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APPLICATION OF AN ELECTRONIC COUNTER IN THE EVALUATION OF SIGNIFICANT BACTERIURIA

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THE DETERMINATION of the bacterial count of urine specimens is a tedious and time consuming procedure. Therefore, a need for an automatic device for making these determinations would be of considerable value. Two years ago we considered the possibility of using an electronic instrument for the purpose of assaying the bacterial population of urine specimens. The Coulter Counter[1] appeared to have some possibilities. This apparatus was originally designed with an aperture tube of 100 microns in diameter to count red blood cells at rates exceeding 5000 per second,[2] an order of magnitude more rapid than most optical counters.[3] Kubitschek[4] in 1958 modified the aperture tube and the circuitry of an electronic unit and showed that it could be adapted for the counting and sizing of bacteria. The theoretical advantages outlined by the Coulter Bulletin T-1[5] strengthened our decision to make an attempt to devise a method for counting bacteria in urine specimens. It was obvious at the onset that several obstacles had to be overcome. Among these were such interfering substances in the urine as crystals, crystalloids, epithelial, red and white cells, etc.

MATERIALS AND METHODS

In order to explore the possibilities of using the electronic instruments for the detection of "positive urines", a variety of procedures were undertaken. The methodology will be discussed under the following headings:

1. Instrumentation.
2. Measurement of particle volume.
3. Pure and mixed cultures.
4. Dilution media.
5. Urine specimens.
6. Filters.
7. Lysing agents.

1. Instrumentation

The experiments were first performed with the Coulter Counter Model A* and subsequently with the Model B and plotter.* Both instruments have similar attachments for the sample stand which consists of the aperture tube flow control elements, electrodes and the sample beaker. Each unit has an oscilloscope which provides relative size distribution. The instruments also contain threshold level controls which provide cell size distribution data.

*The apparatus was kindly supplied by the Coulter Electronics Company. The technical details can be obtained from this firm located at 590 West 20th Street, Hialeah, Florida.

2. Measurement of particle volume.

The majority of counts with the Model A were conducted at a current setting of 5 and gain of 6. The Model B current setting was 1.0 and the amplifications were 0.125, 0.25 or 0.5. Once the instruments were properly installed, the only settings that required possible manipulation were the aperture current setting (A. C. S.), amplification and the threshold response. Occasional adjustments for improving the oscilloscope pattern were necessary and conveniently accomplished. If the smaller particles being counted were too close to the "background noise level", a more satisfactory separation can be accomplished by increasing the aperture current setting. The threshold setting knobs controlled the level of response so that for a given threshold position, only pulses that exceeded that level were counted. The procedure for our experiments consisted of taking repeated counts on the same specimen (culture or urine) starting at the lowest threshold setting and progressively raising the response level.

The particle volume was determined by counting the number of particles in a given size range, multiplying the number of particles in a given size range by the average volume of this range and adding the products for the various size ranges. This sum gave the total cell volume of the particles in the 0.05 ml. Division by the total cell count yielded the average volume per cell or minimal particle threshold (mpt).

To test the accuracy of the instruments, latex polystyrene particles were suspended in saline and successive counts at increasing threshold heights were obtained (Table I). Size distribution curves were frequently plotted since a relationship exists between pulse height and particle size.[5] Furthermore, in principle, the other calibrations which are necessary involve the volumetric calibration of the mercury syphon and determinations of coincidence loss at various count levels for a given aperture size. The occurrence of coincidence can be reduced to a minimum or point of improbability by diluting the test sample. The coincidence loss due to doublets and triplets, etc., can be expected to follow the Poisson distribution as discussed by Mattern **et al.**[6]

3. Pure and mixed cultures.

In the study of bacterial size and number during various growth phases the following groups of organisms were examined in pure culture: **Aerobacter, Alcaligenes, Bacillus, Escherichia,** enterococci, micrococci, pneumococci; alpha and beta streptococci. The cultures were grown in T-Soy broth at 37°C for periods of 4, 18 and 72 hours. The electronic studies were conducted on saline dilutions ranging from 10^{-3} to 10^{-12}. Some specimens were washed with saline and standardized at various transmittance readings (0-100 per cent).

The majority of studies on mixed cultures were performed on dilutions of urine containing the following groups of organisms: (a) coliform and **Proteus,** (b) coliform, diphtheroid and **Proteus,** (c) coliform, enterococci and **Proteus,** as well as many other combinations.

4. Dilution media.

The medium used for preparing the dilutions must be an electrical conductor. At the same time it must not deleteriously affect the particles to be counted. Our experiences have shown that Abbott's normal saline* had a low particle count and was preferable to the saline routinely prepared in the laboratory unless it was filtered by means of fritted or Seitz filters. T-Soy** broth filtered through 0.45 u. millipore or other filters (fritted or Seitz) was also extensively used as a diluent at final concentration of 10-20 per cent.

5. Urine specimens.

The urine samples were first collected from the bacteriological laboratory then from various Inpatient and Outpatient Services, as well as the Urinalysis Laboratory. The specimens examined by the Coulter Counter procedure were also examined by the usual bacteriological methods for identification and quantitation and frequently by the Urinalysis Laboratory. Undiluted and saline diluted (1:10, 1:50, 1:500) specimens were examined during our early studies until it was decided that a 1:50 dilution was the most suitable. As the experiments progressed, the dilutions were made in T-Soy broth and incubated at 37°C in order to increase the prediction on specimens whose counts ranged from 25,000 to 700,000 cells per ml.

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*The normal saline was obtained from Abbott Laboratories, North Chicago, Illinois.

**The T-Soy broth in the dehydrated state was produced by Baltimore Biological Laboratories, Baltimore, Maryland.

Table I

Multiple specimen counts of saline, latex and coliforms using Coulter Counter Model A

	5	10	20	30	40	50	60	70	80	90	100	30*
SALINE THRESHOLD												
Counts												
1.	104	58	26	20	24	17	12	9	6	12	6	22
2.	116	58	33	17	22	10	8	8	9	7	8	18
3.	115	57	26	23	21	13	13	11	12	5	7	22
AVERAGE	112	58	28	20	22	13	11	9	9	8	7	21
COLIFORM THRESHOLD												
10^8 18 hours												
1.	5781	3548	1765	1218	991	736	709	611	514	484	433	1173
2.	4243	3430	1802	1247	1049	742	688	607	546	484	453	1251
3.	4825	3173	1763	1209	913	763	627	605	530	500	445	1149
AVERAGE	4950	3384	1777	1225	984	747	675	608	530	489	444	1191
LATEX THRESHOLD												
1.	5159	1536	1299	1260	1167	972	814	724	741	564	504	1276
2.	4378	1553	1246	1308	1157	957	816	776	702	573	485	1247
3.	3107	1551	1361	1293	1198	1005	787	783	708	602	510	1216
AVERAGE	4215	1547	1302	1287	1174	978	806	761	717	580	500	1246
COLIFORM-LATEX												
1:1 THRESHOLD												
1.	4281	2392	1705	1432	1225	981	836	810	739	611	595	1387
2.	4012	2536	1648	1434	1197	1007	889	810	736	607	571	1424
3.	4055	2569	1795	1427	1209	985	880	835	774	618	571	1439
AVERAGE	4116	2499	1716	1431	1210	991	868	818	750	612	579	1417

The data illustrates a cross-section of the readings obtained with the Model A at various thresholds. The results at the threshold readings of 5 show greater variation due to background noise which may also be prominent at settings of 10 and 15.

*Note—at the completion of each series, a threshold setting of 30 was selected at random in order to check on the degree of variation on the same specimen.

6. Filters.

Since blocking of the aperture was frequent with the undiluted specimens especially with the 19 and 25 u. tubes, it was decided to filter the specimens which were cloudy or showed considerable sediment on standing. A 10 u. clarifying filter was used but plugged readily and proved to lack uniformity. As a result the counts were erratic and frequently showed 20-30 per cent of the original cell count. At present, we are experimenting with 3, 5 and 10 u. millipore filters.

7. Clarifying procedures.

In an effort to remove the large particulate structures in urine, the following agents have been considered: (a) saponin[7] or 0.1N HC1[8] for stromatolysis of red blood cells, (b) Streptolysin[9] for the lysis of epithelial cells, (c) Allen **et al**[10] have described such lysants as urea, sodium lauryl sulfate, Triton X-100, X-114 and X-102 and sulfonic N-95 for the destruction of red blood cells and progressive lysis of leukocytes. Although these agents[10] are being considered, we have not had the opportunity to test these as yet.

Results

The evaluation of the Coulter Counter Models A and B for the routine screening of "positive urine" was based on many thousand of counts on saline, latex polystyrene particles, pure and mixed cultures, as well as a wide variety of urine specimens. In order to determine the reproducibility of the counts at the onset, the replicate studies were performed chiefly on saline, latex and cultures of coliform. Considerable variation was observed during the first six weeks. The reasons for the erratic counts were due to the following: (a) variation in line voltages due to centrifuges, elevators, refrigerators, etc., (b) airborne noises, such as doors being slammed, (c) fluorescent light disturbances, (d) possible deterioration of the cementing substance surrounding the aperture, (e) excessive brushing of the aperture tube in removing debris. The problems were gradually solved by a combination of events. The instruments were moved to four different areas in an attempt to avoid electrical interferences and percussion-type noises. The former were also improved by the installation of a Sola voltage stabilizer* and the noises and lighting problems were improved by moving to more select locations. The aperture tubes that were fused rather than cemented seemed more desirable if not absolutely necessary. Cleaning the debris was best achieved by a downward rather than a circular brushing motion.

When all variables attributable to instrumentation had been stabilized, the data showed greater reproducibility on replicate counts of the same specimen (Table I). Our results before standardizing the procedure showed variations of 10-25 per cent in successive counts, whereas one will observe that the data in Table I usually showed only a 1-2 per cent difference in comparable counts at the same threshold settings except at the initial positions which always demonstrated background noise. Typical curves for saline and latex particles are illustrated in Plate 1.

The pure and mixed culture data at various growth phases and dilutions showed tremendous variations. The information as yet is not complete enough to predict the pulse height profiles for all population changes. Table I shows the consistent counts obtained with coliforms for a threshold interval of 5 and 10 on the Model A using a 30 u. fused aperture with the current setting of 5 and a gain position of 6.

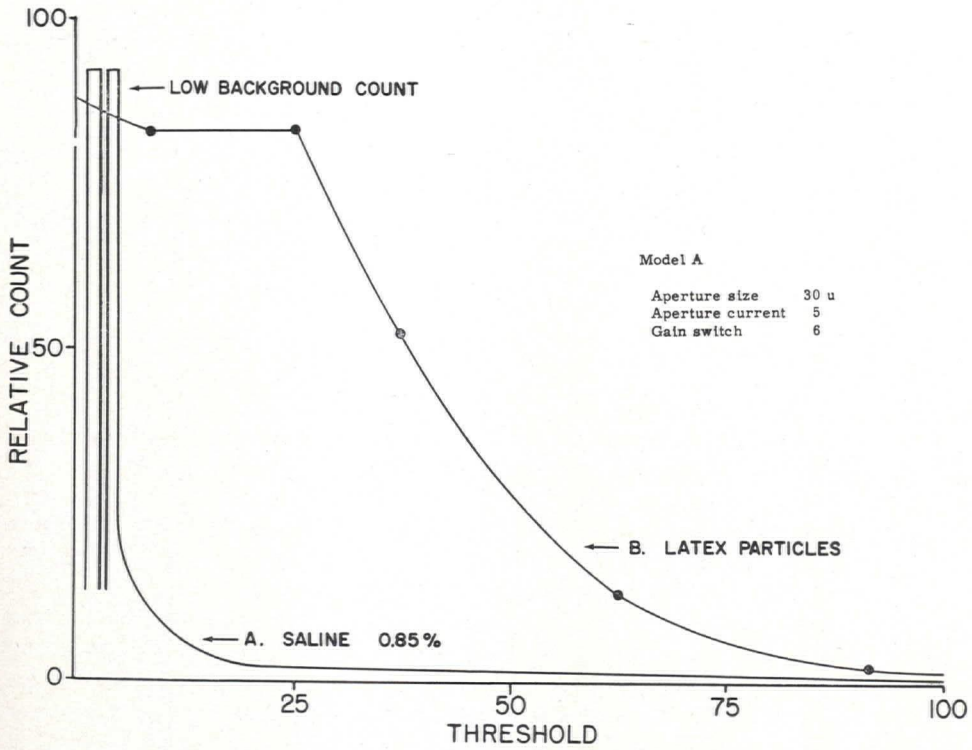
*The Sola voltage stabilizer (Type CVN-1) is a constant voltage transformer produced by Sola Electric Company, Elk Grove, Illinois.

The population age has been observed by us, as well as others, to have an effect on the shape and size characteristics of bacteria which necessarily adds to the uncertainty of mixed populations of pure and compound cultures. Since our final goal was to apply the instruments to the identification of "positive urine specimens", we pursued this avenue at the same time as the pure culture studies.

A series of 300 urine specimens submitted to the Bacteriology Laboratory for routine and quantitative cultures were examined routinely with the Coulter Counter Model A. The specimens were examined at full strength, as well as the following dilution: 1:10, 1:50 and 1:500. The experimental results were most reliable at dilution of 1:50. Many of the more concentrated (i.e., 1:10) specimens produced frequent plugging of the instrument and the more dilute material did not identify the known positive specimens as frequently as the 1:50 dilutions.

The data (total counts) obtained with the Model A at the 0-100 threshold settings was plotted on simple graph paper, as well as unto two and three cycle logarithmic paper. The plottings as shown by the heavy lines (2,3,4,5) in Plate 2, Figure 1, identify the type of curves obtained for culturally positive specimens.

Plate 1



Sample threshold curves with Coulter Counter Model A showing
A. Normal physiological saline — 0.85 per cent
B. Latex polystyrene particles.

Note: As the threshold is increased, the smaller particles are eliminated in the count and the latter decreases.

The observer will note that specimens number 2 and 4 extend out from the initial baseline at a rapid rate and plate counts have shown them to be positive. These two curves are typically representative of culturally positive specimens. Although these two positive specimens were readily detected by the plottings, it will be noted that specimens such as 3 and 5 could not be suspected by these criteria.

Plate 2

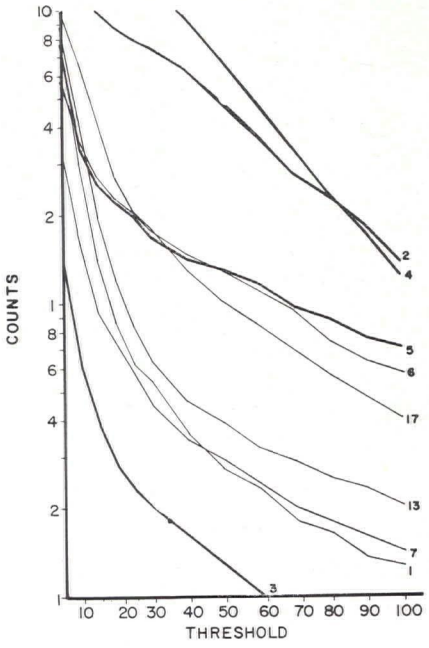


Figure 1

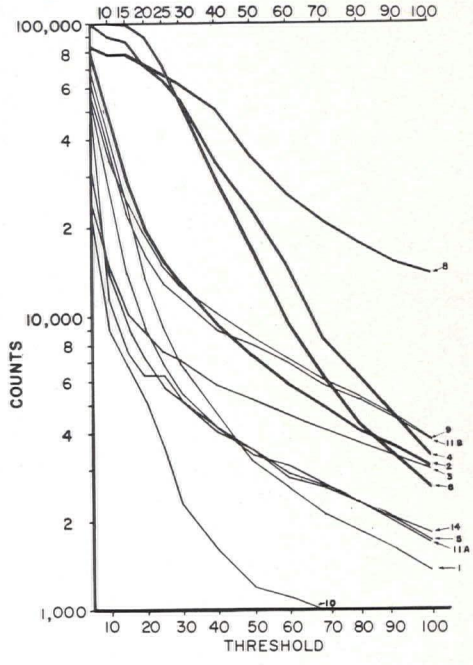


Figure 2

The curves were drawn from urine specimens obtained with the Coulter Counter, Model A. The plottings of total counts were made on 2-cycle semi-logarithmic graph paper.

Figure 1 shows the location of the curves for positive urine specimens (#2, 3, 4 and 5) "darker" lines. Note location of #3 which was not detected by the electronic method.

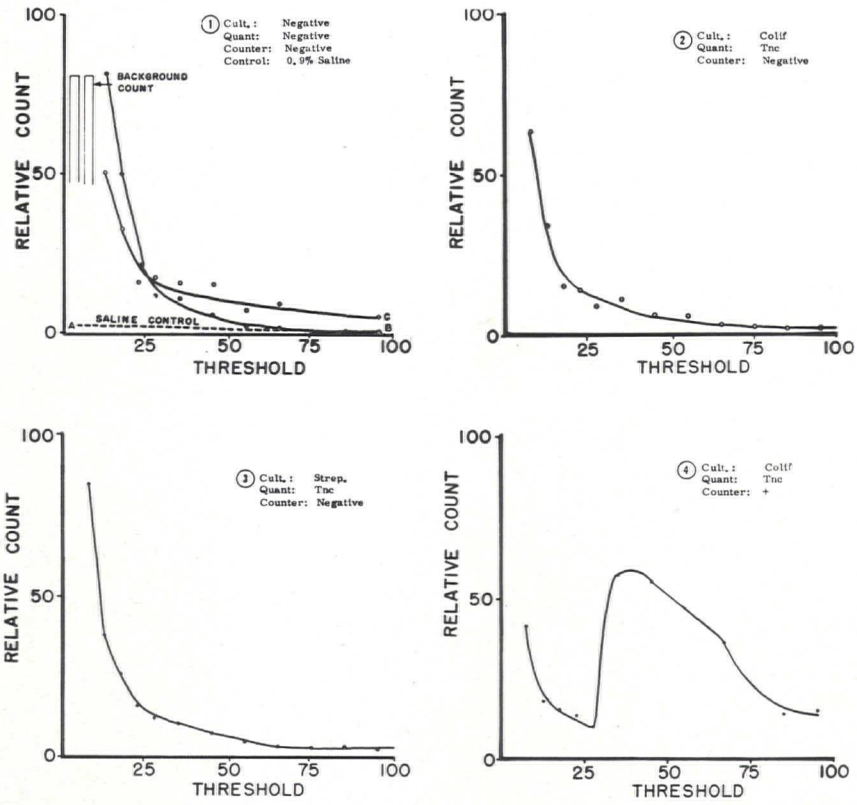
Figure 2 shows the curves for positive urine specimens (#2, 4, 6 and 8) in darker lines. No. 2 had an equivocal quantitative count. The negative urine count data are plotted in "lighter" lines.

Another typical experiment (see Plate 2, Figure 2) clearly illustrates how three culturally positive specimens (Curves No. 4,6,8) were predicted by plotting the "coulter counts". Line No. 2 represents the plottings of a specimen, not detected by us but containing 93,000 cells (equivocal range) of *Proteus,* micrococci and diphtheroids per ml. of urine. In this experiment it was possible to predict 100 per cent of the negative urines, as well as the three positives which contained more that 100,000 cells per ml. of specimen. Results as favorable as these were not obtained routinely. The usual findings showed accurate (usually 100 per cent) prediction of the negative urines and a 50-75 per cent correct classification of positive specimens. Our experiences using T-Soy broth rather than saline as the dilucent with the Model B were more favorable.

KEY FOR LEGENDS (of plates 3-7 inclusive)

amp.	—	amplification
Colif.	—	Coliform
Cult.	—	Culture
dipth.	—	diptheroids
Entero.	—	Enterococci
Quant.	—	Quantitative culture
Micro.	—	*Micrococcus*
Neg.	—	Negative
Pseudo.	—	*Pseudomonas*
S. aureus	—	*Staphylococcus aureus*
Strep.	—	*Streptococcus*
Tnc	—	Too numerous to count (2 to 3 million cells)

Plate 3



This data was obtained with the Coulter Model A, by plotting difference in threshold counts. Figure 1 shows two curves (B and C) of negative urine specimens and their plottings in relation to the control saline sample (A).

Figures 2 and 3 illustrates the curve obtained from two positive urines (coliform and streptococci, respectively) which remained undetected by the electronic method.

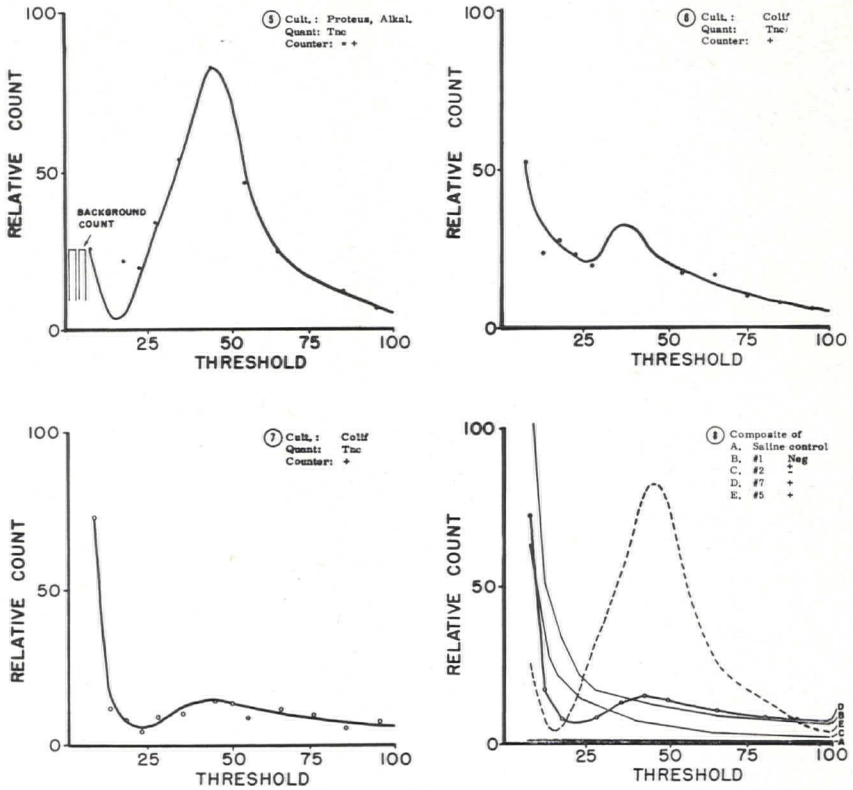
Figure 4 demonstrates the bell-shaped curve obtaind by plotting the counts from a positive urine specimen.

The curves on Plate 3 illustrate a cross-section of the types of curves which were obtained with counts on urine specimens which were either bacteriologically negative or positive. The plottings on Plate 4 demonstrate a cross-section of the variations which were obtained with either pure or mixed bacterial urine specimens. Figure 8 of Plate 4 shows a composite of curves and the correct predictions made by interpreting the "plotted data". It should be stressed at this point that these curves (Plates 1-4) were plotted on the basis of the differences in counts at different threshold settings (0-100). For example, substract the reading at threshold 25 from the count at 20 to give to number of particles at the average threshold of 22.5.

Data obtained with the Coulter Counter Model B.

Since the Coulter instruments were used for experimental and developmental procedures, all the data on over 1000 specimens were evaluated on the basis of parallel bacteriological studies. The graphs in Plates 5, 6 and 7 are intended to give

Plate 4

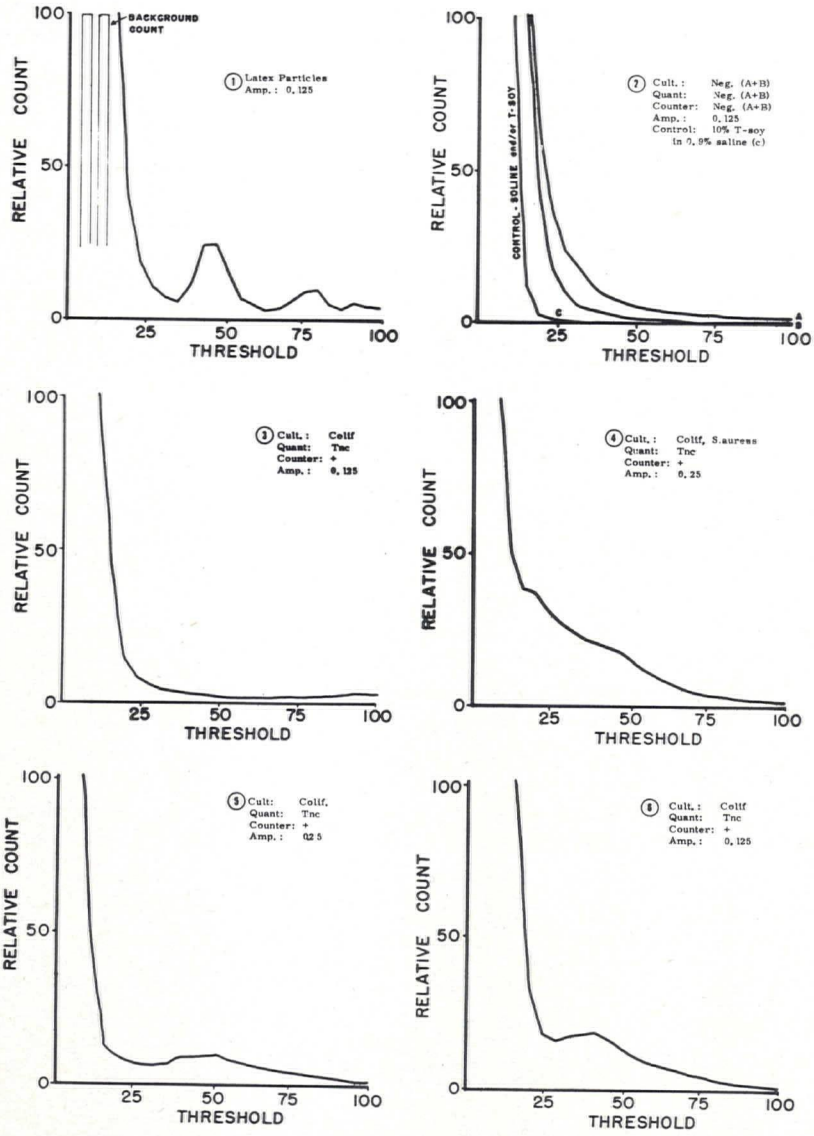


This data was obtained with the Coulter Counter Model A, by plotting difference in threshold counts. Figure 5 shows the type of curve obtained with urine specimens containing two types of organisms. Figures 6 and 7 illustrate the slight activity which may occur with positive specimens containing coliforms.

Figure 8. This shows a composite of curves on all types of urine specimens ranging from the negative to the strongly positive material.

a cross-section of the information obtained from all types of urine specimens using the Model B and plotter. Each figure contains the basic information related to the condition of experimentation. The pertinent data relative to organisms, conditions and results are summarized in the upper right hand corner of each figure. The

Plate 5



This series of graphs illustrate the curves obtained with the Coulter Counter Model B.
Figure 1 shows a typical latex curve.
Figure 2 demonstrates the pattern seen with negative urines.
Figure 3. Note the slight elevation at the end of the curve for this positive specimen.
Figures 4, 5 and 6 show the variations in curve patterns seen with different positive urine specimens.

curves were drawn from the total count data at each threshold range as recorded by the plotter of the Model B.

The latex curve (Figure 1 of Plate 5) was obtained with a current setting of one which was used routinely for all the studies to be discussed in this section. The curves of Figure 2, Plate 5, show the slope of the lines for saline, T-Soy and negative urine specimens. Figure 3 illustrates the slight "peaking" at the end of the curve which enabled the investigator to detect a positive specimen. The small variations in the "humping curves" (Figures 4,5,6) at different threshold readings served to detect the specimen which contained a significant number of organisms. Whenever a suspicious curve appeared, the oscilloscope patterns were examined closely for evidence of positivity. It was found helpful at times to change the amplification on the doubtful positive specimens in an attempt to improve the sensitivity of the instrument (see Table 2). The difficulty encountered with both Models A and B in predicting the positive urines having a range of 100,000 to 800,000 cells per ml. by both Models A and B led the authors to the use of 10-20 per cent T-Soy as a diluent in the hope that specimens with this "bacterial count range" would multiply in a two to three hour incubation period. This theory was proven to be true (see Table II).

The curves in Plate 6 illustrate the variation in the height and location of the peaks due to following reasons: (a) the generic types, (b) total numbers, and (c) physiological age of the cells, etc. Note, for example, the variation with three coliforms (Figures 7, 8 and 11) showing the peaks at different ranges of threshold settings.

The curves seen in Plate 7 are shown in an attempt to complete the illustration of the more outstanding types of data obtained with the Model B and plotter. Figures 13 and 15 of Plate 7 show the doubtful positives by plottings but strongly

Table II

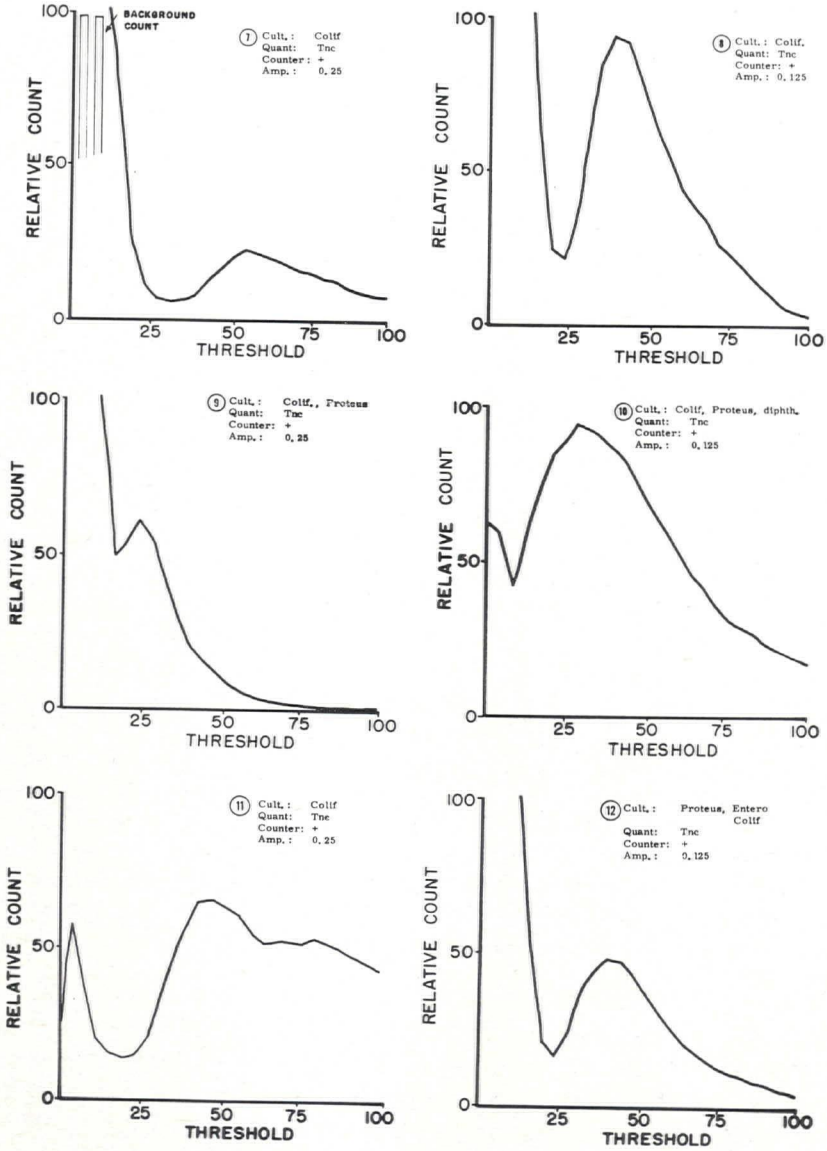
Comparative studies showing the superiority of T-Soy broth over saline as the diluent.

1962 urine specimen no. and diluent	Routine culture	Quantitative culture	Coulter Model B Graph Oscilloscope	
2-22-10 saline	Coliform	3 million	−	−
2-22-10 broth	Coliform	>3 million	+	+
3-2-2 saline	*Proteus*	1,958,000	±*	±
3-2-2 broth	*Proteus*	>1,958,000	+	+
3-3-1 saline	*S. albus*	768,000	±	±
3-3-1 broth	*S. albus*	>768,000	+	±
3-3-19 saline	Entero.	70,000	−	−
3-3-19 broth	Entero.	>70,000	±	±

*The ± denotes doubtful or suspicious positive results. If the specimens were positive in saline, they were always positive in T-Soy broth.

suspected as positives by the oscilloscope pattern, etc. These two specimens were substantiated as being positive by the quantitative cultural data. Figure 14 illustrates a specimen which contained two isolates and remained undetected by the Coulter

Plate 6



The curves were plotted with the Coulter Counter Model B.

Figures 7, 8 and 11 show the graphs of three different specimens containing significant number of coliform.

Figures 9, 10 and 12 illustrate the types of curves with different mixtures of organisms.

Counter Model B. An interesting example of the changes in the height of the curves due to a ten-fold difference in the dilution factor is shown in Figure 16. However, increasing the concentration from a 1:50 to a 1:10 dilution only resulted in more frequent plugging of the orifice and inaccurate readings. The curves in Figure 17 demonstrate the variation in the height and location of the peaks due to a change in amplification. Note the greater sensitivity at settings of 0.125 and 0.25 as compared to the curve obtained with the 0.5.

Red blood cells on occasion have presented a blocking or masking effect to conceal the presence of bacteria in the urine (Figure 18). When erythrocytes and bacteria are present in sufficient numbers at an amplification of 0.25, the erythrocytes, although they are not counted, prevent the accurate expression of the bacteria by the counter (Curve A). When the amplification is changed to 8, the RBC curve becomes apparent (Curve B). Addition of a few drops of 5 per cent saponin solution lyses the red blood cells and essentially results in a negative count shown by Curve C which follows the threshold baseline. When the amplification is returned to 0.25, the bacterial peak (shown by Curve D — represented by circles joined by broken lines) becomes prominent at threshold range 65-90. Experiences with such other lytic agents as HC1[8], stretolysin 0[9], etc., are inconclusive. Attempts to remove debris (epithelial cells, leukocytes, red cells, etc.) from very cloudy urines by means of filters and lytic agents have not been extensive enough to draw firm conclusions. Consequently, we plan to pursue these investigations with the hope of simplifying the determinations on the cloudy urine specimens.

Discussion

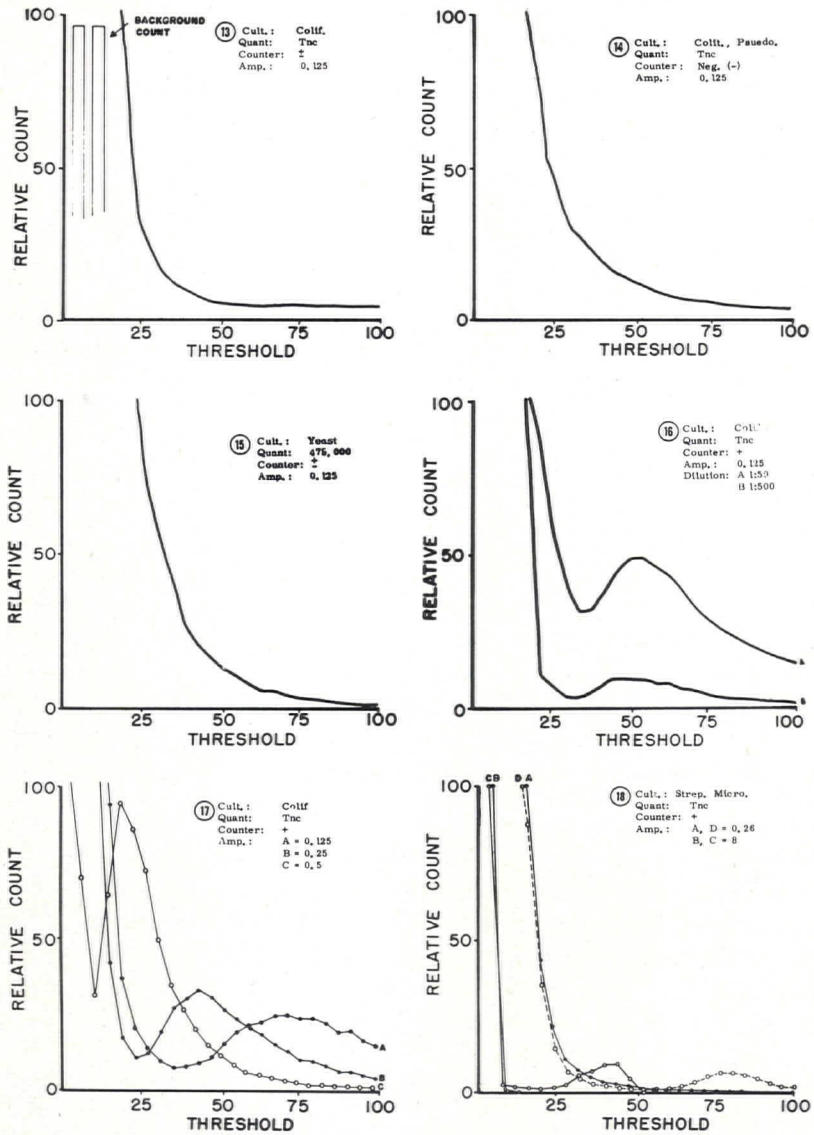
In the experiments described above, we have studied the several parameters of the quantitative urine problem. Both Coulter Models A and B have been successfully used in examining urine specimens. The data with Model A has been plotted on both simple graph paper, as well as 2 and 3 cycle semi-logarithmic paper. Although reliable methods for predicting negative and highly positive specimens (Plates 2, 3, 4) have been devised, the method was time consuming. Counts could be obtained with relative speed but plotting the data was a very tedious procedure.

This difficulty encouraged us to the use of the more automatic Model B and plotter. The studies with this model were conducted with urine diluted in both saline or broth. It soon became apparent that the tedious and time-consuming plotting problems had been eliminated since the graphs for each specimen usually required only 100 seconds.

Small differences in numbers were recognized by the variations in the height of the peaks (Plates 5, 6, 7). Observation of the impulses on the oscilloscope screen were helpful in determining possible positive specimens. When an interruption occurred in the oscilloscope pattern, the operator realized that the orifice had become plugged.

The sample results (Table II) obtained with either Model A or B indicated that the one could routinely predict positive specimens which had counts designated

Plate 7



Curves obtained with the Coulter Counter Model B.

Figure 13. The operator predicted this doubtful positive from slight rises in the plotted curve not seen herein.

Figure 14. This positive specimen was not predicted by the operator.

Figure 15. The yeast cells were barely detected on the initial curve.

Figure 16. Shows the difference in the curves by varying the dilution. (Read: Cult: Colif.).

Figure 17. Illustrates the difference in the curves with a change in amplification.

Figure 18. Demonstration of RBC and bacteria in urine (see Text).

as Tnc (too numerous to count — 2 to 3 million organisms per plate). Specimens which contained 100,000 to 500,000 cells per ml. of urine were more difficult to detect in saline than in broth.

Advantages and disadvantages of the Coulter Counter in the examination of urine specimens for significant number of bacteria.

These instruments offer a number of advantages for the bacteriologist interested in the quantitation of bacteria in urine specimens. The Model A Counter is capable in 15 seconds of counting cells of a given size range. Such a sampling at a large number of threshold settings leads to excellent reproducibility. Counts seldom vary more than 1-2 per cent from the mean value of replicate counts on the same specimen at similar threhold settings. The Model B with plotter is capable of computing the data through the low and high threshold ranges within a 100 second period. If one has sufficient experience with the instrument and methods, the procedure with the Model B may well be more economical if a sufficient number of specimens are to be screened daily.

There are inherent problems which arise intermittently in maintaining the apparatus in operating condition. The many electronic components do give rise to occasional interruptions. In addition, the orifice does plug readily and requires constant surveillance and unplugging procedures. The aperture tube, capillary tubing and manometer require some attention. Adequate calibration curves and controls (i.e., electrolyte, latex counts, positive and negative reference data on urines, etc.) must be performed. The urines frequently require dilution and/or subculturing in broth at a 1:50 dilution. The initial cost and maintenance of the apparatus may be a question of some concern to the supervisor of laboratories.

The instrument's potentialities for counting and sizing particles in urines and pure cultures make its continued usage highly desirable. Further studies on the differences in impedance between viable and non-viable organisms have been planned. Additional experimental procedures designed to simplify the methodology for detecting "positive urine specimens" will also continue.

Summary

1. Both the Coulter Counter Models A and B with plotter can be used for screening positive urine specimens.

2. The Model B with plotter is more desirable than the Model A because specimens can be processed much more rapidly with considerably less technical assistance and reduction of fatigue.

3. The data shows that T-Soy broth is superior to saline if the former is used as the diluent together with a two to three hour incubation period.

4. The identification of positive specimens by this electronic method agreed reasonably well with the cultural data.

5. Specimens which were considered to be negative by cultural methods (0-100,000 cells per ml.) were always reported as being negative on the basis of the Coulter Counter procedures.

6. The instruments definitely increased the rapidity and accuracy of particle sizing pure and mixed cultures at various population ages. The accuracy and reproducibility of results with the electronic counter were usually of the order of 2 per cent, as judged by replicate counts and dilution curves.

7. Counts cannot be expected to be meaningful unless the investigator stabilizes all variables attributable to instrumentation.

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