RESOLVING BACTERIA WITH THE COULTER COUNTER

by

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I. INTRODUCTION

The enumeration and sizing of bacteria has always been a tedious and haphazard task. No matter what method is employed, extensive preparations of materials and cell cultures are usually necessary. To make cell counting practical, methods must be employed for representing a large population of bacteria by a relatively small count. This is usually accomplished by making a series of dilutions from one sample until the appropriate dilution for the particular method is obtained. With each successive dilution the probability of getting a representative sample of the original population is decreased. Pipettes and glassware also provide sources of error since adsorption of cells on these surfaces might occurr.

Current techniques used to determine the amount of bacterial growth are numerous (31). Direct microscopic counts of bacterial cells can be obtained with smears (which might be stained slide preparations) or with suspensions of bacterial cells in counting chambers such as the Petroff-Hausser Chamber. Using the microscope for direct counts has obvious limitations and undesirable characteristics such as tedious labor, small counts resulting in poor statistical validity, and human errors. Automation of counting stained bacterial cells has been accomplished

experimentally with a device called the "Partichrome" which scans a microscopic field and reacts to a characteristic color of the dyed bacteria (27). A plate preparation of bacteria is necessary for this process.

Cell counting can also be done by dilution and plating methods. A small sample of proper concentration is added to a Petri dish of growth inducing medium. After incubation the enumeration of the colonies serves as an approximate count. A colony counter, consisting of a light and a magnifying glass, may be utilized for counting the colonies. To promote easy and accurate counting and to minimize the probability of one organism interfering with the growth of another, a dilution which yields a plate count between 30 and 300 colonies is considered optimum. A modification in this method is the use of Millipore filters. The bacteria are filtered out of the diluent and the filter pad is placed upon the growth inducing medium. The counts from any plate counting method are usually undersized because cells might be damaged in the process of dilution or some colonies might represent more than one cell.

Other methods are used for quantitative measurements of bacterial growth. Among these are turbidimetry, nitrogen content, nucleic acid determinations, dry weight of cells, and the monitoring of specific chemical change (31).

Although these methods indicate bacterial growth, no information regarding the actual number of cells is obtained.

The actual volume of a cell is estimated mainly by direct cell observation where the observer must approximate the depth of the cell from two dimensional observations. This method is slow and inaccurate.

It is obvious that an improved method of counting and sizing of bacterial cells is desirable. This method should be accurate, easily performed, quick, and reliable. The Coulter Counter, first introduced in 1956, has been demonstrated to meet these qualifications in the counting and sizing of larger biological cells such as blood cells (7,6, 9,25,21,23,20,24,19,30). However, it has not been extensively tested in regards to the counting and sizing of bacteria. Significant literature does not exist to attribute a great degree of reliability to this method of sizing and counting of bacteria.

The following investigation presents information which will be of value to the operator who uses the Coulter Counter for the sizing and counting of bacteria. An attempt is made to reveal the many variables inherent in this method. By controlling these variables, the results of future experiments with the Coulter Counter should be more reliable.

II. REVIEW OF LITERATURE

The Coulter Counter (see Appendix D) was first introduced by Wallace H. Coulter in 1956 (9). His talk, entitled "High Speed Automatic Blood Cell Counter and Cell Size Analyzer", emphasized the efficiency of this non-optical scanning system in the electronic detection of red blood cells at rates in the range of 6000 per second. Since this meant that the number of cells counted electronically was 100 times greater than the usual microscopic count, he declared that the statistical error would be reduced by a factor of approximately 10.

Kubitschek (16) in 1958, first presented information regarding the counting and sizing of bacteria with the Coulter Counter. Size distribution graphs of Escherichia coli strain B and of spores of Bacillus megaterium were given. In 1960 he also presented a size distribution of Bacillus terminalis spores and mention was made of counting virus particles of feline pneumonitis (17). An orifice of 10-micron diameter was used for these determinations.

Lark and Lark (19) utilized the Coulter Counter to study cell division of a sychronized culture of Alcaligenes facalis in 1960. With a 27-micron orifice they obtained size distributions of successive samples taken periodically during the experiment. Counts were presented as a relative

percentage of the total count. Except for a slight increase in cell volume just before cell division, the cell volume of A. fecalis remained relatively constant during the log phase of growth. Volume measurements of late log phase cells and stationary phase cells with the hematocrit did not correspond to Coulter Counter results. Volume ratios of the two types of cells were approximately equal for both methods. Streptococcus faecalis and Escherichia coli were monitored with a Coulter Counter using a 30-micron orifice by Toennies (37) in 1961. During the exponential growth phase, bacterial mass doubled every 32 minutes while number doubled every 26 minutes. Samples of Streptococcus faecalis were taken periodically from a broth culture in the exponential phase and size distribution curves of these samples were prepared. The remaining data of Toennies' work is reported by giving the percentage of total cells in each of three classes: small, medium, and large.

Boone (5) reported that he was in the process of calibrating the Coulter Counter for bacterial measurements. His only presented data, obtained with a 30-micron orifice, was a size distribution curve of Escherichia coli. He also stated that he was working with Hemophilus influenza.

Allison (3) showed the effects of chloramphenicol on the growth and multiplication of Escherichia coli B/r. Total counts were obtained with the Coulter Counter and

30-micron orifice. Two size distributions of Escherichia coli B/r are given which indicate a shift in the population to larger sizes as a result of a 90 minute contract period with chloramphenicol. He also presented the results of an experiment which differentiated total (Coulter) cell counts from viable (colony) counts. It appears that agreement was obtained for counts from both methods whenever agreement was expected.

The electronic counter was used experimentally in the analysis of urine samples by Traunt (38) in 1962. With a 30-micron orifice he reported detection of coliforms in the samples following an incubation in t-soy broth. Several graphs of different urine samples were presented. He indicated that distribution studies were made on pure cultures of Aerobacter, Alcaligenes, Bacillus, and Escherichia but presents no results of these studies. He did report that he was hardly able to detect yeast cells. This is hard to understand in the light of other published articles (10,34).

Using a 30-micron orifice, Swanton (36) obtained responses on the Coulter Counter with samples of viable and heat killed cells of Staphylococcus aureus SM, Escherichia coli, and Serratia marcescens. Total Coulter counts of S. aureus and S. marcescens were compared with optical wet counts and plate counts. Electronic counts were consistent

and close but always slightly lower. This is possibly due to emphasis on maximal gain instead of maximal aperture currents (22). Pictures of oscilloscope patterns of E. coli and S. aureus were also given to illustrate qualitative differences in live and dead cells.

Shindala (34) reported in 1964 on the commensalism of Proteus vulgaris and Saccharomyces cerevisiae. Size distributions for the bacteria and for the yeast are presented. By adjusting the counter to two predetermined settings according to the original size distributions, total counts for the two different species were obtained. Counts obtained by using a Petroff-Hausser Chamber were about 20 per cent higher than counts obtained with the Coulter Counter and the 19-micron orifice.

Only these few articles were found on bacterial measurement with the Coulter Counter. Since so little work has been done, it is perhaps in order to review other literature which deals with the measurement of biological cells by using the Coulter Counter. Results dealing with other biological cells could possibly be generalized to apply to bacterial cells.

Considerable work has been done concerning the Coulter Counter and the measurement of red blood cells (7,6,9,25,21, 23,20,24,19,30). The first evaluations of the Coulter

Counter (7,26) were done in regards to red blood cell enumeration and sizing. The electronic counter has also been used to count yeast cells (10,34),mitochondria (11), and algal cells (32). These reports will be considered together in the following discussion.

Many particles distributions follow the logarithmic form of the Gaussian statistical law of errors (15). Brecher (6) points out that normal red blood cells follow this distribution when graphing cumulative counts against volume. Microcytes do not follow the log-normal distribution found for healthy red blood cells. Lushbaugh (21) indicates that the healthy erythrocyte population consists of two normally distributed overlapping populations when considering cumulative frequency and volume. These subpopulations can be distinguished by their different sensitivity to various hemolysins. By analysing the red blood cell distribution, anemia (25) can be detected. Distributions of diseased blood cells which were obtained with the Coulter Counter have been shown to follow distributions determined by Price-Jones in 1910 (26). Computer programming was necessary to fit two normal distributions to the one red blood cell distribution obtained with the Coulter Counter (21).

Another biological cell distribution which follows the Normal Gauss Curve is Saccharomycodes ludwigii Hansen (10). This occurrs when the cumulative per cent is plotted against

the diameter of the yeast cell. Beysson (10) obtained these results by using the Coulter Counter and a 50-micron orifice.

Using a 19-micron orifice, Kebicki (11) was unable to show that mitochondria followed the normal size distribution as theorized by Pauly (29). Whether plotting in terms of volume or particle diameter, the distribution had skewness towards the larger sized particle. This could have been the result of inaccurate measurements because one or more of the many variables involved in electronic counting was not controlled.

The biological cells, the diluent, and the electronic circuitry are the three main classes of variables involved when determining size distributions with the Coulter Counter. In the following review each main class will be considered.

Biological cells in their natural environment do not necessarily have a constant volume. During cell division (19) and logarithmic growth (37) the volumes of bacterial cells do not remain constant. Red blood cells are larger in acidosis with a pH less than 7.35 than in normal arterial blood having a pH of 7.4 Approximately a ten per cent change in erythrocyte volume is caused by a change of one pH unit. Rushbaugh (20) comments that this swelling might be due to a change in osmotic pressure outside the cation impermeable membrance of the red blood cell. Similar phenomena were observed in 1922 by Warburg (39).

Pulse responses initiated by chains of bacteria do not differentiate each bacterium. The Coulter Counter interprets the chain as one cell with approximately the volume of the entire chain. Lark and Lark (19) report the formalin caused the splitting of a large portion of double cells of bacteria. Toennies (37) commented that pH was not a factor in chain length of Streptococcus faecalis. However, he did say that with a lower pH the small trend towards larger units was not consistent with results obtained from bacteria in the growth phase. He suggests that L-tryptophan might play a role in chain separation since it acts as an inhibitor of the chain separating mechanism. When the concentration of L-tryptophan is decreased in the medium smaller units of Streptococcus faecalis appear.

Clumping of biological cells presents another problem. Ethylenediaminetetraacetic acid tetrasodium salt is used as an anticoagulant in blood samples (14). Prolonged shaking of a yeast culture with glass beads dissociated the clumps (10). It has been reported that heparin was used to prevent clumping of mitochondria (11).

Similar to the electrical response due to the close association of two or more cells in chains or possibly in clumps is the single response caused by two or more particles traveling close together through the electrical sensing zone of the counter. Coincidence loss due to doublets and trip-

lets follows the Poisson distribution as pointed out by Mattern in 1957 (26). This caused the true distribution to shift upwards since a doublet is registered as one cell approximately equal in volume to the sum of the two cells (4). The appropriate calibration curve (26) or formula (8) is used to correct for this error. While using 27- and 19-micron orifices, Lark (19) and Gebicki (11) experimented to see if significant coincidence was occurring. By reducing the volume of a sample by one-half, the count was reduced by one-half. When obtaining a constant ratio of volume to count upon succeeding dilutions, they concluded that coincidence was negligible. Brecher (6) cautions the use of high dilutions to reduce coincidence since sampling error due to the Poisson distribution might become too large. This can be corrected by using longer counts. The occurrence of doublets in a dilution of one particle per 20 sensing zone volumes is about five per cent (28).

Differences in biological conductors of electricity appear to exist and thus represent a possible variable in electronic enumeration and sizing of cells. Swanton (36) has reported that killed and viable Staphylococcus aureus cause different electrical responses on the Coulter Counter. For this viable bacterium the pulse pattern changes when the aperture current setting is changed. When S. aureus is heat killed it behaves like an inert particle which displays the

same pulse pattern at different aperture current settings. Swanton suggests that some viable bacteria do not react independently of current flow. Allison (3) stated that while exposing bacteria to chloramphenicol their conductivities changed and thus made determinations of bacterial volume impossible. Irani (15) also suggests that the response of the Coulter Counter to particulate matter is not solely dependent upon volume but also upon the physical and chemical properties of the particle. Gebicki (11) indicates that the membranes of the red blood cells and mitochondria are responsible for the particle's electrical resistance. Kubitschek (16) states that cells have a very high specific resistance to direct current. Berg (4) contends that all particles are effective non-conductors since voltages across the particles are low and momentary (0.1 millivolts to 10 volts, for 0.01 to 1.0 millisecond). Pulse signals are supposedly unaffected by the conductivity and dielectric properties of the particles. He does point out that some "noisy" pulses of unusual particles (for example "activated" powders) must be controlled by some suitable surfactant.

The diluent serves as the electrolyte in the Coulter Counter system. It also serves as the final environment of the biological cells prior to counting and sizing. Commercially prepared 0.9 per cent isotonic saline is the most

commonly used diluent for small biological cell analysis. Lushbaugh (20) reports that 0.9 per cent saline is not isotonic to red blood cells because it causes an apparent increase in cell volume when the cells are transferred into saline from plasma or Eagle's solution. Mitochondria display an immediate rise in count upon dilution with either potasium chloride or 0.9 per cent saline (11). Gebicki believes this is caused by rapid swelling of the smaller particles. After about three minutes the counts would reach a steady state. Toennies (37) reports that bacterial samples are more stable in culture medium than in saline dilutions. It might be noted that gram positive bacteria are permeable to salt ions while gram negative cells are only slightly permeable (12). Abram (1) noted that following a formaldehyde-heat treatment: Halobacterium halobium and Halobacterium cutirubrum (both gram negative) were impermeable to the ions in saline and thus would swell and shrink with changes in salt concentration. Lushbaugh (20) points out that commercial saline is unbuffered and that the pH can vary from 5.8 to 6.0. After prolonged storage commercial saline might sometimes have a pH of 4.0. He suggests the use of phosphate as a buffer which will not change in isotonicity nor in conductivity. Other diluents which have been used with bacteria are hydrogen chloride (16,17,37), t-soy broth (38), and formalized citrate-saline solution (5).

It is interesting to note the discovery that citrate prevents the adsorption of bacteriophage to the bacterial cell wall by creating a cation deficiency due to removal of calcium ions (2).

Kubitschek (17) pointed out that the electronic circuity of the Coulter Counter was designed primarily for rapid counting of pulses rather than voltage measurement. He stated that the resolution power of the counter is not maximum (18). This situation was also observed by Lushbaugh (22). Maximum resolution is obtained when there is a complete integration of pulse voltage directly proportional to the particle volume. The particle must remain in the electrical sensing zone long enough for the rising pulse to reach a plateau. Considering an orifice 25-microns in diameter and 100-microns long, and a flow rate of 300 centimeters per second, a particle might remain in the sensing zone for 30 microseconds. If the cut off frequency is about 30 kilocycles, there would only be partial integration of the voltage pulse. The residence period of particles in an 11-microns in diameter by 40-microns long orifice varies from 50 to 250 microseconds, dependent upon the particles' locations while traveling through the orifice. Because of the variable passage time, identical particles in a passage that is too short for complete response of all particles could possibly give pulses of different amplitude (18).

Aperture current and the volume of the orifice have a bearing on the size of the sense zone. The greater the aperture current or the larger the volume of the orifice the greater the sensing zone (22). This critical volume is larger than the volume of the orifice (26). Lushbaugh (22) indicates that resolution of the Coulter Counter can be improved by prolonging the residence of particles in the orifice. This can be accomplished by: 1) slowing down the flow rate, 2) physical elongation of the orifice, 3) forcing all particles into the moving peripheral area of the orifice by obstructing the central portion of the passage, 4) using high aperture settings. The latter is the most easily varied. Lushbaugh advocates the highest aperture current for the best resolution while Brecher (4) advocates the lowest aperture current because large currents affect cell volume.

Kubitschek (17) reports that pulse amplitude is not strictly independent of particle shape. However, a certain proportionality would be maintained for particles of a common shape but different size. When the cross section of a particle exceeds 10 per cent of the cross section of the orifice, pulse amplitude becomes a poor approximation of volume and particle shape becomes significant.

As previously indicated, little work has been done using the Coulter Counter to enumerate and size bacteria. Electronic counts of bacteria have been shown to be con-

sistent and close to optical and plate counts. Investigations of blood cells have contributed a great deal of the present knowledge regarding electronic detection of biological cells. Many variables are inherent in the Coulter counting method and the relevant importance of each must be checked to improve confidence and validity.

III. OBJECTIVES OF INVESTIGATION

The objectives of this investigation were to:

1. Obtain distributions of various bacteria of different size.
2. Resolve two different species of bacteria in a mixed suspension.
3. Determine the resolving power of the Coulter Counter for mixed microorganisms.
4. Evaluate the orifices prepared in this laboratory.

The long term aims were studies of population dynamics in mixed bacterial cultures.

IV. MATERIALS AND METHODS

Materials

Cystine Tryptic Agar, Dehydrated, (Difco). Used for stock cultures.

Ethyl Alcohol, (National). Used as cleaning solvent for orifice.

Formaldehyde Solution, 36.3 per cent, (Baker). Used to inactivate bacteria.

Millipore Filter, HAWG 47 0.45 microns, (Millipore). Used to filter broth.

Nitric Acid, 70.3 per cent, (Baker). Used as cleaning solvent for orifice.

Nutrient Agar, Dehydrated, (Difco). Used with t-soy broth for slants.

Polystyrene Latex, 0.557 microns in diameter, LS-063-A, (Dow). Used for calibration.

Polystyrene Latex, 0.796 microns in diameter, LS-449-E, (Dow). Used for calibration.

Polystyrene Latex, 1.305 microns in diameter, LS-464-E, (Dow). Used for calibration.

Polyvinyltoluene Latex, 2.9956 microns in diameter, EP-1358-38, (Dow). Used for calibration.

Saline, 0.9 per cent (Baxter). Used as diluent.

7X, Non-toxic for tissue culture, (Linbro). Used for clean-

ing glassware.

Stopcock Grease, (Dow Corning). Used on glass joints.

Sulfuric Acid, 97.0 per cent, (Baker). Used as cleaning solvent for orifice.

Trichloroethane, (Fisher). Used as cleaning solvent for orifice.

Tryptic Soy Broth, Dehydrated, (Difco). Used as broth culture medium.

Wescodyne, General purpose detergent and germicide, (West). Used as germicide for pipettes.

Apparatus

Constant Voltage Transformer, (Sola).

Coulter Counter, Model A, (Coulter).

Magnetic Stirrer, Pyro-Magnestir, (Cole-Parmer).

Vacuum Tube Voltmeter, Model V-74, (Health).

Key to Manufactures

Baker: J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Baxter: Baxter Laboratories, Inc. Morton Grove, Illinois.

Cole-Parmer: Cole-Parmer Instrument and Equipment Company, 7330 North Clark, Chicago 26, Illinois.

Coulter: Coulter Electronics, 590 West 20 Street, Hialeah, Florida.

Difco: Difco Laboratories, Detroit 1, Michigan.

Dow: Bioproducts Department, The Dow Chemical Company, Midland, Michigan.

Dow Corning: Dow Corning Corporation, Midland, Michigan.

Fisher: Fisher Scientific Company, Fair Lawn, New Jersey.

Health: The Health Company, Benton Harbor, Michigan.

Linbro: Linbro Chemical Company, Inc. New Haven, Connecticut.

Millipore: Millipore Filter Corporation, Bedford, Massachusetts.

National: U. S. Industrial Chemicals Company Division, National Distillers and Chemical Corporation, New York, New York.

Sola: Sola Electric Company, Chicago, Illinois.

West: West Chemical Products Inc., 42-16 West Street, Long Island City, New York.

CLEANING OF GLASSWARE

The following procedure was carried out for the cleaning of all glass beakers and test tubes:

1. Soaked in a solution of 7X and Wescodyne for at least 24 hours.
2. Scrubbed in hot water and 7X.
3. Rinsed at least six times with hot tap water.
4. Rinsed twice with distilled water.
5. Drip dried.

While keeping the beakers inverted, they were transferred to a cabinet. The beakers were isolated within the cabinet by lining the interior with meat paper.

The plastic caps for the test tubes were soaked with the tubes, rinsed in hot tap water, and rinsed in distilled water. They were left inverted upon a wire screen until needed.

The pipettes were soaked in a one percent solution of Wescodyne for at least 48 hours. They were then placed in a continuous rinsing bath of soft water for approximately two hours. The pipettes were then put in an aluminum canister and sterilized in an oven at 200°C for two hours.

CULTURES AND THEIR MAINTENANCE

All cultures were obtained from the Bacteriology Department of Virginia Polytechnic Institute. A sample of each species consisted of the bacterium in a cystine tryptic agar tube and in a t-soy slant. Fresh t-soy slants were inoculated once a week from the CTA cultures. Broth cultures were innoculated from the t-soy slants. This minimized the possibility of contaminating the CTA stock cultures. Transfer of stock cultures to fresh CTA tubes was performed once a month. CTA cultures were incubated at room temperature. T-soy slants and broth cultures of Staphylococcus epidermidis 75, Proteus vulgaris Laboratory strain 3, and Pasteurella multocida ATCC7228 were incubated at 37°C while Sarcina lutea UMd and Azotobacter chroococcum (Fletcher) were incubated at 30°C.

All t-soy broth that was used in this investigation was filtered while hot with 0.45-micron Millipore filters. Each sample tube contained 10 milliliters of broth. Media preparations were autoclaved for approximately 20 minutes at a pressure of 15 pounds.

OPERATING TECHNIQUE

Ten milliliter broth cultures, incubated for various periods of time, were inactivated by adding approximately 0.1 milliliter of formaldehyde solution (see Materials) to each culture. A contact period of two hours was considered sufficient for the inactivation of the cells. Between one and three milliliters of a sample were added to approximately 100 milliliters of saline which was being agitated by the mechanical stirrer of the counter. Counting began about 20 seconds later.

During the counting procedure, the condition of the orifice was continually determined by observing the pattern on the oscilloscope. Frequent checks were made to see if air bubbles would pass through the orifice and into the orifice tube when the sample was lowered. If there were: a) consistent patterns, b) bubbles during checks, and c) only small deviations in counts at the same settings; it was assumed that the orifice was operating properly.

When the orifice ceased to operate properly, one or more of the following methods was employed to correct the situation. While using the 12-micron orifice, the most convenient way to correct apparent plugging was to jack up the current from an "I" of six to an "I" of nine for fifteen seconds. Theoretically this burned out any particles which

were blocking the orifice. This method appeared very effective but was discontinued for fear of damaging the glass orifice. Gebicki (11) used a similar procedure with a 19-micron orifice.

Another means of correcting apparent blackage was simply to touch the orifice very lightly. Bubbles would usually appear immediately if this method was going to work.

The most common procedure necessitated removal of the orifice. Following removal, the orifice was filled with saline and closed with a rubber stopper. By applying pressure on the stopper with the thumbs, a small jet of saline from the orifice would usually appear. After the stopper had been removed and about half of the saline in the tube disgarded, the vacuum pump of the counter was attached to the orifice tube. The appearance of bubbles from the orifice would be a second indication that the orifice was in good condition. If none of these methods worked, cold applications of the following solvents were tried: sulfuric acid, nitric acid, ethyl alchol, and trichloroethane. Before attaching the orifice tube to the counting apparatus, it was rinsed in saline. All saline was wiped from the ground glass joint, and stopcock grease was applied.

After using the orifice it was rinsed in clean saline and stored in a humidor containing a solution of 7X. This supposedly prevented the formation of salt crystals on the

orifice and promoted particle free surfaces.

The sampling apparatus of the Coulter Counter was contained in a wooden box with a vertically sliding plexiglass door. The door was opened only enough to permit access to the controls. Constant circulation of filtered air within the box supposedly reduced the presence of particles from the atmosphere exterior to the box.

Size distributions which were determined with the 12-micron orifice had to be obtained within 30 minutes when using an aperture current setting of six. The count reset switch would cease to function properly after the counter had been in operation for 30 minutes. This was caused by overheating a defective relay. Another run was not made until the machine had been off for 12 hours. This situation was corrected by removal and cleaning of the aperture current relay, and no problem of this sort was experienced with the 7-micron orifice.

Another problem occurred when the voltage between electrodes was not identical upon successive count resets. This was probably caused by leakage paths due to dried saline on the apparatus. Alternate counts would be approximately equal while the differences between successive counts were considerable. In order to correct this situation the stopcocks, plastic bridge, and ground glass joints were washed with distilled water and dried. If this didn't reduce the

polarity effects, thorough cleaning of the box and apparatus was begun.

V. RESULTS AND DISCUSSIONS

Calibrations

The manufacturer's recommended procedure (8) for calibration was used for the 12- and 7-micron orifices. This consisted of correlating a particular gain, threshold, and aperture current combination with the mean size of a known distribution of latex particles. From this determination a conversion constant "K" was calculated which was used to convert any machine setting combination to the diameter of the smallest particle that could be detected at that setting.

Calibrating the 12-micron orifice (which was made in this laboratory) appeared impossible. Table 1 gives the "K" values which were experimentally determined for the respective machine setting combinations. The great degree of variability among these values is possibly caused by several factors. One factor might have been poor technic in determining the mean of the known particle distribution according to Coulter's method. This is evident from Table 1 where two different threshold values were determined as representative of the mean of particles the same size while the aperture current was set at five. One threshold value, 9.2, was close to the noise level (which was always present). This determination was possibly influenced by the noise and

Table 1

Calibration determinations for the 12-micron orifice

Gain	Thres-hold	Aperture Current	Diameter of Partical (microns)	"K"	Resistance (ohms)	Voltage
4	54	1	2.956	0.782	298000	3.5
4	55	1	2.956	0.777	298000	3.5
4	25	3	1.305	0.700	279000	12.5
4	25.5	4	1.305	0.860	242000	21
4	28	4	1.305	0.837	254000	22
4	9.2	5	0.557	0.637	248200	40
4	23	5	0.557	0.467	248200	40
4	15.6	6	0.557	0.640	225000	65

thus was not truly representative of the mean of the size distribution.

Another factor which was probably the cause of differences among "K" values was the variability of the sensing zone volume with respect to the aperture current setting. Since the laboratory prepared orifices (see Appendix C) were designed with the minimum channel length possible, sensing zone volume would appear to be strictly dependent upon aperture current setting; when the aperture current was increased the sensing zone volume would be increased. A larger sensing zone would increase the probability that each particle volume would be properly recognized by total pulse amplification (18).

Because of the great degree of variability among calculated "K" values, there appeared to be no one "K" value. An attempt was made to determine a uniform "K" and resistance by comparing threshold and aperture combinations that gave the same count. Different combinations of"K's" and resistances were used to determine tables of particle diameters and appropriate machine settings. These tables were checked with Coulter Counter results. Of the combinations that were tried a resistance of 320000 ohms and "K" values between 0.64 and 0.70 were found to yield the most favorable results. A "K" equaling 0.676 and a resistance of 320000 ohms was assumed.

For the calibration of the 7-micron orifice (which also was made in this laboratory) a new sample of latex particles was obtained. While using the 12-micron orifice it was observed that the modes of the bacterial cell distributions were between 0.75 and 1.05 microns. Because of the previous difficulties in calibrating, it was thought that calibrating with a particle in the range of 0.75 to 1.05 microns would possibly increase the accuracy of measuring bacterial cells in this range. Polystyrene latex particles of 0.796 microns in diameter were used for the calibrations shown in Table 2. Since an aperture current setting of four gave the best oscilloscope pattern of the size distribution of Pasteurella multocida, the "K" which was calculated at an aperture setting of four and determined with 0.796-micron particles was assumed.

Another method of calibration is presented by Lushbaugh (25). Complete size distributions of particles of known volume are obtained with different aperture currents. By plotting the threshold setting against the volume of a known particle, a calibration graph similar to Figure 1 is obtained. When assuming a constant gain, different aperture and threshold settings can be determined for a given volume. It is interesting to note the linear relationship between threshold settings and volumes of particles while an exponential relationship appears to exist between thres-

Table 2

Calibration determinations for the 7-micron orifice

Gain	Thres-hold	Aperture Current	Diameter of Partical (microns)	"K"	Resistance (ohms)	Voltage
4	52	3	1.305	0.544	459000	20
4	18	4	0.796	0.585	452000	37
4	13	5	0.557	0.584	404000	60
4	29	5	0.796	0.603	404000	60

Table 3

Volumes of samples and coincidence factors

Orifice	Time to collect 50 micro-liter sample (seconds)	Counting Interval (seconds)	Volume of timed sample (Microliters)	Coincidence factor
12-micron	125	10.0	4.0	0.539
7-micron	147	10.0	3.4	0.126

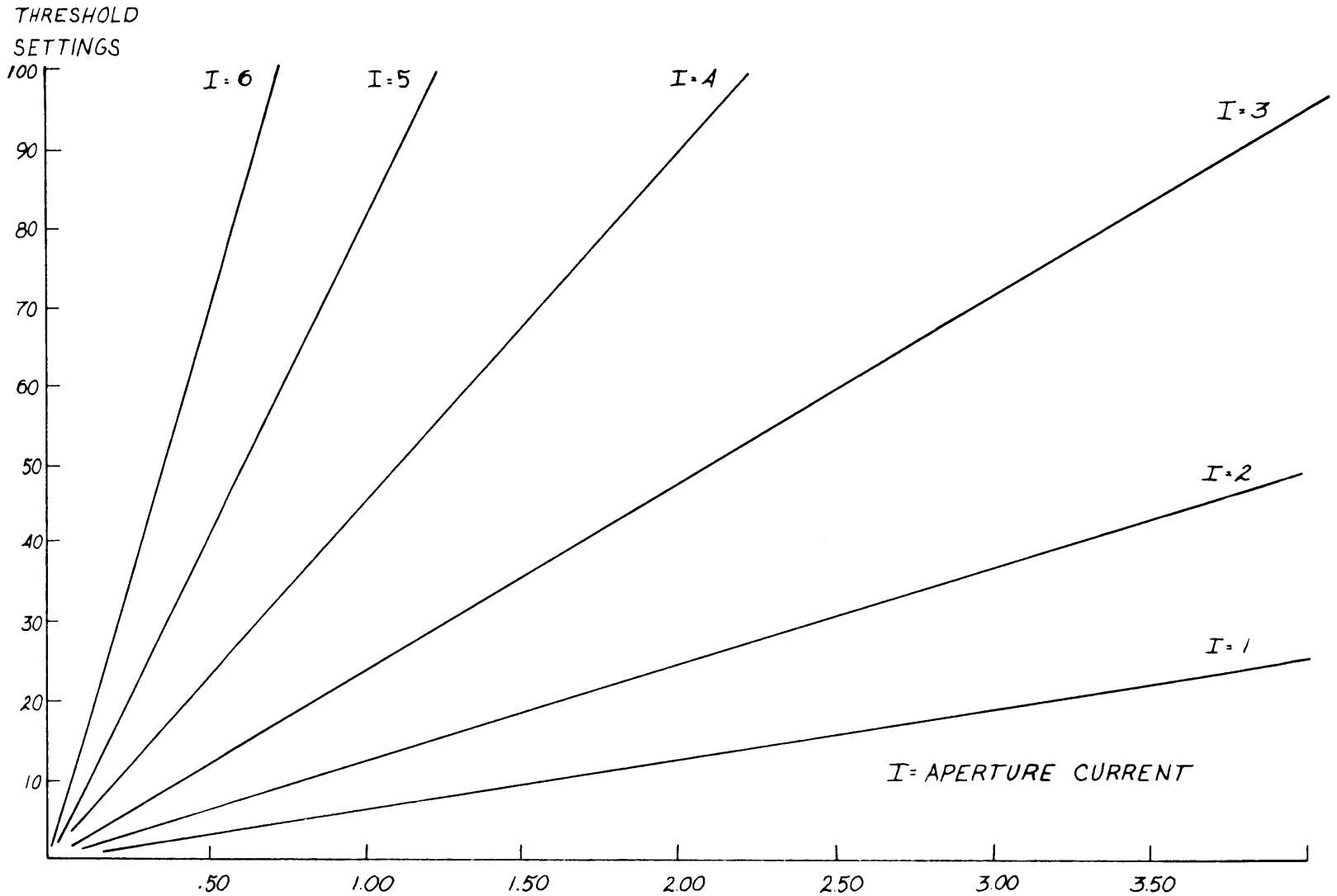

FIGURE 1 CALIBRATIONS FOR 12-MICRON ORIFICE

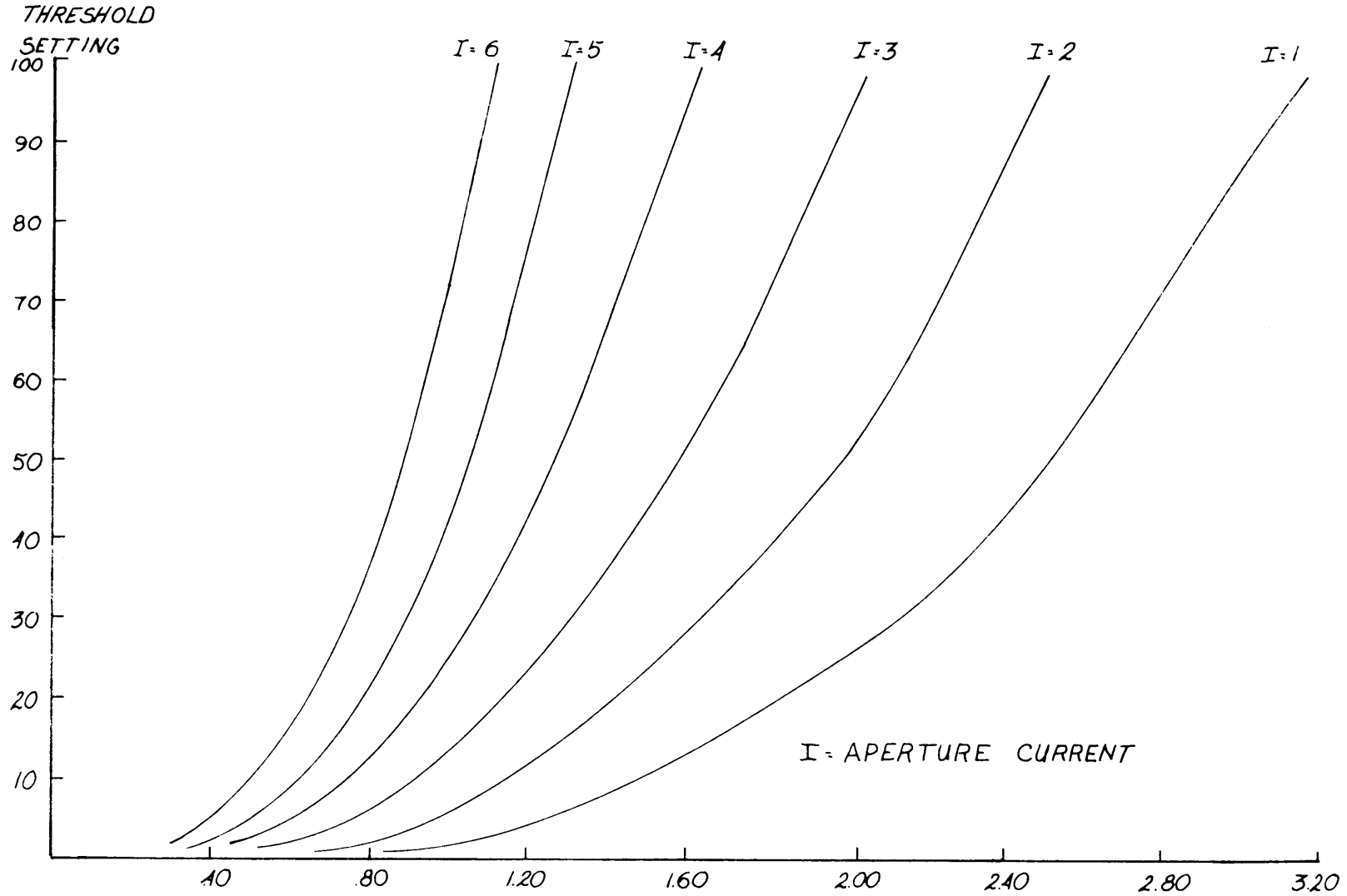

FIGURE 2. CALIBRATIONS FOR 12-MICRON ORIFICE

hold settings and diameters of particles as illustrated in Figure 2. Since this exponential relationship supposedly exists, it is necessary to calibrate the orifice if a size distribution is to be determined from equal increments of diameter. However, calibration is not necessary if the same gain and aperture settings are used and just equal increments of volume are desired. Since classes of equal diameter were desired in this work, calibration was necessary. Figures 1 and 2 were determined from results of Coulter's method of calibration assuming the calibration constants of the 12-micron orifice and a gain of four.

The final determination necessary for each orifice was the sample volume taken during the count. With this the correction factor for coincidence was determined according to Coulter's method (8). Sample volumes and coincidence factors are given in Table 3 for the 12- and 7-micron orifices.

Size Distributions

Size distributions were obtained for *Proteus vulgaris*, *Pasteurella multocida*, *Staphylococcus areus*, *Sarcina lutea*, and *Azotobacter*. All graphs of the size distributions were determined from cumulative counts obtained at each class boundary. The counts observed in each class were compared with the total count for all classes observed. These relative per cents were then plotted at the midpoint of each class. Thus the method of presentation is analagous to connecting the midpoints of classes of a histogram.

When two distributions appeared to be closely related, a paired t-test was used to test for equality. In order to strengthen the assumption that the relative per cents obtained were from a normally distributed population and in order to get away from comparing relative per cents, the observations were transformed to normally distributed scores (see Appendix A). The normal scores were then compared.

The 12-micron orifice was used to obtain size distributions of a 24-hour culture of *Pasteurella multocida* (Figure 3). Distributions were determined for two different samples of this culture. When testing the hypothesis that the mean of the differences between the two distributions was equal to zero, a significance level of five per cent was assumed. The calculated t value of -0.240 was within the

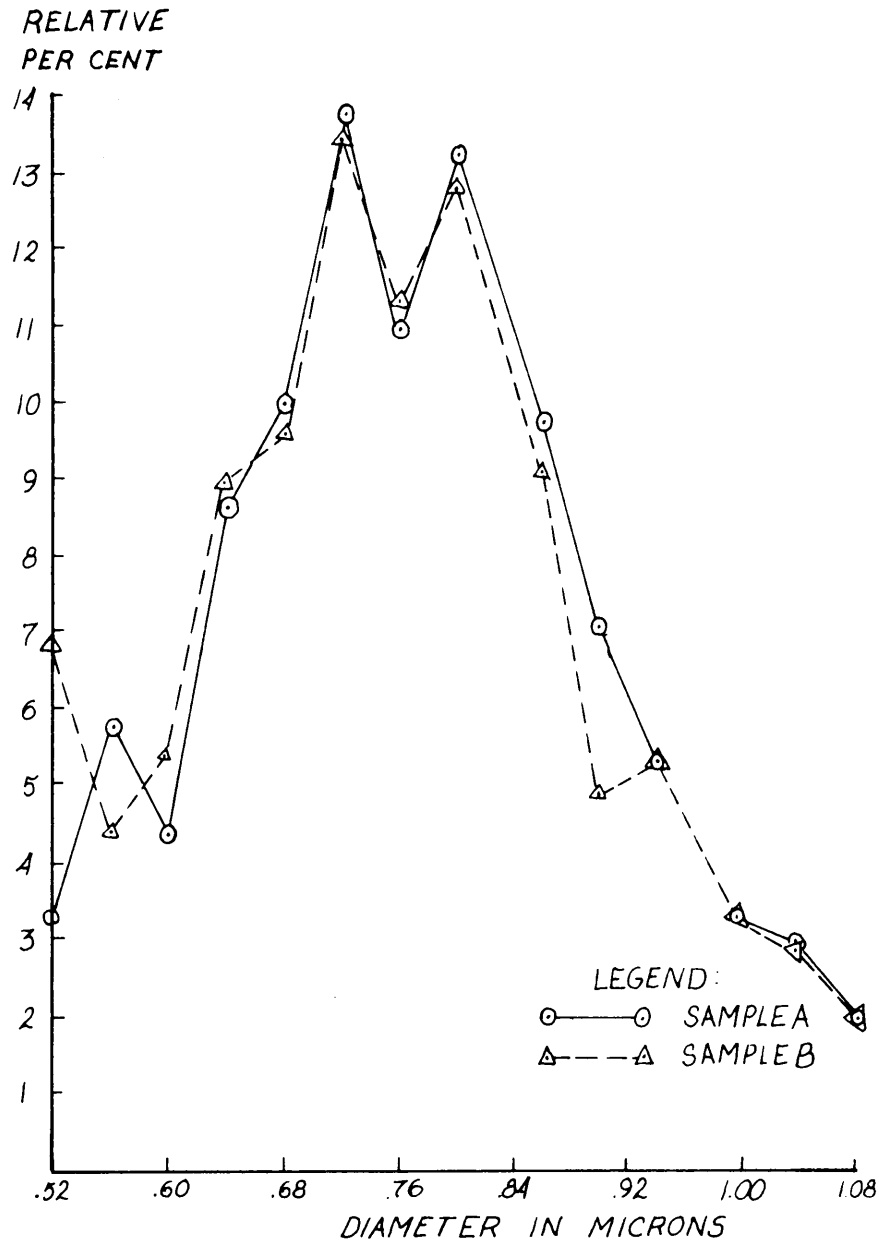

FIGURE 3. SIZE DISTRIBUTION OF *PASTEURELLA MULTOCIDA*, 24 HOURS GROWTH. TWO DIFFERENT SAMPLES FROM SAME CULTURE.

acceptance region determined for 14 degrees of freedom. It was concluded that the mean of the differences between the two distributions was equal to zero. Thus it was possible to reproduce results with the Coulter Counter. Both curves indicate a drop in relative per cent at 0.76 microns. This is possibly caused by an unequal class width about this point. Since it was not possible to correlate a specific threshold setting with every diameter, approximate threshold settings were used to determine the end points of each class.

The possible normality of the size distributions of Pasteurella multocida (Sample A of Figure 3) and Proteus vulgaris (Run A of Figure 9) is illustrated in Figure 4 where cumulative per cent is plotted against the diameter of the bacteria. Deviation of points from a straight line might result from several factors; among these are experimental error, inaccurate calibration of the orifice, and incomplete size distribution. Since the slopes of both lines are about equal, it is possible that the two distributions have the same standard deviation. This could be approximated from the graph as about 0.14 micron.

Figure 5 presents size distributions of Proteus vulgaris, 7 hours growth, which were determined with the 12-micron orifice. The distribution of Sample B was obtained two days after that of Sample A. Results of a paired t-test,

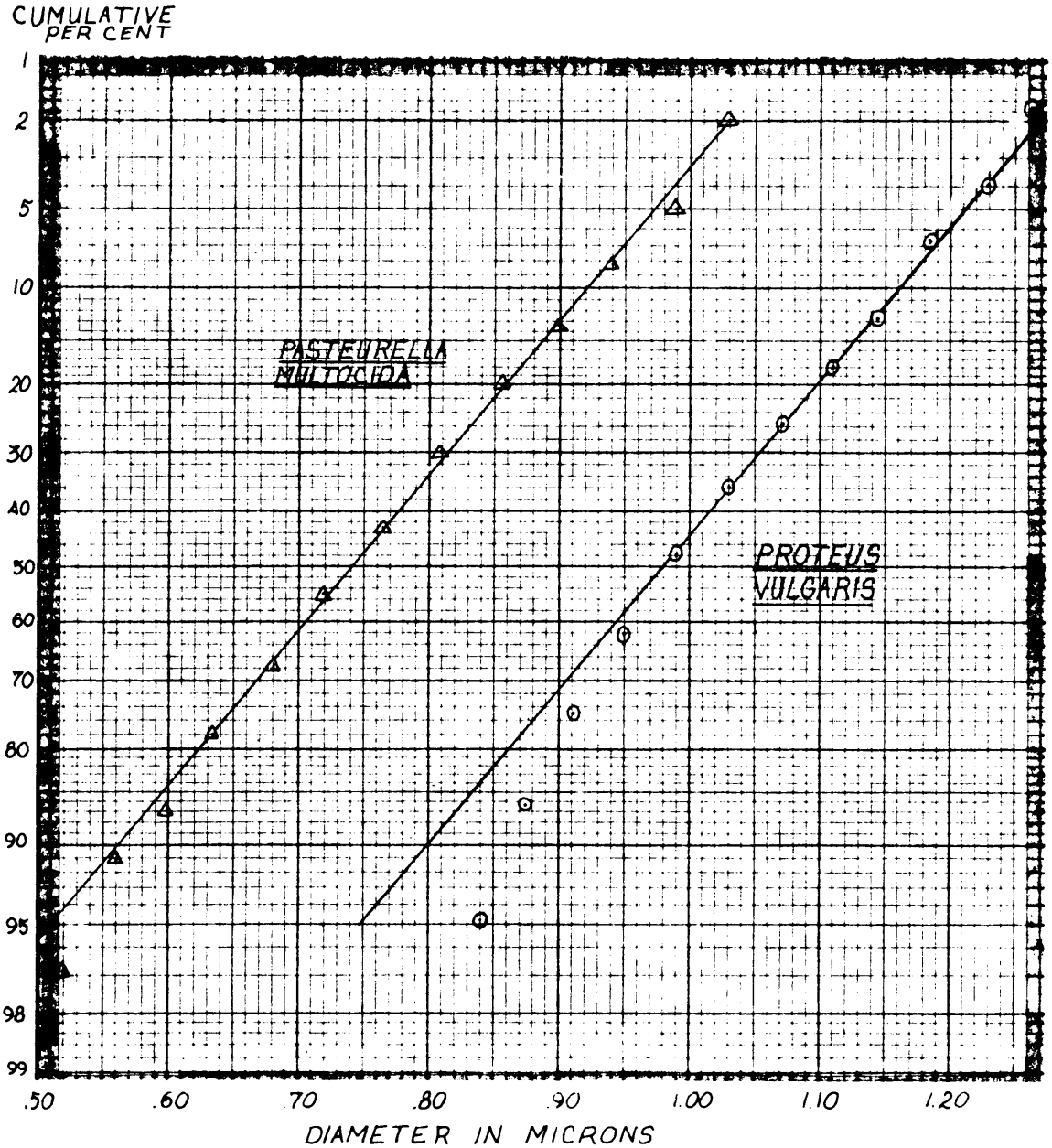

FIGURE 4. SIZE DISTRIBUTIONS OF PASTEURELLA MULTOCIDA AND PROTEUS VULGARIS PLOTTED ON NORMAL PROBABILITY PAPER.

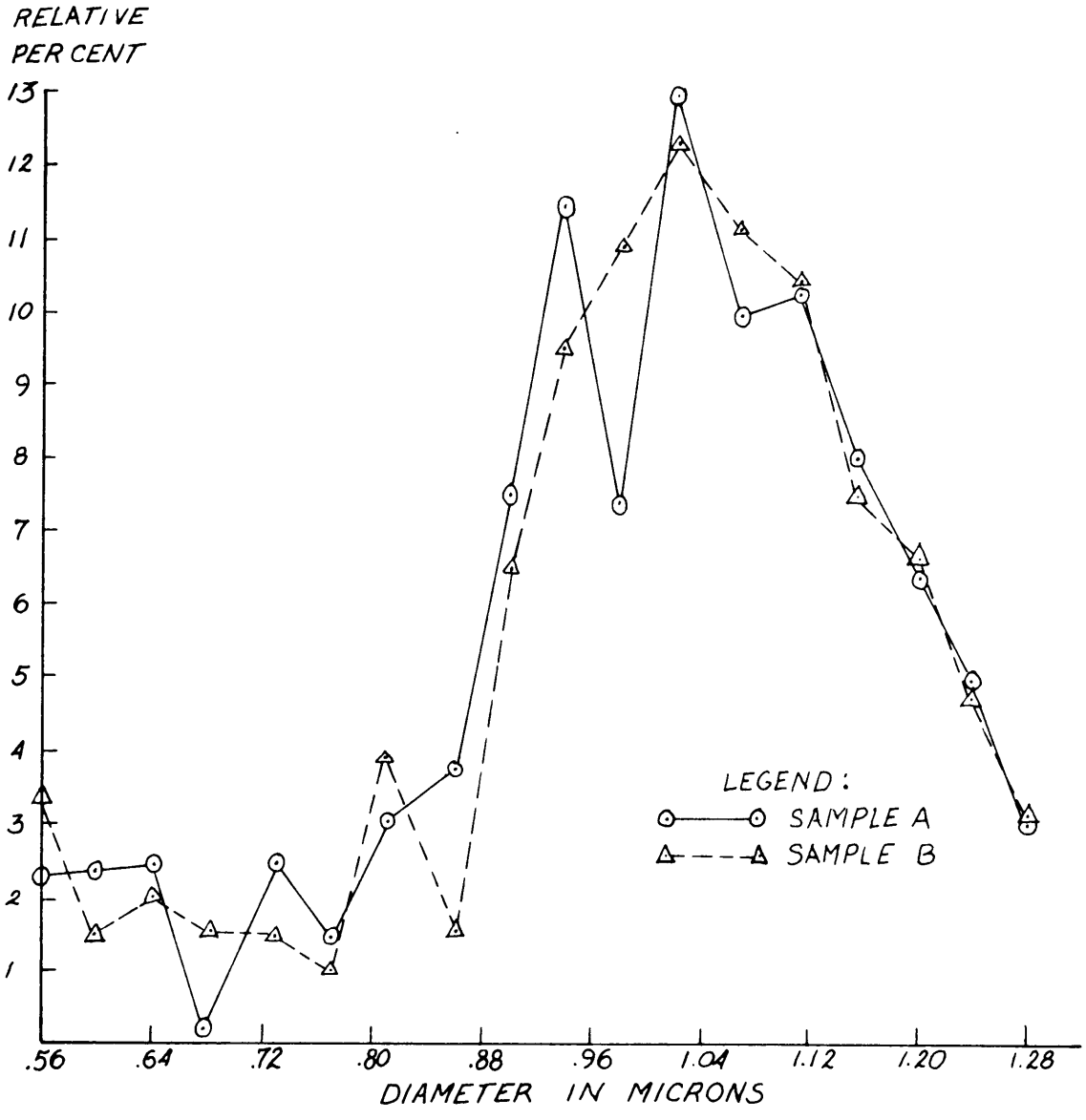

FIGURE 5. SIZE DISTRIBUTIONS OF PROTEUS VULGARIS, 7 HOURS GROWTH. TWO DIFFERENT SAMPLES FROM SAME CULTURE.

"t" equaling -3.099 for 17 degrees of freedom, indicated that the mean of the differences was not equal to zero and that Sample B tended to be smaller than Sample A. This small difference but apparent trend could have resulted from the orifice becoming partially plugged. This would cause the amplitude of the pulses to increase and thus move the entire population up. If it is assumed that both curves were accurate representations of the culture at the time they were taken, it would appear that osmotic effects changed inactivated cells in the culture medium and formaldehyde environment.

Figure 6 represents the results with the 7-micron orifice of sizing Proteus vulgaris, 8 hours growth. Two distributions were determined from the same sample within 40 minutes. A "t" equaling -0.190 was calculated for 21 degrees of freedom. At the significance level of five per cent it is probable that the mean of the differences between the two distributions is equal to zero. Two items are of specific interest when comparing these distributions with those of Figure 5. The mode of the populations seems to be of a larger diameter in Figure 6 than in Figure 5 and the range of the distributions in Figure 6 is larger than that of Figure 5. This is possibly caused by inaccurate calibration of two orifices of different size. It should be noted that the 7-micron orifice only required an aperture

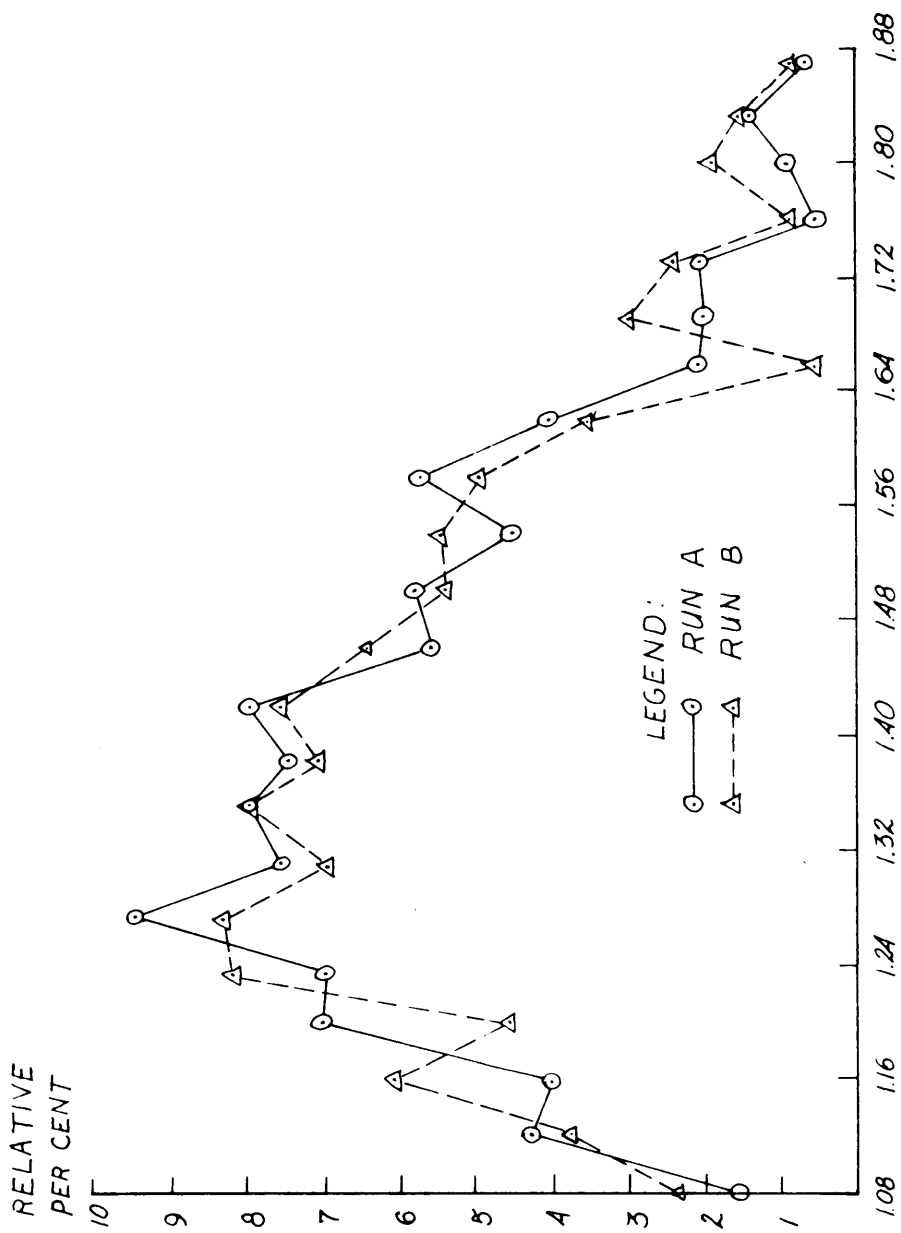

FIGURE 6. SIZE DISTRIBUTIONS OF *PROTEUS VULGARIS*, 8 HOURS GROWTH. TWO DIFFERENT RUNS ON THE SAME SAMPLE.

current of three to obtain the distribution of Proteus vulgaris while the 12-micron orifice required an aperture current of five.

If it is assumed that the Coulter Counter worked properly, Figure 7 illustrates a shift in the size distribution of Proteus vulgaris, similar to that in Figure 5. Sample A was taken one day before Sample B with the 7-micron orifice. Thus the population appeared to become smaller with time because of extended exposure to formaldehyde and the culture medium. This would seem to agree with Larks' contention (19) that formalin split double cells. Splitting of doubles and chains also could have occurred just before sampling at which time it was a practice to shake the suspension of cells vigorously while in the culture medium.

The Azotobacter distributions (Figure 8) appear to display the effects of coincidence in a 12-micron orifice. The total count for Sample A in the ten second count was about 21000 while for Sample B it was 50000. Because of coincidence, more doublets and triplets would be registered as single cells and thus cause the shift of the population upwards. If this is true, the utilization of the correction for coincidence factor did not accurately correct the obtained data. Another item of interest at this time was apparent increase in population number during the first seven minutes of sampling. Although successively higher

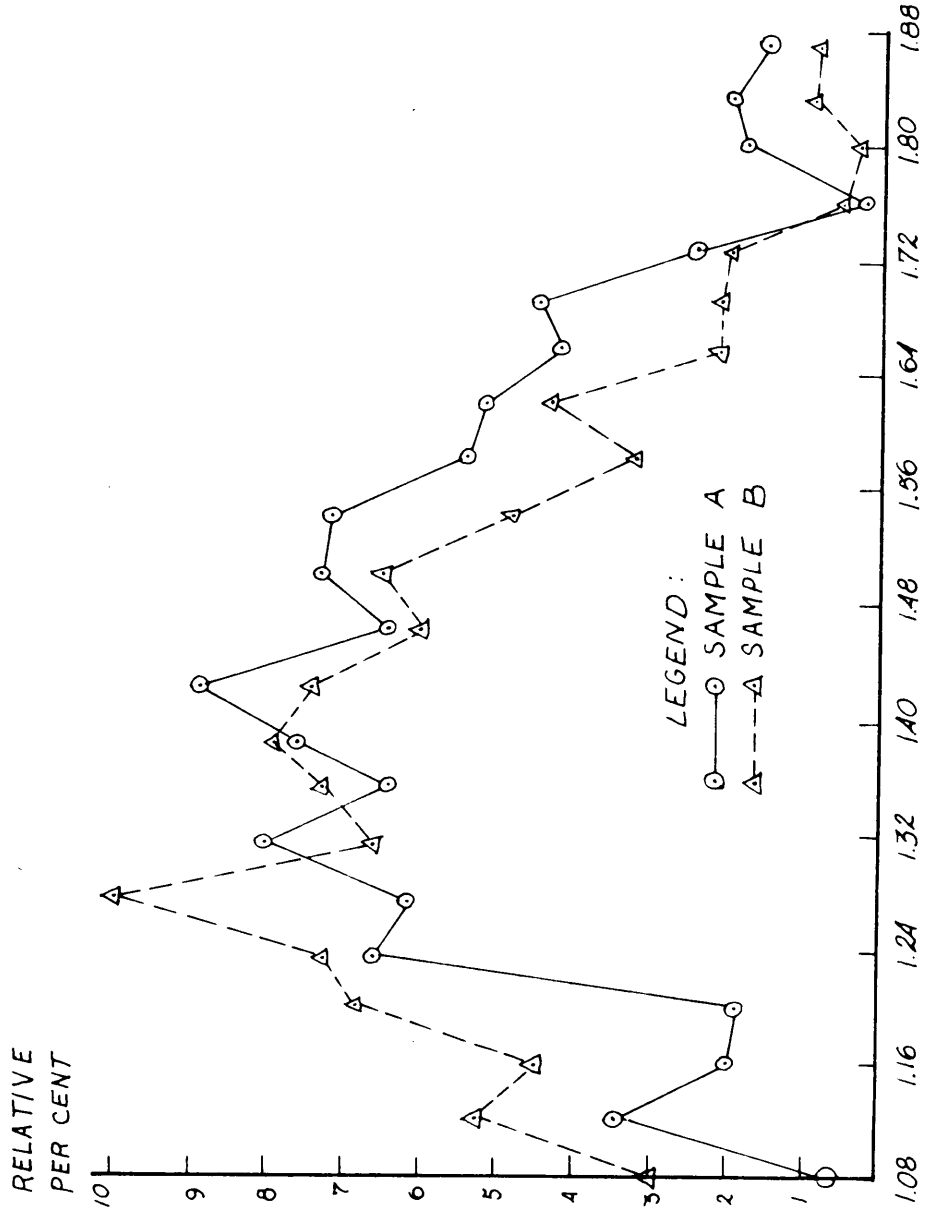

FIGURE 7. SIZE DISTRIBUTIONS OF *PROTEUS VULGARIS*, 8 HOURS GROWTH. TWO DIFFERENT SAMPLES OF THE SAME CULTURE.

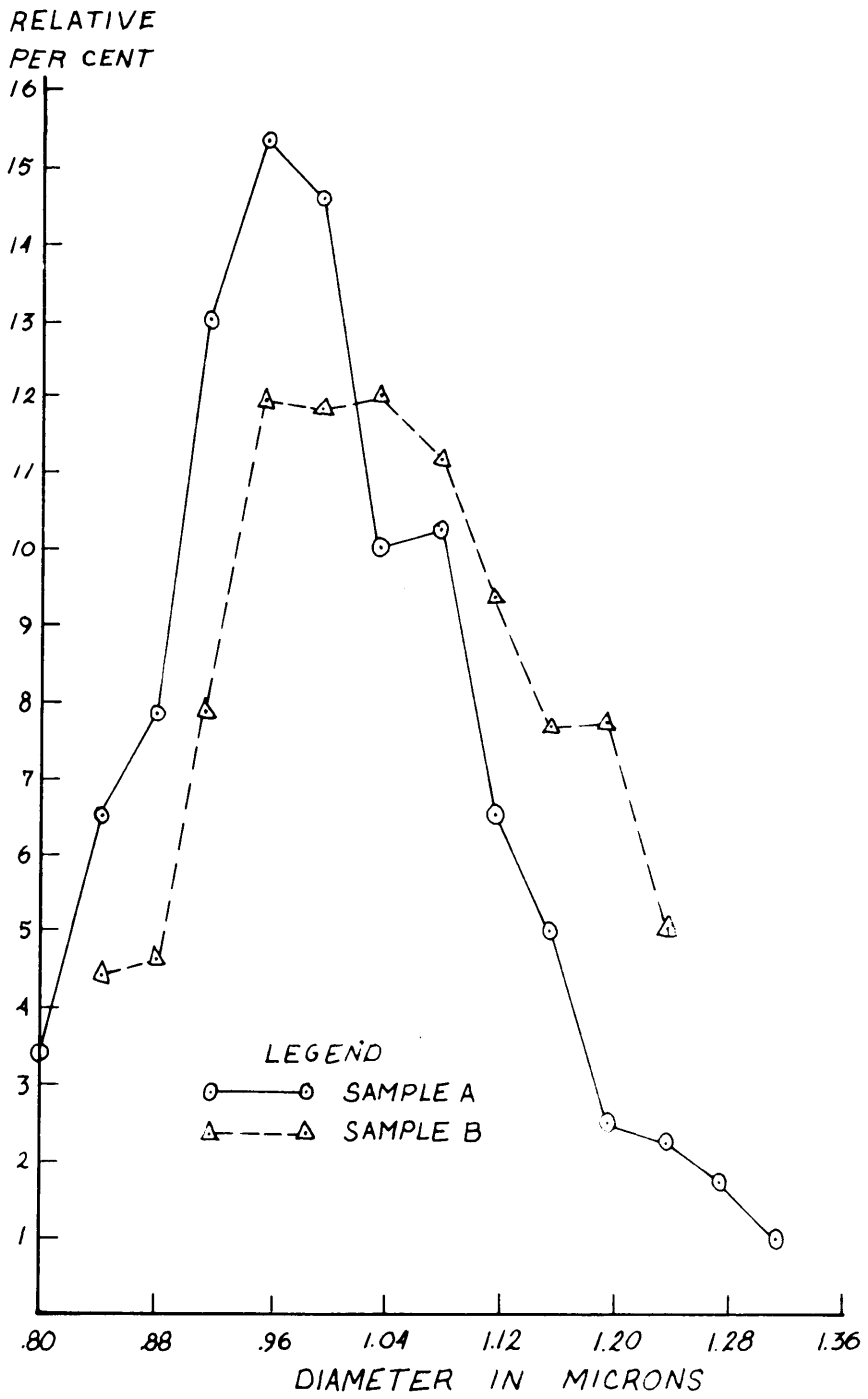

FIGURE 8. SIZE DISTRIBUTIONS OF AZOTOBACTER, 6 HOURS GROWTH. TWO DIFFERENT SAMPLES OF THE SAME CULTURE

threshold settings were used, the counts which were recorded were larger each time. This is possibly a result of the reaction of the cells to a hypotonic solution. Gebicki (11) noticed this period of adjustment with mitochondria particles. It seems that the smaller particles which are undetectable swell enough to cause a pulse on the counter thus giving an appearance of growth to an inactivated sample of bacteria cells.

Figure 9 shows the distributions of live Proteus vulgaris, 8 hours growth, which was determined with the 12-micron orifice. Cell multiplication was evident by the counts increasing at the same settings throughout the experiment (see Appendix A). Background counts of this experiment tended to decrease at the same settings. This was true of counts of inactivated bacterial cells. The reduction in count was probably due to particles adhering to the glass, settling out of suspension, or decreasing in volume. The paired t-test for the distributions of Figure 9 yields a "t" value of 5.615 for 12 degrees of freedom. This would indicate that the mean of the differences does not equal zero and that the distribution of Run B tended to shift down. Thus it would appear that the increasing population consisted of a greater number of smaller cells in proportion to the total number of cells. The hump in the curve at 1.19

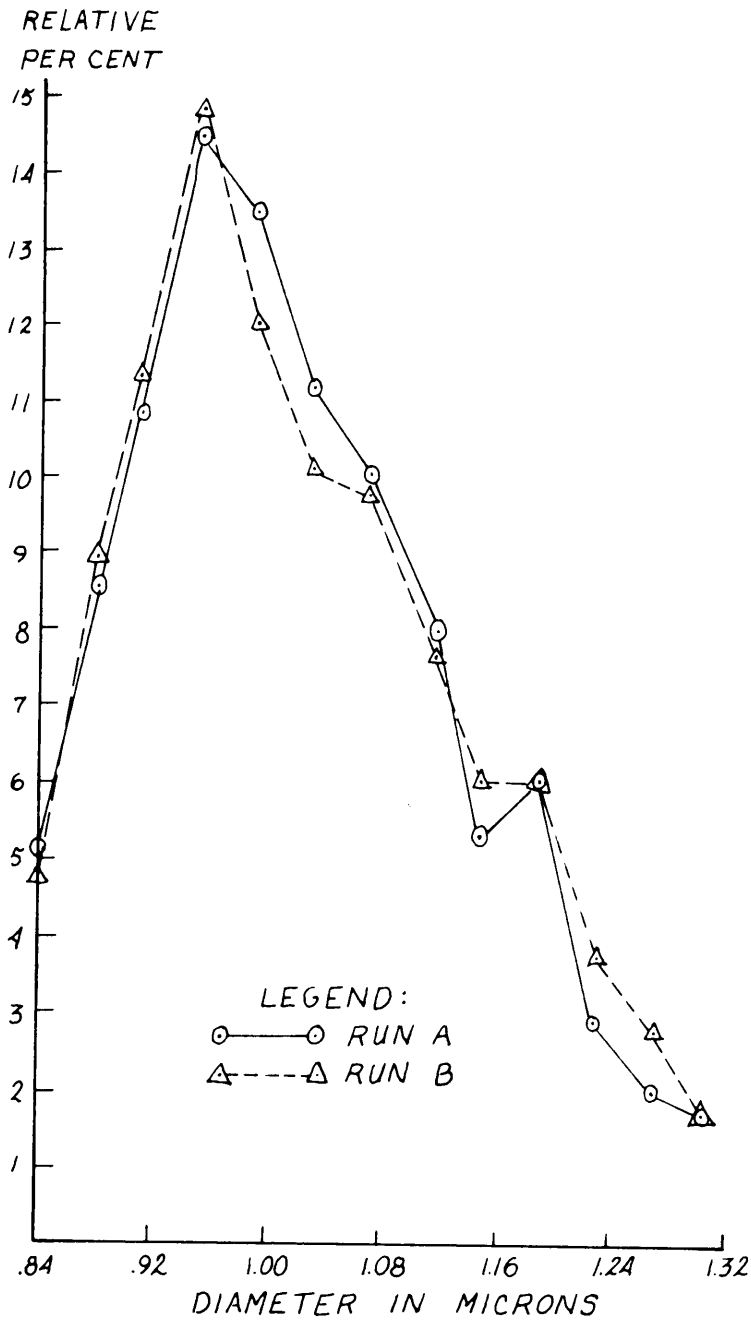

FIGURE 9. SIZE DISTRIBUTIONS OF *PROTEUS VULGARIS*, 8 HOURS GROWTH. TWO DIFFERENT RUNS ON SAME SAMPLE.

microns may represent doublets of the particles representing the mode at 0.95 microns since 0.95-micron particles are equal to one-half the volume of the particles at 1.19 microns.

Figure 10 represents the only distribution obtained of Sarcina lutes. Two milliliters of sample were diluted with about 100 milliliters of saline. Since the total count which was obtained at the maximum setting which would detect the smaller particles was only 9100, it is possible that the majority of the size distribution graph is beyond the sensitivity region of the 12-micron orifice. The apparent trend to a distribution in Figure 10 is probably a result of experimental error which is indicated by the great variability between successive points.

Figure 11 shows a comparison of size distributions of Proteus vulgaris and Staphylococcus areus which were obtained with the 7-micron orifice. Each distribution was obtained separately. From this figure it would appear that there is little possibility of separating these bacteria in mixed culture.

Figure 12 indicates that there might be a possibility of resolving two distributions of bacteria which are this close in size. The abscissa represents an aperture current and threshold range from four, nine to three, ninety-four.

This would suggest using the 7-micron orifice for resolving two or more distributions of bacteria in mixed culture.

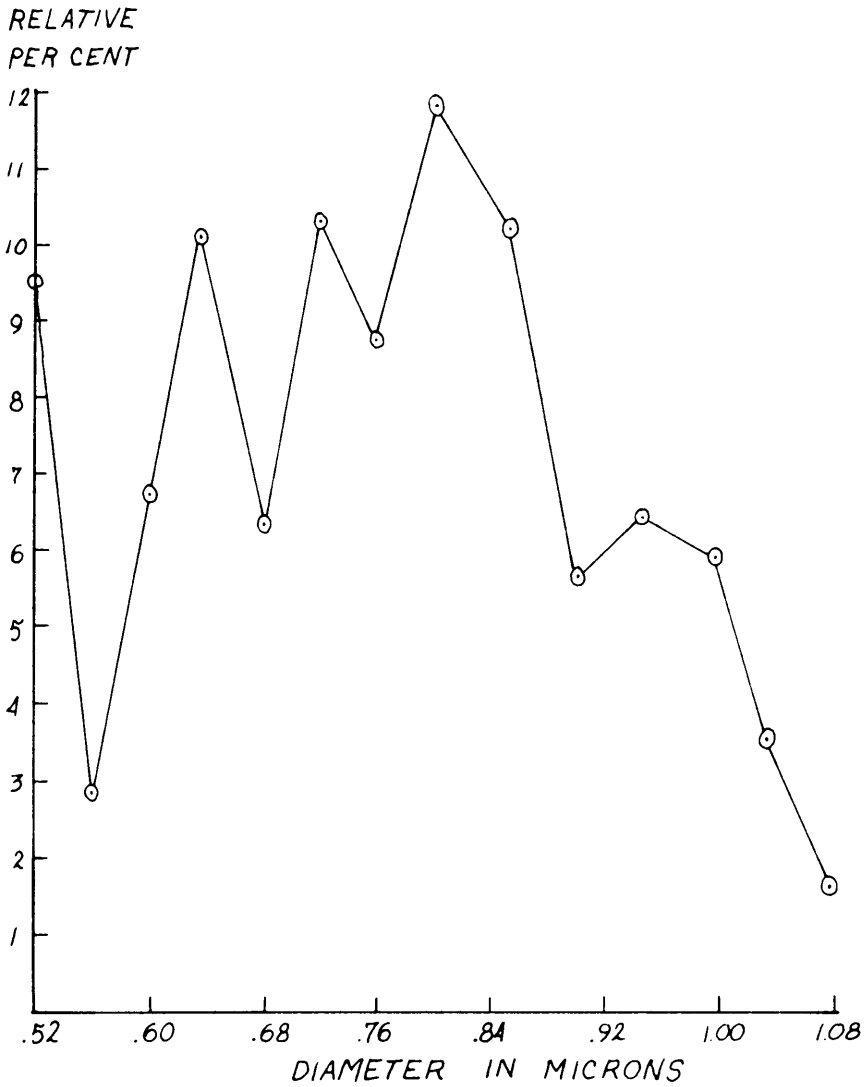

FIGURE 10. SIZE DISTRIBUTION OF SARCINA LUTEA, 34 HOURS GROWTH.

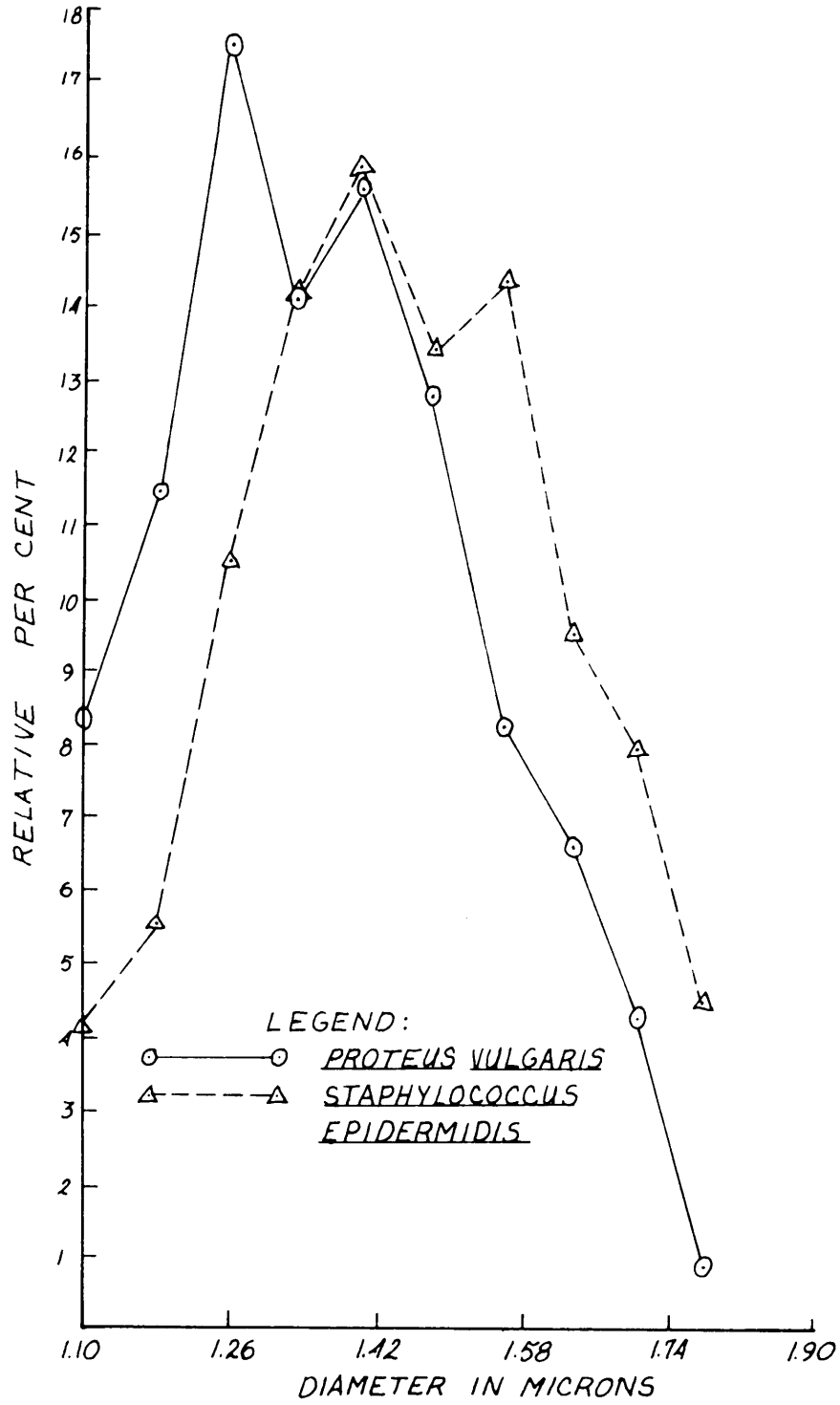

FIGURE 11. SIZE DISTRIBUTIONS OF *PROTEUS VULGARIS*, 8 HOURS GROWTH, AND *STAPHYLOCOCCUS EPIDERMIDIS*, 21 HOURS GROWTH.

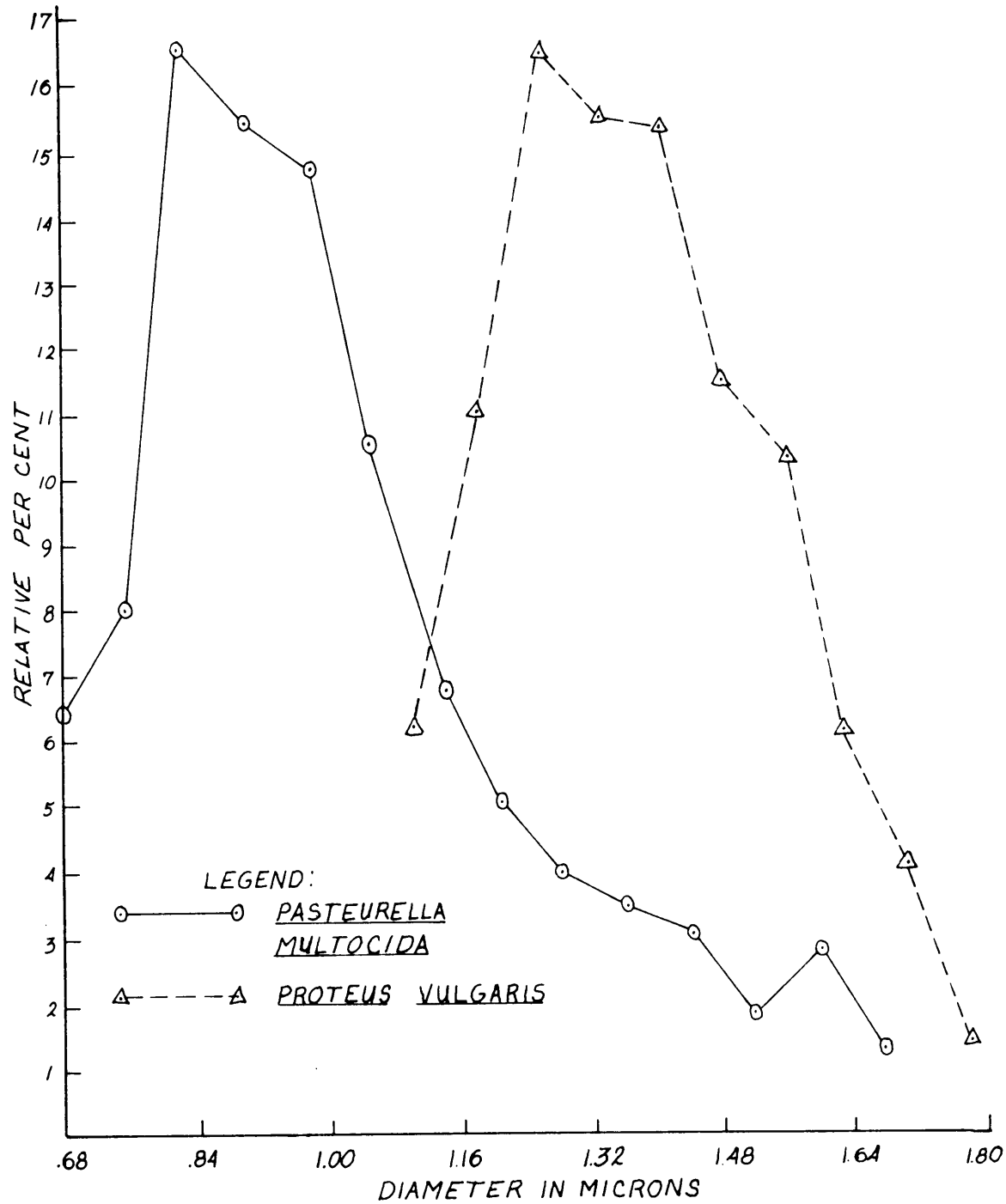

FIGURE 12. SIZE DISTRIBUTIONS OF *PASTEURELLA MULTOCIDA*, 24 HOURS GROWTH, AND *PROTEUS VULGARIS*, 8 HOURS GROWTH.

VI. CONCLUSIONS

From this investigation it is apparent that there are several variables inherent in the electronic particle counting method. Among these variables are the biological cells, the diluent, and the electronic circuitry. Variability in the volume of bacterial cells was apparently demonstrated when they were introduced into 0.9 per cent saline or when they were retained in formaldehyde and the culture medium. Variability in electronic circuitry was demonstrated by using the laboratory prepared orifices. This apparently caused the determination of accurate and constant calibration factors, "k's", to be impossible.

Although accurate size distributions may not have been obtained, distributions which were characteristic of different bacteria were obtained and were reproduced in some cases. The modes of the characteristic distributions of Pasteurella multocida and Proteus vulgaris appeared significantly different to warrant future attempts at separating these bacteria in mixed suspension.

Evidence was also given which favors the conclusion that distributions of Pasteurella multocida and Proteus vulgaris follow the normal distribution when relative counts are plotted against diameter.

The use of the Coulter Counter in sizing and counting

of bacterial cells appears promising. However, an understanding of the variables and the methods of controlling these variables is necessary.

VII. SUMMARY

Current techniques used to count and size bacteria usually are tedious and often yield results which have low statistical validity. An improved method of counting and sizing of bacterial cells is desirable.

The Coulter Counter has been successfully used to obtain better results in blood cell analysis. Because of its apparent success in other biological studies, it appears worthwhile to consider it for use in the sizing and counting of bacterial cells. Its adaptation for the study of bacteria has not been extensively investigated.

The objective of this investigation was to evaluate laboratory prepared orifices and to investigate the feasibility of counting and sizing of bacteria with the Coulter Counter.

Calibration of the orifices which were prepared in this laboratory appeared to be impossible. This was probably caused by variable sensing zone volumes which were always too small for total pulse amplification.

Size distributions were obtained for Pasteurella multocida and Proteus vulgaris which appeared to follow the normal distribution. The difference between the modes of these distributions appeared to be significant enough to warrent future attempts at qualitatively separating P.

vulgaris and P. multocida in mixed suspension.

Many variables appear to be inherent in electronic particle counting and sizing of bacteria. Among these variables are bacterial cells, diluents, and electronic circuitry. In order to improve future results of bacterial studies with the Coulter Counter, more research is necessary to reveal the variables involved and to acquire the methods to control these variables.

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X. APPENDICES

APPENDIX A

Example of Data and Calculations

At least 15 equal classes were considered for covering the entire size distribution. Machine settings were determined for these class endpoints and sampling was begun.

Data for each distribution was recorded as shown in Table 4 (data for Figure 9). Background counts (number of particles in the saline without the sample of bacteria) were obtained for each setting. In order to expedite the counting procedure only two sample counts were recorded for each setting. Since each count was at an opposite polarity, there was a possibility for effects caused by unequal voltage (see Operating Technique). The direction of flow was not monitored, and it was assumed that the voltage for both directions was equal. A better statistical analysis would include these possible effects.

Following the recording of background counts, counts of the sample of bacteria were obtained. The time at which each series of counts began and ended was recorded at the top and bottom of the columns.

Table 5 shows the method of obtaining normal scores from the observed data. The average of each pair of sample counts was corrected for coincidence and background. The highest corrected count obtained was assumed to be the total

Table 4

An Example of Experimental Data

Gain	Threshold	Aperture Current	Run A (start 5:28PM)	Run B (start 5:54PM)	Background (start 5:13PM)
4	12	5	20803 20556	24430 24854	855 846
4	14	5	20024 20424	24494 24994	680 689
4	17	5	19844 19931	24499 24696	424 456
4	20	5	20467 20610	24581 24926	290 287
4	23	5	20623 20350	24141 24302	189 202
4	27	5	19591 19223	23281 22868	137 120
4	30	5	17833 17535	21049 20943	81 104
4	34	5	15762 15227	18595 18119	47 70
4	39	5	12697 12611	15150 14674	36 47
4	44	5	10217 9738	11938 12281	32 37
4	49	5	7902 7600	9581 9919	27 -
4	55	5	5864 5597	7274 7683	24 -
4	61	5	4309 3958	5597 5763	21 -
4	67	5	3154 2991	4187 4329	13 22
4	75	5	1943 1758	2811 2886	17 -
4	82	5	1276 1240	1904 2032	14 -
4	90	5	898 812	1331 1293	12 -
4	98	5	490 533	866 926	10 15
4	12	5	- -	25406 25826	803 744
			(Stop 5:45)	(Stop 6:10)	(Stop 5:25

Table 5

Normal scores for Run A based on normal deviates representing the means for the classes of size of bacterial cell

Threshold Interval	Precentage remaining at beginning interval	Mean of the class of terms of normal deviates	Normal score
Total count at 23	100.00	-2.0558	4.401
23 to 27	94.95	-1.3418	3.687
27 to 30	86.56	- .8902	3.235
30 to 34	75.73	- .4937	2.838
34 to 39	61.89	- .1338	2.478
39 to 44	48.72	.1696	2.175
44 to 49	37.80	.4450	1.900
49 to 55	27.90	.7086	1.636
55 to 61	20.09	.9367	1.408
61 to 67	14.91	1.1835	1.161
67 to 75	8.95	1.4416	.903
75 to 82	6.07	1.6381	.706
90 to 98	4.11	1.8450	.500
remainder	2.43	2.3446	.000

count. Counts obtained at settings corresponding to a diameter less than the total count setting were eliminated from further consideration. Percentages of total counts were than determined for each upper limit of the classes remaining.

The method of converting these percentages to normal scores was that used by D. F. Cox*. The mean of the areas of a normal curve in terms of standard normal deviates was determined from values of the normal distribution. For example, the mean, $\bar{x}_{12}$, of the area which is bounded by the ordinates z_1 and z_2 that represents a fraction, b, of the total population is given by:

$$\bar{x}_{12} = \frac{z_1 - z_2}{b}.$$

The normal score was then obtained by setting the remainder class equal to zero and using the positive difference between the mean of this class and the other classes in the table.

* D. F. Cox, "Description and Measurement of Rates of Early Mortality in the Pig", Agricultural and Home Economics Experiment Station, Iowa State University, Research Bulletin 500, January, 1962.

Final conclusions were based upon results of paired t-tests of the normal scores of different sets of data.

Other methods of presenting data obtained with the Coulter Counter were discussed by Brecher (6), Toennies (37), and Deysson (10).

APPENDIX B

Computer Programs

The Calibration Program was used to determine the machine settings for respective diameters. Input quantities were: FK = "K" (calibration constant), QAR = aperture resistance, GI = gain used during calibration, and FM = size of increments desired for threshold setting (FM = 1 gives all values 1 to 100). Only one input card was used for this program. When sense switch one was on, combinations with aperture currents one, two, and three were given. When it was off, combinations with four, five, and six were given. The threshold setting, aperture current setting, diameter, and volume of particle were given in the output.

The Normal Score Program was used to convert raw data or percentages to normal score. Input quantities were: COR = correction factor, R1(I) and R2(I) = raw counts of particle sample, C1 and C2 = background counts, and R2(I) = percentage (with proper sense switch setting). One card with the correction factor was always the first input card. With sense switch one off, the remainder of the input cards each had two raw counts and two background counts. With sense switch one on, the remainder of the input cards each had a percentage similar to the percentages in Table 5. Output was similiar to Table 5. Hasting's approximation

(13) was used to obtain standard scores from areas of the standard normal curve.

The Interpretation Program was used to convert raw data into useful quantities. Input data consisted of: P = correction factor, PK = "K" (calibration constant), QAR = aperture resistance, GI = gain used during calibration; R1, R2, R3, and R4 = raw data and C1, C2 = background (with appropriate sense switch setting). The first input card had the correction factor, calibration constant, aperture resistance, and gain. The next input card had the machine setting (gain, threshold, and aperture current) at which data was recorded. The following input card was the data card related to the previous machine setting card. Input format for data cards varied with sense switch setting. Machine setting and data cards would be alternated in the remainder of the input deck. Output data is self-explanitory in the output format except for the last column which is the percentage of correction due to coincidence.

```
C       CALIBRATION PROGRAM
C
        DIMENSION DIA(10,6)
        READ 10, FK, QAR, GI, FM
10      FORMAT(4F11.0)
        M = FM
        IF (SENSE SWITCH 1) 19, 18
19      LBJ = 1
        GO TO 20
18      LBJ = 4
20      JB = LBJ + 2
        DO 11 J = LBJ,JB
        QRN = (25600000.)*(1./(2.**(J-1)))
        QR1 = 25600000.
        QC = 15000.
        QG = 1000000.
        QX = 100000.
        FW = (QRN + QAR + QC)/(QR1 + QAR + QC)
        FX = (1. + QAR*(1./QG + 1./QRN))/(1. + QAR*(1./QG
             + 1./QR1))
        FY = 1. + (1./(QX*(1./QRN + 1./QAR + 1./QG)))**2
        FZ = 1. + (1./(QX*(1./QR1 + 1./QAR + 1./QG)))**2
        FV = SQRTF(FY/FZ)
        FE = FW*FX*FV
        DO 12 K = 1,100, M
        AK = K
        T = AK*FE
        DUERR = (LOGF(T))/3.
        RON = EXPF(DUERR)
12      DIA(K,J) = FK*RON + .00005
11      CONTINUE
        PUNCH 13, FK, QAR, GI
13      FORMAT(18HCALIBRATIONS   K =F11.6,7H   AR =F11.0,6
             H   G =F4.0)
        J = LBJ
        L5 = J+1
        L6 = J + 2
        PUNCH 14
14      FORMAT(/14H  T    I   DIA14X12HT    I   DIA14X12
             HT    I   DIA)
        DO 17 K = 1,100,M
        X = (DIA(K,J)/2.)**3*4.189
        Y = (DIA(K,J+1)/2.)**3*4.189
        Z = (DIA(K,J+2)/2.)**3*4.189
        PUNCH 15,K,J,DIA(K,J),X,K,L5,DIA(K,J+1),Y,K,L6,DIA
             (K,J+2),Z
15      FORMAT(I5,I3,F7.4,F9.4,I5,I3,F7.4,F9.4,I5,I3,F7.4,
             F9.4)
17      CONTINUE
```

```
STOP
END
```

```
C       NORMAL SCORE PROGRAM
C
        DIMENSION R1(31), R2(31), DG(31)
        READ 1, COR
1       FORMAT (F11.0)
        I = 0
        IF (SENSE SWITCH 1) 21,3
3       I = I + 1
        READ 2, R1(I), R2(I), C1, C2
2       FORMAT(4F11.0)
        C = (C1 + C2)/2.
        AN = (R1(I) + R2(I))/2.
        R1(I) = ((AN/1000.)**2)*COR + AN - C
        R2(I) = R1(I)/R1(1)*100. + .005
        IF (SENSE SWITCH 9) 4,5
21      I = I + 1
        READ 22, R2(I)
22      FORMAT(F11.0)
        IF (SENSE SWITCH 9) 4,21
4       N = I + 1
        M = I
        R2(N) = 0.
        DG(1) = 0.
        DO 13 I = 1,M
        DIF = R2(I) - R2(I+1)
        AREA = ABSF(R2(I+1)-50.0)
        AREA = (ABSF(R2(I+1)-50.0))/100.
        AREA = .5 - AREA
        IF (AREA) 93, 93, 92
93      TV = 4.5
        GO TO 94
92      FNN = SQRTF(LOGF(1./AREA**2))
        A1 = 2.515517
        A2 = .802853
        A3 = .010328
        B1 = 1.432788
        B2 = .189269
        B3 = .001308
        TV=FNN-((A1+A2*FNN+A3*FNN*FNN)/(1.+B1*FNN+B2*FNN
             *FNN+B3*FNN**3))
94      DG(I+1)=(1./SQRTF(6.2831853))*(EXPF(-TV**2/2.))
13      R1(I) = (DG(I) - DG(I + 1))/(DIF/100.)
        DO 31 I = 1,M
        WAY = R1(M) - R1(I) + .0005
31      PUNCH 32, R2(I), R1(I), WAY
32      FORMAT (F10.2,3H   F8.4,F10.3)
        STOP
        END
```

```
C       INTERPRETATION PROGRAM
C
        DIMENSION COR(30), FN(30), DI(30), DIC(30), AT(30),
              REL(30), T(30)
        DIMENSION DA(30), CUM(30), SSD(30), THS(30)
        I=0
        TOT = 0.
        RCE = 0.
        READ 10, P, FK, QAR, G
10      FORMAT(4F11.0)
67      I = I + 1
        N = I
        READ 20, G, TP, FI
20      FORMAT(3F11.0)
        THS(I) = TP
        J = FI
        QRN = (256000000.)*(1./(2.**(J-1)))
        QR1 = 256000000.
        QC = 15000.
        QG = 10000000.
        QX = 10000000.
        FW = (QRN + QAR + QC)/(QR1 + QAR + QC)
        FX = (1. + QAR*(1./QG + 1./QRN))/(1. + QAR*(1./QG
             +1./QAR1))
        FY = 1. + (1./(QX*(1./QRN + 1./QAR + 1./QG)))**2
        FZ = 1. + (1./(QX*(1./QR1 + 1./QAR + 1./QG)))**2
        FV = SQRTF(FY/FZ)
        FE=FW*FX*FV
        IF(GI-G) 4, 5, 6
4       L = G - GI
        FE=FE/(2.**L)
        GO TO 5
6       L = GI - G
        FE=FE*(2.**L)
5       IF(SENSE SWITCH 1) 89, 90
89      READ 11, R1, R2, R3, R4, C1, C2
11      FORMAT(6F11.0)
161     AC = (C1 + C2)/2.
17      AN = (R1 + R2 + R3 + R4)/4.
        AN = AN - AC
        GO TO 77
90      IF ( SENSE SWITCH 2) 92, 91
92      READ 16, R1, R2
16      FORMAT(2F11.0)
        AC = 0.
        R3 = R1
        R4 = R2
        GO TO 17
91      IF (SENSE SWITCH 3) 13, 96
```

```
13      READ 10, R1, R2, C1, C2
        R3 = R1
        R4 = R2
        GO TO 161
96      READ 10, R1, R2, R3, R4
        AN = (R1 + R2 + R3 + R4)/4.
77      CAN = P*((AN/1000.)**2)
        SP = R1+R2+R3+R4
        SSD(I)=SQRTF(((R1*R1+R2*R2+R3*R3+R4*R4)-((SP*SP)
              /4.))/3.)
        COR(I) = (CAN/AN*100.) + .0005
        FN(I) = CAN + AN
        T(I) = TP*FE
        DUERR = (LOGF(T(I)))/3.
        RON = EXPF(DUERR)
        DI(I) = FK*RON
        FMAX = FN(1)
        IF(FN(I) - FMAX) 32, 32, 31
31      FMAX = FN(I)
32      FMIN = FN(1)
        IF (FN(I) - FMIN) 34, 33, 33
34      FMIN = FN(I)
33      IF (SENSE SWITCH 9) 66, 67
66      TOT = FMAX - FMIN
        M = N - 1
        DO 68 I = 1, M
        DIE(I) = ABSF(FN(I + 1) - FN(I))
        AT(I) = (T(I + 1) + T(I))/2.
        TOY = (LOGF(AT(I))/3.)
        DOG = EXPF(TOY)
        DA(I) = FK*DOG
        REL(I) = ((DIE(I)/TOT)*100.) + .0005
        RCE = RCE + REL(I) - .0005
68      CUM(I) = RCE + .0005
        PUNCH 70
70      FORMAT(4X52HAVE DIA    REL PER    BOUNDARY DI
              A  AVE COUNT  CUM PER/)
        DO 99 I = 1,M
        VOL = (DI(I)/2.)**3*4.189
        VAL = (DA(I)/2.)**3*4.189
        PUNCH 71,THS(I), VOL, DI(I), FN(I), SSD(I), COR(I)
71      FORMAT(3HT =F6.1,5H  V =F8.4,F11.6,F11.3,6
              H  SD =F11.3,F8.3)
99      PUNCH 72,DA(I),REL(I),VAL,DIE(I),CUM(I)
72      FORMAT(F11.6,F8.3,5H  V =F8.4,F10.3,F11.3)
        VOL = (DI(I)/2.)**3*4.189
        PUNCH 71,THS(I), VOL, DI(I), FN(I), SSD(I), COR(I)
        STOP
        END
```

APPENDIX C

Laboratory Prepared Orifices

The procedure for making orifices was:

1. Prepare glass capillary tubes about 300 microns in diameter.
2. Close one end of the capillary tube in a flame in order to give a shape as shown in Figure 13.
3. Use jeweler's rouge to grind the closed end of the capillary to the desired opening A.
4. Mount orifice by cementing to orifice tube.

The main feature of the laboratory prepared orifice was its smaller sensing zone which was designed to lower the chances of particle coincidence. However, the smaller sensing zone might be too small and not allow total particle pulse amplification.

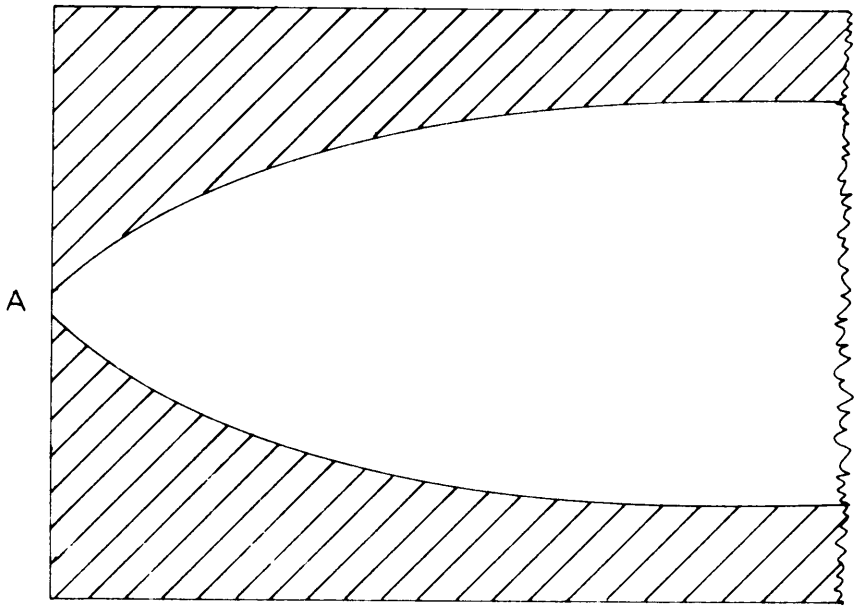

FIGURE 13. SIDE VIEW OF 12-MICRON ORIFICE PREPARED IN THIS LABORATORY.

APPENDIX D

Theory of the Coulter Counter

The Coulter Counter consists of two basic units: the sampling apparatus and the electronic pulse interpretation unit. Particles are suspended in an electrolyte as shown in Figure 14. A direct current passes from Electrode C to Electrode B. Upon successive count resets the direction of the flow of current changes. Between the electrodes the current must pass through the opening in the orifice tube at A. The small size of the orifice causes an electrical resistance in the circuitry of the Coulter Counter which is constant. Because the flow of the electrolyte is from the beaker into the orifice tube, particles in the suspension will be forced to pass through the orifice. When a particle enters the electrical sensing zone of the orifice, a change in resistance occurrs. This change is apparently proportional to the volume of the particle. The pulse interpretation unit at D then detects the change in the circuit and attempts to analyze it correctly. The number of particles analyzed during each sampling procedure is determined by either a constant volume or an electronically timed sampling period.

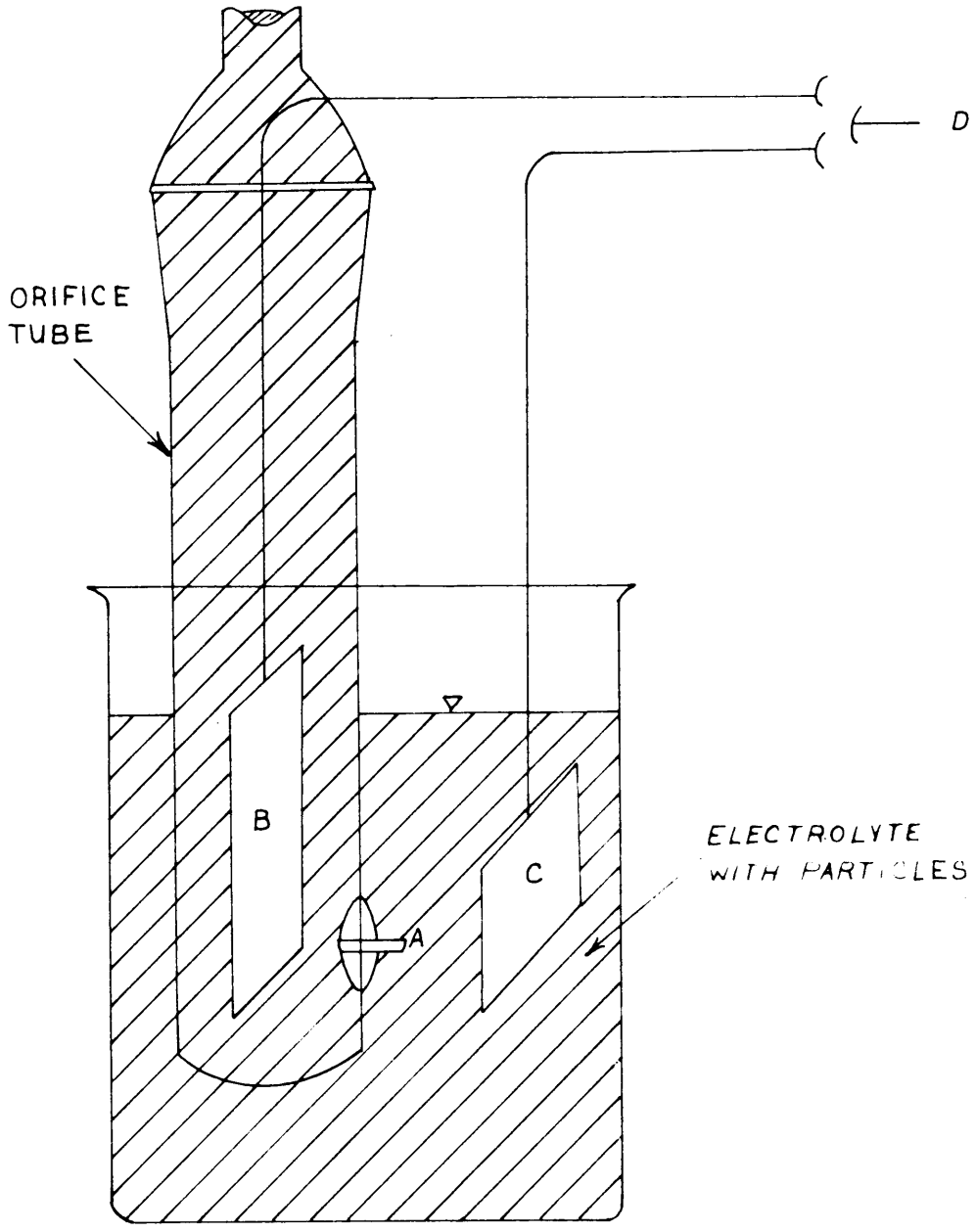

FIGURE 14. SAMPLING APPARATUS OF COULTER COUNTER

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ABSTRACT

Resolving Bacteria With The Coulter Counter

Present methods of counting and sizing of bacteria are usually tedious and often have low statistical validity. The objective of this thesis was to investigate the feasibility of counting and sizing of bacteria with the Coulter Counter and to evaluate orifices which were prepared in this laboratory.

Size distributions of Pasteurella multocida and Proteus vulgaris were obtained with the electronic particle counter. These distributions appeared to follow the normal distribution when relative per cent was plotted against diameter. The two modes of the distributions appeared far enough apart to warrant future attempts to separate quantitatively P. vulgaris and P. multocida in mixed suspensions. Size distributions also were obtained for Staphylococcus epidermidis, Sarcina lutea, and Azotobacter.

Many variables appeared to be inherent in the electronic particle method of counting biological cells; among these are the biological cells, diluent, and electronic circuitry. Apparently the variability in the electronic circuitry caused the calibration of laboratory orifices to be impossible according to conventional methods.