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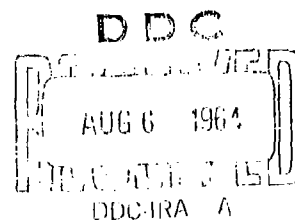
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TECHNICAL MEMORANDUM 40

SYSTEMS FOR COUNTING
AND MEASURING
SMALL PARTICLES IN FLUIDS

JUNE 1964



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FORT DETRICK

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U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MEMORANDUM 40

SYSTEMS FOR COUNTING AND MEASURING SMALL PARTICLES IN FLUIDS

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Technical Engineering Division
DIRECTOR OF ENGINEERING SERVICES

Project IC522301A082

June 1964

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ACKNOWLEDGMENT

The facilities furnished by the former Allied Sciences Division and the assistance given by Mr. Milton Shon are gratefully acknowledged, as well as the several interesting and searching discussions relating to the individuality and vitality of microorganisms.

ABSTRACT

Research into methods of counting viable bacteria in the presence of many dead ones to yield a rapid microbiological assay did not reach that final objective, but advanced to several important milestones:

(a) Methods and apparatus were reduced to practice wherein the light scattered from individual bacteria or clusters in liquid suspension was used for detection and size measurement.

(b) The inhibition of mitosis of organisms in a sample, as a method of culturing the microbial giant form to test viability, proved difficult because of the variability of response among individuals in the population to the inhibiting agents and nutrients tested. However, the exploratory experiments produced samples useful for instrument testing.

(c) Exploratory research indicated that changes in the dielectric constant of a minute sensing zone could be used to detect the presence of particles in a fluid passing through the zone, and that this method might also show the organization, composition, or uniformity of the particles.

(d) We also concluded that the light-scattering apparatus could be used, with minor optical modification, to count fluorescent particles in a liquid.

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I. INTRODUCTION

The work reported is a continuation of work started under a Secretary of the Army research and study fellowship¹ and was supported by a FY 1962 Research Task Proposal of Technical Engineering Division. An earlier report, included here as the Appendix, covered work during the period 1 January to 1 March 1961; this memorandum covers the period 1 March to 27 October 1961, when work was suspended.

The first section of this memorandum describes the development of an instrument with specialized hydraulic, optic, and electronic parts that can examine and count individual bacterial cells. Experiments in giant cell culture of bacteria, proposed as a rapid test of viability, are described, as well as the use of the samples for instrument tests.

The second section deals with an instrument development, based on initial studies of the electrical properties of bacterial cells, that may be useful in testing viability, as well as for counting. Experiments with the "dielectric-constant monitor" apparatus are described.

These two approaches to a solution to the problem of rapidly counting viable bacteria in the presence of dead ones are but two of a number of approaches that were uncovered as a result of study conducted under the Secretary's fellowship.

II. SYSTEM FOR COUNTING SMALL PARTICLES

The first instrument development dealt with the automatic counting and measurement of hydrosol particles in the size range of bacteria and protozoa. It had three major objectives. The first was the development of a liquid handling system and an optical system capable of examining individual particles in a fine flowing liquid stream and converting the light scattered from each particle to an electrical signal suitable for electronic pulse-height sorting. The second was to adapt this "particle revealer system" to the electronic amplifying and size classifying system of an aerosoloscope as a convenient means of data readout. The third was to conduct some exploratory and very elementary microbiological experimentation in the cultivation of giant bacterial forms as a relatively rapid viability test, and at the same time yield realistic samples for instrument testing.

Successful advance toward any of these objectives would further the development of a basic instrument with potential uses in many areas. As a rapid method of microbiological assay, applications within the biological program should be many and varied. As a device of general utility, capable of counting and measuring the size of hydrosol particles, it should be useful in marine microbiology involving studies of life forms in fresh or sea water that range from about one to 200 microns in diameter. A rearrangement of size channel boundaries would permit the counting of the different types of organisms in different size channels.

Similarly, by rearrangement of channel boundaries in the electronic system, a more complete analysis of blood would be possible. The particle size spectrum of the cells in a blood sample is an important datum in medical diagnosis, because the number of certain types of cells changes with radiation damage and certain diseases.² The diameters of human blood cells range from approximately three microns for blood platelets through 7.2 to 7.5 microns for erythrocytes, to the neutrophil and eosinophil that are 12 to 14 microns in diameter.

In our earlier report (see Appendix), hydrosoloscopes Mark I, II, and III were briefly described and the design drawings of a Mark IV were presented. This report will deal exclusively with the development and testing of the Mark IV design and its application to the objectives previously outlined.

A. GENERAL DESCRIPTION

Figure 1 shows a general view of the hydrosoloscope Mark IV, which was the first instrument of this type to be completely functional and capable of counting and measuring the diameter of particles as small as 0.5 micron suspended in a liquid sample. The double rack of electronic chassis will not be described; this unit is the electronic portion of the aerosoloscope that has been described in detail elsewhere.³ In this development, it was used to supply stabilized high voltage for the multiplier phototube, general control, pulse height discrimination, and register of counts in each size channel. It was a means of reading out the information contained in the signals from the particle revealer. The particle revealer system is shown in more detail in Figures 2, 3, and 4. It consists essentially of an optical system and an external and internal liquid-flow systems.

1. Optical System

The optical system shown schematically in Figure 5 has two major parts, the illuminating optics and the viewing optics. The illuminating optics start at the lamp, followed by the condenser lens, which focuses an image of the lamp's ribbon filament onto a one- by three-millimeter aperture. Light passing the aperture becomes the true source of light and is gathered by a projection lens assembly F:1.4, 1 inch E.F., made by Simpson Optical Manufacturing Company, Chicago, that causes the light to fill the aperture of a Cook, Troughton, and Simms Type D.G., 16-millimeter microscope objective. Details of this type of objective are shown in Figure 6. The light is then brought to a focus on the flowing sample stream. The spent light is gathered and absorbed by a cornucopia-shaped black glass light trap. The viewing optics are very similar and consist of a second Type D.G. objective that views the illuminated portion of the sample stream and is backed up by a second cornucopia-shaped light trap. Both objectives have flat front lenses and were specially constructed, sealed, and hydrostatically tested to eliminate moisture leakage. Following the viewing objective is a second F:1.4 projection lens that serves as the eyepiece, another one- by three-millimeter aperture, and a multiplier phototube.*

2. External Liquid Flow System

Figure 7 shows the external liquid flow system and its relationship to the optical system. Water flows from the reservoir, under the influence of the motor-driven circulating pump, through the jet pump and back to the reservoir. In so doing, a pressure is generated that fills the small filtered-water reservoir through the membrane filter unit. The higher level

* Sometimes termed a photomultiplier tube, or preferably a photomultiplier, although actually a phototube.

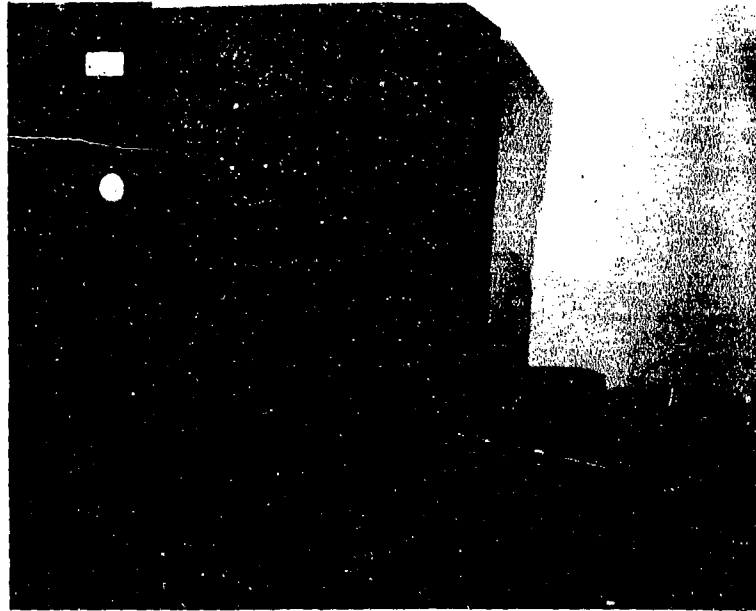


Figure 1. General View of the Hydrosoloscope Assembly.
(FD Neg C-6407)

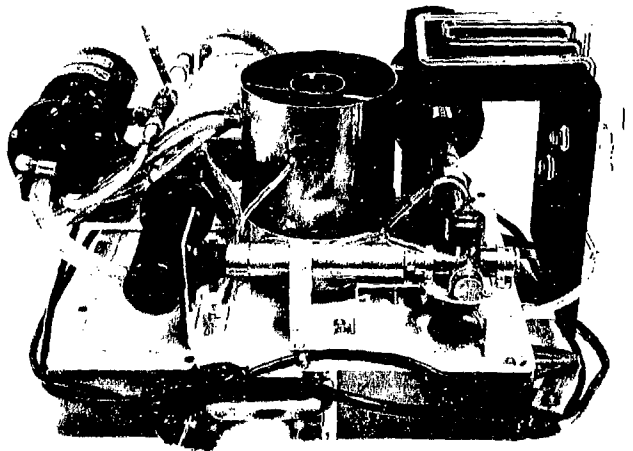


Figure 2. Detailed View of Particle Revealer from
Front. (FD Neg C-6121)

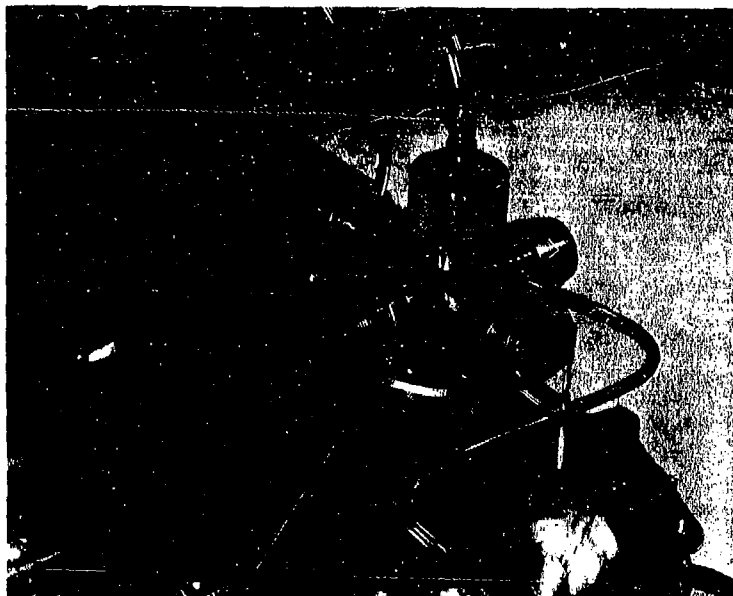


Figure 3. Sensing Head of Particle Revealer.
(FD Neg C-6413)

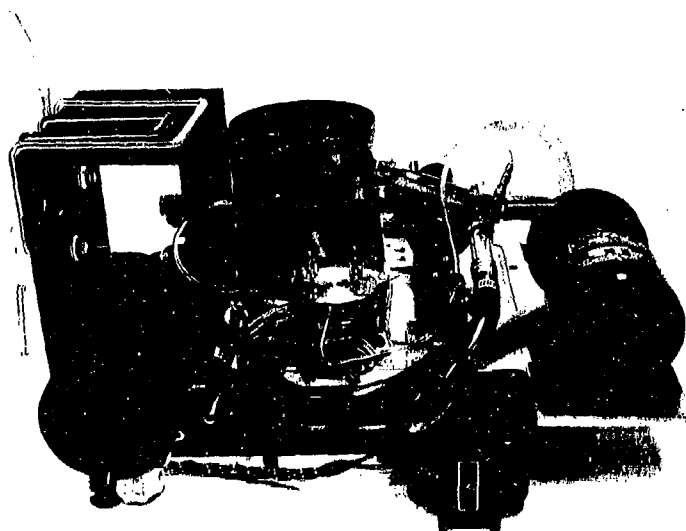


Figure 4. Detailed View of Particle Revealer from
Back. (FD Neg C-6123)

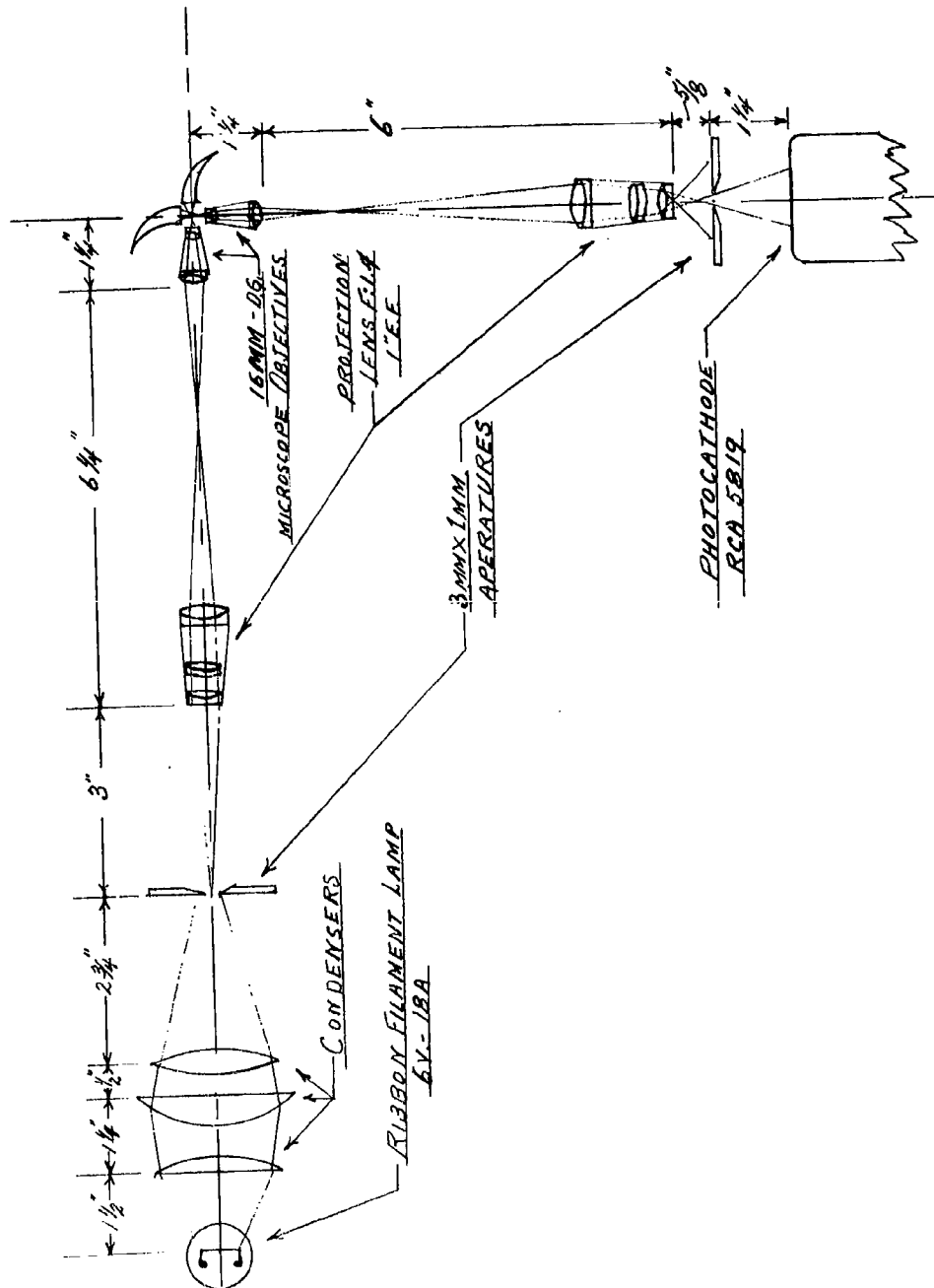


Figure 5. Diagram of the Illuminating Optics and the Viewing Optics.

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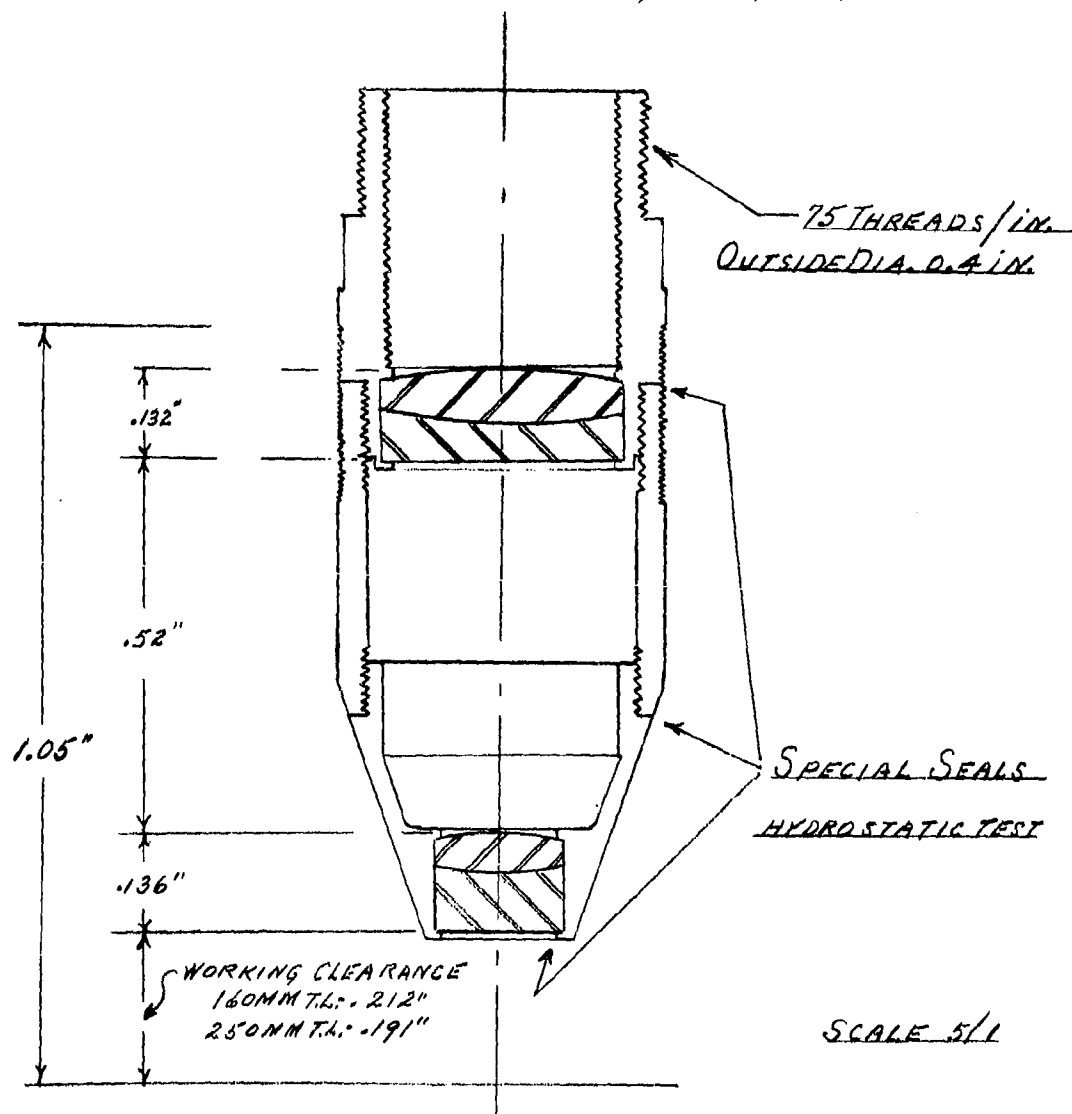


Figure 6. Details of the Illuminating and Viewing Objective.

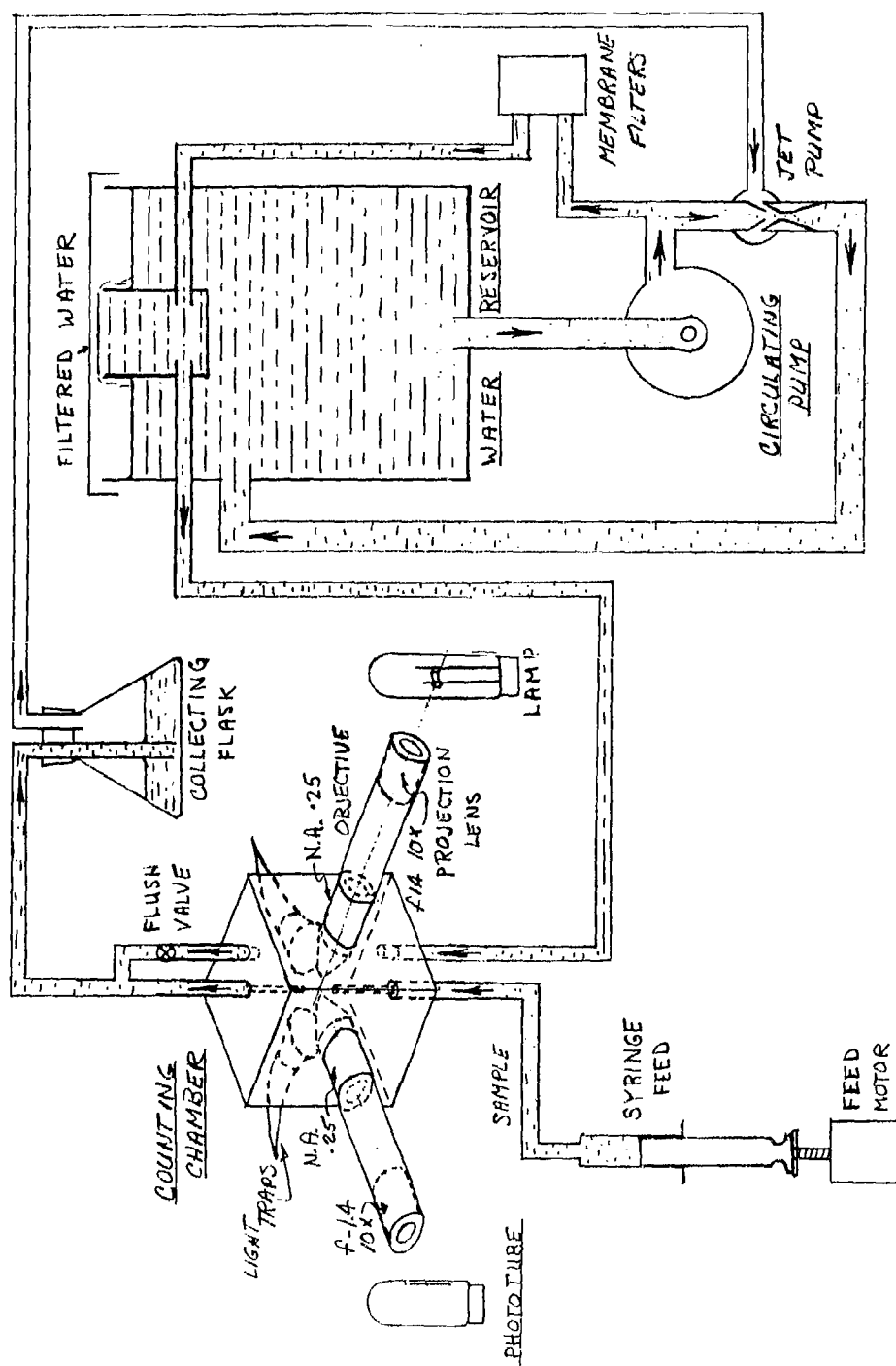


Figure 7. Diagram of the External Liquid Flow System.

in that reservoir maintains a very low but constant pressure on the counting chamber. The jet pump generates a suction that moves the clean water through the counting chamber. The system is similar to that described by Dr. Crosland-Taylor.⁴ Water leaving the counting chamber is collected in a flask to prevent contamination of the reservoir by the sample. For any future work a second membrane filter unit could be installed in place of the collecting flask. This type of filter has been quite successful. Design details of the filter assembly are shown in Figure 8. A membrane filter on each side of a stainless steel fiber disc is sealed with O-rings. Clean liquid is collected from within the disc.

3. Internal Liquid Flow System

Essential features of the internal flow system and sensing zone are shown in Figure 9. The stream of clean filtered water entering the bottom side arm enters the sensing zone through the annular space between the two bottom tubes, and exits at a distance of about six millimeters through a No. 24 hypodermic needle. The sample is injected into this stream of clean liquid through a No. 33 hypodermic needle with an inside diameter of 0.004 inch. The two streams flow in a laminar fashion without intermixing through the very small sensing zone generated by the intersection of the two optical paths (Figure 5). A particle in the sample entering this zone will scatter light from the source to the multiplier phototube. The phototube will generate an electrical pulse proportional to the quantity of light that it receives and with a duration equal to the transit time of the particle through the sensing zone. The height of the pulse, in electrical terms, is proportional to the size of the particle. By proper arrangement of aperture size and rate of flow, it is possible to have the pulse duration fall within the range of pulse lengths accepted by the aerosoloscope electronic system.

4. Factors of System Noise

Initial experiments with this apparatus indicated that the quantity of light scattered from a certain size of particle suspended in water was little, if any, different from that scattered by the same particle suspended in air. This result was somewhat unexpected because the difference in refractive index between the particles tested (polystyrene or bacteria) and water is less than the refractive index difference between these particles and air.

Another unexpected result was that the level of noise caused by light scattered from water molecules in the sensing zone was much lower than that calculated from air data, which predicted an increase by a factor of one thousand. This result is similar to that obtained by Robert W. Wood.⁵

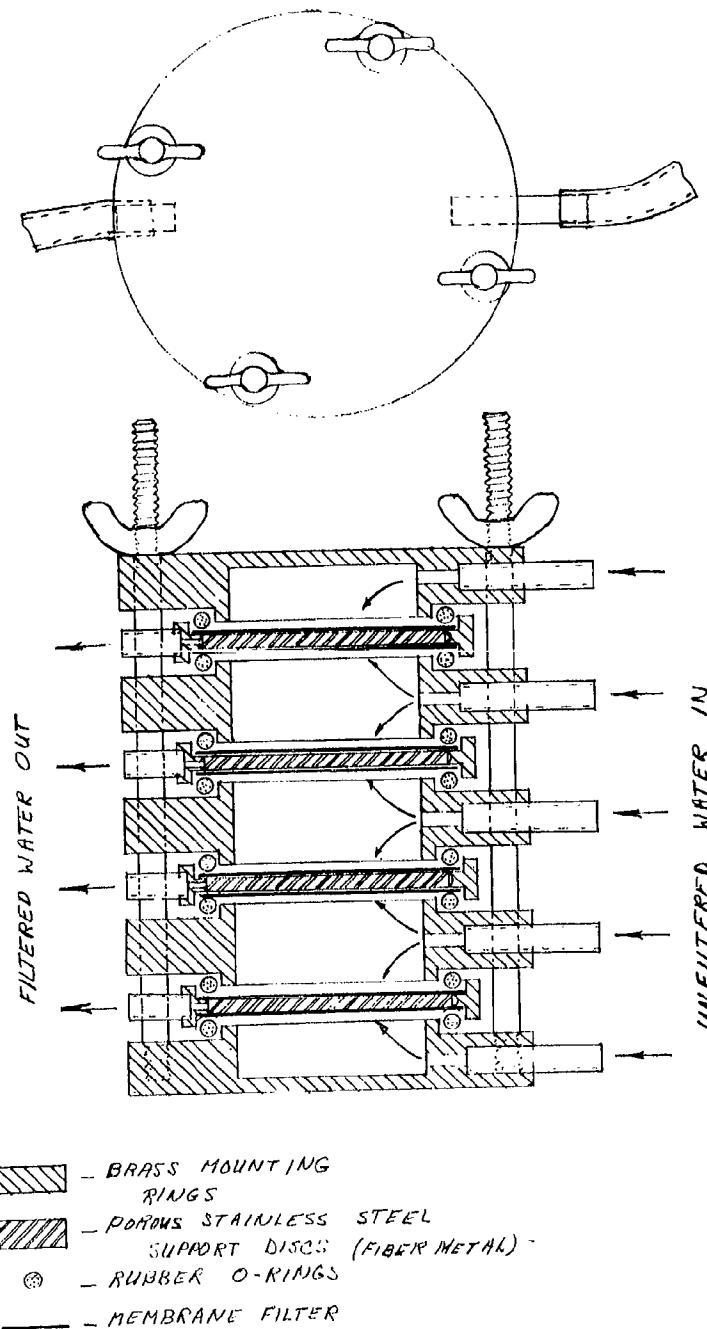


Figure 8. Construction Details of the Multiple Membrane-Filter Holder.

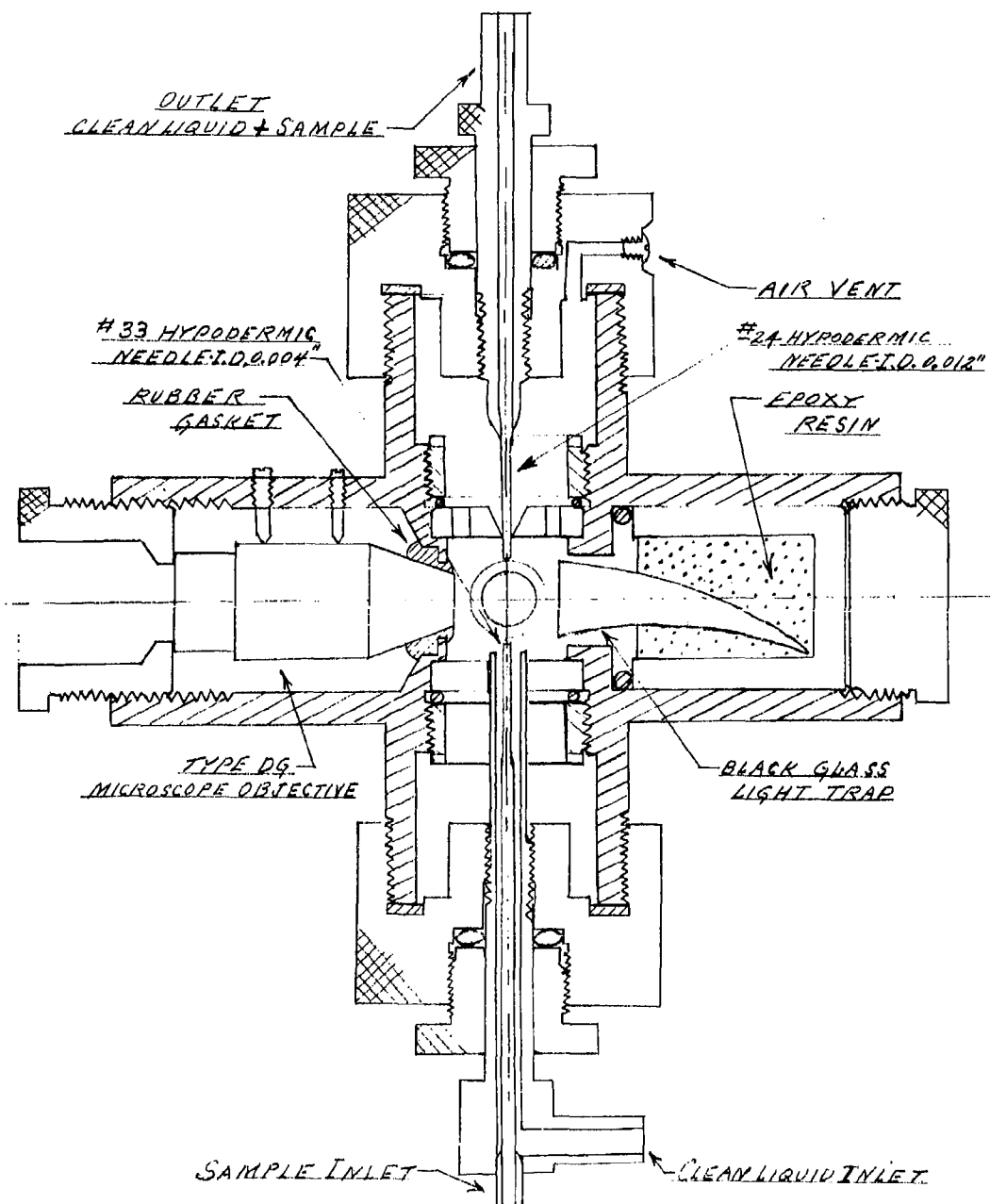


Figure 9. Sectional View of Internal Liquid Flow System and Sensing Zone.

Professor Wood's experiments show that scattering by a given volume of liquid ether is only 50 times greater than the scattering by the same volume of ether vapor. The molecular scattering would have been 1000 times greater if it had been proportional to the density of molecules. "The missing factor of 20 is explained by evanescence (vanishing) of scattering associated with the approach toward a transparent homogeneous medium, the liquid, where there are myriads of oscillators in a cube with dimensions of one wave length of light on an edge."⁶

These initial trials on 16 May 1961 were conducted with 0.8-micron polystyrene microspheres. With a slight increase in electronic gain, developed by an increase in the dynode voltage on the phototube, most counts fell in Channel 1 (1- to 1.4-micron channel) of the aerosoloscope. The trial yielded two very important results; first, the signal-to-noise ratio with 0.8-micron particles was much better than had been expected, and second, the amount of light scattered from the particle in water is similar to the amount of light scattered by the same particle in air.

B. RESULTS AND DISCUSSION

1. Early Experiments

On 24 April 1961 assembly of the Mark IV hydrosoloscope was started. Its immediate purpose was that of a test device or a pilot model. It was not expected that it would function as a completed instrument but rather, would enable us to learn how a better one could be designed.

The entire optical system and the flow system of the hydrosoloscope was mounted on a cast aluminum base to provide the rigidity necessary to maintain alignment of parts. The circulating pump was mounted on rubber to prevent vibration from reaching the rest of the system. The water reservoir was mounted on a felt pad to minimize the transmission of vibration into the water. The manometer sample feed system, Figure 10, was tested and produced a flow so rapid that turbulence was created in the counting chamber. It was discarded in favor of the hypodermic syringe feed that had previously proved satisfactory. The sample flow rate produced by the slowest speed of the large syringe feed mechanism with a 20-milliliter syringe was 0.275 milliliter per minute.

Several incandescent lamps and a carbon arch lamp were tested. The 6-volt, 18-ampere ribbon filament lamp used with the aerosoloscope was found best. A socket for this lamp and a second condenser lens were mounted in a microscope lamp housing. The lens shortened the focus of the condenser so that the lamp assembly, including the transformer, could be mounted on the cast base. The signal filter circuit (Figure 11) was constructed and mounted in a suitable housing on the aluminum base.

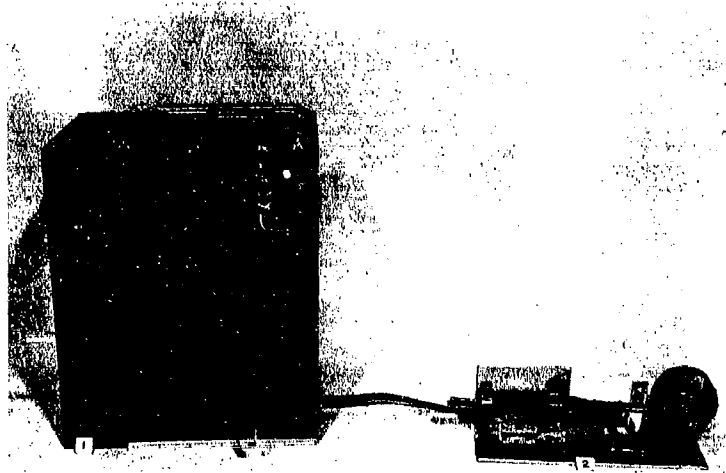


Figure 10. Manometer Feed System (left) and Cam-Driven Piston Feed System (right). (FD Neg C 6408)

Several 1P21 photomultipliers were tested and the most sensitive one was selected. A magnetic shield was added to the phototube housing to reduce pickup from stray magnetic fields.

The aerosoloscope electronic unit was serviced, tested, and the necessary electrical interconnections completed. On 16 May 1961, the first test of a complete hydrosoloscope system was conducted. The particles used were several dilutions of 0.8-micron polystyrene latex. These particles were counted in Channel 1 (1- to 1.4-micron) with an occasional count in Channel 2 (1.4- to 2-micron) caused by coincidence or clusters of particles. The counts obtained were proportional to the dilutions used. The accuracy of the counts could not be estimated because an independent accurate estimate of the number concentration was not available. It was apparent, however, that particles were being counted, although numerous technical difficulties demanded immediate attention. For some unknown reason, the noise level of the apparatus suddenly increased so that an attempt to count a suspension of Serratia marcescens at that time was not successful.

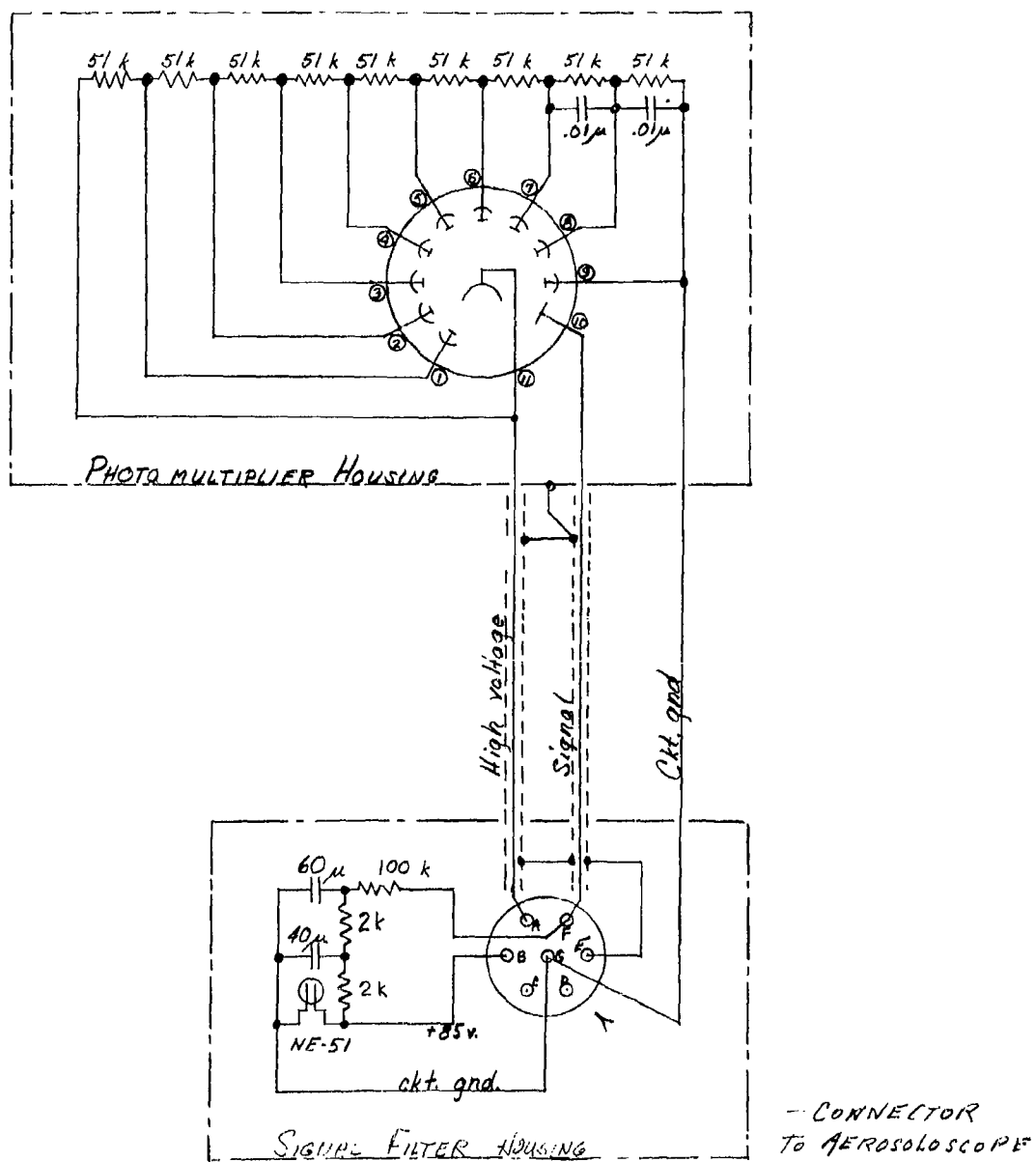


Figure 1. Schematic Diagram of Signal Filter Circuit.

Recheck of the optical alignment and focusing revealed that the light beam was slightly out of focus where it intersected the sample stream because the focal length of the objective was longer in water than in air. The counting chamber had been designed according to the manufacturer's data (Figure 6) for the working clearance in air. The working clearance in water is longer by a factor of 1.33, which should be taken into consideration in future design. The immediate problem was corrected with a thicker water-sealing gasket between the objective and the chamber. The thicker gasket was also helpful in obtaining an improved water-tight seal. The magnification of the optical system was about 25X. Considering this new value of system magnification, it was apparent that the sensing volume was larger than necessary. Brass extensions were designed and constructed that would place the apertures 2 7/8 inches from the eyepiece lenses. With a 0.012-inch circular aperture, the sensing zone at focus was about 30 microns in diameter.

Since the exact magnification of the objective in water was not known, nor was that of the F:1.4 projection lens when used as an eyepiece, some experimentation was required. The best measurements indicated the new magnification to be about 30X. Where the component magnifications are known, the total magnification M can be calculated from the formula $M = M_O \times M_E \times S/250$, where S is the distance from the eye lens to the aperture, and M_O and M_E are magnifications of objective and eyepiece respectively.

Apertures 1 by 3 millimeters were substituted for the 0.012-inch apertures in the illuminating and viewing optics. The sensing zone under these conditions was then about 33 by 100 microns (0.0013 by 0.004 inch).

With these modifications complete, a test was made on 9 June 1961, using dilutions of S. marcescens containing 1×10^4 , 1×10^5 , and 1×10^8 organisms per milliliter. The 1×10^4 dilution was counted with the hydro-soloscope. An average count of 1.3×10^4 was obtained, demonstrating that it was possible to count bacteria with this device. The other concentrations were too high for the machine to count without large error due to coincidence of particles in the sensing zone at the minimum sample feed rate available (0.275 milliliter per minute).

Table I shows the results of counting a suspension of S. marcescens, prepared by Allied Sciences Division on 12 June 1961, with a concentration of 10,000 organisms per milliliter. Since the sample flow rate was 0.275 milliliter per minute and a sampling time of 30 seconds was used, the quantity of sample was 0.137 milliliter. At 10,000 organisms per milliliter, the expected count would approximate 1370. The gain of the pulse height analyzer was adjusted with clean filtered water flowing through the apparatus so that relatively few counts were registered in Channel 1. The sample was then introduced. A series of 30-second counts were taken and the data recorded as shown in the table. The counts obtained in Channel 1 were

TABLE I. COUNTING SERRATIA MARCESCENS WITH THE HYDROSOLOSCOPE^{a/}

Sample	Channel No. 1						Channel Counts						Counts	
	1-1.4	2	3	4	5	6	1-1.4	2	3	4	5	6	Total	Theoretical
Background (filtered water)	9	1	1	1	0	0	9	1	1	1	0	0	12	0
	27	0	0	0	0	0	27	0	0	0	0	0	27	0
	16	0	0	0	0	0	16	0	0	0	0	0	16	0
0.137-ml Sample of <i>S. marcescens</i> 1×10^8 org/ml (prepared with filtered water)	1000	181	32	10	6	1	1000	181	32	10	6	1	1230	1370
	1120	205	37	9	1	1	1120	205	37	9	1	1	1373	1370
	1018	236	34	9	0	1	1018	236	34	9	0	1	1298	1370
	1062	205	37	14	5	1	1062	205	37	14	5	1	1324	1370
	1018	238	33	10	5	1	1018	238	33	10	5	1	1305	1370
	1057	241	34	14	4	0	1057	241	34	14	4	0	1350	1370
	1077	200	39	12	4	2	1077	200	39	12	4	2	1334	1370
	1033	393	38	15	4	1	1033	393	38	15	4	1	1484	1370
	957	213	32	15	4	2	957	213	32	15	4	2	1217	1370
	865	211	36	18	4	3	865	211	36	18	4	3	1137	1370
	917	225	42	13	2	1	917	225	42	13	2	1	1200	1370
Background (filtered water)	1	0	0	0	0	0	1	0	0	0	0	0	1	0
	4	0	0	0	0	0	4	0	0	0	0	0	4	0

a. Dynode voltage 640, 30-second counts, 12 June 1961.

somewhat below the expected count, but the total count in Channels 1 through 6 was not too far from the expected value. It is probable that the particles counted in Channels 2, 3, and 4 were clusters of organisms that would produce a single colony when cultured on an agar plate. After eleven counts, filtered water was again allowed to run through the apparatus and a second background count was taken.

On 14 June 1961, a sample of S. marcescens was received that had been prepared with unfiltered water and was grown in unfiltered medium. The counts shown in Table II indicate large numbers of the larger-sized particles. This sample was then used to test the ability of the apparatus to recognize dilutions of a sample on an order-of-magnitude basis. Four 30-second counts were run on this sample; it was then diluted with an equal volume of filtered water and four more counts were made. This sample was again diluted with an equal volume of filtered water and a third series of four counts was made. The counts registered on the individual channels (Table II), as well as the means of the total counts, indicate that, as a good approximation, the count was reduced by one-half at each dilution. The ability of the instrument to discriminate particles of the several sizes rather consistently is demonstrated by the reproducibility of successive counts at each dilution. An improved instrument and additional care in dilution and sample handling should yield improved precision.

2. Later Experiments

On 14 August 1961, an attempt was made to find a better solution to the problem of introducing and metering the sample. The glass mercury manometer feed system (Figure 10) had been previously tested and found to feed the sample much too fast. This system was repaired, modified, and retested. The new feed rate was approximately 0.3 milliliter per minute, but there were other features that made this system undesirable. It was difficult to change samples without some intermixing of the new sample with the previous one. The system required complete emptying of the manometer, a flush with clean water, and a refill with the new sample.

A feed device using a cam-driven piston was constructed (Figure 10) and several methods of connecting it into the system were considered, such as the use of a pair of ball check valves. Another feed system tried involved a balance between the negative pressure in the counting chamber and the capillary attraction in the feed tube, similar to that used in the Evans Electroselenium Limited apparatus and by Dr. Crosland-Taylor in England. In the course of this work, the negative pressure was 0.25 inch of water. A gravity feed system was also tried by reversing the inlet and outlet tubes and introducing the sample at the top of the counting chamber. The sample was allowed to flow by gravity from a small elevated reservoir into the inlet needle. It was found that the filtered water supply level had to be 20 inches below the sample level to give a feed rate of 0.3 milliliter per minute. The problems of contaminating a sample with residues from preceding samples and accurate metering of the flow are unsolved problems.

TABLE II. DILUTION TEST DATA^{a/}

Sample	Counts at Indicated Channels						Total
	1	2	3	4	5	6	
Sample 1: <u>S. marcescens</u> (prepared with unfiltered water)	1692	1305	529	203	112	41	3882
	1762	1016	739	355	158	69	4099
	1628	1252	459	201	118	36	3694
	1496	1023	450	210	80	50	3309
Mean	1619	1149	544	242	117	49	3721
2:1 Dilution of Sample 1	751	453	262	96	42	14	1618
	842	485	244	109	37	18	1735
	852	480	208	99	33	6	1678
	838	414	226	89	33	6	1606
Mean	821	458	230	76	36	11	1632
4:1 Dilution of Sample 1	258	222	116	54	15	3	668
	238	182	97	51	19	4	591
	244	181	91	38	26	3	583
	236	190	124	44	16	4	614
Mean	244	206	107	49	19	4	629

a. 30-Second counts, 14 June 1961.

None of the feed systems tested was successful in replacing the driven syringe feed system. This device, with a 20-milliliter glass syringe, produced a reliable feed of constant rate and known volume. The hypodermic needle that introduced the sample into the sensing zone was reduced from a 29-gauge to a 33-gauge with an inside diameter of 0.004 inch (100 microns). The spun-down 22-gauge tube originally used at the inlet was replaced by a straight 27-gauge needle with an inside diameter of 0.008 inch. When tested, the entering sample stream tended to billow around the inlet needle and did not flow through the chamber as a rod-like stream. Some further difficulties were encountered with alignment and the focus of the optics. The 27-gauge needle on the liquid outlet was replaced with a larger 24-gauge needle (ID 0.012 inch) that increased flow velocity in the sensing zone, straightening and narrowing the sample stream. The optics were refocused, the alignment was checked, and the extension was removed from the photocell optics; 1- by 3-millimeter apertures were used.

A test run on 6 September 1961 with a sample of S. marcescens (1000 organisms per milliliter) yielded consistent counts, but they could not be correlated with the concentration of the sample. It appeared that this sample was contaminated by fine particles of unknown origin, since the counts tended to be high. The noise level was also high.

A power supply, lamp housing, and starter assembly were modified and assembled to operate a special short-arc xenon lamp made by the Duro-Test Corporation. A 50-watt lamp with a 0.012-inch arc length was borrowed from Vision Warfare Branch, Ft. Belvoir, on 24 August 1961. Initial test of this lamp on the hydrosoloscope gave a pronounced increase in noise level that indicated stray light in the system. To minimize this difficulty, the inside surfaces of the optical tubes were coated with flat black paint. The needle guide disc on the sample inlet was eliminated and a new and simplified inlet for clean water and sample was designed and constructed in the Model Shop. The revised internal liquid flow system is shown in Figure 9.

The 1P21 phototube was replaced with a 5819 phototube and connected to the signal filter circuit (Figure 11).

These changes significantly reduced the apparatus noise level. A test was conducted on 14 September to compare the short-arc xenon lamp and the ribbon filament lamp. The results of counting a test sample of S. marcescens are shown in Table III.

TABLE III. LAMP COMPARISON DATA^{a/}

Lamp	Channel Number							
	1	2	3	4	5	6	7	8
Ribbon Filament	1492	318	81	32	10	2	0	0
	1422	304	64	30	11	4	0	0
Short Arc Xenon	1635 ^{b/}	507	1236	334	54	16	2	2
	1701 ^{b/}	436	1363	365	75	-	1	4

a. Dynode voltage 900, 30-second counts, 14 Sept 1961.

b. Not significant; some counts due to system noise.

Counts that fell in Channel 1 (1- to 1.4-micron) when the ribbon filament lamp was used moved up to Channel 3 (2- to 2.8-micron) when the xenon lamp was used, a shift of two channels. This change is equivalent to doubling the apparent size of the particles. Most of the counts in Channel 1, and probably many of the counts in Channel 2, when the xenon lamp was used, may have been caused by particles less than 0.5 micron in diameter. The Channel 1 counts are not significant, however, since an unknown number of the counts appeared to be due to system noise. It was also found that the dynode voltage on the multiplier phototube could be reduced from 1000 volts with the filament lamp to 800 volts with the arc lamp, with counts remaining in the same channels. This was a good increase in sensitivity, but it was not really needed for bacterium detection. The xenon arc did not appear as stable as the ribbon filament lamp and was put aside in favor of the simpler lamp. It would be very useful, however, in counting fluorescent particles or in the detection of particles 0.5 micron or less in diameter.

A search was made for a method of standardizing and calibrating the apparatus, but none of the schemes considered was satisfactory. It was then decided to use the giant cell culture technique to obtain samples containing both normal-sized cells and elongated cells, then standardize the over-all sensitivity of the instrument so that the normal-sized cells are counted in Channel 1. The giant cells should then be counted in a channel recording larger particles. At this point the counting of clean suspensions of micro-organisms was essentially routine.

3. Giant Cell Culture and Machine Counting Experiments

A technique was worked out in the culture of the microbial giant form in which the diluent and the nutrient were carefully filtered through membrane filters to remove all particles that might be counted by the instrument. Only the inoculum was unfiltered. The results of counting a 2½-hour culture of *S. marcescens* containing 0.2 microgram per milliliter of mitomycin C are shown in Table IV.

TABLE IV. RESULTS OF COUNTING AFTER ATTEMPT
TO CULTURE GIANT *S. MARCESCENS*^a/

Sample	Channel Number					Expected Count
	1	2	3	4	5	
Control 10 ³ org/ml	140	2	3	1	0	137
<i>S. marcescens</i> , 2½-hr culture plus 0.2 µgm/ml of mitomycin C	96	9	1	1	0	No growth of giant forms (Confirmed by microscopic examination)
	107	6	3	0	0	
	120	7	1	0	0	
	206	4	1	0	0	
	111	6	3	0	0	

a. Dynode voltage 900, 30-second count, 0.137-milliliter sample
25 Sept 1961.

It is apparent that very few counts were recorded except in Channel 1, where normal-sized cells should be counted. Microscopic examination revealed that no growth in size had taken place, confirming the indications of the hydrosoloscope.

In the following trials of giant cell culture with filtered nutrients, growth seemed slow and very irregular. Typical results are shown in Table V, Tests A to D. Two samples were used; a control with 1000 organisms per milliliter, and a similar culture of *S. marcescens* to which 0.2 microgram of mitomycin C per milliliter had been added. The control was used to adjust the gain of the hydrosoloscope for the expected count of approximately 137, Test A. The sample containing the mitomycin was then tested, Test B.

TABLE V. GIANT CELL CULTURE EXPERIMENT WITH
MITOMYCIN C, HYDROSOLOSCOPE COUNTING^a

Test Sample	Channel Number					Remarks
	1	2	3	4	5	
A. Control 10 ³ org/ml	137	17	4	0	0	Gain adjusted for about 137 count
	132	15	1	1	0	
B. <u>S. marcescens</u> , culture plus 0.2 µgm/ml of mitomycin C	175	44	11	3	0	Channel 1 and 2 show a small increase over the control
	159	32	6	3	0	
	158	41	5	1	0	
	149	32	7	2	0	
	134	33	3	0	0	
	153	31	3	0	0	
	127	32	2	3	0	
C. Clean water background	5	0	0	0	0	Dynode voltage increased to 1000
	4	1	0	0	0	
D. <u>S. marcescens</u> , culture as above	163	24	2	0	0	No real change on count
	186	15	4	2	0	
	148	15	4	0	0	
	166	24	2	0	0	
E. <u>S. marcescens</u> , culture as above	2089 ^b /	158	7	2	0	Gain increased one full channel
	2048 ^b /	148	8	1	0	
	1988 ^b /	150	9	1	0	
F. <u>S. marcescens</u> , culture as above	11	5	2	0	0	Gain lowered two full channels
	21	3	0	0	0	
	23	3	2	0	0	

a. Dynode voltage 980, 30-second count, 0.137 milliliter sample,
26 Sept 1961.

b. Noise and small particles.

The results indicate some increase in concentration of normal-sized cells due to reproduction and a relatively small count of cells that had enlarged, as indicated by increased counts in Channels 2 and 3 over the control. Following these counts, the dynode voltage on the photomultiplier was increased to 1000 volts. The background count with clean water remained fairly low, Test C. The sample was again counted, but the counts were essentially unchanged in Test D.

As a separate experiment, the gain of the system was then increased one full channel. Qualitatively, the counts that were previously in Channel 1 were then found in Channel 2, as shown in Test E. The gain of the system was then lowered two full channels and it was found that the counts previously falling in Channel 3 were now falling in Channel 1 (Test F). These trials clearly indicate that particles and organisms were actually being measured and counted in their proper relative size classes. The trials demonstrated the feasibility of the instrument design and its ability to recognize particles in a liquid and evaluate their size.

4. Exploratory Experiments in Giant Cell Culture

The poor results obtained in the culture of the giant form as a test of microbial viability, shown in Table V, were not consistent with earlier work^{1*} and led to an exploratory study attempting to define in a little more detail the microbiological problems. *S. marcescens* was grown at room temperature in concentration of approximately 10^5 cells per milliliter. Both trypticase soy broth and synthetic nutrient were used as media. Mitomycin C, Celbenin,** penicillin G, and urea were tried as mitotic inhibitors. Consistent results were not obtained.

Mitomycin was tested at concentrations of 0.2, 2, and 20 micrograms per milliliter of culture. The samples were examined by phase contrast microscope at two-hour intervals. Cultures were allowed to grow for as long as 18 hours. Samples with 20 micrograms per milliliter of inhibitor were observed to grow to giant form in the shortest time, but very irregularly. Some cells would generate long filaments while others retained their normal size. Then the long filaments would disappear with time, apparently becoming too large and bursting. A similar situation was true of the samples at two micrograms per milliliter. Cells took longer to grow to the filamentary form, but the cells were just as nonuniform in size. Little filamentary growth was observed with the cultures at 0.2 microgram per milliliter. Samples of these cultures showed an increase in cell concentration but very little increase in cell size, even after 18 hours, indicating that cell division was not inhibited by this concentration of agent under these conditions.

* Unpublished.

** Beecham Research Laboratories, Ltd., Brentford, England.

The preceding test was repeated using unfiltered trypticase soy broth and a fresh sample of mitomycin C. The results of microscopic examination at 3½, 4, and 5 hours are shown in Table VI. In general, it appears that at low concentrations of mitomycin, reproduction is not inhibited and there is some reproduction, increasing the number of normal-sized cells. At high concentrations, there appears to be considerable variability; some organisms produce long filaments, others grow more slowly to produce medium to short filaments, while others do not appear to grow at all. With time, the long filaments burst and disperse their contents. This variability is probably another example of the normal reaction of a bacterial population to a stress.

C. CONCLUSIONS

The test results indicate that although the instrumentation of a viable bacterium counter appears well under control, the problems involved in differentiating between viable and dead organisms are probably beyond a quick solution through do-it-yourself microbiological research. The facilities available to Technical Engineering Division did not permit further pursuit of a solution to the microbiological problem, although this general approach appears to be a feasible one in spite of obvious technical difficulties.

The culture of giant bacterial forms suitable for machine counting of the viable cells on a size basis will require care to exclude foreign particles. The diluent and nutrient, as well as glassware, stoppers, etc., must be kept clean and free of particles that can contaminate the sample.

The microbiological problem of devising a suitable system wherein all the viable organisms in a sample grow to a larger and uniform size is a difficult one. Future work could involve tests to determine if some important constituent of the nutrient is removed when filtered through a membrane filter, and tests to determine any interaction between the antimitotic agent and the various constituents in the nutrient media. The mitomycin C obtained from Japan for these tests appeared to be variable in its inhibiting activity. It is known to be decomposed by light. The tolerance of the material to various light levels is not known and may have been an interfering factor. The Japanese authors^e found that 0.02 to 0.1 microgram per milliliter of mitomycin C completely inhibited biosynthesis of DNA by Escherichia coli, although that of protein and RNA were unaffected.

TABLE VI. GROWTH TESTS OF *S. MARCESCENS* IN UNFILTERED TRYPTICASE SOY BROTH WITH MITOMYCIN^a/

Incubation Time, hr	Mitomycin Concentration, $\mu\text{g/ml}$	<i>S. marcescens</i> Concentration	Organism Size ^b /	
			% Normal	% Length of Filaments
3½	20	normal	60	40 medium
	2	3X normal	100	
4	20	normal	30	20 long 50 medium
	2	3X normal	90	10 medium
	0.2	4X normal	80	20 short
5	20	Less than normal	40	30 medium 30 short
	2	2X normal	30	10 long 60 medium
	0.2	5X normal	80	20 medium

a. 29 September 1961.

b. Estimated by microscopic examination.

Because it was observed that the giant forms developed much more quickly in synthetic nutrient medium, some work on nutrient composition or with stabilizing agents such as spermine could be productive.

The advances that have been made as a result of this development are directly applicable to the solution of other problems. The most important of these may be the identification and counting of fluorescent particles suspended in a liquid sample. The fluorescent particles could be inert "cloud tracer" particles of zinc cadmium sulfide phosphor, a specific microorganism tagged with a fluorescent antibody, or a microorganism tagged for identification as viable with a vital stain such as acridine orange. Experiments in counting fluorescent particles would require only minor changes to the present hydrosoloscope. Substitution of a xenon or a high-pressure mercury-arc lamp for the incandescent lamp and the installation of suitable optical filters would be about the only changes required.

The successful development of a "fluorosoloscope"-type instrument would permit the counting of viable bacteria in the presence of dead ones by application of the vital fluorescent staining techniques that have proved successful in certain applications, notably those of Dr. E. S. Anderson in England and of Dr. Siegfried Strugger in Germany. A technique for identifying a specific organism by fluorescent antibody in the body of a fluid without fixation of the organisms is under development. When the microbiological techniques are developed, equipment would be quickly available to relieve the experimenter of the arduous and time-consuming task of identifying and counting manually with a fluorescent microscope.

III. MEASURING AN ELECTRICAL PROPERTY OF A BACTERIUM

A. INTRODUCTION

As pointed out previously, a live bacterium is essentially a closed system electrically.¹ When a bacterium is placed in an electric field, only the ionizable surface groups are active, as has been shown by studies of electrophoretic mobility. There is some electron conduction over the surface but little, if any, transfer of charge through the body of the organism. The passage of ions and electrons through the osmotic membrane is under biological control. The individual bacterium may be considered as a dielectric particle with a measurable dielectric constant different from that of the surroundings. It was conceived that bacteria or other small particles could be counted by an instrument sufficiently sensitive to detect the change in capacitance of a sensing zone as individual particles passed the measuring point of a very fine flowing stream of sample liquid being monitored by the instrument.

An electronic device that approaches the required sensitivity is an oscillator-detector that has been used for many years in such diverse applications as dynamic engine pressure indicators,⁷ explosive mine detectors, phonograph pickups, and industrial instruments.⁸

The electronic apparatus has three principal parts: first, a radio-frequency oscillator, and second, a tank circuit having inductance and capacitance of which the capacitance of the sensing zone is a part. The fundamental frequency of the tank circuit as a whole is influenced by the capacitance of the sensing zone. The third element is a sensitive detector that recognizes small changes in the resonant frequency of the tank circuit. Theoretical sensitivity in measurement of capacity is of order of 10^{-11} microfarad.

B. DESCRIPTION OF THE DIELECTRIC CONSTANT MONITOR

The dielectric constant monitor (DCM) has three essential parts: the electronic oscillator-detector, the sensing head through which the sample of liquid flows, and a means of pumping the liquid sample through the sensing head. Construction details of the first experimental sensing head, designed and constructed in December 1960, are shown in Figure 12. The heads were constructed by diamond-drilling a hole about 0.008 inch in diameter through a microscope cover glass and inserting two pieces of 0.001- by 0.004-inch tungsten ribbon through the hole and folding each back on itself so that the ribbons formed a pair of electrodes, one on each side of the hole, as shown in Figure 12a. A wire 0.0015 inch diameter was coated with detergent, for ease of subsequent removal, and inserted through the hole in the cover glass to form the orifice. A ring of epoxy cement (Eccobond 26)

HEAD DESIGNS
DIELECTRIC CONSTANT MONITOR

Figure 12. Design Details of Experimental Sensing Head.

was placed on each side of the cover glass and the ground-off ends of two test tubes were pressed into the cement (Figure 12a). The cement flowed around the wire, filling the hole in the cover glass. When the cement had solidified, the wire was removed, producing an orifice of the type shown in Figure 12b. The oscillator-detector circuit was constructed.

The assembled apparatus is shown in Figure 13. The oscillator-detector unit is shown on the left. The sensing head was mounted within the chassis of the oscillator-detector in an attempt to reduce stray capacitance by making the leads as short as possible. A sample to be tested was pumped from the beaker on the right (Figure 13) by the proportioning pump, then passed through the sensing head and exhausted from the tube shown at the top of the apparatus on the left. The electrical output was read on a voltmeter for tuning. Signal pulses were shown on a cathode-ray oscilloscope.

The oscillator-detector circuit shown in Figure 14 was constructed in two versions, one operating at 46.7 megacycles, as shown, and the other operated at 2.1 megacycles.

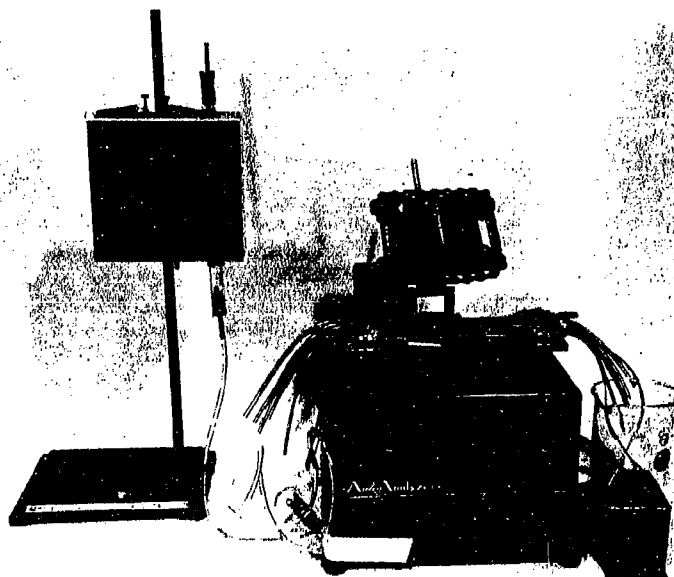


Figure 13. Dielectric Constant Monitor Test Setup.
(FD Neg C-6409)

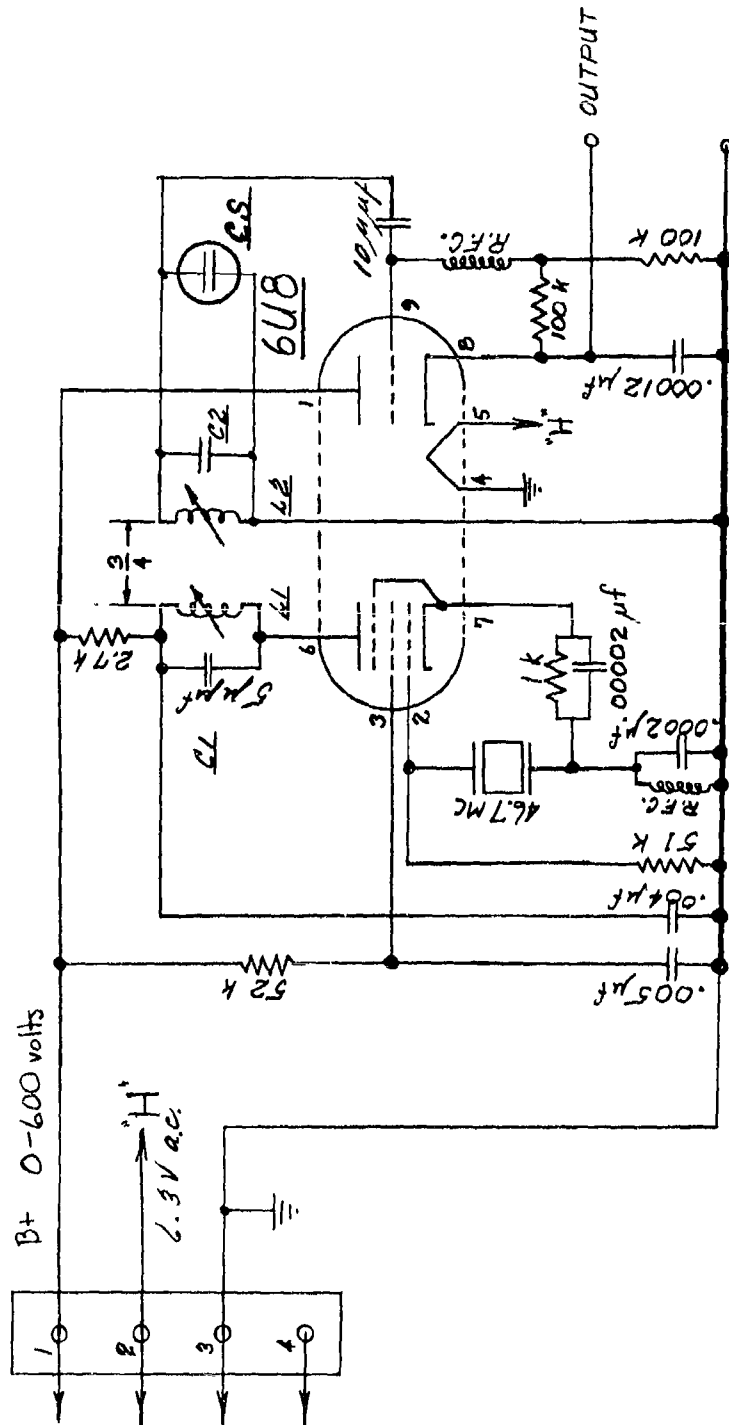


Figure 14. Schematic Diagram of Dielectric Monitor.

C. SENSITIVITY REQUIREMENTS

As originally constructed and tested in June 1961, the sensing head (Figure 15) worked into a remote tank circuit that in turn reacted on the measuring tank circuit. Initial trials indicated low sensitivity. The circuit was modified to eliminate the remote tank circuit, and a test assembly was tried wherein the proportioning pump moved water and air through the sensing head. The dielectric constant of the sensing zone would be changed from that of water, with a value of 78, to that of air, with a value of 1, as a minute bubble would pass. The operating frequency was 46.7 megacycles. When the apparatus was tuned to maximum sensitivity, bubbles caused a change of 10 to 12 volts at the indicator. It was estimated that the ratio of the total sensitive volume to the volume of a single bacterium is about $10^6:1$. The indicator, then, would need a 0.01-millivolt sensitivity to indicate individual bacterial cells. It was further estimated that since the one-millivolt



Figure 15. Dielectric Constant Monitor Sensing Head.
(FD Neg C-6410)

level of a signal is easy to detect, an improvement in the sensitivity of the apparatus by a factor of 100 should open the way to feasibility. Further research and experimentation centered on improving the design of the sensing heads and the sensitivity of the electronic apparatus. During July and August 1961, six different heads and four electrical circuits were tried.

D. RESULTS

During the initial period, experiments were performed with a d.c. pulse detector circuit and with a simple circuit consisting of a battery, the sensing head, and a one-megohm resistor. These input circuits were followed by a special pulse amplifier with clipper.⁹ The pulses were displayed on a cathode-ray oscilloscope. Electrolysis with gas evolution was a problem with d.c. apparatus. These effects produced pulses that under some conditions were difficult to distinguish from signals; consequently, the d.c. systems were put aside in favor of the radio frequency system.

Published information^{10,11} indicated that bacteria should show a maximum difference in dielectric constant as compared with water at frequencies considerably lower than 46 megacycles. On the other hand, the sensitivity of the apparatus decreases with the decreasing frequency of operation. A test conducted with apparatus operating at 2.1 megacycles indicated that the instrument's loss of sensitivity with decreasing frequency was greater than the gain in dielectric constant difference between water and bacteria.

Work with the dielectric constant monitor then centered on the 46.7-megacycle oscillator-detector and on improving the construction of sensing heads. Testing was done with rust spores and 0.8-micron polystyrene microspheres. The techniques previously described were developed for producing the sensing-head types shown in Figure 12. Heads 1, 2, and 3 were constructed as shown in Figure 12b. The construction shown in Figures 12c and 12d was used for heads 4, 5, and 6.

A water suspension of 30-micron rust spores was tested with head 3 and the 46.7-megacycle oscillator; signals that were obtained from the spores ranged from 0.8 to 1.2 volts. It was observed that particles in air appeared to be detected with this apparatus. Modification of the electronic apparatus to the circuit (Figure 14) resulted in the marked increase in sensitivity. However, before experiments could be performed with bacteria, head 3 failed because the epoxy cement loosened, and all attempts to repair it were unsuccessful. This unfortunate occurrence on 4 August 1961 delayed further testing. The hastily constructed heads that followed were not as sensitive or as successful. Head 4 was defective because of some misalignment of the electrodes so that on test it yielded no useful signals. Head 5 had good alignment and a very fine slot, very close to the desired 0.001- by 0.004-inch size, but it clogged almost immediately and after it was cleaned the good results obtained with head 3

could not be duplicated. Head 6 was also plagued with clogging difficulties. A technique for clearing the stoppage by a high-voltage spark was successful, but appeared to damage the head.

The 46.7-megacycle oscillator-detector and the sensing head 5, constructed as shown in Figure 12c, was rather sensitive and was tested with a suspension of 0.8-micron polystyrene microspheres and a suspension of Serratia marcescens with a cell concentration of 1000 organisms per milliliter. The electrical pulses obtained appeared random and a reproducible count could not be obtained with either suspension.

The basic difficulty with the design appears to lie in the large size of the slot-shaped orifice and electrodes, which result in a volume of the sensing zone of about 62,500 cubic microns. The one-micron volume of a single bacterium can cause only a very small change in the effective dielectric constant of this volume. The electronic apparatus was not sufficiently sensitive to detect the change. Clogging problems would probably increase with further reduction in orifice diameter below 0.001 inch, but width and thickness can be considerably reduced. The work was discontinued on 8 September 1961.

E. CONCLUSIONS

These expoloratory experiments with the dielectric constant monitor (DCM) apparatus indicated that it is a feasible approach to detection of small particles for counting. It was not possible to continue the experiments far enough to determine if a difference exists between signals arising from living bacteria and those from dead bacteria. This experiment would require some refinement in apparatus and in the technique of constructing the sensing head.

Future work could center on the circular orifice type of sensing head construction shown in Figure 12b, with a reduction in electrode width to 0.001 inch and the use of thinner glass, mica, or plastic for the electrode support to reduce the depth dimension. If the depth were reduced to 0.001 inch, and the electrode width to 0.001 inch, then the sensing volume would be reduced by a factor of 100 to 625 cubic microns.

The sensitivity of the electronic apparatus could be greatly improved by electrically insulating the electrodes from the liquid stream by a thin insulating film to minimize electrical conduction across the electrodes. Lowered conductivity would improve the "Q" of the resonant electronic circuit and markedly improve the sensitivity. It is believed that the successful head 3 had a thin layer of epoxy over the electrodes that insulated them from the liquid.

Microminiaturization techniques used in electronic construction could produce electrodes and orifices with the small dimensions that are needed. It is not very practical to produce these critical parts by hand.

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APPENDIX

Early Development of a Counting and Measuring System*

The name "hydrosoloscope" implies viewing individual particles suspended in a liquid, just as its forerunner the aerosoloscope implied the examination of individual particles in air. Another potentially useful member of this class of instruments might be called a "fluorosoloscope," which would examine individual particles for the nature and degree of fluorescence.

This class of instruments operates on two important principles. The first is a flow system such that individual particles are examined one at a time, with the flow system marshalling the particles so that they pass through the detecting zone single file. The other is a measurement of the quantity of light scattered from the particle, or originating in the fluorescent particle, which carries information on the particle size. Through special instrument adaptations, it is possible that information on other characteristics such as absorption spectrum and, in the case of larger particles, the refractive index may be obtained. The most useful quantitative information carried by the scattered light from a single particle is the size of the particle, in accordance with recognized laws of optics. It is much easier to measure the intensity of a flash of light as the particle is examined than to apply a measuring scale to the particle itself or to its image to determine the size. The flash of light produced by individual particles as they pass through a sensing zone is readily converted to electrical signal pulses whose height alone, or when combined with other characteristics such as pulse duration, is proportional to the size of the particle. The pulses may then be sorted according to size into various classes and each pulse tallied in its appropriate size class.

The size discrimination provided by the electronic sorting techniques permits the counting of one type of particle, bacteria, for example, in the presence of other types of particles. In the automatic counting of viable bacteria, one of the more promising techniques proposes to inhibit the mitotic reproduction of the individual cells, which, combined with a short incubation time in the presence of nutrients, allows that from all living cells a giant form develops such as the spheroplast or filament. When such a sample was passed through the hydrosoloscope, the enlarged particles would be counted in a different class (living cells) from the smaller particles (dead bacteria).

* To 1 March 1961.

The flow system for a hydrosoloscope is probably its most critical part. During 1960 four experimental models were designed. The Mark I is characterized by the large throat area shown in Figure 1. This apparatus did not make the sample stream sufficiently small and fully stable flow was not achieved.

The Mark II was based on the blood cell counter built by Dr. Grosland-Taylor in England and used optical elements that he loaned us. The forward-scattered light from particles is detected. The flow system employed two hypodermic needles and is described in the literature.⁴ This model was not successful because of a failure of the optical system, causing a liquid leak to develop.

Figure 2 is a sketch of the Mark III sensing head and flow system. In the design the incident light is on the axis and two pickup units with associated light traps and flow system are radial, like spokes of a wheel. The purpose of the two pickup heads for scattered light, which were 10X microscope objectives, was to obtain two independent signals from the passage of each individual particle. These signals could then be added or multiplied electronically to improve the signal-to-noise ratio. Some

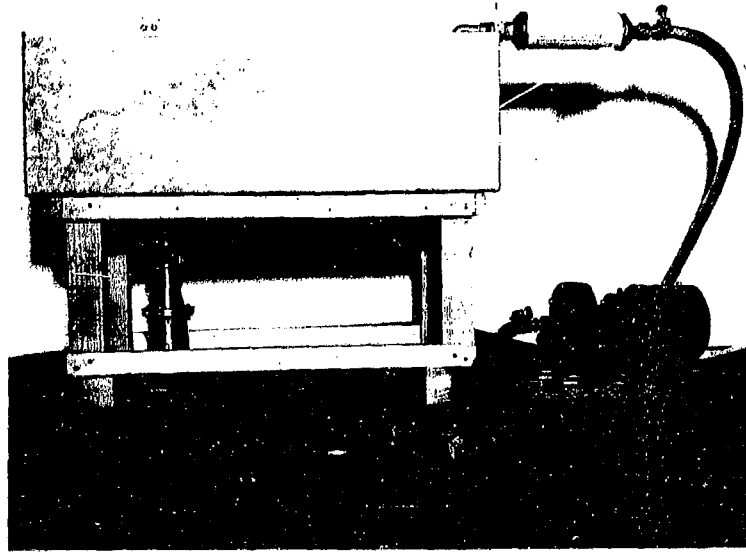
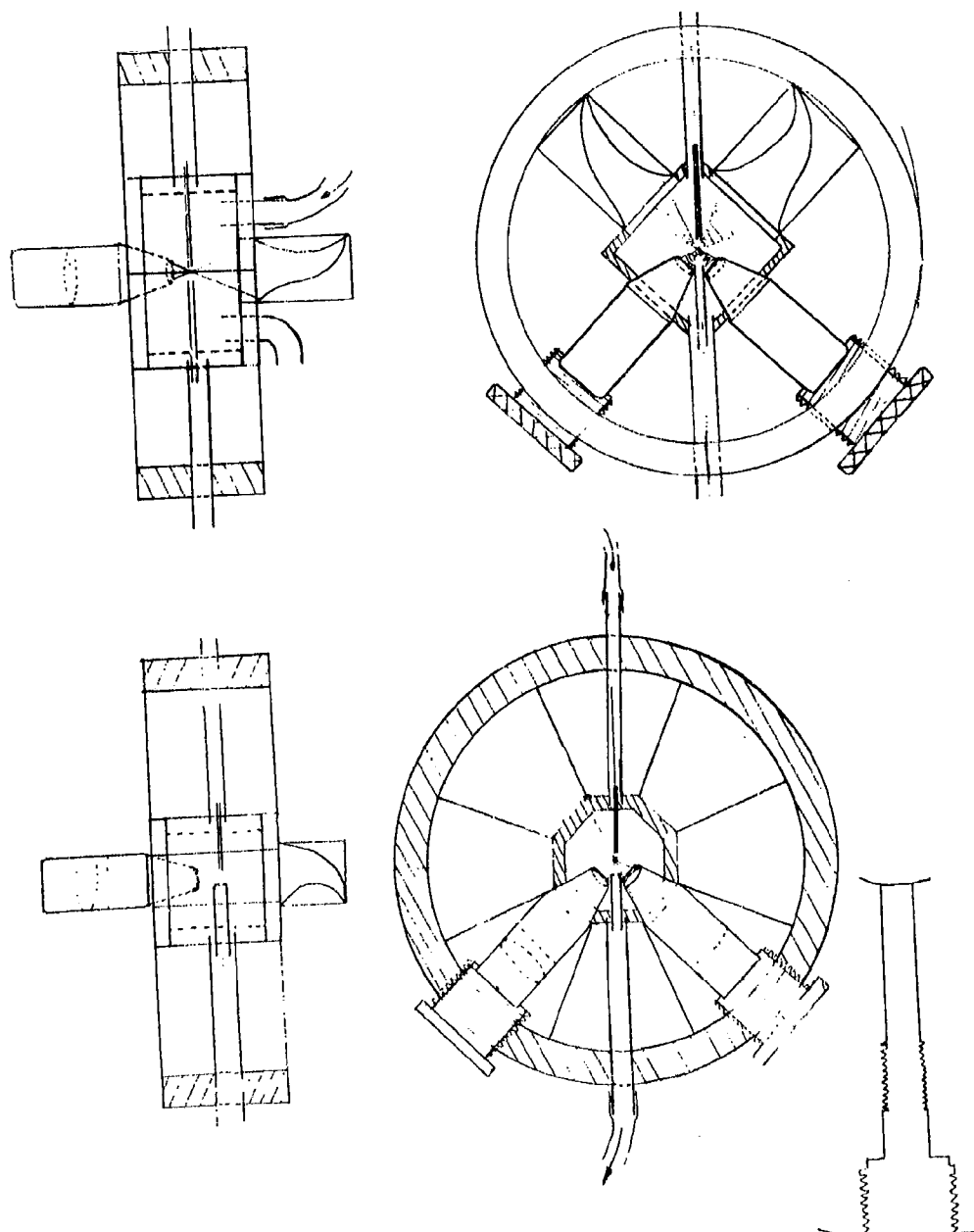


Figure 1. Mark I Hydrosoloscope. (FD Neg C-5356)



Hydrosoloscope Type-3

Figure 2. Mark III Hydrosoloscope Sersing Head and Flow System.

experimentation and testing of the Mark III have been performed* but many technical difficulties arose with alignment of three optical systems and a liquid flow system. This complex design was put aside in favor of the simpler Mark IV design.

In the Mark IV design, the direction of sample flow, illumination, and viewing are mutually at right angles. The Mark IV represents a more flexible and simplified design, better adapted to experiments for improving or to optimize the many complex and frequently interacting parameters of the system.

* Further details of this design are given in the Technical Engineering Division Report, "Viable Bacteria Counting," 1 March 1961.