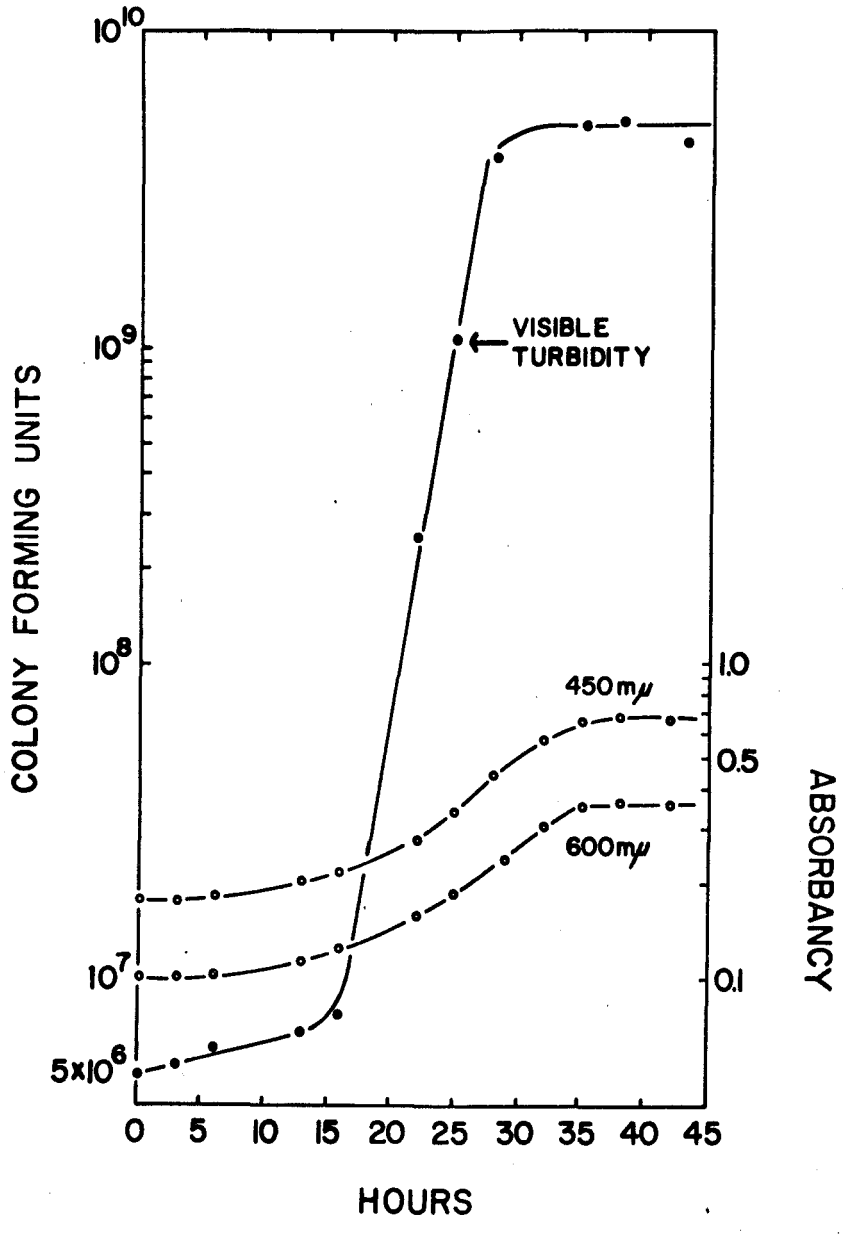

Fig. 1. A typical growth curve of M. laidlawii growing in medium II supplemented with 2% serum.

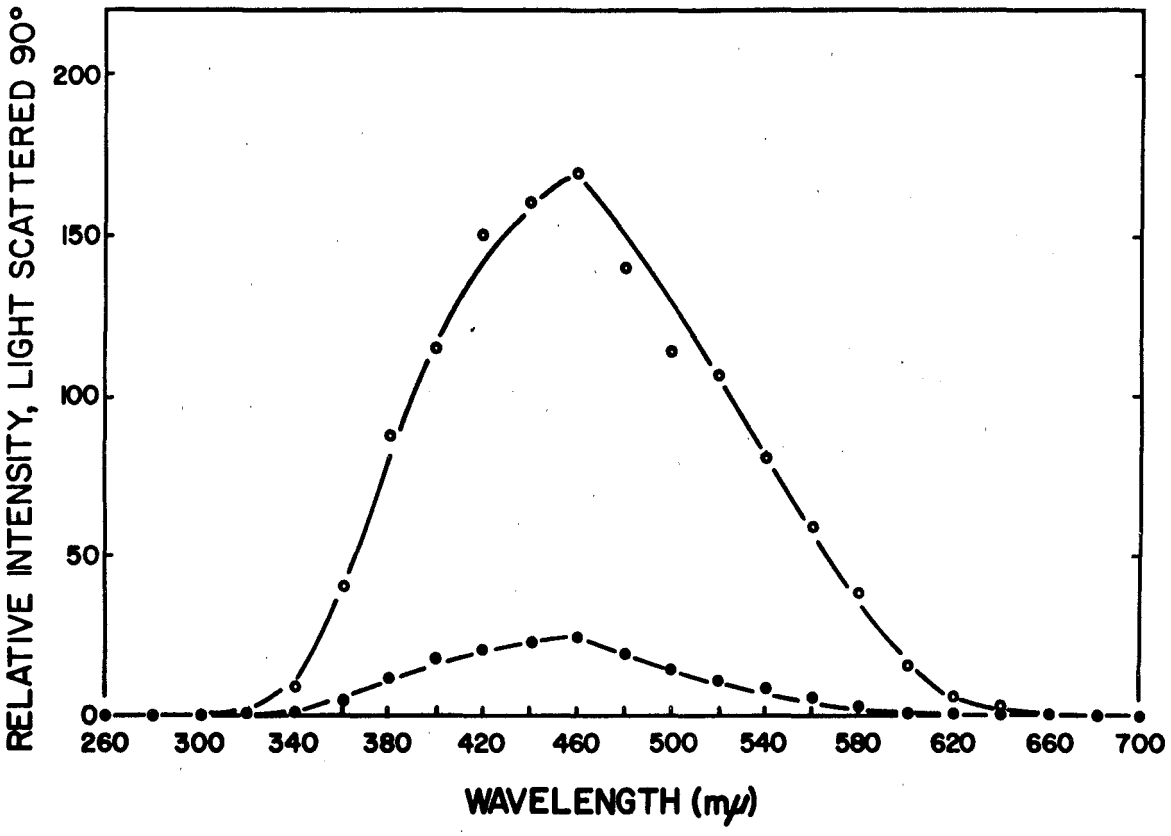

Fig. 2. The relative intensity of light scattered at 90° is plotted as a function of the excitation-emission wavelength.

when the emission-excitation wavelength corresponds to the same wavelength suggests that we are observing light scattering.

In order to rule out the phenomena of fluorescence and phosphorescence, we washed the cells of an exponentially growing culture of M. laidlawii containing about 10^9 CFU/ml, resuspended them in buffer consisting of 0.15 NaCl, 0.05 M Tris·HCl and 0.01 M B-mercaptoethanol at pH 7.4 and scanned the suspension in the Aminco Bowman spectrophotofluorometer. Figure 3 shows the scan obtained. We again see that the maximum intensity is reached when the excitation wavelength is the same as the emission wavelength of the scan. Figure 4 shows that when the maximum intensity for each wavelength is plotted against the corresponding excitation-emission wavelength, the maximum amount of scattering occurs at 460 mu, as with the inoculated culture. There was no indication of a useable phosphorescence or fluorescence pattern.

We disrupted the cells of this suspension by sonication. Figure 5 shows that the scattering patterns obtained are similar to those found with intact cells; neither phosphorescence nor fluorescence was observed. Maximum emission occurred at 460 mu with an excitation wavelength at 460 mu.

We then determined the usefulness of light scattering in the enumeration of Mycoplasma. In Figure 6, M. laidlawii was added to the PPLO broth to give an initial concentration of 10^4 CFU/ml. The upper curve drawn from data marked as open circles is the growth curve as measured by viable count. The lower curve drawn from data distinguished by the closed circles are the light scattering readings. The curves run parallel to each other; it is obvious

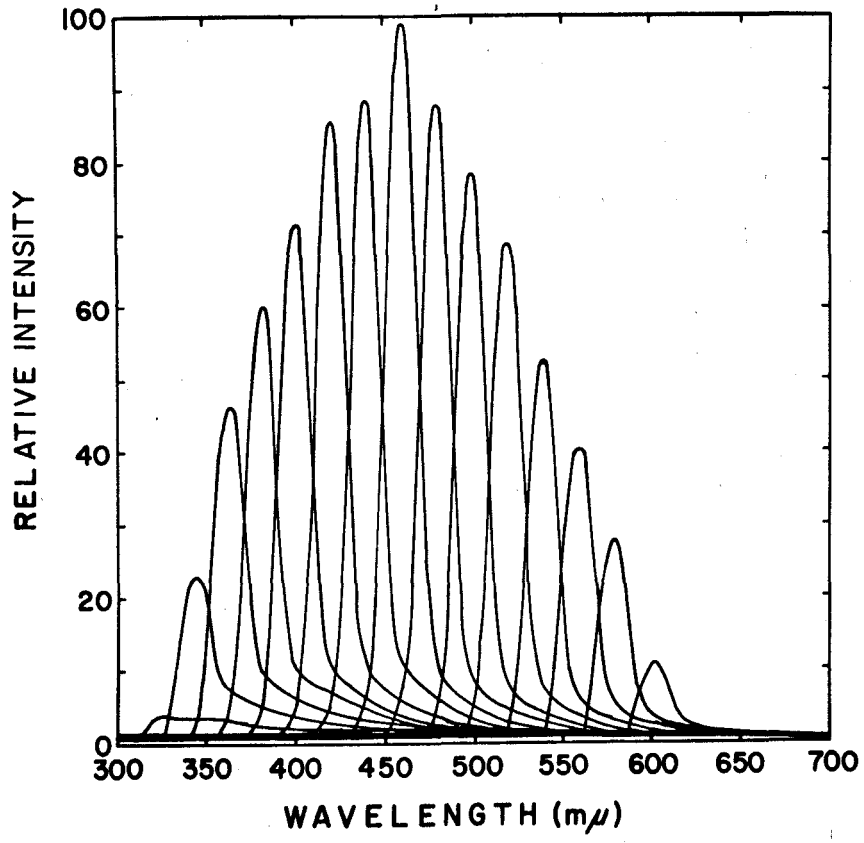

Fig. 3. The relative intensity of light scattered at 90° is plotted as a function of the excitation-emission wavelength for cells washed in buffer.

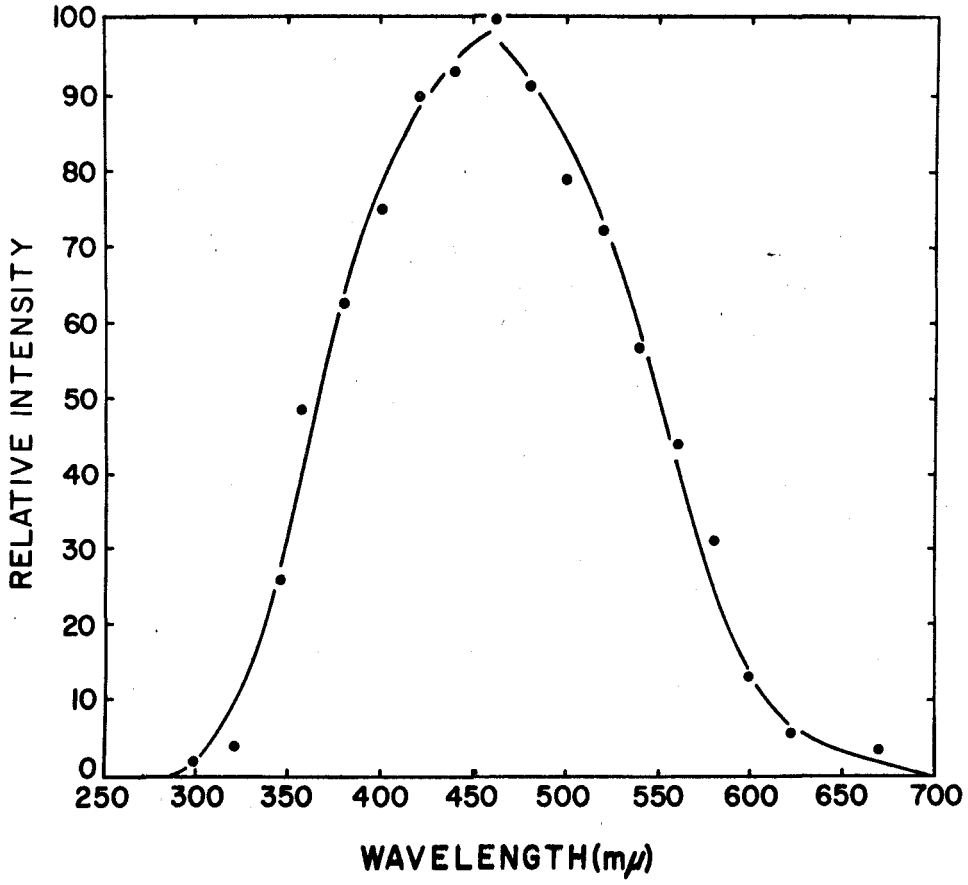

Fig. 4. A plot of the relative intensity of each of the light scattering peaks of the washed cells.

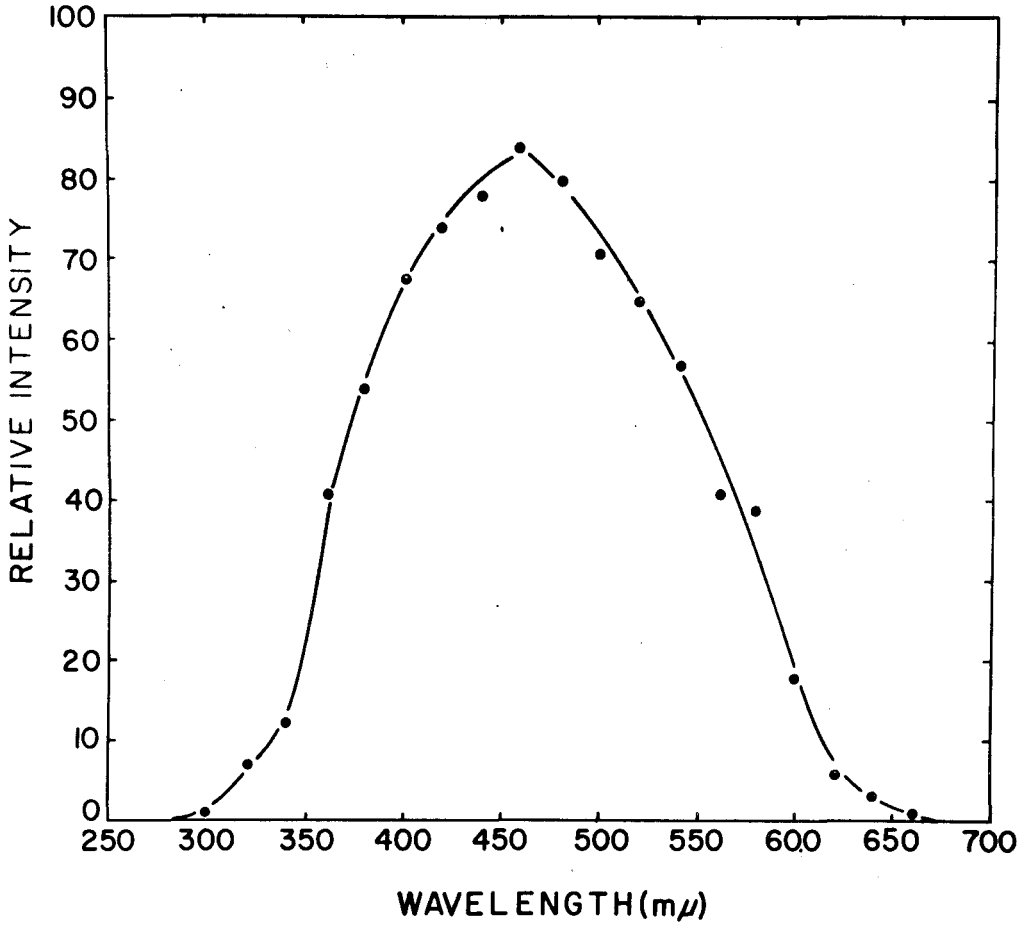

Fig. 5. The relative intensity of light scattered at 90° as a function of the excitation-emission wavelength for a suspension of ruptured cells.

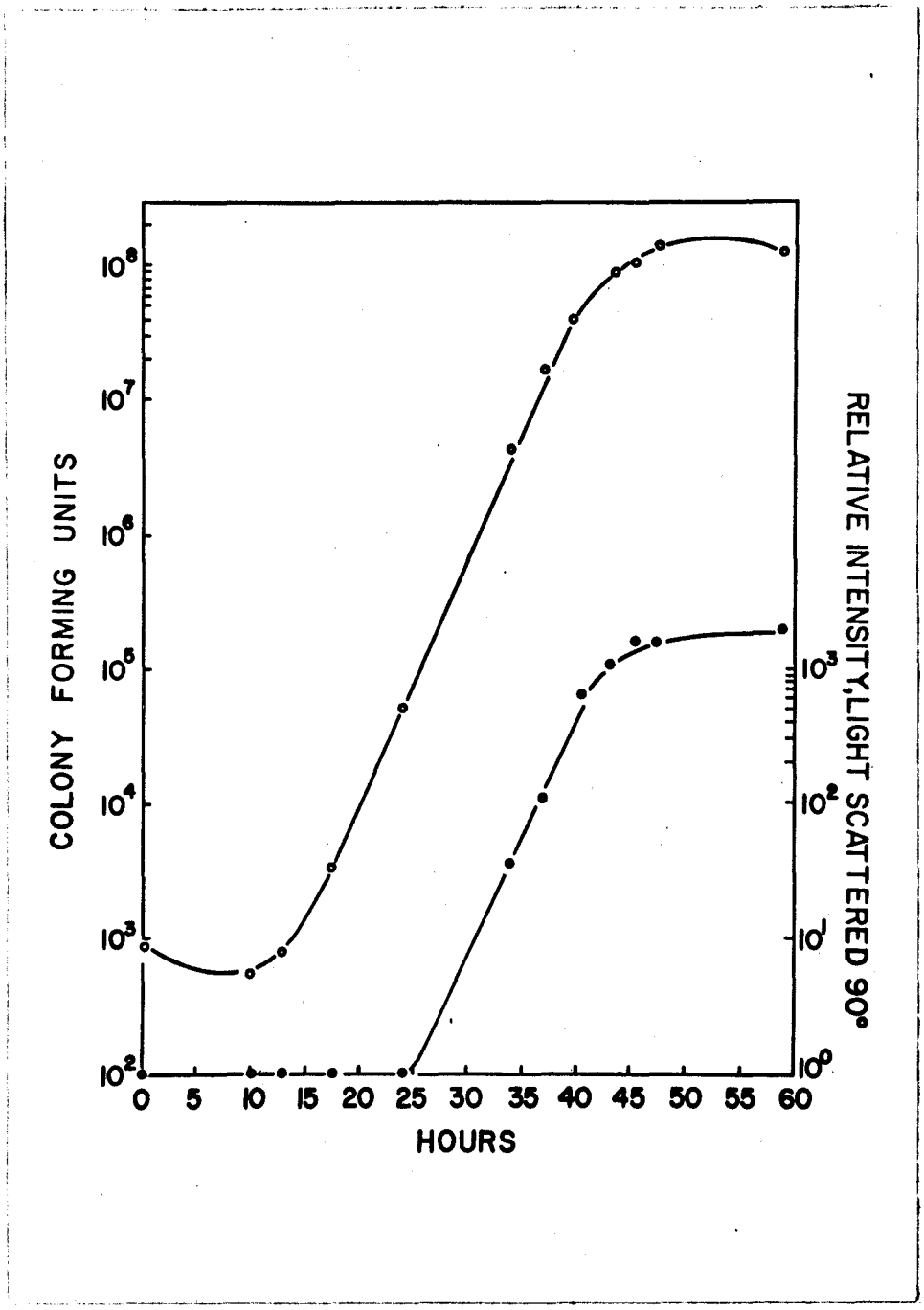

Fig. 6. A comparison of growth curves derived from light scattering and viable counts.

that the light scattering can be used to monitor growth of Mycoplasma during most of the development of the culture. The lower limit of the instrument's sensitivity fell into the 10^5 particle per ml range. Although the plate counts showed that exponential growth began at about the eighth hour after inoculation, we did not detect any evidence of growth by light scattering until about the sixteenth hour; that is, until the cells reach a concentration of 10^5 CFU/ml. The upper limit of sensitivity on the 0.03 scale full range is about 10^6 particles/ml, but of course this problem is obviated by reading dilutions. Light scattering allows us to monitor the culture continuously. We can detect about a 1000-fold change in the range of 10^5 to 10^8 CFU/ml.

Figure 7 shows a similar growth curve in which the medium was prefiltered through a milipore filter, pore size 0.3 u diameter. The culture flask initially contained 10^3 CFU/ml. The culture entered the exponential phase at about the twelfth hour according to the data from the standard plating technique. Again, light scattering was found to be insensitive at cell concentrations below 10^5 ml. This still leaves us a range of 3 to 4 logs of growth to measure. Filtration did not increase the sensitivity of the method nor did it have much effect on growth.

We next tested the effects of varying concentrations of serum on the sensitivity of the instrument. The concentration of serum was varied between 0.5% to 10%. We noted that the sensitivity of the technique decreased as the concentration of the serum increased, until at 10% serum, the sensitivity was reduced by a factor of 10. Even when sensitivity is reduced to this extent, reliable growth curves may be obtained. Since the maximum stimulation occurs

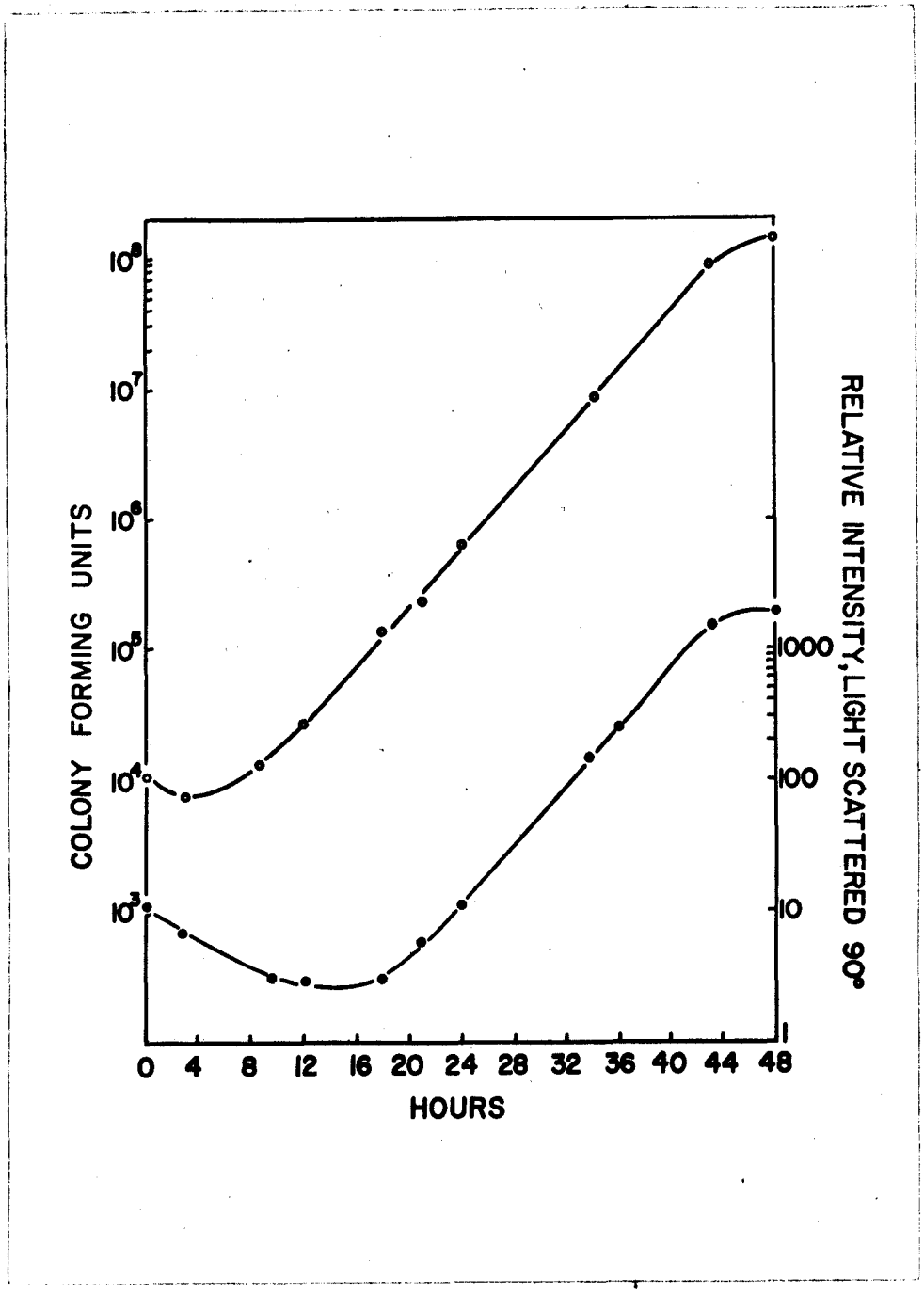

Fig. 7. The effect of prefiltering the medium on the growth of Mycoplasma.

with 0.5% serum, the problem just mentioned is not encountered. Upon the addition of the serum to the medium, the organism immediately enters the logrithmic phase of growth. At about six to eight hours after inoculation, the generation time suddenly changed from three hours to one hour and then continued at the new rate until a final concentration of 10^9 CFU/ml was reached. We examined a hexane extract of cotton seed as a medium constituent and noted results identical to that obtained upon addition of serum to the medium. This suggested to us that one of the main features of serum is to provide readily available lipid material for the Mycoplasma to incorporate.

When we treated PPLO broth cultures with formaldehyde in concentrations of 1.0% to 2.0%, we found that growth continued to its normal maximum. It appears that a functional concentration of formaldehyde is not only dependent on the numbers of the organisms, but also on the medium used. Since the concentration of protein varies with the medium, a separate concentration curve must be worked out for each medium. We found that for PPLO broth with 0.5% serum, a final concentration of 7.5% was sufficient to stop growth.

DISCUSSION

During the growth of the culture of Mycoplasma laidlawii (Fig. 1), there was little, if any, increase in absorbancy for the first twenty-four hours. Then, the absorbance increased for eleven additional hours. This was followed by a period in which there was no further increase in absorbance. The plate count data, on the other hand, suggests that insofar as growth is concerned, this is misleading. Logrithmic growth started at fifteen hours and terminated in the stationary phase at around the thirtieth hour. The cells grew with a generation time of one hour. Obviously, absorbancy data are valueless for the estimation of numbers of Mycoplasma.

Since visual or measureable absorbance only occurs when the organism has entered the later stages of maximum development, it can be concluded that this is due to one of the following: (1) because of the small size of the organisms, numbers large enough to produce absorbance are not reached until late in the growth cycle, or (2) an increase in the size of the organism with "large bodies" production is occurring in this period. In this connection, Kang and Casida (6) have recently reported that the large bodies of Mycoplasma and L-form organisms appear to result from the accumulation of metabolic by-products and/or medium components within or on which minimal reproductive units had become entrapped. We have earlier noted in Figure 1 an increase in absorbancy at the end of the logrithmic phase. This may be attributed to large body formation and to increases in internal density due to accumulation of macramolacules.

Light scattering patterns on the other hand correlate precisely with those of the viable count under our conditions. From inspection of the curves plotted from Figures 6 and 7, it becomes evident that the organisms undergo a typical microbial type of growth cycle. There is evidence of a lag phase (about eight to twelve hours in duration) under the above conditions of cultivation. For the next thirty-two hours, there follows a logrithmic increase in the number of viable organisms. This period terminates in a stationary phase. Thus, the question of the mode of replication arises.

There are at present two schools of thought on this subject. One school holds that small elongated or spherical cells with a diameter in the 0.1 u to 0.3 u range, multiply by extruding a short, thin filament on the end of which a new cell is formed (14). The so-called large bodies arise from multiple centers of growth on a single organism and only disaggregate under certain environmental conditions (15). The other school holds that non-septate filaments extruded from the elementary bodies, are eventually transformed into long chains of regularly spaced, uniformly shaped, elementary bodies (5). When one considers the plasticity of the cell wall, the probability of randomly occuring single or multiple growth centers, as well as species variation, the difference between the two general modes of reproduction appears to be semantic rather than real.

The relatively high cost of the Aminco-Bowman spectrophotofluorometer led us to examine less expensive instruments which would still give reasonable sensitivity. The Coleman Jr. Universal spectrophotometer was inadequate for our purposes because most of the scale was utilized solely to blank out the

optically dense growth medium used for Mycoplasma. The Turner 110 may be of value if square cuvettes and the corresponding holder are employed. It was necessary to reduce the slit width to its narrowest position, however, and this in turn decreased sensitivity somewhat.

SUMMARY

The light scattering technique is an effective means of solving the problem of rapid growth determinations in Mycoplasma. It allows the investigator to follow the development of the Mycoplasma culture after the addition of an inoculum as low as 10^5 CFU/ml up through stationary phase. Since other methods are tedious and time consuming in comparison, it should be a great aid in determining the effects of different medium constituents on the growth rate of this organism.

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