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Permanent Turbidity Standards

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Permanent turbidity reference standards suitable for measurement of microbial suspensions were prepared by suspending finely divided titanium dioxide in aryl sulfonamide-formaldehyde or methylstyrene resins. Turbidities of these standards, adjusted to a useful range for microbiological and immunological studies, were compared with other reference standards in use today. Tube holders for a Coleman Photonephelometer and a Nepho-Colorimeter were modified to eliminate the water well and to allow use of optically standardized 10-, 16-, or 18-mm test tubes. The standards and the tube holders have been used satisfactorily for more than 12 years.

Light-scattering techniques have been utilized for many years to estimate the number of cells in a microbial suspension. The theories and definitions used in light-scattering studies, the application of techniques, and the instruments employed in turbidity measurements have been reviewed by several authors (1, 4, 7, 15, 28, 30, 33, 34, 37, 38; A. A. Terry, PhD. Thesis, Univ. of Florida, Gainesville, 1961). Because of the diversity of applications, the wide range of turbidities measured, and the lack of understanding of the physical laws concerning dense suspensions, no universally acceptable standard for turbidity has been recognized. Many preparations proposed for standards do not remain constant because of aggregation, settling, or physical or chemical changes in the particle or suspending medium.

In microbiological and immunological work, the suspensions are usually dense and in many instances must be diluted for satisfactory measurement. In contrast, control laboratories in the food (sugar, beer, wine, citrus juices, etc.) and chemical (synthetic fibers, polymers, lacquers, etc.) industries are concerned with very faint turbidities. Investigations on air pollution, treatment of water and sewage, or molecular weights of proteins and other macromolecules, for example, generally involve study of solutions or suspensions of relatively low turbidity.

Various ionic compounds such as barium sulfate, calcium carbonate, and silver chloride have been used for many years for turbidity standards. Clays have been used widely. Representative of these are kaolinite, bentonite, illite, and fuller's earth. These are generally unsatisfactory because suspensions must be shaken frequently, particle size changes with time, or turbidity is affected by light. Formazin (condensate of hexamethylenetetramine and hydrazine), Ludox (colloidal silica; E. I. duPont de Nemours and Co., Wilmington, Del.), Pyrex glass, and polystyrene latex (Dow Chemical Co., Midland, Mich.) have been used in recent years (2, 3, 5, 8-11, 13, 14, 24, 25, 29, 31, 36). These materials are more stable, particularly if the proper particle size is used. Particles do not readily aggregate and apparently are not affected by light. The material in suspension gradually settles out, however, and must be resuspended before use. A polymethyl methacrylate-polystyrene interpolymer for use as a permanent turbidity standard in chemical analyses has been described (12).

This report concerns permanent turbidity standards prepared from finely divided titanium dioxide suspended in aryl sulfonamide-formaldehyde or methylstyrene resins. Modifications of the tube holders of Coleman instruments that permit use of standard 10-, 16-, or 18-mm test tubes are described.

MATERIALS AND METHODS

Standard Pyrex test tubes (16 or 18 mm) were used. After a preliminary screening for scratches, air bubbles, and size, tubes were selected for use by measuring the light transmission of a 5% copper sulfate solution with a 590-mμ filter.

Santolite MHP (Monsanto Chemical Co., St. Louis, Mo.), a resin formed by the condensation of aryl sulfonamides and formaldehyde, was the principal resin used to suspend titanium dioxide. The

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resin is hard, friable, and practically colorless. It softens at about 62 C and is fluid at 90 C or above. Prolonged heating above 90 C causes a yellow discoloration that may not be desirable.

Another resin used was an α-methylstyrene polymer, 276-V9 (Dow Chemical Co., Midland, Mich.). Resin 276-V9 is water-clear, has a specific gravity at 60/60 C of 1.04 and viscosity at 60 C of 700 to 1,000 centipoises. At room temperature, it is highly viscous.

The refractive indexes of α-methylstyrene and aryl sulfonamide-formaldehyde resins are 1.5878 and 1.574, respectively. These values were established at 23 C with the sodium D line. The solid formaldehyde resin was dissolved in benzene and indexes of known concentrations were measured; these results were extrapolated to 100% resin.

Titanium dioxide was ground to a small particle size in a special mill designed by H. G. Tanner at Fort Detrick (35). An analysis of particle-size distribution showed 78% $< 1\ \mu$, 61% $< 0.5\ \mu$, and 37% $< 0.35\ \mu$. This preparation (R-100) was used for primary standards first made in 1945. Since that time, a variety of suitable titanium dioxide preparations have become available from the pigment and cosmetic industries. These products are essentially pure, impart no color to the resin when used in a turbidity standard, and have arithmetic mean diameters ranging from 0.32 to 0.18 μ. Representatives of these products are: Zopaques R-55 and LD-C (The Glidden Co., Baltimore, Md.), Unitanes 0-110 and 0-220 (American Cyanamid Co., Bound Brook, N.J.), Atlas white (H. Kohnstamm and Co., Inc., New York, N.Y.) and Ti-Pure, R-900 (E. I. duPont de Nemours and Co., Wilmington, Del.).

The Santolite MHP resin contained in a beaker was placed on a hot plate. After it had softened, a few milligrams of titanium dioxide was added to 100 to 200 g of resin and stirred to a uniform turbidity by hand, mechanically, or with a magnetic stirrer. The turbid resin was then further diluted as desired in clear resin, mixed thoroughly, poured to a depth of 4 to 5 cm in the previously standardized test tubes, and allowed to cool gradually. Because of the coefficient of expansion of the resin, gradual cooling was necessary to avoid deep indentation at the surface of the resin. If an indentation formed, it was of no consequence as long as the indentation did not penetrate into the light path when the standard was used. Upon cooling, the resin forms a solid that is permanent, is not affected by light, and is stable.

Titanium dioxide was mixed with the 276-V9 resin by stirring for 2 hr or more at slow speed at room temperature; a homogeneous suspension was made more rapidly by stirring the resin at 60 to 80 C, however. Any bubbles formed during mixing were gradually driven out by heating the turbidity standard in a test tube at 80 C in an oven. Pyrex glass tubes were closed with a glass blower's torch, forming a rounded, air-tight seal.

RESULTS

In 1952, when this work was started, no universally acceptable permanent turbidity standard existed. Various standards had been proposed for different purposes, however. For example, the "nephelos" standards manufactured by Coleman Instruments, Inc. (Maywood, Ill.), are suitable for water analyses or measurement of turbidities in clarified products such as beers, syrups, lacquers, etc., but not for growth of microorganisms in liquid culture or preparation of standard vaccines. The nephelos standards are of limited use in microbiology because of the low range of turbidities available. The $BaSO_4$ standards of McFarland (26), proposed in 1907, have been rather widely used in microbiology, although the particle size tends to change on standing and the particles must be resuspended frequently during use. In 1953, an international reference preparation for opacity was established by the World Health Organization Expert Committee on Biological Standardization for distribution by the Statens Seruminstitut, Copenhagen, Denmark (25). This preparation is a suspension of Pyrex glass particles of approximately the size of bacteria and is similar to the standard for opacity used by the National Institutes of Health in the United States. These standards are used primarily for preparation of vaccines.

The titanium dioxide standards reported in this paper were prepared primarily for use in bacterial growth experiments. Although absorption of light is an exponential function of the concentration of the solution or the suspended material only when the solutions or suspensions are dilute (Beer's law), nephelometric methods are used satisfactorily for measurement of microbial suspensions over a wide range of densities. In many instances, however, suspensions must be

TABLE 1. *Relationship of various turbidity standards to Roessler-Brewer turbidity units*

Standard	Roessler-Brewer turbidity units[a]
McFarland No. 1	28
McFarland No. 2	64
McFarland No. 3	98
McFarland No. 4	127
McFarland No. 5	162
McFarland No. 6	190
McFarland No. 7	216
McFarland No. 8	235
McFarland No. 9	253
McFarland No. 10	260
U.S. Opacity Standard (10 units)	90
Nephelos, Coleman (251 units)	ca. 15.3
Nephelos, Coleman (77 units)	ca. 5.0

[a] Readings on the Coleman Nepho-Colorimeter, model 9, adjusted for nephelometric measurements. Readings were made without a color filter.

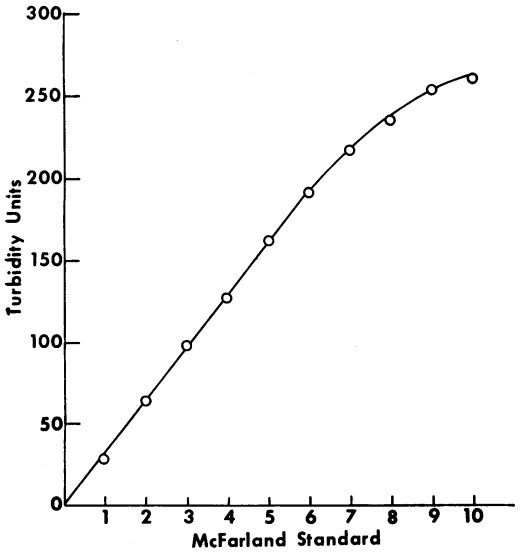

FIG. 1. *Relationship of McFarlands standard to Roessler-Brewer turbidity units.*

diluted before satisfactory measurements can be made. For preparation of various standards, a dense suspension was arbitrarily set at 100 units; a Coleman Photonephelometer or Nepho-Colorimeter was adjusted for a reading of 100 on the nephelometric scale. As dilutions were made of titanium dioxide in the resin, turbidities of other standards were measured relative to the 100-unit standard on the same scale.

Subsequently, these standards were compared with the nephelos unit, the McFarland $BaSO_4$ standards, and the opacity standard of the National Institutes of Health. Turbidity measurements were made with light reflected at right angles from the suspended particles by use of a Coleman instrument; no filter was used. Results of the comparison are given in Table 1.

The 10 opacity units of the National Institutes of Health and the number 3 McFarland standard correspond to 90 and 98, respectively, of our turbidity units. At the other extreme, 90 of our units correspond to approximately 1,400 to 1,500 nephelos units.

When the data in Table 1 are plotted, a straight-line relationship is shown through the first six McFarland standards. Higher standards, however, show a deviation, undoubtedly because of interference of light-reflecting particles (Fig. 1). Turbidity measurements by McFarland's standards are difficult because of the rapid settling of particles. The suspension must be mixed and the instrument reading observed carefully until a reasonable stability from the mixing is achieved. A reading must be recorded before particles begin

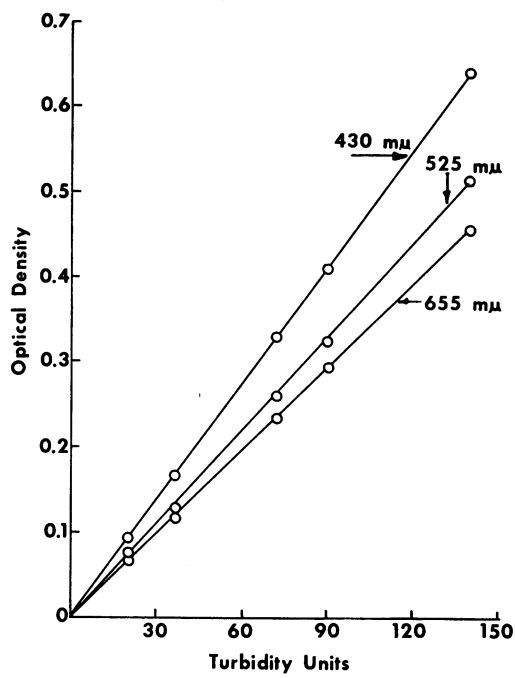

FIG. 2. *Relationship of turbidity units to optical densities.*

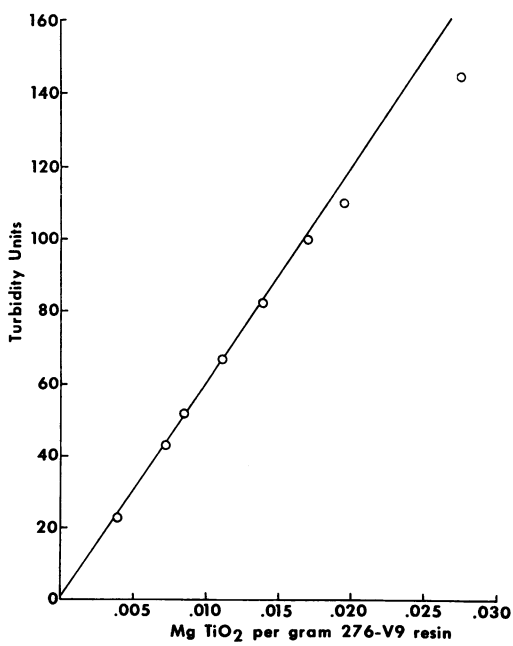

FIG. 3. *Relationship of amount of titanium dioxide suspended per unit of 276-V9 resin to turbidity produced.*

to settle (10 to 15 sec after mixing, depending on turbidity of $BaSO_4$ suspension).

Because many investigators use optical densities to record the amount of growth in a microbial suspension, we investigated the relationship or our turbidity units to optical density. When optical density or light transmittance measurements are made, a filter is generally used to compensate for absorption of light by a blank. For the optical density measurements reported (Fig. 2), wavelengths of 655 (red), 525 (green), and 430 mμ (blue) were used with a Coleman Photonephelometer. The plotted data show a straight-line relationship over the range of turbidities studied. These values will vary somewhat from instrument to instrument depending on response of the photocell, light intensity, and other components of the instruments. In general, values show the relationship of optical densities to our turbidity units.

To demonstrate that a satisfactory relationship exists between the turbidity produced and the amount of titanium dioxide suspended per unit of resin, an experiment was performed with various amounts of R-900 titanium dioxide and 276-V9 resin. A good relationship exists until approximately 100 turbidity units are reached (Fig. 3). Beyond this point, the turbidity increase does not correspond to the increase in titanium dioxide concentration. In our work, accurate measurements of microbial populations are made when the turbidities of the cell suspensions are between 20 and 100 turbidity units. Many cell suspensions obtained in microbiological experiments, therefore, must be diluted to these turbidities for accurate estimates of populations.

The suitability of several commercial titanium dioxides for the preparation of turbidity standards was compared by use of 276-V9 resin. All samples suspended equally well in the resin, but considerable differences were shown in the amount of turbidity obtained. Undoubtedly, these differences result from size of the particle. Particles were mixed in resin for at least 2 hr before distribution into test tubes. This was adequate as evidenced by the straight line that can be drawn from the origin (Fig. 4) when turbidities of heavy suspensions (0.017 mg of TiO_2 per g of resin) and the turbidities of the dilute suspensions (0.010 mg of TiO_2 per g of resin) were plotted. The heavy suspension (10 g) was diluted with 7 g of clear resin, mixed thoroughly, and then distributed to standardized tubes to obtain a dilute suspension.

Design of tube holders. The most commonly used test tubes for microbiological work have dimensions of 16 or 18 mm (outside diameter) by 15 cm (length). The tube holder for Coleman

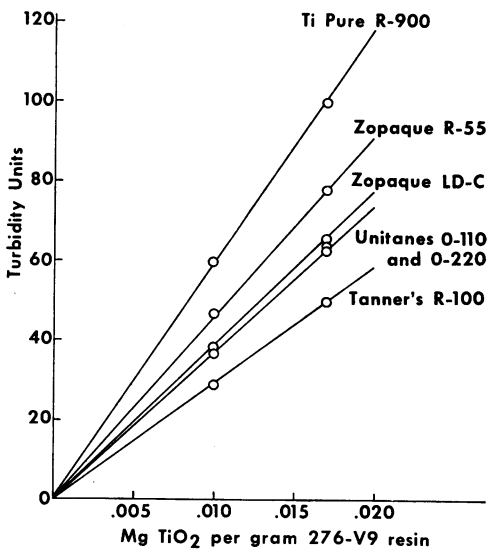

FIG. 4. *Straight-line relationships of turbidities produced by commercial titanium dioxides.*

instruments is designed for 19-mm test tubes positioned in a water well. For work involving cultures of microorganisms, vaccines, or other turbid suspensions, the water well is not necessary. It is, in fact, undesirable from a safety veiwpoint, particularly when infectious or toxic materials are evaluated.

Most turbidity and light-scattering measurements are made with photocells at right angles to the incident beam of light and without a color filter. In the experiments reported, both reflected and transmitted light were used, and many of our instruments are capable of being used for colorimetric as well as microbiological work. Consequently, modifications were made of tube holders for both transmitted and reflected light.

Holders for 18-mm tubes were made of 17S or 24S-T4 aluminum, finished with a matte black anodic oxide coating. A sleeve extending approximately 3 cm down inside the holder allowed them to be used with 10- or 16-mm tubes. Figure 5 shows details of a tube holder used for nephelometric measurements, and Fig. 6 shows a tube holder used when light is transmitted through a sample. Most dimensions were omitted from Fig. 6 because the holder is identical with that shown in Fig. 5 except for openings permitting passage of light.

A small O-ring or circular rubber cushion was placed in the bottom of a tube holder as a safety device in the event a tube slipped from an operator's hand and fell to the bottom of the well. The cushion served also to raise small tubes (10 by 75 mm) off the bottom of the holder,

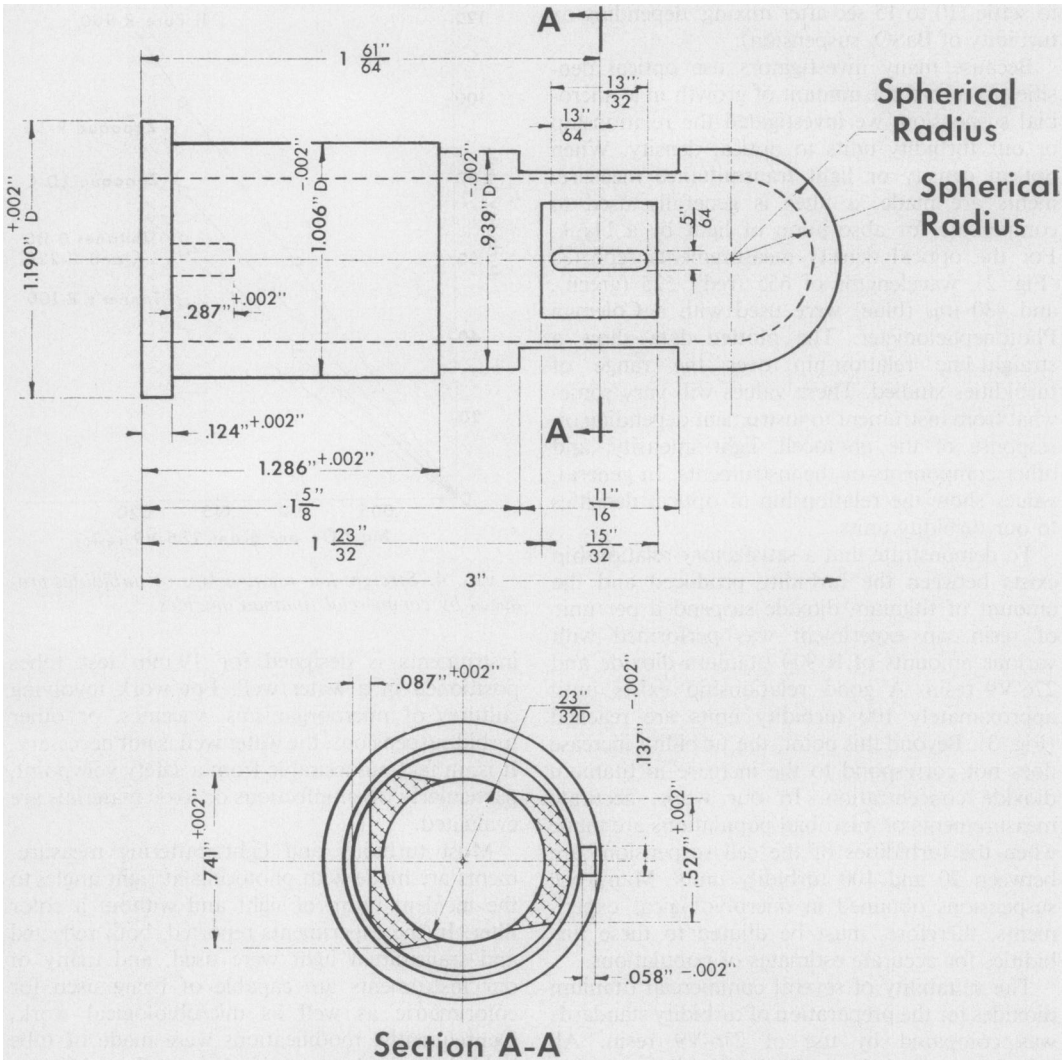

FIG. 5. *Details of tube holder for use in nephelometric measurements with the Coleman Nepho-Colorimeter.*

allowing them to be grasped more easily by an investigator.

DISCUSSION

Turbidity standards made with Santolite resin have been used by various investigators in the field of microbiology at Fort Detrick and elsewhere for over 12 years. Turbidities are constant, and no color change has been observed in that time. As shown in Table 2, they have been particularly useful with nephelometers in studies on the growth of microorganisms (6, 16–23, 27, 32).

Because of stability, ease of preparation, and low cost of readily available materials, turbidity standards have a wide potential use in microbiology, particularly in nutritional and vaccine studies. Units of turbidity were arbitrarily established for such studies. Cell densities of 2×10^{10} to 10×10^{10} per ml exhibit turbidities of 160 to 250 at a dilution of 1:10 and depending upon the type of organism (16, 17, 27, 32). Suspensions of organisms that are less dense, such as leptospira cultures, are conveniently measured with the 20-unit turbidity standard. Investigators in the field of leptospirosis have considered using these standards as international reference standards (*personal communications*, A. D. Alexander, Walter Reed Army Medical Institute of Research, Washington, D.C., and M. M. Galton, Communicable Disease Center,

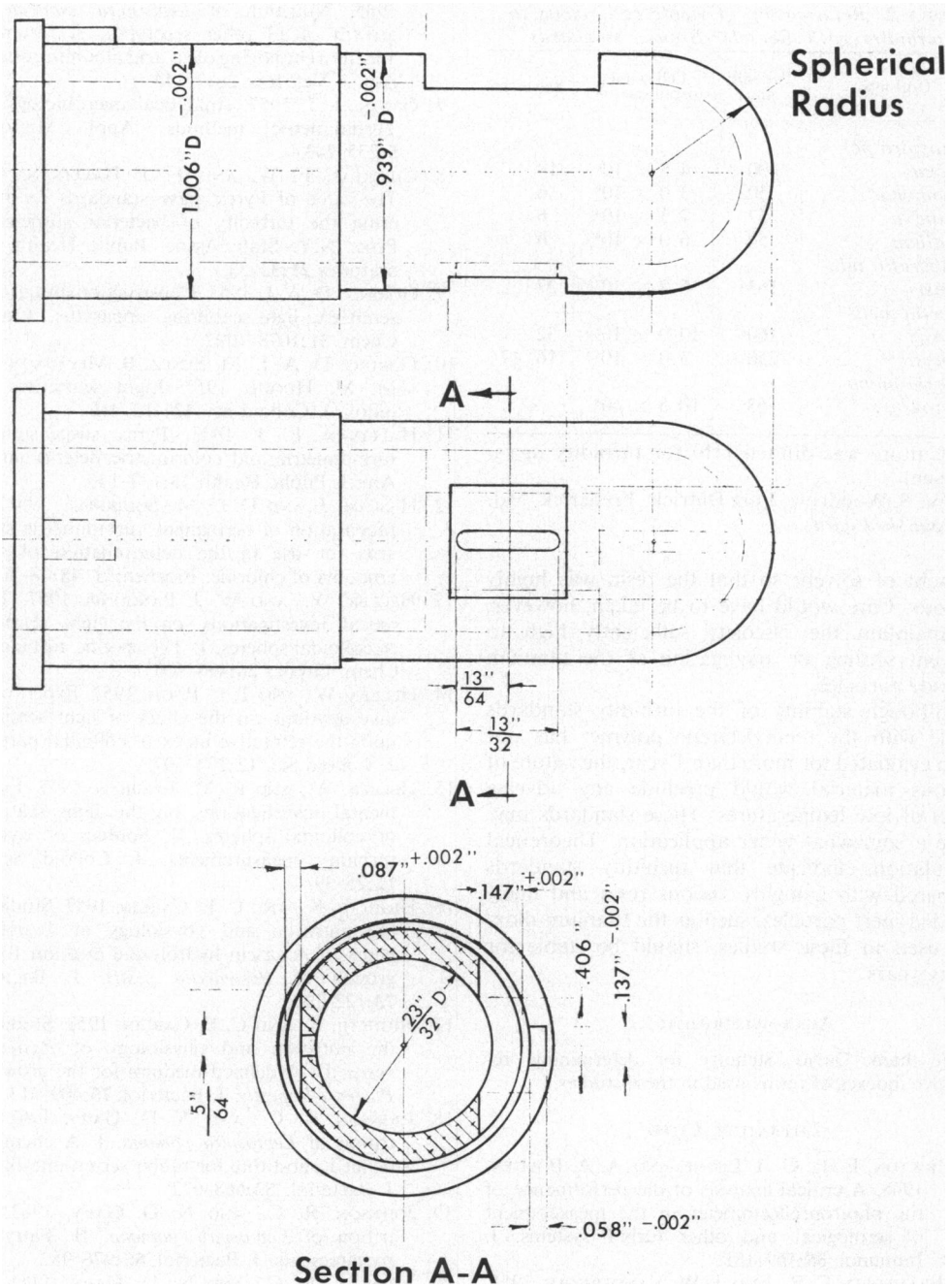

FIG. 6. *Details of tube holder for use in light transmission measurements.*

Atlanta, Ga.). Although not evaluated, it is probable that the lower turbidity standards would be useful in water and sewage analyses if an expanded scale were used.

One disadvantage of the standards made with the solidified Santolite resin is the tendency of the resin to crack at refrigeration temperatures; this presents a problem when tubes are shipped in cold weather or inadvertently placed in the refrigerator. The coefficient of expansion of the resin, in some instances, caused the glass tube to crack. This could be overcome by adding a small

TABLE 2. *Relationship of viable cell counts to turbidity with Roessler-Brewer standards*

Organism	Turbidity units	Cell counts (organisms/ml)	Reference
Leptospira pomona	100	4.8×10^8	18
L. pomona	50	3.0×10^8	6
L. biflexa	67	7.3×10^8	6
L. ballum	54	6.0×10^8	6
Pasteurella tularensis	163[a]	6.2×10^{10}	27
Brucella melitensis	160[a]	10.0×10^{10}	32
P. pestis	250[a]	5.0×10^{10}	16, 17
Listeria monocytogenes	63	10.5×10^8	–[b]

[a] Culture was diluted 1:10 for turbidity measurement.

[b] W. S. Woodrow, Fort Detrick, Frederick, Md. (*unpublished data*).

amount of solvent so that the resin was highly viscous. Care would have to be taken, however, to maintain the viscosity sufficiently high to prevent settling or aggregation of the titanium dioxide particles.

Although stability of the turbidity standards made with the methylstyrene polymer has not been evaluated for more than 1 year, the nature of viscous material would preclude any adverse effect of low temperatures. These standards may have a somewhat wider application. Theoretical calculations indicate that turbidity standards prepared with a highly viscous resin and finely divided inert particles, such as the titanium dioxide used in these studies, should be stable for many years.

ACKNOWLEDGMENT

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