AUTOMATIC METHODS IN DIAGNOSTIC BACTERIOLOGY

by

ROBERT EDWARD TROTMAN

Wright-Fleming Institute,
St. Mary's Hospital Medical School,
London, W.2.

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ABSTRACT

A brief review of equipment for use in serological procedures and equipment for isolating and identifying bacteria is followed by a study of basic laboratory test procedures used in medical diagnostic bacteriology, an analysis of the problems of developing automatic methods in diagnostic bacteriology and a discussion of possible fields of further study. The conclusions are stated.

Extensive investigations of some diagnostic procedures that lend themselves to mechanization and automation, and evaluations of the performance of definitive diagnostic test equipment are reported and discussed: the procedures investigated include a method for serially diluting antibiotic and serum, as required for the measurement of the minimal inhibitory concentration of antibiotic and the serum antibiotic level, and a method for distributing the reagents for the Wassermann reaction.

Detailed investigations of some techniques that may lead to the development of further definitive diagnostic test equipment are reported and discussed. The techniques include automatically spreading a culture over an agar plate, and an r.f. induction heating method for sterilizing vessels with which infected material comes into contact in situ, and in a reasonable time: our inability to sterilize vessels in situ has hitherto been a major obstacle to the

further development of automatic methods in diagnostic bacteriology.

The conclusions drawn from the investigations are stated and many suggestions for further work are made.

C O N T E N T S

Chapter 4 - EXPERIMENTAL METHODS (contd.)

Chapter I

Introduction

Automatic methods have hitherto been very little used in diagnostic bacteriology, despite the fact that the work load in many laboratories has been increasing at a rate of about 10% per annum - not much less than the rate of increase in chemical pathology laboratories. This seems surprising, particularly in view of the serious shortage of technical staff, which necessitates increasing productivity, and the quantity of automatic apparatus used in chemical pathology and haematology. Some of this apparatus is reviewed.

The auto-analyzer (Skeggs, 1957) is widely used in chemical pathology, particularly for electrolyte analysis (Marsh, 1963). In principle, a sample of serum is pumped along flexible tubing, by means of a peristaltic pump, and mixed with a stream of diluent. The mixture is pumped through a dialyser so that the diffusable constituents are separated, and each constituent is mixed with appropriate reagents. Each mixture is then fed to one or more of a variety of instruments, such as a heating bath, a colorimeter or a flame photometer, depending on the analysis being carried out. The electrical outputs of the colorimeter or flame photometer are manipulated for presentation in either analogue or digital form, or fed to a computer. Many different analyses may be carried out

simultaneously on a single specimen, and it is frequently necessary to do this. The auto-analyzer is a continuous flow device: after each specimen has passed through the system, a washing fluid is passed through followed by a short air space and then the next specimen. The maximum work load depends on the analyses, but it can be as high as 60 specimens per hour. The apparatus is fully automatic, the operator has only to place the specimens in cups in the machine which then carries out the analyses and presents the results, on paper, without further manual intervention.

The Coulter cell-counter (Coulter, 1953) is widely used in haematology for counting red blood cells (Brecher, Schneiderman and Williams, 1956) and white blood cells (Richar and Breakell, 1959). In principle, a sample of blood is diluted in an electrically conducting fluid (usually saline) and the beaker containing the mixture is placed on a platform so that a probe is immersed in the mixture. The probe consists of a flat-plate electrode and a glass tube enclosing a second flat-plate electrode. There is a small orifice in the wall of the glass tube and an electric potential is applied across the electrodes, thus producing an electric field across the orifice. The diluted sample is drawn through the orifice, and as each blood cell passes through it displaces some of the conductive fluid, thus raising the resistance of the orifice contents and producing a voltage pulse: the amplitude of the pulse is proportional to cell size. The output signal may be displayed on an oscilloscope or it may be processed to give the total count in

digital form, but no print-out is produced by the standard apparatus. Pulse amplitude discrimination techniques may be used to count cells within a pre-determined size range, and size distribution curves can be obtained in a few minutes: it takes 20 seconds to count a sample.

A microscope slide staining machine (Shandon Ltd.) is also widely used in haematology, particularly for the Leishman, Jenner-Giemsa and May-Grunwald-Giemsa staining techniques (Dacie, 1956): the apparatus is also used in histology for haematoxylin and eosin staining (Culling, 1957). A rack containing a batch of slides is attached to the underside of a circular rotary table. Baths of stains and washing fluids are placed under the circumference of the table, in the order appropriate to the particular staining technique to be carried out, and the apparatus is programmed so that the rack of slides is lowered, in turn, into each bath for a pre-determined period. The period may be different for each bath, but if the immersion times are the same, many batches of slides may be processed simultaneously. There are machines that take a maximum of 8 baths and 15 slides per rack, machines that take a maximum of 12 baths and either 16 or 46 slides per rack and those that take a maximum of 23 baths and either 16 or 46 slides per rack. The slide staining machine is much simpler than either the auto-analyzer or the Coulter counter, but is nevertheless very useful. Once loaded, the apparatus carries out the staining procedure automatically, but it does not measure and record the results automatically: slides have to be examined visually and the results recorded manually.

The reason the auto-analyzer, the Coulter counter and other automatic apparatus have been very little used in diagnostic bacteriology is elucidated in the following review of equipment used in bacteriology laboratories: the slide staining machine has been used in some laboratories.

Bacteriology may be divided into two branches, serology, in which the procedures are very similar to chemical pathological procedures, although the indication systems are more complex, and the isolation and identification of bacteria, in which the indication systems are much more complex and in which the specimens are infective and the spread of infection, and also contamination of the specimen, must be avoided; the problems of handling infective material are considerable (Sect. 1.2.5).

1.1. Equipment for use in Serological Procedures

It is obvious that the auto-analyzer - so widely used in chemical laboratories - would be introduced into bacteriology, or at least serology, and it has been used for performing the Wassermann reaction (Pugh and Gaze, 1965) and other complement-fixation procedures (Vargues, 1965).

Pugh and Gaze performed 900 Wassermann reactions on the auto-analyzer and also by the manual method, and compared the results. They found that the automated method gave an error of 9.7%, 5.6% being false positives and 4.1% false negatives; in some cases the discrepancy was attributed to the sera being turbid, fat or grossly haemolysed. Improved results were obtained when the Maltaner antigen

was used instead of the crude heart extract antigen obtained from the V.D. reference laboratory: a discrepancy of 2.6% was obtained in this case. In a subsequent paper, Pugh and Gaze (1966) described a modification to the apparatus to permit samples to be tested at a rate of 60 per hour, and indicated that after each run, sera that were found to be positive were run again without antigen, for an anti-complementary check.

It has been found, however, that the washing of the apparatus by the standard volume of washing fluid is insufficient to remove all traces of antibody after the passage of a sample of serum with a high antibody titre: there is some "carry-over" to the next specimen. It seems likely that the "carry-over" phenomenon represents a major obstacle to the use of the continuous flow system in diagnostic serology (Taylor, 1968): in fact, there is a tendency for chemical pathologists, who have been using the auto-analyzer for the past decade, to consider using discrete analysis methods (Loebl, 1966).

Sequeira (1964) developed hand operated apparatus for performing serological titrations. In principle, the apparatus consisted of a table, containing racks of reservoirs of reagents and test tubes, which could be moved so that any row of test tubes or any reservoir could be placed under a row of 12 plastic pipettes: the pipettes were lowered into the test tubes or reservoir. Each pipette was permanently connected to a syringe and the 12 syringe pistons were operated, simultaneously, by a single lever to suck up and expel fluid: the volume dispensed depended on the position to which the

lever was turned and, therefore, on the skill of the operator. Each syringe piston was connected directly to the lever, and the stroke length of each piston, and hence the volume dispensed, was not independently variable: the accuracy of dispensing depended, therefore, on the tolerances of the syringes as well as on the operator.

It was necessary for the operator to know the test procedures, because he controlled the order in which the reagents were dispensed, the volumes dispensed and the row of tubes into which the reagents were placed. The apparatus was, however, very flexible in that variations of the order in which the reagents were dispensed, the volumes dispensed and the row of tubes into which the reagents were placed could be introduced without difficulty.

Sequeira's apparatus was very similar to that produced by Baron, Burch and Uhlendorf (1961), who claimed to be able to sterilize the pipettes by flushing them six times in boiling demineralised water: they did not discuss the technique. Sequeira's apparatus was also a simplified modification of Weitz's machine (1957). The only essential difference between Sequeira's machine and Weitz's machine was that in the latter, the volume each pipette-syringe system dispensed was independently variable, even though a single lever operated them all simultaneously, and therefore inaccuracies due to tolerances in the apparatus could be reduced at the cost of making the apparatus very complicated.

1.2 Equipment for Isolation and Identification of Bacteria

1.2.1. Counting bacteria in suspension

The Coulter counter was first used for counting bacteria in suspension by Kubitschek (1958). He modified the standard cell-counter, by replacing the 100 μ orifice with a 10 μ orifice, and counted Escherichia coli (strain B) and Bacillus megaterium spores: 4 hour cultures of each were diluted in 0.1N hydrochloric acid. To calibrate the apparatus, he suspended 1.13 μ and 3.2 μ polystyrene latex spheres in saline and counted them on a haemocytometer, to obtain an absolute count, and compared the count with that obtained on the electronic counter: agreement to within 2% was obtained.

Kubitschek also used the electronic counter for sizing the same organisms, but gave very little information about the technique; he simply stated that he used a pulse height analyser to give the cell volumes.

A more detailed investigation was carried out by Curby, Swanton and Lind (1963). They grew Staphylococcus aureus S.M., E.coli (Sias) and an E.coli variant, and E.freundii (8454) and an E.freundii variant in brain heart infusion broth. Cultures inoculated from an agar slant and from a broth culture were diluted 0.9% in sodium chloride solution and counted on a Model A Coulter counter, with a 30 μ orifice, at 1 hour intervals after inoculation; the inoculation techniques were not discussed. Similarly, pour plates were made of each culture.

Curby and his co-workers found that the electronic count depended on the magnitude of the electric field applied across the orifice of the counter, and that the relationship between count and electric field depended on the organism. The phenomenon was not investigated in detail, but the authors concluded that "it was not related to a charge on the organism but was determined by an active process within the organism just before cell division". In general, the electronic count obtained for the E.coli and its variant agreed with the pour plate method for the culture inoculated from an agar slant, but poor agreement was obtained for the culture inoculated from a broth culture. With the E.freundii and its variant, poor agreement was obtained for both cultures and with the Staph.aureus, the electronic count was always higher than the pour plate count.

The discrepancies were not discussed in detail, but there are various possible explanations. The electronic counter cannot distinguish live from dead bacteria or bacteria from other particles which may be in the suspension, and consequently, the total viable count obtained by the pour plate method, which is taken to be the number of viable colony-forming units, could well be lower than the electronic count. Also, the electronic counter cannot distinguish single bacteria from aggregates and this is likely to cause errors that offset errors due to the aforementioned factors: when counting colonies on a pour plate one has to be cautious of aggregates, but they are usually clearly recognisable. The effects due to all these factors must vary considerably from specimen to specimen, and it is likely to prove difficult to formulate general criteria for relating

the electronic count to the total viable count.

Toennies, Izard, Rogers and Shockman (1961) used the cell-counter to obtain the distribution of particle sizes in growing cultures of E.coli and Streptococcus faecalis, for cell-multiplication studies. They plotted a curve of total count versus threshold orifice current for each of two suspensions; they did not say if the suspensions were of the same or different organisms. The two curves were significantly different but this was not discussed.

Manor and Haselkorn (1967) used the cell counter for the size fractionation of exponentially growing E.coli. In principle, different size fractions of bacteria in a culture were prepared in a zone centrifuge, and a sample of each fraction was counted to give information about the age of the organisms in the culture; the technique was not discussed in detail.

The evidence indicates that much more work needs to be carried out before the Coulter counter, or any similar apparatus, can be used as a diagnostic aid. Apart from the difficulties referred to above, in none of the papers discussed was there reference to the problem of "carry-over" (Scots. 1.1 and 1.2.5) which may be important in diagnostic work. However, if the problems can be overcome, cell-counters might be utilized for an actual diagnostic test, such as urine analysis, or for detecting growth. If one could count very small numbers of bacteria accurately, this could be a very sensitive method for detecting growth and, possibly, for measuring antibiotic sensitivities: the apparatus could also

be used in experimental work for measuring growth curves.

Photometric methods (Snell and Snell, 1948) have been widely used in experimental bacteriology for many years, at least since 1933 when Alper and Sterne measured growth curves of Salmonella gallinarum, and Pulvertaft and Lemon measured opacity curves of B.coli growing in beef broth and in Lemco broth: they did not define opacity. Brief reviews of the applications of photometric methods to bacteriology are given by Norris (1959), Kavanagh (1963) and Meynell and Meynell (1965). Many devices, such as the Vitatron universal photometer - densitometer (Vitatron Scientific Instruments) and the Unicam spectrophotometer (Unicam Instruments Ltd.), are commercially available and new instruments are still being devised: Forrest and Stephen (1965) built a device in which the lamp/photo-cell unit was mounted in a water bath, and growth curves were permanently recorded in analogue form.

It appears that photometry is much more likely to become a diagnostic technique for measuring concentrations of bacteria in suspension than electronic cell-counting techniques. Many of the technical problems have been solved and one does not necessarily have to handle infective material directly, although Haney, Gerke and Pagano (1963) described a device for antibiotic assays in which the specimens were transferred by a single pipette to a tube containing the bacterium which was then incubated, killed with formaldehyde and transferred to the cuvette: the cuvette and transfer device were simply rinsed through with water before the next specimen was processed.

One of the biggest disadvantages of photometric methods is that their sensitivity is poor: the minimum concentration of organisms in suspension that can be detected is of the order of 10^6 organisms/ml.

Bowman, Blume and Vurek (1967) developed a much more sensitive method for counting viable bacteria, and also for determining antibiotic sensitivities, than classical photometric methods. Specially prepared agar was melted and the specimen was added to the agar and mixed thoroughly. The end of a capillary tube was dipped into the agar, the tube was filled by capillary action and both ends of the tube were sealed: approximately 150 ul. of agar was needed to fill the tube which was left until the agar had solidified.

The capillary tube was scanned by a linear tungsten filament lamp which was focussed on to the centre of the capillary. An objective collected the light scattered within the capillary and the light was passed through a stop to a photo-multiplier tube: the image of the source was 50 μ mm. wide. The instrument counted, electronically, light pulses scattered from growing colonies and from other points, such as the joint between the agar and the capillary. The tube was scanned at intervals, and growing organisms produced new or larger pulses than those obtained on a previous scan but other scattering points produced constant pulses. The background scatter was such that it obscured small signals and, therefore, the organism had to divide several times before it could be detected: the threshold level was set at four times the background and the minimum number of organisms that formed a recognisable micro-colony

was of the order of 20.

At high concentrations the true count was higher than the measured count because two or more colonies in the area formed by the image of the source were counted as one: the effect becomes significant at a concentration of 10^5 E.coli. Also, at high concentrations the supply of nutrients is exhausted more quickly than if fewer organisms are present. Growth curves for E.coli, streptococcus, proteus and staphylococcus were obtained, and in the case of E.coli, a quantitative estimate of the number of viable cells present in a capillary was made within 5 hours: a rough estimate of the number of viable cells present was made in 2 hours. Longer periods were required for the other organisms.

To determine antibiotic sensitivities, the antibiotic was mixed in the agar during its preparation and before the culture was added. Growth curves for klebsiella in different concentration of Na-colistimethate were obtained, and the difference in growth rates of specimens with large differences in concentration of antibiotic was easily measured, but the resolution was poor.

Bowman and his co-workers found that the preparation and handling of the capillary tubes was very laborious, and possible modifications to the technique are under investigation.

Whilst this work is in its very early stages, it has considerable potential. Not only is it a very sensitive method for detecting growth of bacteria in suspension, it may lead to means of producing some bacteriological test results on the same day as the specimen is sent to the laboratory.

1.2.2 Counting colonies on agar plates

Apparatus for automatically counting bacterial colonies on solid agar plates was described by Alexander and Glick (1958). This was an electronic scanning device in which a cathode ray tube flying spot was focussed on to a culture, and variations of light transmitted during a scan were measured on a photo-multiplier. Two light/photo-multiplier channels were used. The first channel light spot traversed the culture and the photo-multiplier output signal was recorded immediately. The second channel light spot traversed the culture simultaneously with the first light spot, but the photo-multiplier output signal was delayed the time the spot took to traverse its span. Therefore, a channel 2 signal could be compared with the channel 1 signal of the next line scanned, and the apparatus was arranged to record a count only if there was a pulse on channel 1 and no pulse on channel 2.

Despite the two channel anti-coincidence networks, the chief source of error is still due to coincidence. Bouffant and Soule (1954) discussed such errors in detail. Briefly, they fall into four categories, fractional interception errors, when one particle is intercepted by two or more passages of the scanning beam, overlap errors, when particles overlap in the projected image of the scanning beam and are counted as one, coincidence errors, when two particles are so close together that the apparatus cannot discriminate between them, and sensitivity errors, when the apparatus cannot distinguish a particle from the background.

Alexander and Glick's apparatus was evaluated by Halligo in 1965: the time between publication of these two papers suggests many difficulties were encountered. Halligo used B. subtilis and Serratia marcescens plated on peptone agar and tryptone agar respectively: the agar had to be transparent and, therefore, blood agar could not be used. Plates were prepared so that the number of colonies ranged between 30 and 300, and the colonies were kept away from the edge of the plate. Halligo counted 3,000 plates, for each species, on the machine and visually. In general, the machine gave a lower count than the visual method if more than 100 colonies were present and a higher count than the visual method if less than 100 colonies were present. These discrepancies were attributed to, colonies being on the periphery of the scanning area, touching and overlapping colonies, and optical imperfections in agar plates. Constant relationships between machine and visual counts were sought and an equation, for each species, that related the machine count to the visual count within the 95% confidence limit was derived.

The difficulties that must have been encountered during this work were not discussed, but it appears that within the obvious limitations of the method, one can count colonies on an agar plate in this way. A much more detailed investigation is necessary before one can decide if this is likely to become a useful diagnostic technique, and the fact that so few papers have been published may well be indicative of the problems involved.

1.2.3 Identifying bacteria by chromatography

An early attempt to use paper chromatography for identifying bacteria was made by Proom and Woiwod (1949) who examined culture filtrates of 300 strains (27 genera) of bacteria grown in acid-hydrolysed casein medium. They examined the amino-acid and polypeptide composition of the culture filtrates, and found marked differences in the chromatograms of different genera.

In 1956, Sellers, Mitchell and Davis examined the supernatant fluid of centrifuged cultures and found differences in the chromatograms of several species of micrococci, and Cummins and Harris (1956) examined the composition of the cell-wall of 60 strains of corynebacteria, lactobacilli, streptococci and staphylococci: the chromatogram of cell wall components of each bacterial genus appeared to have a characteristic pattern. Mattick, Cheeseman, Berridge and Bottazzi (1956) examined 4 species of L. plantarum, 4 species of L. casei, 2 species of L. acidophilus, 8 species of Strep. cremoris and 7 species of Strep. lactis and were able to distinguish all these species by differences in their chromatograms. Cheeseman and Silva (1959) examined 91 strains of lactobacilli and subdivided them into groups according to certain characteristics of their chromatograms: the constituents of the groups were identical to those constituents given by a biochemical grouping method.

Henis, Gould and Alexander (1966) obtained different chromatograms for various genera and species, and for strains of the same species: B. subtilis (11838), B. licheniformis (8190),

B. circulans (4515), B. subtilis (6051), B. licheniformis (6598) and B. circulans (61) were identified, and qualitative and quantitative differences between strains were observed.

The first attempt to use pyrolysis combined with gas chromatography was made by Reiner (1965) who investigated possible chemical differences between bacterial strains of similar antigenic or pathogenic character: he examined 18 strains of E. coli, 1 strain of Shegella sp., 4 types of group A Strep. pyogenes and 10 forms of mycobacteria and each strain had its own distinctive pyrogram (chromatogram of the pyrolysis products), but some differences in the pyrograms were very slight and difficult to detect. This study was extended (Reiner and Ewing, 1968) to see if closely allied organisms could be distinguished: consistent differences in the pyrograms of E. coli (0111a, 0111b: B4: H12) and E. coli (0111a, 0111c: B4: H12) were obtained.

Whilst taxonomists may well find chromatography and pyrolysis combined with chromatography useful techniques, they are unlikely to be used in diagnostic bacteriology: a great deal of preparative work, sometimes taking more than a day, is necessary before the specimen is ready for chromatography.

1.2.4 Simple aids to manual methods

1.2.4.1 Multipoint inoculators

Multipoint inoculating devices are usually designed for one specific purpose, but they can almost invariably be used in two ways. The device can pick up many different inocula from an array of tubes

or dishes and distribute the inocula successively to a series of tubes or test plates, or the device can pick up from one tray of inoculum and distribute it to different plates or tubes.

Lidwell (1959) developed a device for phage typing of Staph. aureus. A set of 27 loops, as designed by Tarr (1959), was mounted at each end of a rod which rotated in a horizontal plane, about a vertical pillar, and stopped at 4 positions: the rod was depressed by means of a lever. In position 1, the loops were sterilized by a 27 jet gas burner and then the rod was turned to position 2 to allow the loops to cool. In position 3, they were lowered into the phage inocula, which were in a drilled perspex block, and in position 4 the charged loops were lowered on to the surface of a plate previously flooded with the organism to be tested. Since there were two sets of loops, many plates could be inoculated very rapidly.

A more complicated device for applying phage inoculum was devised by Zierdt, Fox and Norris (1960). Twenty-six syringe barrels were fixed to a plate, and the syringe pistons were fixed to a second plate which was attached to a lead screw. An appropriate volume of each bacteriophage was dispensed, on to a previously inoculated agar plate placed under the apparatus, for each turn of the lead screw.

Another device for phage typing of staphylococci was developed by Simon and Undseth (1963). These workers used stainless steel pins mounted in a base plate that moved up and down two vertical guide rods, and claimed that if the plate was lowered so that the

pins touched the bottom of the plastic tray type reservoirs, "0.003 ml. of phage inoculum was delivered": they did not give evidence to support this claim. Various other devices, based on similar principles, have been devised.

An aid to inoculating liquid cultures was developed by Quadling and Colwell (1964). In principle, the apparatus consisted of a brass base plate into which 60 stainless steel needles were fixed at right angles: each needle was 4 in. long by 3/16 in. diameter. A guide rod was fixed in each corner of the base plate, and the rods fitted into corresponding tubes attached to a holder containing racks of culture tubes, thus ensuring that each needle was properly aligned over the appropriate culture tube. A tray (or rack of tubes) containing the inocula was placed over the culture tubes and the needles were lowered, by hand, until each needle touched the inoculum. The base plate was raised, the inoculating tray was removed and the base plate lowered until the needles were in contact with the sterile broth in each of the culture tubes: the needles were then raised and the caps placed over the culture tubes.

Hale and Inkley (1965) developed a similar inoculating device specifically for the multiple inoculation of agar plates. The inocula were placed in 'Oxoid' aluminium test tube caps: 27 caps were placed in a jig so shaped as to keep them within the area of a 4 in. petri dish. 1 in. stainless steel needles were mounted, in positions corresponding to those of the 'Oxoid' caps, in a detachable stainless steel plate which was attached to a lever

operated press. The plate was lowered until the pins touched the inocula, it was raised, the inocula were removed and an agar plate (4 in. petri dish) was put in position under the pins which were lowered to inoculate the agar: the detachable plate was removed for sterilization purposes.

1.2.4.2 A dilution device

A simple device for titrating small measured volumes of liquid was developed by Takatsy (1955) and used for serological titrations by Sever (1962). The device, known as the microtitrator loop, consisted of a tightly wound wire spiral, closed at each end. One loop titrated 0.025 ml. of liquid and another titrated 0.05 ml. of liquid: one simply dipped the loop into the liquid, rotated the loop a few times, removed it from the liquid and the appropriate volume was held in the wire spiral by capillary action.

Sever found many difficulties. The loop, which was not made of stainless steel, tended to rust and it was easily distorted, thus becoming very inaccurate. Also, in order to ensure that it transferred the correct volume, the loop had to be pre-wet and all particles and grease had to be removed before use. These factors suggest that the loop is very delicate and likely to become inaccurate very easily.

Marymont and Wentz (1966) used a 0.05 ml. loop for serially diluting antibiotics for sensitivity testing: they used 0.05 ml. volumes of diluent and, with an initial antibiotic concentration of 200 µg./ml., serially diluted the antibiotic twelve times.

The minimal inhibitory concentrations of 60 bacteria, for 3 antibiotics, were measured by this method and also by the standard tube dilution method: a total of 111 minimum inhibitory concentrations were measured by each method. In 10 cases, the tube dilution method gave a minimal inhibitory concentration which was either one or two tubes higher than that given by the microtitrator method, and in the remaining 4 cases, the tube dilution method gave a minimal inhibitory concentration which was 3 or more tubes higher than that given by the microtitrator method. These results confirm Sever's statements about the possible sources of inaccuracies in the spiral loops, and it is evident that exceptional care in handling them is vital.

1.2.4.3 Other simple aids

An aid to counting colonies on a roll tube culture was made by Barclay (1965): the method was specifically for counting colonies under a microscope. The device was a simple hand operated mechanism for rotating the roll tube and simultaneously moving it across the field of view: the tube moved in a spiral curve. The various gears were so arranged that, when the tube was turned through one complete revolution it moved laterally exactly one field of the microscope. Barclay counted 150 colonies on a roll tube culture in approximately two-thirds of the time taken to count 150 colonies on a petri dish culture, by the visual method. He found no significant difference between counts, of soil bacteria and bacteria of plant and root surfaces, obtained by the roll tube and petri dish methods.

An aid to scanning microscope slides was developed by Phipps (1966) who was concerned with scanning dried milk films to determine leucocyte contents: the count was estimated by spreading 0.01 ml. over a 1 cm. x 1 cm. area and counting the number of leucocytes in a total of 10 strips across the film. The apparatus moved the slide very slowly and smoothly across the field of view by means of three fixed syringes. The pistons were attached to a brass block which, in turn, was attached to the carriage of the microscope. One syringe was filled with silicone oil, to provide hydraulic damping, and air was directed into one of the other two syringes to move the brass block: the direction of motion depended on which of the two syringes was filled with air and the speed of motion was controlled by a valve in the silicone fluid lines.

1.2.5 Discussion

There are some complicating factors pertaining, exclusively, to all procedures for isolating and identifying bacteria. The specimens are infective and the spread of infection must be avoided. Contamination of the specimen must also be avoided and this is particularly difficult because bacteria, even more than a serum antibody, stick to surfaces: in making serial dilutions of serum, it is adequate to use a pipette and wash it out, by sucking fluid up and down 10 times, after each dilution has been made, but if one does this when diluting bacteria one gets nonsensical results. Therefore, to avoid "carry-over", it is necessary either to discard any vessels with which the infected specimen comes into contact and replace them with freshly

sterilized vessels before the next specimen is processed, or, to sterilize the vessels before subsequent use. In consequence, continuous flow apparatus, such as the auto-analyzer, cannot be used for processing infected materials, and fully automatic apparatus for such procedures is likely to be significantly more complicated than apparatus at present in use in chemical pathology, haematology or serology: to our knowledge, the methods for counting bacteria in suspension (Sect. 1.2.1), for counting colonies on agar plates (Sect. 1.2.2) and for identifying bacteria by chromatography (Sect. 1.2.3) have not been used in diagnostic bacteriology, and the simple aids to manual methods (Sect. 1.2.4) can hardly be described as automatic methods.

The difficulties of handling infective materials are such that it may not be possible to develop more automatic methods for use in diagnostic bacteriology, but we felt that the benefits of so doing, which are well shown in the development of automation for clinical chemistry, are such that one should at least investigate the work of a routine laboratory to discover if one should attempt any further developments: the proliferation of automation in recent years has been such that one is reluctant to reject the possibility of making further advances in this field. We proceed to study bacteriological techniques as applied in our routine laboratory.

1.3 Brief study of Routine Diagnostic Bacteriological Procedures and Conclusions

1.3.1 Serological procedures

On average, 100 sera a day are tested for syphilis antibody. The procedures occupy one senior and one junior technician very nearly full time and a substantial proportion of this time is taken performing the Wassermann reaction. This is a procedure that other investigators have studied (Sect. 1.1), but, in view of the difficulties encountered with the only automatic method hitherto developed, we could usefully devote time developing an alternative automatic method for performing the Wassermann reaction, or at least certain parts of it, on a relatively small number of specimens, say of the order of 50 to 250 per day. A detailed discussion is given in Sect. 3.3.

No other single test is used so much, but simple aids, such as automatic diluters and dispensers, might be of assistance.

1.3.2 Procedures for isolation and identification of bacteria

The nature of the work of the laboratory is such that it is very difficult to apply work study techniques without the aid of a large number of experienced investigators. Many procedures involve a period during which a specimen is incubated and, consequently, many different tasks are performed by one person. Additional difficulties are that most procedures are performed in stages and it does not necessarily follow that each stage is performed immediately on completion of the previous stage, and that specimens are received

in the laboratory throughout the day.

Nevertheless, it is obvious that four procedures take up a substantial proportion of the working day. These are, sterilising an inoculating loop, spreading a culture over an agar plate and examining the culture after incubation, and reporting the findings to the clinician who requested the test: none of these procedures is a complete diagnostic test procedure, but each is merely one of a series of stages that constitute a complete procedure.

Reporting the findings is a very tedious and time consuming procedure, and one that may well lend itself to mechanization in view of the complex data processing techniques becoming available, but we have not investigated the possibilities and do not discuss them further.

Spreading a culture over an agar plate is a technique that should be mechanized, because there are obvious advantages in so doing. For example, all swabs, numbering approximately 50 per day, are spread on a total of 100 agar plates: this takes approximately 1 hour. In addition to spreading swabs over an agar plate, a suspension or colony is spread over a plate for antibiotic sensitivity testing by the disc method: on average, 25 suspensions or colonies are spread each day. Also, a measured volume of suspension is spread for measuring the surface viable count of an organism.

An inoculating loop is often used for spreading a culture and for other purposes, such as inoculating a row of tubes, and the loop has to be sterilized very frequently. There are obvious advantages

in mechanizing the sterilization of the inoculating loop. Similarly, it would be advantageous to be able to repeatedly use pipettes for inoculating agar plates with suspension.

The agar cultures fall into three main groups, cultures with a large number of colonies to be counted, cultures with inhibition zones to be measured, and cultures with single colonies to be identified. A comparatively small number of cultures are examined in a given manner, and methods for examining them that we can visualize, such as Alexander and Glick's electronic scanning method for counting colonies on transparent agar plates, would be very complicated.

We process, on average, approximately 50 urine specimens per day and the procedure takes approximately 2 technician hours per 50 specimens. This time may not be great, but an attempt to develop an automatic method for examining urines should be made, although it will not be easy to develop a simpler method than the existing manual method.

Two other procedures that one should investigate are, the measurement of the minimal inhibitory concentration of an antibiotic by the tube dilution method, and the measurement of the serum antibiotic level: the greater part of these procedures does not involve infective material but, nevertheless, they have to be carried out under aseptic conditions. The number of each test performed is not great, but both tests involve repeatedly dispensing fixed volumes of diluent, and serially diluting a mixture of broth, or serum, and antibiotic. Both processes are very tedious and lend themselves to automation.

The final stage of both the above procedures is to inoculate a row of tubes from the same culture. The inoculation is almost invariably carried out with a glass pipette, so manufactured that it produces drops of equal volumes, usually 0.02 ml. Similar pipettes are often used for transferring liquid cultures, and an automatic method for dispensing small volumes of infective liquids would be of considerable value, because it would save the time and expense involved in making the pipettes, which are often used only once and then disposed of: approximately 700 pipettes are used each week in serological procedures alone.

One might also consider methods for transferring a solid culture, other than by the inoculating loop, because one requires to pick a colony off an agar plate and inoculate a broth culture.

The examination of broth cultures, usually to discover whether there is any growth or to produce growth curves, is also a procedure worth investigating. This technique is used comparatively infrequently, but since photometric techniques are well established (Sect. 1.2.1), the development of automatic techniques for examining broth cultures may be a practical proposition.

1.3.3 Discussion

Despite the simplicity of our investigation, the need for further development of automatic methods in diagnostic bacteriology was obvious: when we commenced work, the only automatic apparatus in use in our laboratory was the slide staining machine (Shandon Ltd.) for the Gram staining technique. We were satisfied that advances could be made and concluded, therefore, that it was worth examining

in greater detail many of the procedures discussed, with a view to developing automatic methods for performing them.

1.4 Possible Fields of Study

It is convenient to divide the possibilities into two fields, namely, the measurement and presentation of the results of the test procedures and the manipulation of the specimens and other materials.

1.4.1 Measurement and presentation of the results of the test procedures

1.4.1.1 Measurement of results

The biggest problem in measuring results is acquiring a transducer that converts the parameter to be measured into an electrical or pneumatic signal.

It is established that the Eh of a bacterial culture varies during growth of the organism (Hewitt, 1950). It may be possible, therefore, to use a Mackereth Eh electrode (Mackereth, 1964), which is more stable and reliable than those hitherto available, for automatically detecting growth in liquid cultures. Whether differences in the growth curves of the organisms commonly occuring in diagnostic bacteriology are great enough for the technique to resolve them, is unknown.

In Sect. 1.2.1 we referred to photometric techniques and the possibility of using them in an automatic method for detecting and measuring growth in broth cultures. Provided one could overcome the difficulties involved in feeding the cultures into the apparatus automatically (Sect. 1.4.2), automatic growth measuring methods

based on photometric techniques might be possible: we have made a simple modification to the 'Spekker' absorptiometer (Hilger and Watts Ltd.) for use in a kinetic study of the bacteriolytic and bactericidal action of human serum (Glynn and Milne, 1967 - see Appendix 1.1).

The application of the Coulter cell-counter, and other similar devices (Particle Data Instrument Co.), to diagnostic bacteriology could be investigated, but the available evidence indicates that the cell-counter is unlikely to become a diagnostic tool for some time (Sect. 1.2.1).

Similarly, electronic scanning methods for counting colonies on a culture plate could be investigated, but this is also unlikely to become a diagnostic tool in the near future.

An electronic scanning technique for measuring areas was developed by Tattam (1966), and it may be possible to adapt the technique for measuring sensitivity disc zone diameters. One might also use optical projection techniques for this purpose, and it is probable that the resulting apparatus would be significantly simpler than apparatus utilizing Tattam's technique.

It may be possible to use optical techniques to detect lysis in the Wassermann reaction test automatically, and to detect the end point in the measurement of the minimal inhibitory concentration of an antibiotic and of a serum antibiotic level.

1.4.1.2 Presentation of results

If one can develop methods for measuring test results, it may be comparatively easy to display them in a suitable form. A multitude of electronics techniques for processing electrical signals have been developed, and these may well be suitable: it is probable that one would merely have to purchase the appropriate equipment. The availability of equipment for processing pneumatic signals is limited, and displaying such signals would, therefore, present more difficulties.

1.4.2 Manipulating specimens and other materials

1.4.2.1 Non-infected materials

A multitude of techniques for handling mechanically both solids and liquids are widely used, and it should be comparatively easy to develop automatic methods for handling such materials. For example, handling antibiotics under aseptic conditions, for the measurement of minimal inhibitory concentrations of antibiotics, should not be difficult, and if one can devise an automatic device for spreading a culture over an agar plate, one can visualise a conveyor-belt system for transporting the plates from the spreading machine to the incubator and on to the reporting bench. However, "carry-over" of serum antibody (Sect. 1.1) indicates that the handling even of non-infected materials is not always straight-forward.

1.4.2.2. Infected materials

The biggest problem in handling infected material mechanically is the fact that bacteria stick to surfaces and, therefore, one

cannot simply rinse through a vessel that has contained bacteria and use the vessel for handling another sample: it is almost invariably necessary to re-sterilize the vessel or to discard the vessel and replace it with a freshly sterilized one (Sect. 1.2.5). An investigation of the problem of bacteria sticking to surfaces would be worthwhile. Some materials may not be wetted so easily as others, but it is likely to prove impossible to find a material that could be used repeatedly without re-sterilizing it.

We have, therefore, to develop methods for feeding pre-sterilized disposable vessels into the apparatus and discarding them after use automatically or for sterilizing the vessels in situ. The former is probably basically simple, but the safeguards required to ensure that neither the vessel nor the apparatus become contaminated are such that the apparatus would be very complicated. Automatically sterilizing components in situ also presents problems, particularly as chemical sterilization is unacceptable: in any case, the time taken to sterilize chemically is too long for the technique to be of much value in automatic methods. Steam sterilization is a possible method, but is not very suitable for a routine laboratory. However, despite the difficulties, it will be essential to be able to sterilize vessels in situ before much progress is made in handling infective materials mechanically, and r.f. induction heating is a possible method. As an intermediate step, one might make more use of pre-sterilized disposable vessels and, also, of simple mechanical aids to manual methods that are automatically sterilized.

1.5 Conclusions

Both the fields of study discussed in Sect. 1.4 are interesting and will have to be investigated in due course. We concluded, however, that we should first investigate the possibilities of manipulating specimens and other materials mechanically, for the following reasons.

We think it unlikely that any known method for measuring the results of test procedures could be developed into an economic, automatic method in the near future. In any case, the ability to handle infective specimens mechanically is an essential pre-requisite for a fully automatic diagnostic system, and, finally, so few automatic methods are in use in routine bacteriology laboratories that, in a comparatively short period, one might make a useful contribution to the problem caused by the rapidly increasing work load, and the serious shortage of technical staff.

We also concluded that whilst it may be possible to devise fully automatic methods for performing a given test procedure, it is unlikely that such apparatus would be economic in the average routine bacteriology laboratory. However, this should not deter one from developing automatic methods, because the ever increasing work load and the shortage of technical staff makes it necessary to increase productivity: the availability of automatic methods may also enable bacteriologists to perform tests that should be performed but which hitherto could not be performed. In due course, it may become desirable to process all specimens in relatively few very large

laboratories, because this may be the only economic way of processing the total work load with the simplest fully automatic apparatus that will be developed.

The development of fully automatic methods will not be completed in the immediate future, and in the meanwhile it is desirable to design apparatus suitable for use in the average routine laboratory. Since some stages of many procedures are common to other procedures, it may be possible to assemble apparatus from a few basic units, each of which may, in principle, be utilized for a variety of different procedures: this may be the only way of producing an economic diagnostic test system suitable for processing small numbers of specimens.

In any automatic equipment for use in a bacteriology laboratory, simplicity of operation is essential, even at the cost of complicating the control mechanism, but it is rarely necessary for automatic apparatus to perform its function more quickly than it can be performed by hand: what is important is that the total time the operator spends performing the task be minimal.

For many test procedures it is unnecessary to achieve very high orders of accuracy, and one should aim at producing as high an order of accuracy as is consistent with any other criteria that have to be satisfied, but reproducibility is important. In general, an accuracy of $\pm$ 2% is probably adequate and, as we shall see, this is significantly better than many manual methods and easily achieved.

I shall describe, in detail, several automatic methods that have been devised and evaluated.

Chapter 2

Simple, Definitive Methods

2.1 The Inoculating Loop

The inoculating loop has been used in bacteriological laboratories for many years, and there is little doubt that it will never become redundant because it is very simple, cheap and suitable for a variety of tasks. Usually, the loop is sterilized after use by 'flaming' it, a task which takes approximately 5 seconds. This is not in itself very long, but when multiplied by the number of times a loop is 'flamed' in the average laboratory, we see the total time spent on this procedure is significant: the organisation of the work of a routine bacteriology laboratory is such that it would be very difficult indeed to determine an accurate estimate of the man hours involved. Nevertheless, it is clearly desirable to develop an automatic method for sterilizing inoculating loops and two possible methods come to mind. One could devise a holder into which the loop is placed suitably positioned over a bunsen burner, the main jet of which is automatically ignited, and, one could pass a suitable electric current through the infected part of the platinum wire, thus heating it. Both methods have been attempted and are described below.

2.1.1 Automatically 'flaming' an inoculating loop

Fig. 1 is a drawing of the apparatus used. Briefly, it consisted of an adjustable stand and a standard bunsen burner with

pilot jet which was fixed to the base plate in the optimum position: the stand could be moved vertically and horizontally to accommodate different lengths of wire. The stop-cock lever of the burner was replaced by an electrically operated rotary solenoid and this, in turn, was operated by a simple timing device which switched on the main gas supply for 5 seconds every 15 seconds: the cycle was simply varied if required. The loop was placed in the holder as shown and on actuating a switch, the 'flaming' cycle commenced. We continuously re-sterilized the loop to ensure that it was always ready for use, but the cycle allowed little time for the loop to cool, and if such a system were adopted for use in a routine laboratory, some indication of the time elapsed since the loop was last heated would be necessary.

The main problem with this technique was that since the platinum wire is so flexible it was very difficult to arrange for all parts of the wire to rest in the flame without spending time adjusting the position of the wire, which defeated the object of the device.

No doubt one could have improved considerably on this simple apparatus, but we had doubts of finding a really satisfactory solution to the problem by this method. We therefore considered the possibilities of sterilizing the loop electrically.

2.1.2 An electrically heated inoculating loop

It would be very convenient if the standard platinum loop could be sterilized by passing an electric current through it. There are, however, two difficulties. Since a current of the

order of amperes has to be passed through the wire to produce sufficient heat, it is essential to make a high pressure connection between the wire and the power supply, to prevent arcing, and this would not be easy with such flexible wire as that ordinarily used for an inoculating loop. In addition, the current would have to be passed through the whole length of wire one end of which was contaminated. Connection would have to be made with that end, thus contaminating the connector. One could have partially overcome these difficulties, but a much more suitable form of loop is one in which the loop is formed at the centre of a piece of platinum wire, both ends of which are permanently attached to thicker wires which form a handle, as shown in Fig. 2. The standard cautery burner (Fig. 3) is very similar to what is required, and the materials, 21 gauge platinum wire with copper handles, are suitable from the sterilization point of view: the suitability of the standard cautery burner in general is discussed on page 47.

For experimental purposes, the electrical power was derived from a continuously variable transformer, the output of which was connected to an a.c. current meter, a 1Ω resistor and the loop, all in series, as shown in Fig. 4: this enabled one to increase the current passing through the loop slowly up to approximately 9A, at which point the platinum wire became red hot. The loop was permanently connected to the apparatus by copper braid.

The current required to sterilize the loop was determined by the following technique. The current passing through the loop was increased until it became red hot. The current was passed for an

additional 10 seconds and then removed: the loop was left to cool. The wire was contaminated by picking a colony off a plate, heated by passing a specific current through it for 5 seconds and then streaked on to a blood agar plate: the wire was heated to red heat and the procedure was repeated 25 times. The results obtained for two organisms, E. coli and Staph. aureus, are shown in Table 1. The current required to sterilize the loop when it was contaminated with Bacillus stearothermophilus was determined by a slightly modified technique: the loop was dipped into fresh tryptone soy broth and not streaked on to an agar plate. The results obtained are shown in Table 2. A similar series of tests, in which the time the sterilizing current was passed through the loop was varied, was carried out using Staph. aureus, and the results are shown in Table 3.

All these results show that the exact current required to sterilize the loop varies, as would be expected since no attempt was made to control the size of the inoculum. However, a current of 6A for 5 seconds was usually adequate, and a current of 6.5A for 5 seconds was adopted for routine use.

The circuit diagram of the power supply used in the routine laboratory is shown in Fig. 5. The power is derived from the secondary of a tapped transformer: the secondary circuit is identical to that used in the experimental apparatus (Fig. 4). The apparatus contains appropriate warning lights and a simple control device that automatically switches off the power at the end of a pre-determined but variable time: the operator has only to initiate the process by

TABLE 1

Sterilization of ½ in. inoculating loop by varying currents passed for 5 seconds

Current passed in amperes	Number of failures in 25 tests	
	E.coli	Staph. aureus
1	25	25
2	25	25
3	11	20
4	14	15
5	0	5
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0

TABLE 2

Sterilisation of ½ in. inoculating loop by varying currents passed for 5 seconds

Current passed in amperes	Number of failures in 25 tests *B.stearothermophilus*
1	25
2	25
3	25
4	16
5	9
6	0
7	0
8	0
9	0
10	0

TABLE 3

Sterilisation of $\frac{1}{8}$ in. inoculating loop after various times of heating

Time in seconds	Number of failures in 10 tests Staph. aureus 4A	5A	6A
3	10	3	0
4	7	1	1
5	4	1	0
6	0	1	0
7	0	0	1
8	0	0	0
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	0

momentarily pressing a switch, thus effecting a considerable saving of time. Any significant variations in the current passing through the loop, due to ageing of the loop or differences between various loops, can be corrected by altering the tap on the transformer: 1V steps to a maximum of 10V is adequate.

For use in the routine laboratory, it was necessary to protect the handle of the cautery burner to ensure that it is safe to hold during sterilization, and we therefore moulded a silicone rubber sheath round the copper handle, as shown in Fig. 6: the copper braid is permanently connected to both the cautery burner and the power supply (Fig.7).

Whilst the standard cautery burner is suitable for use with solid cultures on agar plates, and thus has a place in a diagnostic laboratory, the $\frac{1}{8}$ in. length of platinum wire makes it unsuitable for use with liquid cultures or when the loop has to be put in a narrow tube, because there is a danger that the joint of the platinum wire with the copper handle, and the handle, will become contaminated. A further series of tests showed that a minimum current of 9A for 5 seconds is required to sterilize the joint between the platinum wire and the copper handle. This is nearly the maximum current that can be passed through the platinum wire without damaging it and is such that the life of the wire would be significantly reduced. A special loop was therefore manufactured for us. This has a standard copper handle and the platinum wire is approximately 2 in. long (Fig. 8).

The current required to sterilize this loop was determined as

before (p42): a current of 9A was passed until the loop became red hot, the wire was contaminated, heated by passing a specific current for a fixed period, and then streaked on to a blood agar plate. The results obtained with Staph. aureus are shown in Table 4. In addition, a series of tests were performed in which the loop was contaminated by dipping it into a liquid broth culture and the efficacy of sterilization was tested by dipping the loop into sterile nutrient broth; this was necessary to ensure that sufficient heat is developed to remove, completely, any liquid that is held between the two straight lengths of wire. The results of this series of tests, conducted with Staph. aureus, are shown in Table 5. The results are very similar to those obtained with the ½ in. loop but, if anything, a slightly longer time is required to sterilize the 2 in loop: a current of 6.5A for 6 seconds was adopted for routine use.

The 2 in. loop is more convenient and is almost as versatile as the conventional inoculating loop. We find the 21 gauge platinum rigid enough to withstand normal usage with negligible danger of the two sides of the loop touching each other: should this happen, the current that would flow is limited by the 1Ω resistor, thus safe-guarding the power supply. The loop can be 'flamed' in the normal manner, but the silicone rubber sheath, whilst not flamable, disintegrates if held in a flame.

It may be inconvenient to have the loop permanently connected to a pair of wires, however flexible they may be. Consequently, we have also used a pair of single pole 10A miniature connectors,

TABLE 4

Sterilization of 2 in. inoculating loop by varying currents passed for varying times: inoculation from solid culture

Current passed in amperes	Time in seconds	Number of failures in 20 tests *Staph. aureus*
4	1	20
4	2	20
4	3	19
4	4	18
4	5	13
4	6	11
4	7	6
4	8	8
5	1	20
5	2	19
5	3	18
5	4	15
5	5	8
5	6	5
5	7	6
5	8	4

Continued ...

TABLE 4 (continued)

Current passed in amperes	Time in seconds	Number of failures in 20 tests *Staph. aureus*
6	1	20
6	2	20
6	3	17
6	4	4
6	5	4
6	6	1
6	7	0
6	8	0

TABLE 5

Sterilization of 2 in. inoculating loop by varying currents passed for varying times; inoculation from liquid culture

Current passed in amperes	Time in seconds	Number of failures in 24 tests *Staph. aureus*
4	3	24
4	4	24
4	5	24
4	6	21
4	7	10
4	8	11
5	3	24
5	4	14
5	5	3
5	6	1
5	7	0
5	8	0
6	3	3
6	4	2
6	5	0
6	6	0
6	7	0
6	8	0

as shown in Fig. 9. This type of loop has its advantages, for in addition to its portability one may have a variety of different loops, each of which may be plugged into the same power supply. However, the plugs have to be located in the sockets and this takes time and attention which partially offsets the advantages. On balance, therefore, we feel that it is better to have one or more loops permanently connected to the power supply by insulated copper braid.

Our experience demonstrated that both loops are perfectly satisfactory from the bacteriological point of view. They may be used in some environments where a flame is unusable, such as in an anaerobic cabinet (Drasar, 1967), or in field work. Whilst this method is unlikely to supersede the classical inoculating loop, it has a place in a diagnostic bacteriology laboratory.

2.2 Dispensing Nutrient Broth

There are so many liquid dispensers commercially available today (Broughton, 1965) that one hesitates to design and construct yet another. However, our requirements are unusual in that the apparatus is unlikely to be used frequently, those parts with which fluid comes into contact must be sterilizable by heat, and it must be a simple matter to remove all parts with which the fluid comes into contact quickly and to replace these parts without affecting the calibration of the system. Most of the devices readily available are unsuitable, as they have to be sterilized chemically and they utilize a pin or ball valve which would probably stick due

to the formation of crystallized broth. One could dismantle the apparatus and clean it after use, but this is undesirable, particularly as most equipment would have to be re-calibrated. We have, therefore, developed apparatus based on the peristaltic pump.

2.2.1 The peristaltic pump

The peristaltic pump (Fig. 10) operates by squeezing a trapped volume of liquid along a flexible tube with some form of rollers or mechanical fingers. The volume of fluid transferred depends on the bore of the tube (the volume of fluid trapped between rollers) and the speed with which the rollers rotate. A variety of pumps are available today, some single-channel with or without variable speed control (Fig. 11) and some multi-channel also with or without speed control (Fig. 12). Flow rates as low as 0.015 ml./h. and as high as 200 gal./h. are easily obtainable.

The advantages of the peristaltic principle are that the pumps are valveless and that one can use any flexible material that is sterilizable by heat, such as silicone rubber. One can have a completely closed circuit (Fig. 13) and all parts with which the fluid comes into contact can be removed as one entity and replaced without re-adjusting any controls. The disadvantage is that continuous squeezing of the flexible tube slowly distorts it and consequently the volume of fluid trapped in the tube gradually varies with time.

Peristaltic pumps are almost invariably used for continuous

flow techniques, such as continuous infusion chemotherapy (Pegg, Trotman and Pierce, 1963), but they can be used as simple dispensers of discrete small volumes of liquids and are particularly suitable for our purposes.

2.2.2 The dispenser

There are three ways of adjusting the quantity of liquid that is dispensed, by varying the bore of the tube, by varying the speed of rotation of the rollers and by varying the time they rotate. It is inconvenient to have to change the tubing and in any case, consequential changes in both the speed of rotation of the rollers and the time of rotation would follow. We decided, therefore, to fix the bore of the tubing and to find the combination of speed of rotation of the rollers and time of rotation that gives 1 ml. of broth, the volume of chief interest to us, with the greatest accuracy.

The technique adopted for finding the optimum conditions was as follows. Using 1/16 in. bore, 1/16 in. wall silicone rubber tubing, the pump was operated at a speed of 160 rev/min. for approximately 1.5 seconds to dispense 1 ml. of broth into a pre-weighed test tube: this process was repeated 48 times, keeping both pump speed and time of rotation constant. The test tubes were re-weighed and the difference in weight of each tube was taken to be the volume dispensed. The difference between the highest and lowest volume dispensed was called the spread of the readings in ml. about the mean value. This procedure was repeated for a variety of different speeds, the time for each speed being that required to produce a

volume of 1 ml. The results are plotted in Fig. 14, and a typical set of figures, those obtained at a speed of 70 rev./min., is shown in Table 6 and in the accompanying histogram. The highest volume dispensed in the 48 samples was 1.0452 ml. and the lowest 1.0210 ml. giving a spread of 0.0242 as plotted in Fig. 14. If we take the mean $(\bar{x}) \pm 2.5x$ standard deviation (S.D.) points (these values are exceeded in only 0.75% of trials) for estimating the accuracy of dispensing, we see it is approximately $\pm$ 1.5%. Fig. 14 shows an unmistakable trend, namely that as the speed of rotation is reduced, the repeatability is improved. We did not pursue this further because the time required to dispense 1 ml. at a speed of 70 rev./min. is approximately 3.8 seconds, which is as long as practicable. Since an accuracy of $\pm$ 1.5% is sufficient, we adopted a speed of approximately 70 rev./min. for routine use. The mean of the readings given in Table 6 is slightly above 1 ml. and minor adjustments were necessary: either the speed of rotation or the time could be reduced slightly to give a mean reading of 1 ml. and we reduced the time to 3.5 seconds.

The complete apparatus is shown in Fig. 15. The control unit is based on a commercially available timer and the unit is arranged so that one can run the peristaltic pump continuously, for priming, or one can press a spring-loaded switch to dispense a single volume, or one can dispense a series of volumes; there is just sufficient time to move the nozzle from one receptacle to the next. The full circuit diagram of the control unit is given in Appendix 2.1. The variable speed control on the pump is not

TABLE 6

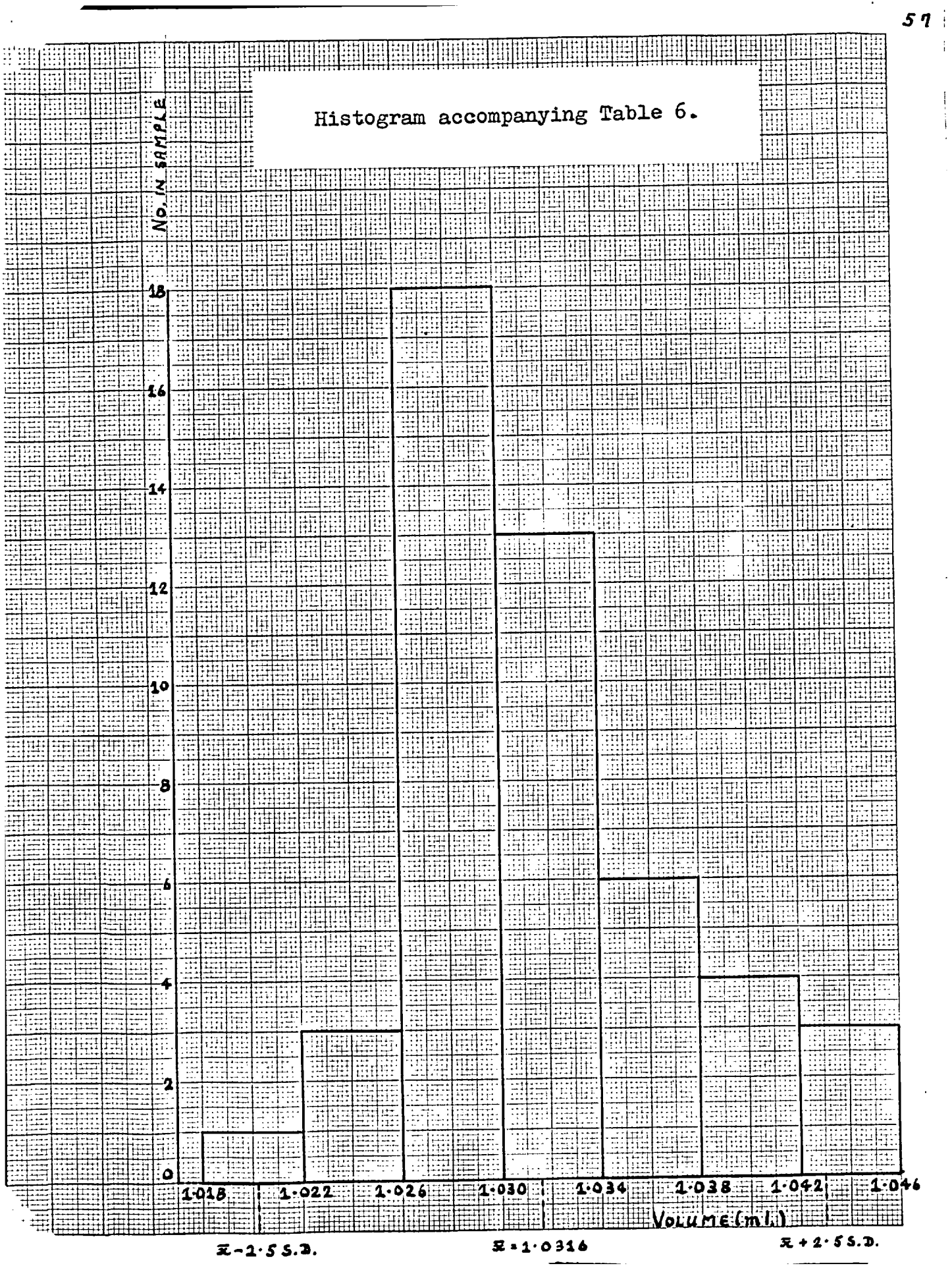
Accuracy of the peristaltic pump dispenser

Speed of rotation of rollers = 70 rev./min., time of rotation of rollers = 3.8 secs., bore of tubing = 1/16 in.

Tube number	Volume dispensed in ml.	Tube number	Volume dispensed in ml.
1	1.0291	25	1.0284
2	1.0302	26	1.0263
3	1.0343	27	1.0324
4	1.0298	28	1.0342
5	1.0344	29	1.0307
6	1.0380	30	1.0299
7	1.0428	31	1.0352
8	1.0328	32	1.0275
9	1.0369	33	1.0298
10	1.0330	34	1.0330
11	1.0265	35	1.0280
12	1.0350	36	1.0295
13	1.0436	37	1.0401
14	1.0452	38	1.0327
15	1.0301	39	1.0302
16	1.0291	40	1.0286
17	1.0328	41	1.0252
18	1.0276	42	1.0366
19	1.0271	43	1.0293
20	1.0320	44	1.0295
21	1.0311	45	1.0258
22	1.0310	46	1.0210
23	1.0388	47	1.0245
24	1.0281	48	1.0296

$\bar{x}$ = 1.0316 S.D. = 0.00498

Histogram accompanying Table 6.



calibrated directly in revolutions per minute and consequently independent calibration was necessary (Fig. 16).

The method has its disadvantages, it is not easy to significantly alter the volume dispensed and small changes have to be compensated for by trial and error. In addition, care has to be taken to ensure that the small drop of broth that sometimes forms at the end of the nozzle is not knocked off. However, we have found that the repeatability is maintained over long periods and that the broth remains sterile for at least two weeks. One could have devised a valve type dispenser (Sect. 3.3.3.1) which would not have the disadvantages of this method, although it would be unsuitable for infrequent use (Sect. 2.2). The advantages and disadvantages of each type of dispenser are fairly evenly balanced and the final decision depends to a large extent on the circumstances, but the method described above has a place in bacteriology laboratories.

2.3 Final Comments

Two very simple devices that have been evaluated in our laboratory have been described. Both devices are very useful, particularly in that they save substantial time, and the dispenser is far more consistent and at least as accurate as manual methods.

The very simplicity of these devices illustrates the need for the introduction of automatic methods in diagnostic bacteriology, but it is necessary to introduce a much greater degree of automation. In Chapter 3 we discuss some more sophisticated methods.

Fig. 1. Drawing of apparatus for automatically 'flaming' an inoculating loop: A_1, A_2 - adjustable sections of stand, B-solenoid operated bunsen burner, L - loop resting on stand.

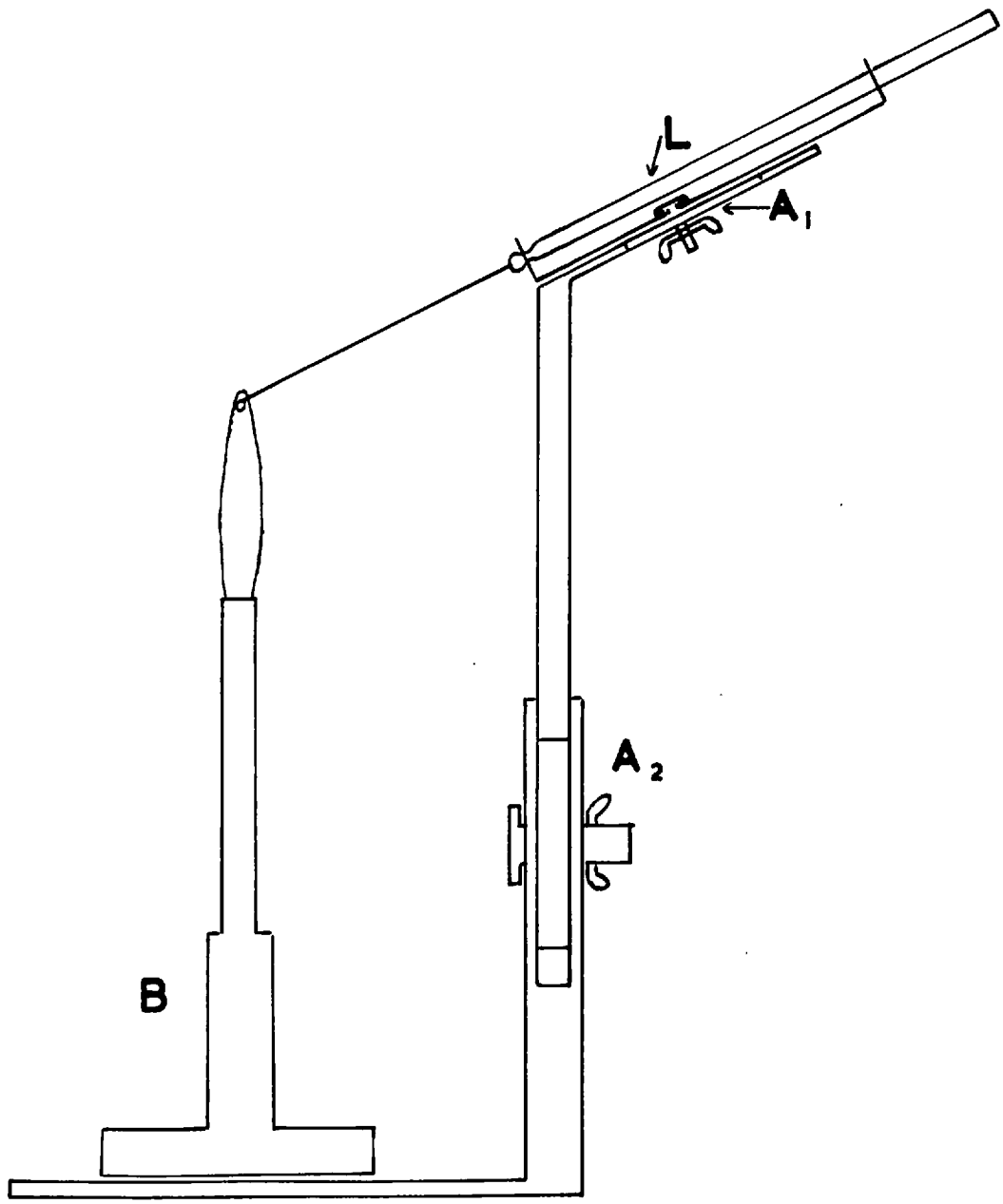


Fig. 2. Sketch showing form of inoculating loop suitable for electrical heating: P - platinum wire, the ends of which are permanently joined to a pair of robust wires H which form a handle (the wires H are insulated from each other).

Fig. 3. A standard cautery burner.

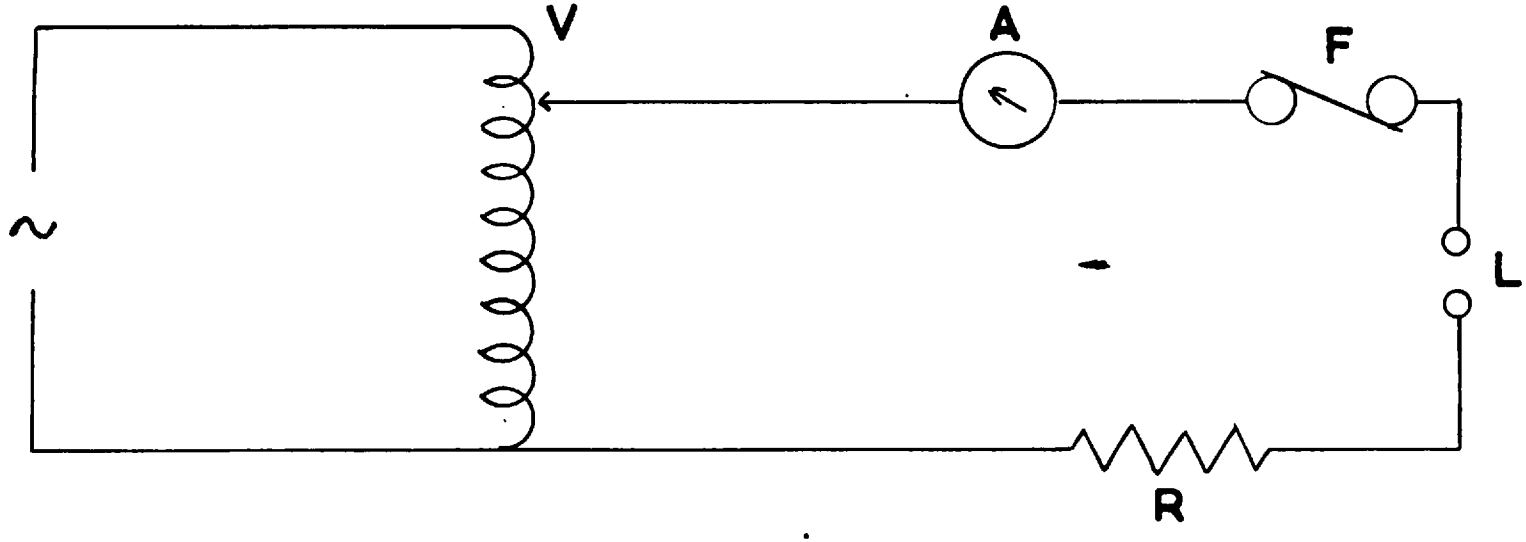


Fig. 4. Circuit diagram of power supply used in experiments with the electrically heated inoculating loop: V - Variac (0-15V), F - fuse (10A), A - ammeter (10A), L - connections to loop, R - 1Ω resistor (150W).

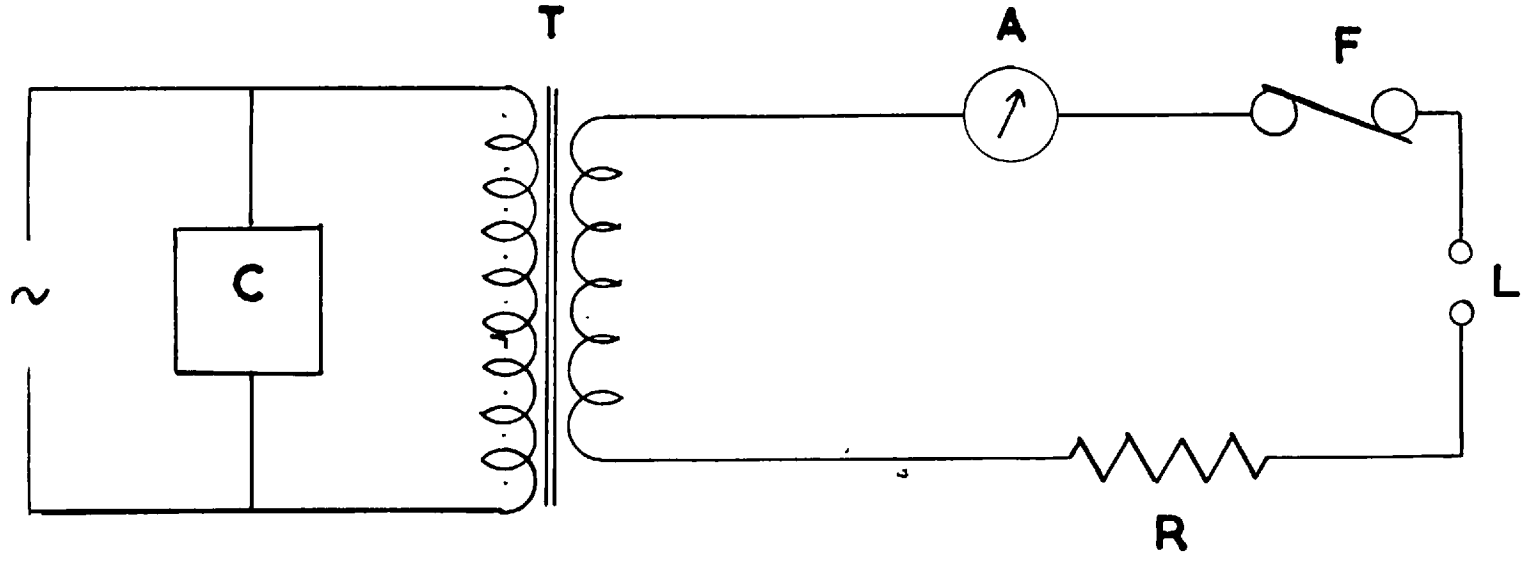


Fig. 5. Simplified circuit diagram of power supply for the electrically heated inoculating loop used in the routine laboratory: T - tapped transformer (1V stages to 10V), C - timing and control circuit, F - fuse (10A), A - ammeter (10A), L - connections to loop, R - 1Ω resistor (150W).

Fig. 6. A standard electrically heated inoculating loop with moulded silicone rubber handle.

Fig. 7. The inoculating loop and the power supply currently in use in the routine laboratory.

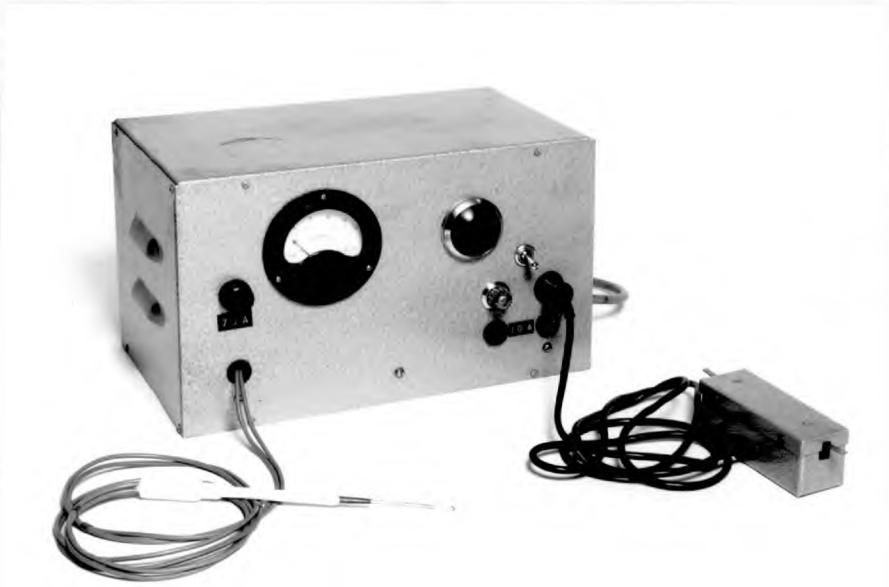


Fig. 8. A 2 in. inoculating loop with moulded silicone rubber handle.

Fig. 9. A standard inoculating loop with moulded silicone rubber handle and with plugs attached.

Fig. 10. Illustration of the principle of the peristaltic pump - the three rollers rotate in an anti-clockwise direction, thus squeezing the flexible tube and transfering the fluid along the tube from left to right.

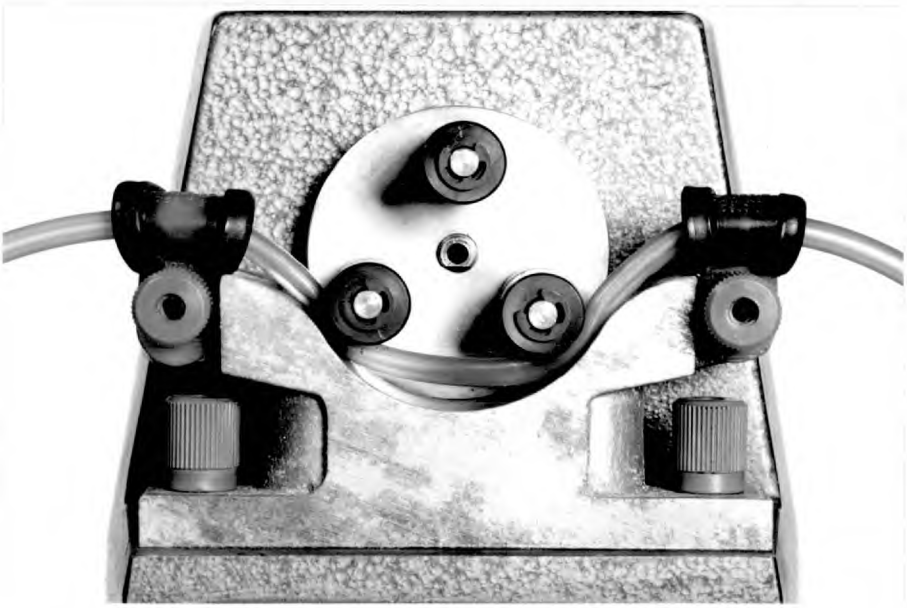


Fig. 11. A single-channel peristaltic pump (with variable speed control).

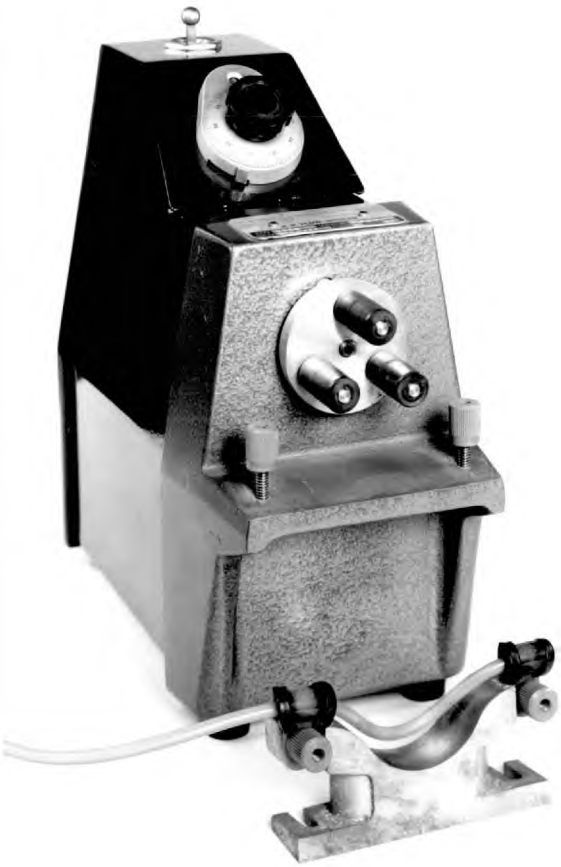


Fig. 12. A multi-channel peristaltic pump (without variable speed control).

Fig. 13. The peristaltic pump, the fluid reservoir R and the output nozzle O, illustrating that the system through which the fluid passes is completely closed.

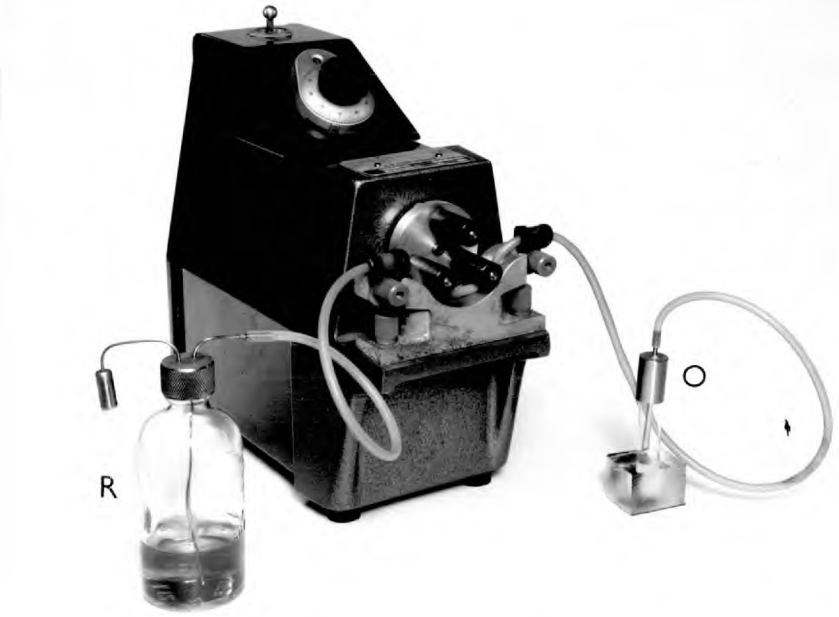


Fig. 14 — Graph of the difference between the highest and lowest volumes of a set of 48 nominal 1ml. volumes dispensed by the peristaltic pump dispenser (Sp.) vs. speed of rotation of the rollers (S).

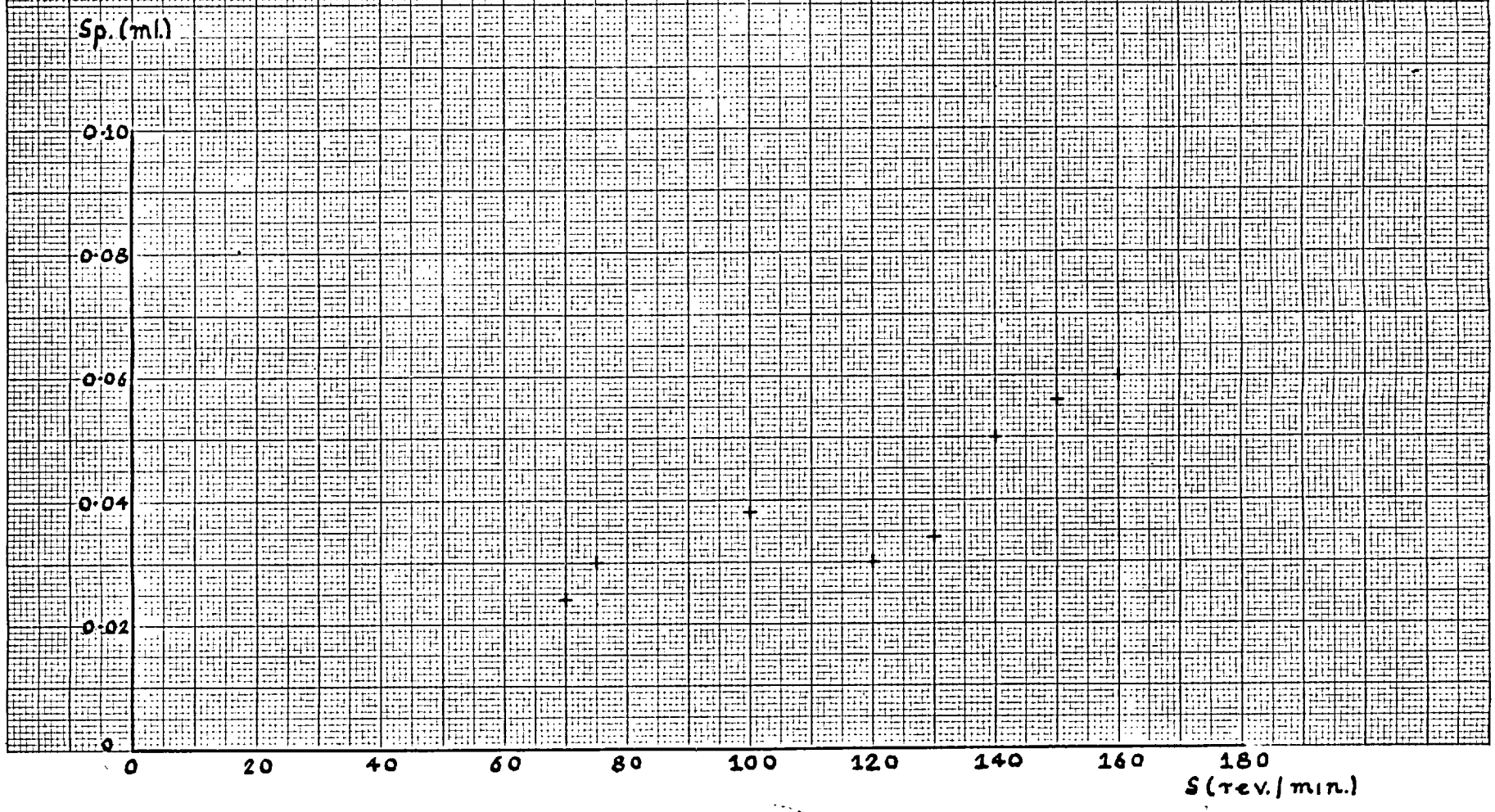
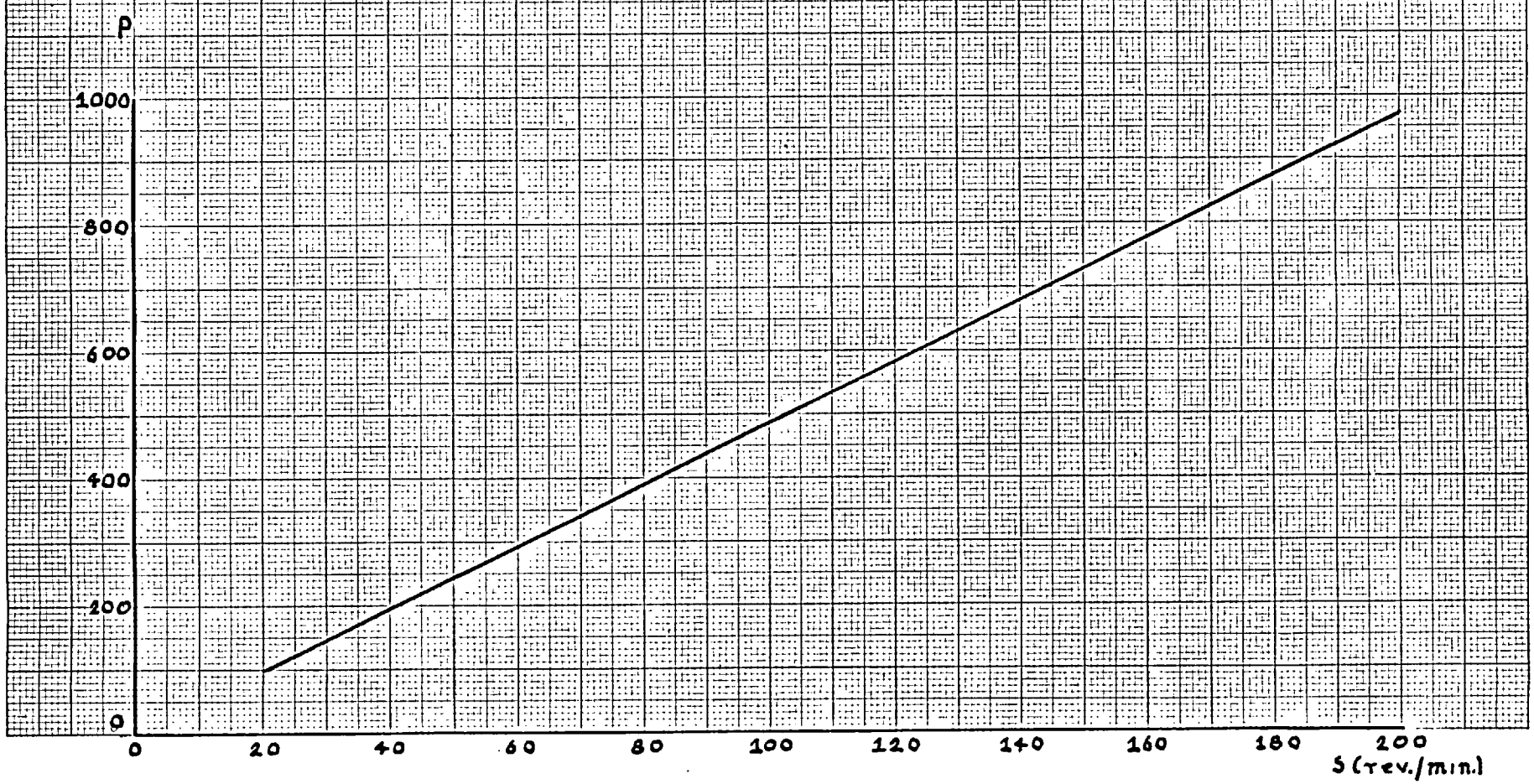


Fig. 15. The peristaltic pump dispenser.

Fig. 16 Calibration curve of the peristaltic pump: instrument potentiometer reading (P) vs. speed of rotation of the rollers (S).



Chapter 3

More Sophisticated, Definitive Methods

3.1 Serial Diluting

Two procedures often used in diagnostic bacteriology are, measurement of the minimial inhibitory concentration of an antibiotic (M.I.C.) and measurement of the serum antibiotic level.

The method used for determination of the M.I.C. in our laboratory is as follows. 2 ml. of appropriately concentrated antibiotic solution in nutrient broth is placed in a test tube and 1 ml. of broth is placed in each of a series of 10-12 tubes. 1 ml. of the antibiotic containing broth is transferred from the first tube to the 1 ml. of broth in the second tube and mixed thoroughly. This transfer is repeated 9 or 10 times to give a series of two-fold falling dilutions. Subsequently, a standard suspension of the bacterium under test is added to each tube. After incubation one observes the lowest concentration of antibiotic that inhibits growth. For a serum antibiotic level, serum is similarly diluted and each tube is inoculated with a standard organism: a control M.I.C., in human serum, is also performed. After incubation, one observes which dilution of serum contains sufficient antibiotic to inhibit growth of the organism and the corresponding concentration of antibiotic is calculable from the M.I.C.

There are three parts of the procedure that lend themselves to mechanization, the distribution of initial volumes of diluent, the serial diluting and the final addition of culture. We use the method described in Sect. 2.2 to distribute the initial volumes of diluent and final addition of culture is discussed in Sect. 3.2.

3.1.1 Evaluation of manual method

Two sources of error in the serial diluting technique are, in transferring the 1 ml. volume of antibiotic containing broth to the next tube in the series, and in mixing the solution with diluent; the accuracy with which the initial volumes of diluent are dispensed is also relevant, but we do not here regard that procedure as part of the serial diluting technique. To investigate the magnitude of the errors we measured, the accuracy with which a manual operator performs the transfer of solution from one tube to the next and the concentration of the solution left in each dilution tube at the end of the procedure. We also measured the time taken to perform a series of dilutions.

The procedure used to measure the accuracy with which an operator performs the transfer of solution is as follows. A set of 12 tubes was taken and 2 ml. of broth was dispensed into the first tube and 1 ml. of broth was dispensed into the remaining 11 tubes; very great care was taken to eliminate errors in dispensing. Each of the 12 tubes was then weighed. The set of tubes, together with a pipette, was given to a member of the routine laboratory staff who was asked to perform a set of serial dilutions using the technique normally

adopted; the whole procedure was timed. The tubes were then re-weighed and the volume of broth left in each tube calculated: it was assumed that the initial volumes had been dispensed with 100% accuracy. A typical result is shown in Table 7. If we take the highest and lowest values and calculate the difference between these and 1 ml., the volume that should be left in each case, we see that the errors vary between +7.6% and -1.0%. The results of 19 similar tests are given in Table 8.

The procedure used to measure the concentration of the solution left in each dilution tube is as follows. We diluted a solution of Armor bovine albumin powder V in distilled water, and measured the optical density of each dilution at 2800 Å with a Unicam S.P.500 spectrophotometer, using 0.5 ml. silica cells, a 12 mm. x 4 mm. slit and the collimating lens. To calibrate the apparatus, a series of standard solutions in the range 100 to 1600 µg/ml. was prepared as accurately as possible and the optical densities measured. The calibration curve is shown in Fig. 17.

With a solution of 4000 µg/ml. in the first dilution tube (dilution 1), a series of six serial dilutions was produced and the optical densities of dilutions 3 to 6 were measured. Twelve such series were produced as accurately as possible, not under normal routine laboratory conditions as before, and the measured optical densities (Table 9) were plotted on the calibration curve (Fig. 18): the concentration of the solutions was derived. It was found to be impractical to measure more than four dilutions of a series because, of doubts about the linearity of the calibration curve at

TABLE 7

Accuracy of the manual serial dilution procedure:

12 successive dilutions

Operator number 9 - see Table 8

Tube number	Volume of broth left in tube in ml.
1	1.0761
2	1.0075
3	1.0029
4	0.9899
5	1.0108
6	0.9896
7	0.9940
8	0.9909
9	1.0095
10	0.9920
11	0.9915
12	1.0050

$\bar{x}$ = 1.0049 S.D. = 0.0238

TABLE 8

Speed and accuracy of manual operators performing a series of 12 serial dilutions

Operator number	Percentage error in volume transferred		Time taken for 12 dilutions	
	Max. positive	Max. negative	min.	secs.
1	6.9	2.3	1	15
2	2.5	1.9	1	17
3	6.0	3.2	1	17
4	6.5	1.1	1	35
5	7.7	2.1	2	0
6	8.0	7.4	2	0
7	5.1	1.7	2	0
8	6.0	4.1	2	5
9	7.6	1.0	2	5
10	3.3	1.3	2	30
11	5.0	1.1	2	30
12	8.7	4.5	3	0
13	4.7	2.7	3	0
14	3.2	1.6	3	0
15	4.2	4.7	3	5
16	6.4	5.2	4	0
17	0.7	1.1	4	40
18	4.8	1.1	5	0
19	4.3	5.5	7	30

TABLE 9

Concentration of hand-made dilutions of albumin

Dilution number	Measured optical density: 12 sets of dilutions		
3	0.650	0.688	0.580
	0.625	0.656	0.590
	0.650	0.672	0.591
	0.665	0.672	0.550
4	0.322	0.357	0.260
	0.347	0.368	0.279
	0.340	0.351	0.292
	0.341	0.353	0.272
5	0.155	0.182	0.108
	0.184	0.194	0.144
	0.188	0.184	0.140
	0.198	0.184	0.235
6	0.094	0.096	0.023
	0.107	0.109	0.031
	0.103	0.101	0.054
	0.118	0.101	0.069

intermediate concentrations, its slope is very small at high concentrations and the initial solution would have to be much more highly concentrated and would, therefore, be far too viscous. Further discussion of this point is given in Sect. 3.1.5.

Fig. 18 shows that, despite the fact that great care was taken, the technique is very poor: there is a very wide spread in the concentration of the solution left in each tube and many dilutions are more than 25% inaccurate. If we assume that the errors in transferring the liquid were no greater than $\pm$ 3%, which is easily achieved with grade A pipettes, it is interesting to speculate how much poorer the results would have been had the test been carried out under normal routine laboratory conditions: Table 8 shows that under those conditions the volume transfer can be as inaccurate as $\pm$ 7.5%. The time the manual method takes under routine conditions is discussed in Sect. 3.1.5.

3.1.2 Criteria for an automatic method

Any automatic technique should not only save time but should also produce more accurate dilutions than those above provided this can be achieved simply, because the number of M.I.C.'s and serum antibiotic level tests performed in the average laboratory does not justify very complicated and costly apparatus. The most important part of the apparatus is the mechanism for transferring the broth/antibiotic mixture or serum, and whereas the absolute accuracy does not have to be of the highest order, reproducibility is most important; an accuracy of approximately $\pm$ 2% is adequate. The

parts the liquid touches must be sterilizable by heat, and, since a variety of different antibiotics and sera will be used, the pipettes must be readily removed from the apparatus and replaced without affecting the calibration of the apparatus; the pipettes often have to be changed between series of dilutions. One can use 3 in. x $\frac{1}{2}$ in. test tubes, and the racks and covers must be sterilizable by heat and detachable from the apparatus for incubation purposes. The number of series of dilutions that one requires to perform simultaneously is purely arbitrary and depends on circumstances. We felt that it was undesirable to use a rack significantly larger than those in general use and this places a limit of 4 sets of 12 tubes per rack; up to 12 dilutions are sometimes required and 4 sets are quite suitable for our purposes.

3.1.3 The apparatus

3.1.3.1 The liquid transfer assembly

Some manually operated and automatic devices for pipetting liquids are commercially available (Broughton, 1965), and Weitz (1957) published details of a manually operated multi-volume pipetting device. Sequeira (1964) has also produced a similar hand operated machine (p.12). However, it is neither convenient nor economic to incorporate these devices in a multi-volume automatic pipetting device and many of them do not satisfy our requirements. It was decided, therefore, to use a different system and we decided to examine first the efficacy of an extremely simple method. The apparatus consists of a pipette, made of a

straight length of polytetrafluorethylene (p.t.f.e.) tubing, connected by a flexible tube to a 1 ml. syringe which is operated mechanically to draw up and expel the fluid. The principle of the apparatus used for evaluation purposes is shown in Fig. 19. The syringe is connected by a Luer connector to a length of silicone tubing and this, in turn, is connected to a piece of p.t.f.e. tubing A by a Luer lock connector B. The female part of the Luer lock connector is linked to the p.t.f.e. tube by a short length of silicone tubing C. The syringe barrel D is mounted between two fixed brackets E, with the aid of device F which is adjustable to allow for tolerances in the external dimensions. The syringe piston G is connected to a 1 in. diameter double-acting pneumatic cylinder I, the inward stroke length being varied by device K.

The p.t.f.e. tube, which has a $\frac{1}{8}$ in. internal diameter and a 3/16 in. outside diameter, is approximately $6\frac{1}{2}$ in. long, so that 1 ml. occupies approximately three-quarters of its length; there is therefore no risk of the fluid being drawn into the flexible connecting tube. The apparatus is shown in Fig. 20.

The procedure used to measure the accuracy with which this device performs the transfer of 1 ml. of solution is the same as that used to evaluate the manual method (Sect. 3.1.1), but in this case we performed 40 dilutions. A typical set of results is given in Table 10 and in the accompanying histogram. Taking the $\bar{x} \pm 2.5$ S.D. points as before (p.55), we can quote an accuracy of $\pm 2\%$.

TABLE 10

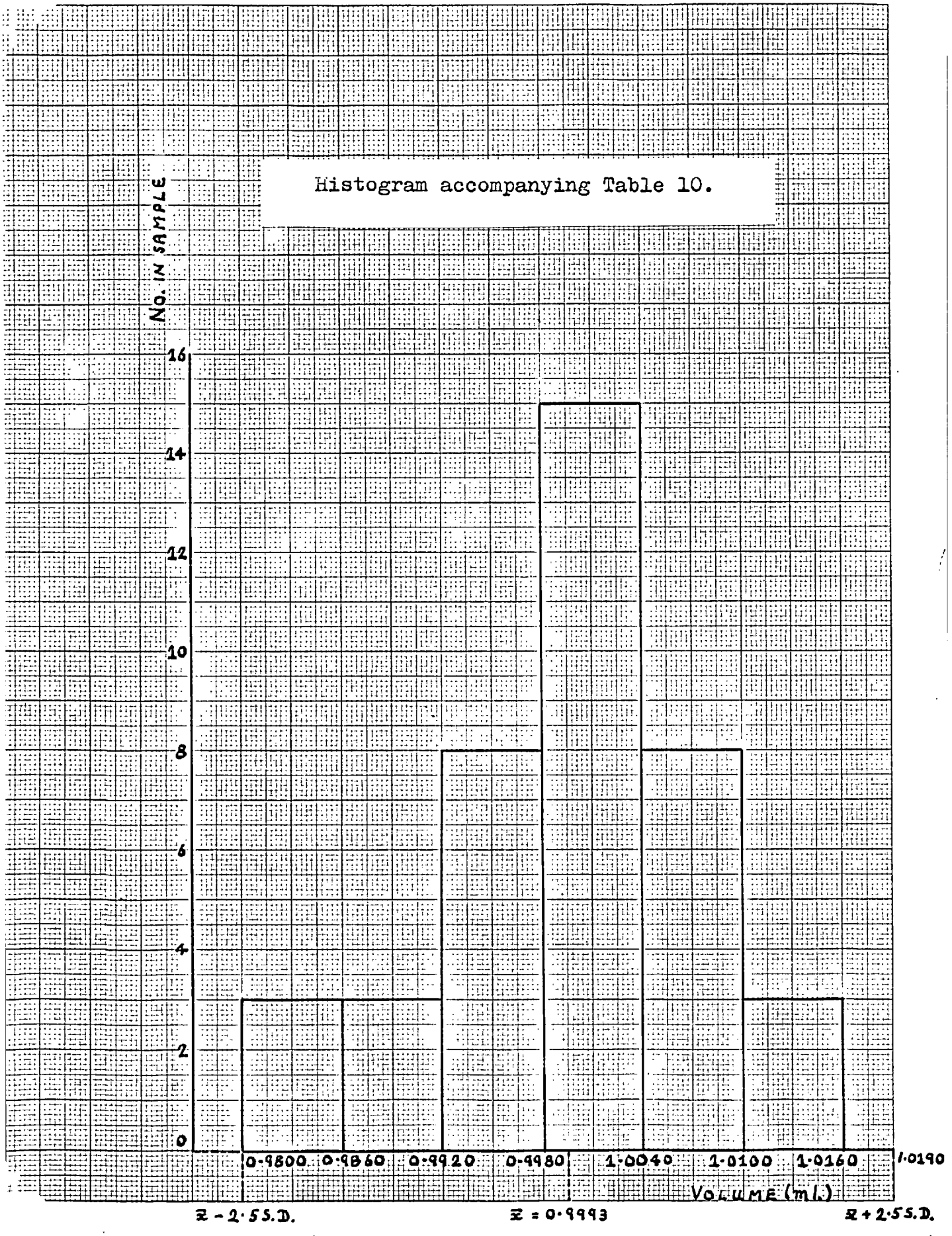
Accuracy of automatic method of transferring 1 ml. of liquid:

40 successive dilutions

Tube number	Volume of broth left in tube in ml.	Tube number	Volume of broth left in tube in ml.
1	0.9994	21	1.0074
2	0.9849	22	0.9923
3	1.0151	23	1.0006
4	0.9990	24	1.0059
5	0.9930	25	0.9994
6	1.0041	26	0.9953
7	0.9839	27	0.9995
8	1.0091	28	1.0077
9	1.0047	29	0.9953
10	0.9858	30	1.0040
11	0.9951	31	0.9947
12	1.0010	32	1.0026
13	0.9986	33	0.9995
14	0.9894	34	1.0003
15	1.0063	35	0.9866
16	1.0020	36	1.0146
17	0.9878	37	0.9962
18	1.0153	38	0.9994
19	1.0001	39	0.9980
20	0.9971	40	1.0036

$\bar{x}$ = 0.9993 S.D. = 0.0078

Histogram accompanying Table 10.



We required four independent transfer systems and it was desirable to use a single pneumatic cylinder to actuate all four syringes. It would be difficult to arrange for the stroke length of each syringe to be adjusted independently, and so we had to discover if the absolute difference between syringes had any significant effect. The apparatus used is illustrated in Fig. 21. The system was tested in exactly the same way as the single transfer system, four sets of 40 dilutions being performed simultaneously. The highest and lowest values of the volumes left in each tube were:

Syringe No. 1:	1.0031 ml.	Syringe No. 2:	1.0046 ml.
	0.9839 ml.		0.9846 ml.
Syringe No. 3:	1.0124 ml.	Syringe No. 4:	1.0147 ml.
	0.9829 ml.		0.9829 ml.

Despite the fact that we are unable to adjust each syringe independently, there is little difference in the repeatibility and the absolute difference between syringes is negligible. The overall accuracy is $\pm$ 2%; the Sumit 1 ml. tuberculin syringes were not specially selected.

The above experiments show that this transfer system is adequate; it is too early to assess the effect of wear in the syringes, but this is unlikely to be a major problem because the syringes can easily be replaced. In addition, the pipettes, the syringes and the connecting tubes can all be sterilized by heat and the pipettes can be removed without affecting the stroke length of the syringes. The syringes can be removed without affecting the calibration which is

set by device K, a completely independent component. Consequently, this system was adopted for use in our apparatus.

3.1.3.2 Automatic feeding of dilution tubes

There are a number of 'fraction collector' type devices readily available today, but it is surprisingly difficult to find a cheap, compact system that will accommodate only a small number of tubes in a rack that is both small and removable. Consequently, it was decided to build one especially.

It would have been advantageous to use the rectangular racks in common use. This, however, requires a device for moving and accurately positioning the rack in two different directions. Whilst this is not impossible, the mechanism would be far more complicated than that required to rotate a circular rack and position it correctly. This entails making special racks, but the rectangular racks now in use easily become distorted and it is likely that they would, in any case, be unsuitable because the tube has to be positioned accurately; the outside diameter of the pipette is 3/16 in. and this is only approximately half the internal diameter of the test tubes. On balance, therefore, we felt that it is better to make circular racks and build a simple mechanism for rotating them.

Fig. 22 is a drawing showing the principle used which is briefly as follows. An induction motor A runs continuously and its shaft is connected to the armature of an electro-magnetic clutch B. The rotor of the clutch is connected to another shaft C which runs through an electro-magnetic brake D and thrust-race E. The armature of the

brake is attached to this shaft and the fields of both clutch and brake are fixed. A disc with 12 grooves round the circumference, F, is mounted on this shaft and actuates a micro-switch M which, in turn, operates the clutch/brake system in such a way that the shaft will stop at 12 positions per revolution. Attached to the top of the shaft is a splined rod G. There are 12 splines and a lug H fixed on the racks I (figs.22 and 23) will fit any of the splines, thus enabling the rack to be mounted in any position.

The brake is necessary to prevent the rack overshooting its correct position and to ensure that the splined rod is not rotated when removing or replacing the racks.

This mechanism has a simple basic principle and by varying the number of grooves on disc F, the shaft C can be made to stop at any number of positions per revolution. Consequently, any number of tubes can be accommodated facilitating the mechanism's use in a variety of applications (see Sect. 3.3.3.2). With a device for moving a dispensing head, it makes a versatile fraction collector.

3.1.3.3 The complete apparatus

Fig. 23 shows the lay-out of the racks; the inner two rows, a and b, each contain 12 tubes and the outer row, c, contains 24 tubes. The pipettes are mounted in a holder (Fig. 24) in such a way that we have, in effect, four series of 12 tubes (or, say, 8 series of 6 tubes). The overall diameter of the rack is 7 in.

Fig. 25 shows the complete apparatus. The mechanism for feeding the dilution tubes is mounted in the centre of the right-hand unit and the liquid transferring mechanism is in the right background.

On the left-hand side of the motor is the chassis containing the solenoid valves and the uni-directional air-flow regulators that control the pneumatic cylinders. Fig. 26 is a drawing of the air control circuit for each of the cylinders. In the right foreground (Fig. 25) is the mechanism for raising and lowering the pipette holder.

The left-hand unit is the control system which produces the following sequence of events. On starting, after having placed the prepared rack and pipettes into position, the pipettes are lowered into the first tube of each series and 1 ml. of broth and antibiotic is sucked into each pipette. After a short delay, the 1 ml. is put back into the tube, sucked up and replaced twice and sucked up again. The pipettes are now raised, the rack rotated, the pipettes lowered into the second tube of each series and the procedure is repeated; as no bacteria are present, the same pipettes are used throughout. The number of times that the cycle is repeated is pre-selected by a 12 position switch and the machine stops with the pipettes raised and containing 1 ml. of the final dilution. When the pipette holder is rotated in an anti-clockwise direction to the position shown in Fig. 27, the final 1 ml. is automatically ejected to waste; Fig. 27 also shows the rack and the four syringes which are mounted together and driven by a single pneumatic cylinder. Fig. 28 shows the apparatus in use. Full circuit diagrams and drawings are in Appendix 3.1.

3.1.4 Evaluation of automatic method

The accuracy with which the solution is transferred from one tube to the next was measured using the technique described in Sect. 3.1.1; we here performed four sets of 12 dilutions. The results are given in Table 11; the first tube in each series was used to find the absolute volume transferred, which was between 0.91 and 0.94 ml., and the remainder of the tubes were used to find the repeatibility. The results show that the accuracy with which the apparatus transfers the solution is within $\pm$ 2%. The complete cycle takes approximately 3.0 minutes.

The concentration of solution left in each dilution tube after the serial dilution procedure had been carried out on the machine was measured as described in Sect. 3.1.1. Twelve series of dilutions were made, after the apparatus had been adjusted so that the volume transferred was 1 ml., and the results are given in Table 12 and plotted in Fig. 29.

Since the tubes are left uncovered whilst the machine is working, we had to ensure that they do not become contaminated during this time. We therefore performed a series of dilutions under aseptic conditions, exactly as one would normally except that the 2 ml. of broth in the first tube did not contain any antibiotic. The tubes were incubated for 48 hours. This experiment was repeated ten times and no tube showed any growth; there was no evidence of contamination due to the apparatus and no evidence that using a single lid to cover all 48 tubes in a rack introduces a greater disposition to contamination than using single lids for each tube.

TABLE 11

Accuracy of automatic method of transferring 1 ml. of liquid:

4 sets of 12 serial dilutions

Tube number	Volume of broth left in tube in ml.			
	Set no. 1	Set no. 2	Set no. 3	Set no. 4
2	0.9944	0.9964	1.0098	1.0030
3	0.9960	1.0036	1.0014	1.0013
4	1.0030	0.9960	1.0035	0.9992
5	0.9998	1.0009	1.0033	1.0030
6	0.9987	1.0013	1.0012	0.9958
7	0.9994	0.9966	1.0016	1.0006
8	0.9892	1.0012	1.0002	0.9990
9	0.9978	0.9984	0.9937	0.9874
10	0.9971	1.0006	1.0005	0.9983
11	0.9998	0.9956	1.0023	1.0034
12	0.9941	0.9954	1.0026	0.9970
Absolute volume transferred	0.9400	0.9322	0.9158	0.9290
$\bar{x}$	0.9972	0.9987	1.0018	0.9989
S.D.	0.0037	0.0028	0.0037	0.0045

TABLE 12

Concentration of machine-made dilutions of albumin

Dilution number	Measured optical density: 12 sets of dilutions		
3	0.542	0.632	0.611
	0.562	0.620	0.605
	0.588	0.597	0.582
	0.583	0.600	0.601
4	0.304	0.302	0.325
	0.354	0.301	0.322
	0.299	0.311	0.290
	0.290	0.300	0.322
5	0.154	0.178	0.178
	0.159	0.170	0.161
	0.146	0.158	0.163
	0.144	0.174	0.165
6	0.063	0.110	0.088
	0.066	0.075	0.088
	0.068	0.074	0.090
	0.072	0.081	0.098

3.1.5 Comparison of automatic method with manual method

To facilitate comparison, the results given in Figs. 18 and 29 are plotted together on vertically displaced axes in Fig. 30.

The machine-made dilutions are more closely bunched together than the hand-made dilutions, indicating that the machine produces more consistent results. This would be expected since the machine consistently transfers 1 ml. to within $\pm$ 2% (Tables 10 and 11), whereas manual operators are much more erratic (Table 8). In the case of dilutions 4 to 6, the machine-made dilutions are closer to the calculated concentration than those made by hand, indicating that the machine produces more accurate dilutions than those made manually. Machine-made dilutions 3 appear to be systematically displaced from their calculated positions. However, if the machine introduces a systematic error, dilutions 4 to 6 would be similarly displaced. This is not the case, and it is reasonable to assume therefore that these dilutions are more consistent than hand-made dilutions 3 but not significantly more accurate; the displacement is presumably due to a sampling error. One can explain this by the fact that manual operators can perform the transfer as accurately as the machine (Table 8), but being far less consistent soon make at least one very inaccurate transfer, thus introducing a large error. We measured dilutions 3 to 6 only, but as the results are satisfactory, it is reasonable to assume that the mixing of antibiotic solution with diluent is satisfactory and, since the machine maintains its accuracy, that dilutions 7-12 would also be satisfactory; we have demonstrated that two mixing stages only are adequate when the

meniscus always comes up to the same point in the pipette (Sect. 3.1.3.3).

The machine performs four series of 12 two-fold dilutions in approximately 3 minutes. From Table 8 it can be seen that 11 out of 19 people performed a single series of 12 two-fold dilutions more quickly than this. Since the operators knew why they were asked to perform the task and were doubtless trying very hard, it is reasonable to assume that this is the best performance they are likely to achieve in the normal course of events. But the machine performs four series of dilutions simultaneously and, therefore, it will perform 3 or 4 series more quickly than they can be performed by hand, assuming the preparative work takes the same time. Considerable saving of time is achieved, however, if 5 or more series have to be performed because whilst the machine is diluting four series more dilution tubes can be prepared.

3.1.6 Final discussion

Whilst the machine is not very sophisticated, it satisfies the criteria against which we estimate its suitability for use in a diagnostic bacteriology laboratory (Sect. 1.5); it is suitable for processing small numbers of specimens, all parts that come into contact with the liquid can simply be dismantled and sterilized by heat and it performs a task which is a necessary part of at least two routine diagnostic procedures. Although our machine is arranged to transfer 1 ml. of solution and to accommodate four series of 12 tubes, it is, in principle, very flexible and it may well find

applications in other disciplines.

One may well ask if the overall accuracy of the method is adequate. It is possible to design apparatus that performs each part of the procedure more accurately, or to design apparatus that produces a series of dilutions in such a way that errors are not cumulative. However, in either case, the apparatus would be much more complex and costly, and would probably not be economic in view of the comparatively small numbers of specimens to be processed. In the case of M.I.C.'s, it is necessary to decide, therefore, whether the tube dilution method or one of the alternative methods is the most suitable in any given circumstance, but note that, with machine-made dilutions, there is no overlapping of points as in hand-made dilutions; thus, despite the inaccuracy of the dilutions, none has produced fallacious results. If one decides to use the tube dilution method, it is necessary, as Branch, Starkey and Power (1965) have pointed out, to standardize the procedure. A machine, such as the one described here, could well be the nucleus of such a procedure.

3.2 The Distribution of Liquid Culture

There is clearly a need for an automatic method for distributing liquid culture, because one often requires to inoculate agar plates and tubes or bottles with one or more drops of culture.

3.2.1 Evaluation of manual methods

In 1913 Donald described a device for producing measured small volumes of liquids and he gave more details of its performance in 1915.

The apparatus is a glass pipette with a perfectly cylindrical capillary, from which a fixed number of drops gives 1 ml. of liquid. The actual number depends on the outside diameter of the capillary, and Donald described those devices giving from approximately 130 drops/ml. to 10 drops/ml. Wilson (1922 and 1935) and Miles and Misra (1938) have exhaustively investigated various applications of the pipettes.

A pipette still in common use today is one which gives 50 drops/ml.; it is known as the 50 dropper. Donald showed that if the difference in outside diameter varies from 1.016 mm. to 0.914 mm, the number of drops/ml. varies from 50 to 54, which is an error of $\pm$ 4%; this, doubtless, applies only if the capillary is cylindrical and if the tip of the pipette is smooth and cut at right angles to the cylinder.

There is no reason to doubt that, when correctly manufactured, 50 droppers are as accurate as this, but it is most unlikely that they are manufactured that accurately under modern routine laboratory conditions. Consequently, we took a 50 dropper from those supplied by our central supply department for routine purposes, and checked its accuracy by repeatedly dispensing ~~0.02 ml.~~ drops into pre-weighed tubes, which were re-weighed to obtain the volumes dispensed: the results are shown in Table 13. The mean of the 12 volumes was 0.020 ml. but the volume varied between 0.0237 ml. and 0.0175 ml. To investigate the differences between different droppers, the above experiment was repeated with 12 different droppers. The results

TABLE 13

Accuracy of a "50 dropper"

Drop number	Volume in ml.
1	0.0184
2	0.0205
3	0.0197
4	0.0190
5	0.0188
6	0.0175
7	0.0227
8	0.0211
9	0.0194
10	0.0227
11	0.0237
12	0.0177

$\bar{x}$ = 0.0201

S.D. = 0.0021

are given in Table 14. The mean for dropper number 3 is 0.0172 ml. and for number 12 it is 0.0244 and the remainder are distributed between these two extremes (Fig. 31). The results are hardly surprising, when one considers the difficulty in keeping the capillary cylindrical during manufacture and also the difficulty in cutting the tip smoothly to within 0.1 mm. in diameter, which is necessary for an accuracy of $\pm$ 4%.

One may argue that one should pay more attention to the manufacturing process, but this is a very time-consuming and, therefore, costly procedure.

3.2.2 Criteria for an automatic method

It is very difficult to produce generalised criteria for an automatic method of distributing culture, because of the wide variety of applications. If one simply requires a single drop of a given culture, and accuracy is not important, it is unlikely that one could devise an automatic method that would be more practical than the 50 dropper: such a device would certainly be much more expensive. Similarly, even if accuracy was important, the same would probably be equally true. However, if one requires to inoculate a row of tubes by adding one drop of culture to each tube, as required in the final stage of the measurement of the M.I.C., it may be possible to produce an economic apparatus for performing this task. Our discussion is therefore limited to such apparatus.

Simplicity is essential, because the number of rows of tubes

TABLE 14

Accuracy of "50 droppers"

Drop number	Volume in ml.			
	Dropper number			
	1	2	3	4
1	0.0184	0.0224	0.0171	0.0158
2	0.0205	0.0231	0.0172	0.0172
3	0.0197	0.0229	0.0187	0.0132
4	0.0190	0.0232	0.0140	0.0232
5	0.0194	0.0228	0.0149	0.0198
6	0.0188	0.0227	0.0170	0.0160
7	0.0175	0.0216	0.0163	0.0211
8	0.0227	0.0244	0.0170	0.0155
9	0.0211	0.0223	0.0188	0.0231
10	0.0227	0.0220	0.0172	0.0222
11	0.0237	0.0237	0.0189	0.0227
12	0.0177	0.0194	0.0191	0.0196
$\bar{x}$	0.0201	0.0225	0.0172	0.0191
S.D.	0.0021	0.0012	0.0016	0.0035

Continued .....

TABLE 14 (continued)

Drop number	Volume in ml.			
	Dropper number			
	5	6	7	8
1	0.0197	0.0253	0.0210	0.0244
2	0.0240	0.0258	0.0195	0.0177
3	0.0192	0.0220	0.0189	0.0268
4	0.0178	0.0250	0.0200	0.0277
5	0.0247	0.0210	0.0217	0.0213
6	0.0249	0.0239	0.0198	0.0247
7	0.0253	0.0234	0.0219	0.0253
8	0.0262	0.0192	0.0196	0.0272
9	0.0248	0.0234	0.0206	0.0236
10	0.0217	0.0226	0.0197	0.0228
11	0.0256	0.0249	0.0150	0.0224
12	0.0280	0.0212	0.0223	0.0232
$\bar{x}$	0.0234	0.0231	0.0200	0.0239
S.D.	0.0031	0.0026	0.0019	0.0028

Continued....

TABLE 14 (continued)

Drop number	Volume in ml.			
	Dropper number			
	9	10	11	12
1	0.0220	0.0269	0.0274	0.0202
2	0.0207	0.0256	0.0246	0.0215
3	0.0249	0.0204	0.0250	0.0236
4	0.0256	0.0232	0.0172	0.0265
5	0.0249	0.0235	0.0253	0.0217
6	0.0210	0.0220	0.0246	0.0272
7	0.0234	0.0198	0.0260	0.0257
8	0.0223	0.0205	0.0240	0.0260
9	0.0260	0.0196	0.0232	0.0253
10	0.0251	0.0207	0.0241	0.0257
11	0.0233	0.0197	0.0231	0.0234
12	0.0208	0.0204	0.0260	0.0258
$\bar{x}$	0.0233	0.0218	0.0242	0.0244
S.D.	0.0019	0.0024	0.0025	0.0023

that have to be so inoculated is very small, and one should aim at achieving as high an order of accuracy as the other criteria permit. Since different cultures need to be handled, it is necessary to either, sterilize all components with which a culture comes into contact in situ, or to remove all such components and replace them with freshly sterilized components. This suggests that one should automatically feed pre-sterilized components into the apparatus and automatically remove them after use, but we have not yet found a way of doing this economically and aseptically. It is clear, therefore, that until one can devise a method for sterilizing components in situ (see Sect. 4.3), the only economic method is to feed, manually, pre-sterilized components into the apparatus and to remove them, manually, after use; it is also necessary that this procedure in no way affects the calibration of the apparatus.

One could feed the culture tubes automatically under the dispensing head (Sect. 3.1.3.2), but the apparatus could be used for inoculating a set of sugars. Since the sugar test is performed in screw-capped bottles, it would be necessary to unscrew the caps automatically, and this would be complicated. We decided therefore, that in the first prototype the tubes should be fed manually.

3.2.3 The apparatus

3.2.3.1 The dispensing mechanism

To our knowledge, no commercially available apparatus is suitable for distributing a few drops of each of a variety of cultures. A device known as the Repette (Jencon's Ltd.) is suitable for dispensing

volumes of liquids of the order of 0.02 ml. but the liquid comes into contact with a glass syringe (Fig. 32); the device is therefore unsuitable. However, the mechanism used can be adapted and we therefore decided to examine the efficacy of a method for dispensing drops of culture based on the Repette mechanism.

This mechanism, shown in Fig. 33, functions in the following manner. The base B is fixed and on pressing button A, the arm to which it is attached pivots about point E and therefore the inner end of the arm presses against the rod D. This end of the arm is serrated and grips the rod, thus moving it downwards until the collar F touches the p.t.f.e. bush G, which is fixed to the base B. On releasing A, the spring H forces the mechanism back to its original position and, provided sufficient frictional forces are applied to rod D, the rod remains in its final position. The screw-thread device C controls the height the mechanism is raised above G and hence the movement of the rod D.

For experimental purposes, we attached the lower end of rod D to the top part of a syringe holder W (Fig. 34) into which fits the piston of a 1 ml. disposable syringe. The barrel fits into a fixed holder X. A p.t.f.e. disc Y is attached to the rod and fits tightly into tube Z to provide frictional forces. The button A is operated by a pneumatic cylinder P. In this arrangement the lower limit of the movement of rod D depends on the stroke length of the cylinder P and is fixed: the screw-thread C still controls the upper limit of the movement of collar F and hence the distance the syringe piston moves.

The syringe and needle are placed in situ, the liquid culture is held over the needle tip and the rod D is raised, manually, to fill the syringe. Each time the cylinder P is operated, the syringe piston moves down a pre-determined distance and a drop of culture is dispensed.

To evaluate the accuracy of the apparatus, we plugged in a syringe and adjusted C (Fig. 33) until a volume of approximately 0.02 ml. was delivered and then proceeded as follows. Forty-eight test tubes were weighed and a drop of distilled water was dispensed into each tube through a 21 gauge (i.d. = 0.019 in.) needle. The tubes were re-weighed and the volume of each drop calculated. The results are given in Table 15 and in the accompanying histogram. Taking the $\bar{x} \pm 2.5$ S.D. values we see that this gives an error of approximately $\pm$ 4%. However, the volume dispensed was larger than that required and so this was adjusted and the experiment repeated. The results are given in Table 16 and in the accompanying histogram and give an error of approximately $\pm$ 5%; we repeated this experiment many times with remarkably consistent results.

Any differences between syringes is also important because it is not easy to recalibrate the apparatus and, therefore, one cannot do this each time a different syringe is used. We therefore took five more syringes, at random, and repeated the above experiment with each syringe, without altering the calibration. The highest and lowest volumes dispensed in each case were:

TABLE 15

Accuracy of method for dispensing liquid culture

Tube number	Volume dispensed in ml.	Tube number	Volume dispensed in ml.
1	0.0231	25	0.0230
2	0.0223	26	0.0230
3	0.0228	27	0.0226
4	0.0220	28	0.0224
5	0.0225	29	0.0224
6	0.0221	30	0.0226
7	0.0222	31	0.0231
8	0.0225	32	0.0222
9	0.0227	33	0.0226
10	0.0225	34	0.0230
11	0.0225	35	0.0227
12	0.0225	36	0.0226
13	0.0227	37	0.0231
14	0.0223	38	0.0229
15	0.0229	39	0.0226
16	0.0218	40	0.0227
17	0.0219	41	0.0225
18	0.0222	42	0.0221
19	0.0221	43	0.0233
20	0.0229	44	0.0223
21	0.0228	45	0.0228
22	0.0230	46	0.0232
23	0.0231	47	0.0226
24	0.0230	48	0.0222

$\bar{x} = 0.0226$ S.D. = 0.00037

Histogram accompanying Table 15.

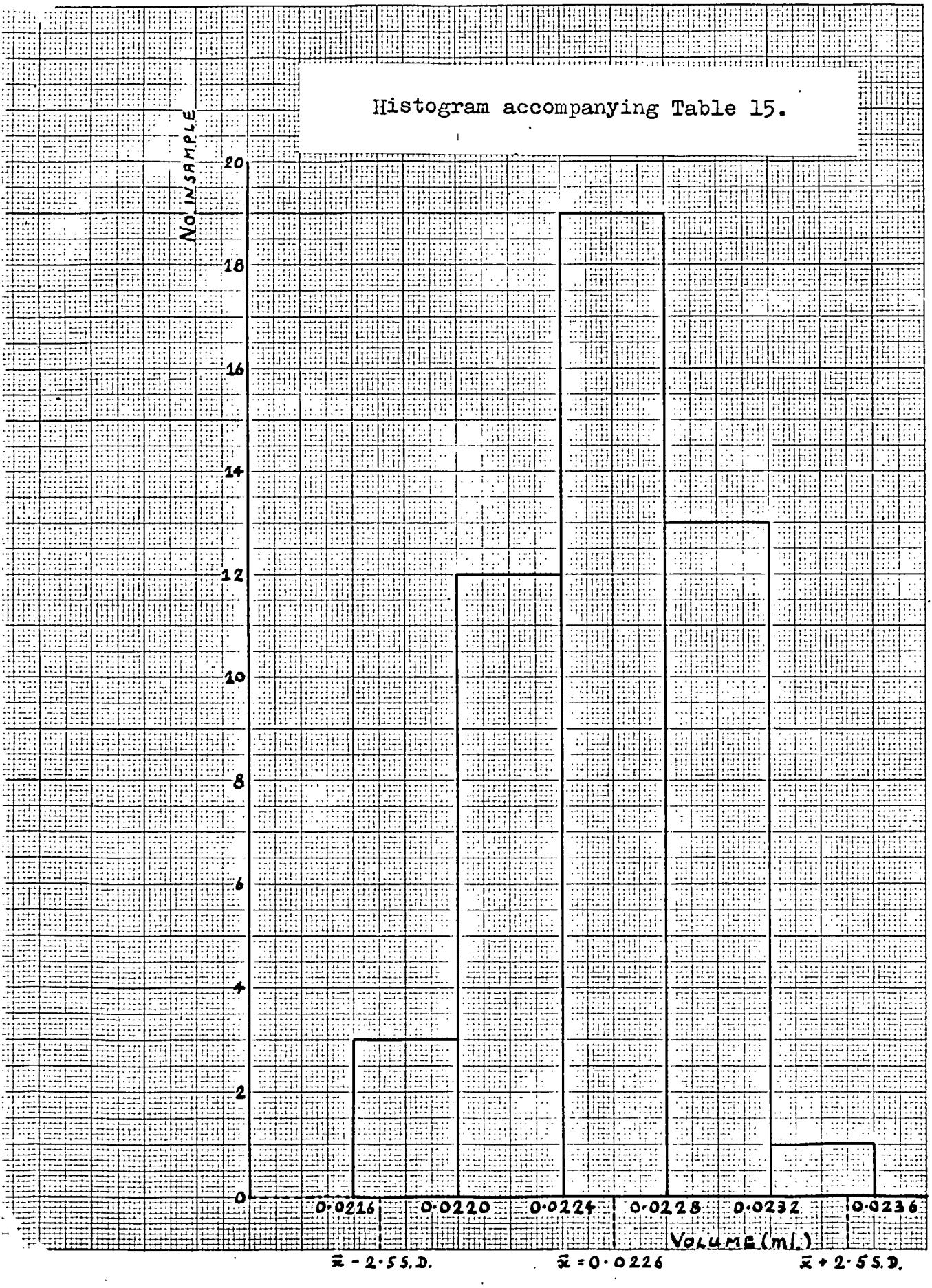


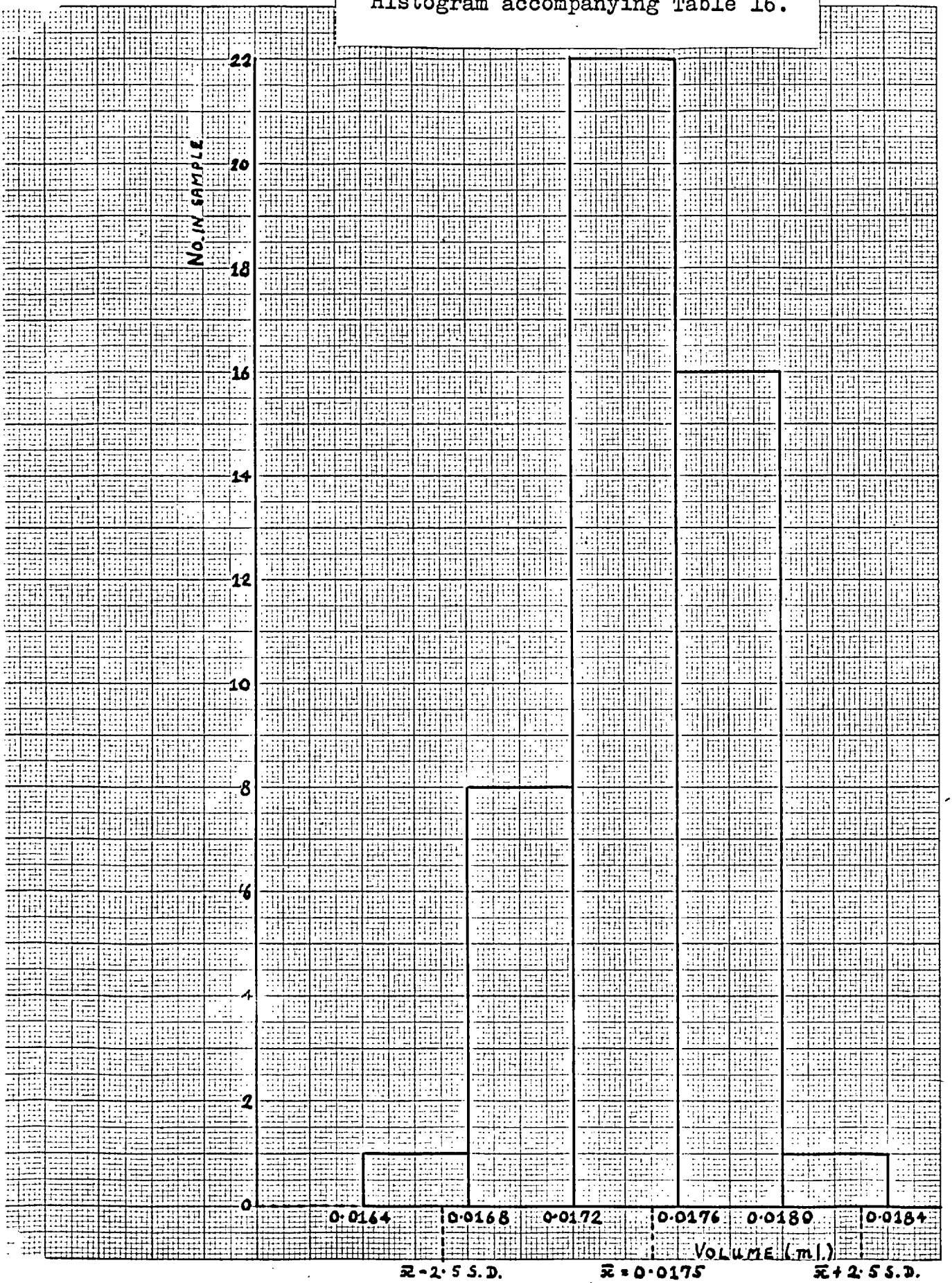
TABLE 16

Accuracy of method for dispensing liquid culture

Tube number	Volume dispensed in ml.	Tube number	Volume dispensed in ml.
1	0.0175	25	0.0178
2	0.0171	26	0.0179
3	0.0174	27	0.0179
4	0.0177	28	0.0177
5	0.0178	29	0.0179
6	0.0174	30	0.0176
7	0.0172	31	0.0177
8	0.0173	32	0.0175
9	0.0172	33	0.0176
10	0.0172	34	0.0176
11	0.0168	35	0.0177
12	0.0172	36	0.0183
13	0.0173	37	0.0176
14	0.0174	38	0.0179
15	0.0175	39	0.0177
16	0.0170	40	0.0176
17	0.0171	41	0.0178
18	0.0175	42	0.0180
19	0.0175	43	0.0176
20	0.0173	44	0.0175
21	0.0176	45	0.0178
22	0.0174	46	0.0178
23	0.0176	47	0.0175
24	0.0172	48	0.0177

$\bar{x}$ = 0.0175 S.D. = 0.00029

Histogram accompanying Table 16.



No. 1	No. 2	No. 3	No. 4	No. 5
0.0182 ml.	0.0178 ml.	0.0171 ml.	0.0179 ml.	0.0177 ml.
0.0168 ml.	0.0166 ml.	0.0163 ml.	0.0170 ml.	0.0165 ml.

Together with the results in Table 16, these results indicate that different syringes make a little difference and one can quote an overall accuracy of $\pm$ 6% when volumes of the order of 0.0175 ml. are dispensed. As this is smaller than the minimum volume it is intended to dispense, namely 0.02 ml., and as the accuracy improves as the volume increases, it is evident that this technique gives an accuracy of better than $\pm$ 6% which is adequate; it is certainly an improvement on the manual method (Sect. 3.2.1). We thought it unlikely that one could improve on this without a significantly more complicated system which would be unjustified. Consequently, this principle was adopted for use in our apparatus.

3.2.3.2 The complete apparatus

The dispensing unit is shown in Fig. 35. The centre rod (D, Fig. 34) is connected to a 2" stroke pneumatic cylinder for filling and emptying the syringe: this cylinder also provides frictional forces to the rod D (Fig. 34) and, therefore, disc Y and tube Z are not now required. Two dispensing mechanisms are used in order that one might dispense two different volumes, presently 0.02 ml. and 0.05 ml. respectively; each is operated by an independent cylinder which is pre-selected manually. The control unit, Fig. 36., produces the following sequence of events. Having placed the syringe and needle in situ, the liquid culture

is held over the needle tip and a hand switch is operated; this actuates the 2" stroke pneumatic cylinder and approximately 1 ml. of culture is drawn into the syringe. On depressing a foot-switch, the pre-selected dispensing cylinder reciprocates, thus repeatedly dispensing drops of culture. On releasing the foot switch the process stops and one then discharges the remaining culture to waste and discards the syringe and needle. The complete apparatus is shown in Fig. 37 and detailed circuit diagrams are given in Appendix 3.2.

3.2.4 Evaluation of automatic method

In this method, the speed with which the culture is ejected from the needle, whilst more consistent, is much greater than in the manual method. There is, therefore, a possibility that aerosols are produced and that splashing occurs, and both possibilities were investigated in the following manner.

Special roll tubes were prepared, as follows:-

(a) a sterile 100 ml. bottle was cooled for at least $\frac{1}{2}$ hour at $-15^{\circ}C$

(b) approximately 3 ml. of sterile molten nutrient agar was placed in the bottle

(c) the bottle was rotated, under running cold water, until the whole surface was covered with a layer of partially solidified agar

(d) the bottle was placed in a $+4^{\circ}C$ refrigerator for at least 4 hours to allow the agar to set

(e) before use, the roll tube was left to slowly warm up to room temperature.

A culture of Staph. albus was drawn into the syringe and a roll tube containing 2 ml. of sterile nutrient broth was placed over the needle, so that the tip was approximately 1 in. above the surface of the broth. 0.02 ml. of culture was dispensed and the bottle was left to stand for 2 minutes. This procedure was repeated five times with 0.02 ml. drops and five times with 0.05 ml. drops, and the 10 bottles were then incubated.

There was some growth on the side of some bottles (Table 17), suggesting that either the speed of ejection was too high for the size of needle used and that splashing occurred, or that aerosols were produced; we suspected splashing. On reducing the speed of ejection, we found that the accuracy of dispensing deteriorated slightly and that it was necessary to use a 19 gauge needle (i.d. = 0.025 in.). We therefore again measured the accuracy with which the drops are dispensed in the same way as before (Sect. 3.2.3.1); the results are given in Tables 18 and 19 and these show that the performance is as good as the experiments described in Sect. 3.2.3.1 indicated. Further experiments to detect aerosols and splashing were carried out. A total of 10 roll tubes were inoculated with 0.02 ml. drops and 10 with 0.05 ml. drops and no growth was obtained in any bottle, indicating that aerosols are not present and that if splashing occurs, it is not dangerous. We therefore adopted these operating conditions for routine use.

TABLE 17

Evaluation of automatic method for distributing liquid culture

Number of roll tubes containing growth after one 0.02 ml. drop of culture had been dispensed into nutrient broth in roll tube, in 5 tests:

5

Number of roll tubes containing growth after one 0.05 ml. drop of culture had been dispensed into nutrient broth in roll tube, in 5 tests:

4

TABLE 18

Final evaluation of liquid culture dispensing apparatus:

0.02 ml. mechanism

Tube number	Volume dispensed in ml.
1	0.0205
2	0.0196
3	0.0194
4	0.0211
5	0.0198
6	0.0206
7	0.0193
8	0.0197
9	0.0201
10	0.0199
11	0.0198
12	0.0198

$\bar{x}$ = 0.0199

S.D. = 0.0005

TABLE 19

Final evaluation of liquid culture dispensing apparatus:

0.05 ml. mechanism

Tube number	Volume dispensed in ml.
1	0.0494
2	0.0494
3	0.0499
4	0.0504
5	0.0505
6	0.0505
7	0.0516
8	0.0499
9	0.0501
10	0.0500
11	0.0500
12	0.0497

$\bar{x}$ = 0.0501

S.D.= 0.0006

3.2.5 Comparison of manual method with automatic method

The automatic method is not as accurate as the manual method when performed with properly prepared pipettes, but is far more accurate than the manual method when performed with pipettes currently in routine use.

The manual method is extremely simple and one would not use the automatic method if one only required a single drop of culture. In the automatic method, the increased accuracy, convenience of handling and the reduction in demand of 50 droppers, all of which have to be made, plugged, packed and sterilized in the department, are all advantageous. However, a 50 dropper costs approximately ¾d whereas a syringe and needle costs approximately 6d. The convenience of handling is an important factor, particularly as it reduces the likelihood of accidents but the automatic method is no quicker than the manual method.

3.2.6 Final discussion

This appears to be the simplest apparatus likely to have any worthwhile advantages over the manual method but, nevertheless, it is quite complicated and its usefulness is limited. We now believe that it is unlikely that any significant advance in handling infective material will be made until components with which infective material comes into contact can be sterilized in situ.

3.3 The Serological Diagnosis of Syphilis

A common method for the diagnosis of syphilis is the complement-fixation reaction using the Wassermann antigen (Cruickshank, 1965).

To summarise, the reactions are:

A. Serum + antigen + complement - Incubate for 1 hour at 37°C
(If antibodies present in serum - test positive - complement removed, if antibodies not present in serum - test negative - complement remains).

B. To above mixture add:
sheep cell antigen + horse anti-sheep cell serum antibody.
Incubate for ½ hour at 37°C.
(If no lysis of cells - no complement present - test positive, if lysis of cells - complement present - test negative).

3.3.1 The manual method

The complete procedure for the diagnosis of syphilis used in our laboratory involves testing the serum against the Wassermann antigen and, for confirmation, also against the Reiter antigen (Cruickshank, 1965); neither is 100% specific for syphilis but both give a very reliable indication. The test is quantitative and the details are as follows.

A quantity of each patient's serum is dispensed into four tubes together with various reagents, as shown in Fig. 38. After incubation for 1 hour at 37°C, 0.1 ml. of sensitised sheep cells (sheep-cell antigen+horse anti-sheep cell serum antibody) is added to each tube, and after incubating for a further ½ hour one observes if lysis has taken place.

The minimal haemolytic dose (M.H.D.) of complement (Fig. 38)

is the minimal concentration of complement in sensitised sheep cells that will lyse red cells, and this has to be measured separately before the above procedure can be commenced.

A series of eight dilutions of complement is prepared, and 0.1 ml. of each dilution is put into each of three tubes together with the appropriate reagents, as shown in Fig. 39. The 24 tubes are incubated for 1 hour at 37°C, and 0.1 ml. of sensitised sheep cells is then added to each. After further incubation for ½ hour one observes the minimum concentration of complement that has lysed the cells: this concentration is 1 M.H.D.

The procedure for performing the Wassermann and Reiter reactions is set out as in Fig. 38 to stress the number of operations performed, namely, an operator pipettes four different reagents into each of three tubes and three different reagents into a fourth tube; two operations on tube 1 could be combined but it is more convenient to have two separate operations.

On average it takes an experienced skilled technician approximately 4 minutes to dispense 12 specimens of sera into the tubes and approximately 10 minutes to dispense the other reagents into the tubes; the apparent disparity is due to the fact that when dispensing the sera, the pipette has to be rinsed through after each serum has been dispensed to avoid "carry-over".

The accuracy with which the reagents have to be dispensed is very difficult to determine, because there are so many variables that a very large quantity of each of many different sera would be required.

However, simple tests on the pipettes normally used in our laboratory showed that the accuracy of dispensing is normally not better than $\pm$ 3%.

The accuracy with which 0.02 ml. of serum can be dispensed is poor. One could dispense the sera more accurately by pre-diluting a larger quantity of serum than 0.02 ml. (say 0.1 ml.), and dispensing 0.1 ml. of diluted serum into each of the first three tubes. There is, however, no evidence that incorrect results due to the inaccuracy of dispensing the sera have hitherto been obtained.

It may be considered that the time involved in performing the procedure is not very great. Nevertheless, we process, on average, 100 specimens a day and it is no longer necessary to perform such a mundane procedure manually; even with very simple automatic pipetting devices the procedure can be made much less tedious and time consuming.

3.3.2 Criteria for an automatic method

The complement titration has to be performed only once each week, and whilst one could mechanise this procedure, one's time is far more usefully spent considering the Wassermann and Reiter reactions: the complete procedure lends itself to mechanisation.

Some workers (for example Pugh and Gaze, 1966) have attempted to use the auto-analyzer for performing the Wassermann reaction, but many difficulties have arisen due mainly to the fact that the auto-analyzer utilizes a continuous flow system and the problem of

"carry-over" is troublesome (p.11). The apparatus is also very expensive and we decided, therefore, to attempt to devise a method, based on a 'discrete' system, which would require significantly cheaper apparatus and which would be particularly suitable for the number of specimens we handle.

Sequeira (1964) produced a suitable hand operated apparatus. However, whilst this has the advantage of being flexible, and is therefore particularly suitable for performing a variety of different procedures, our circumstances are such that it is preferable to have a greater degree of mechanisation than his apparatus gives at the cost of lack of flexibility.

One should dispense the reagents automatically, but whether one should dispense the sera automatically is a different matter. One could devise apparatus for doing this which has adequate safeguards against "carry-over", but in view of the additional complication this introduces, we decided to continue to dispense the sera manually: if the number of specimens to be processed was significantly higher than 100 per day, it would be worth considering automatic methods for performing this part of the procedure.

The accuracy of the dispensing mechanism should be at least as good as the accuracy achieved by manual methods, namely $\pm$ 3%. The mechanism must be simply removed from the apparatus and replaced without affecting the calibration of the system. It is not necessary to be able to sterilize the mechanism but merely to be able to keep it clean, and there is no reason why a pin or ball valve device should not be used.

In an automatic method it is not necessary to have the same number of operations as in the manual procedure (Fig. 38) because some of the reagents can be combined before dispensing. There are a number of possible combinations and those given in Fig. 40, which are based on the principle that 0.02 ml. of serum will be dispensed manually, are convenient: in case it is decided to predilute the serum, the volumes the automatic dispensing mechanisms dispense must be simply varied. For purely technical reasons it is convenient to have four valves dispensing 0.1 ml. volumes (all driven by the same mechanism), one valve, which operates twice per cycle, dispensing 0.18 ml., and one valve dispensing 0.28 ml: the latter valves must be driven by independent mechanisms. The additional complication involved in having only one valve dispensing the Reiter antigen into tubes 3 and 4 is such as to make this impractical: having two valves for the same reagent admittedly makes additional work for the operator and wastes more reagent, but this appears to be the best compromise.

3.3.3 The apparatus

3.3.3.1 The reagent dispensing mechanism

The number of devices suitable for dispensing volumes of the order of 0.1 ml. is limited, in fact to our knowledge there is only one valve mechanism commercially available that dispenses volumes of this order and is also suitable for building into a multi-volume pipetting device. We therefore evaluated this valve mechanism (Becton Dickinson and Co.) which is a simple pin type valve and

is combined with a 1 ml. syringe (Fig. 41): the syringe piston is actuated by a pneumatic cylinder to fill the valve and then to dispense the liquid. The apparatus is shown in Fig. 42: the stroke length of the piston, and hence the volume dispensed is easily varied as before (Sect. 3.1.3.1).

We weighed 50 test tubes and then dispensed approximately 0.1 ml. of distilled water into each. The tubes were re-weighed and the volume dispensed into each tube calculated. The results are given in Table 20 and the accompanying histogram. The accuracy of dispensing was approximately $\pm$ 2.5%, the mean being higher than the optimum 0.1 ml.

Since the accuracy may deteriorate as the volume dispensed is decreased, we re-adjusted the stroke length of the pneumatic cylinder and repeated the experiment, taking care to ensure that the liquid reservoir and the dispensing nozzle were nearly level: we found that, since the pin valve is gravity seated, there is a tendency for a syphoning effect if there is a large difference in height between these two points. The results of this experiment are given in Table 21 and the accompanying histogram. Here the mean is approximately 0.095 ml. and the accuracy $\pm$ 1% which is more than adequate.

Similar experiments were performed dispensing 0.18 ml. and 0.28 ml. volumes to confirm that the performance does not deteriorate at these volumes, and we found this to be the case: the accuracy is within $\pm$ 1%.

TABLE 20

Accuracy of valve/syringe dispensing mechanism

Tube number	Volume dispensed in ml.	Tube number	Volume dispensed in ml.
1	0.1143	26	0.1146
2	0.1143	27	0.1151
3	0.1143	28	0.1143
4	0.1136	29	0.1145
5	0.1141	30	0.1148
6	0.1142	31	0.1118
7	0.1140	32	0.1135
8	0.1147	33	0.1147
9	0.1152	34	0.1136
10	0.1144	35	0.1129
11	0.1134	36	0.1146
12	0.1137	37	0.1126
13	0.1133	38	0.1133
14	0.1133	39	0.1130
15	0.1130	40	0.1131
16	0.1131	41	0.1133
17	0.1126	42	0.1119
18	0.1138	43	0.1096
19	0.1150	44	0.1127
20	0.1141	45	0.1128
21	0.1126	46	0.1130
22	0.1128	47	0.1112
23	0.1135	48	0.1136
24	0.1126	49	0.1127
25	0.1143	50	0.1126

$\bar{x}$ = 0.1134 S.D. = 0.0011

Histogram accompanying Table 20.

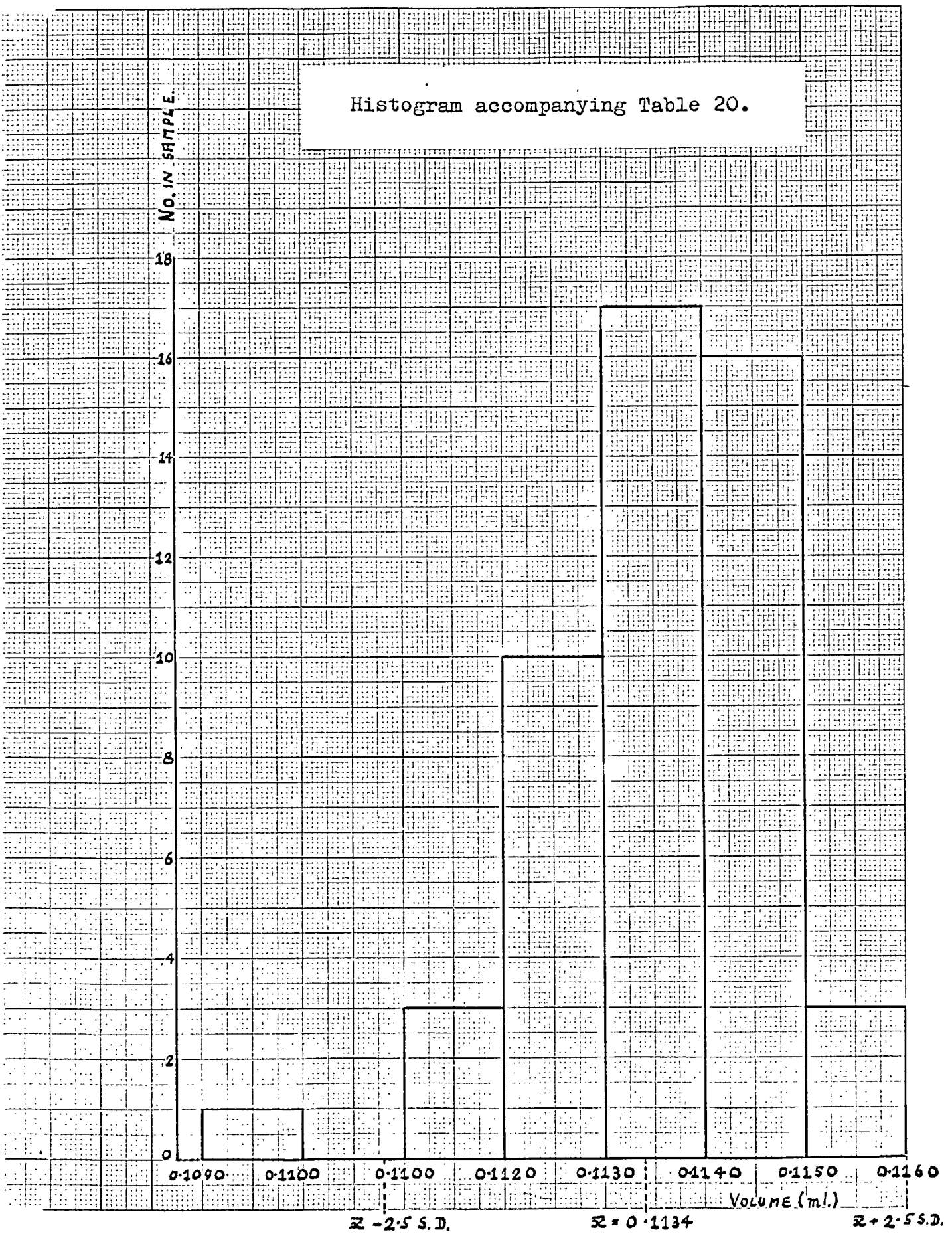


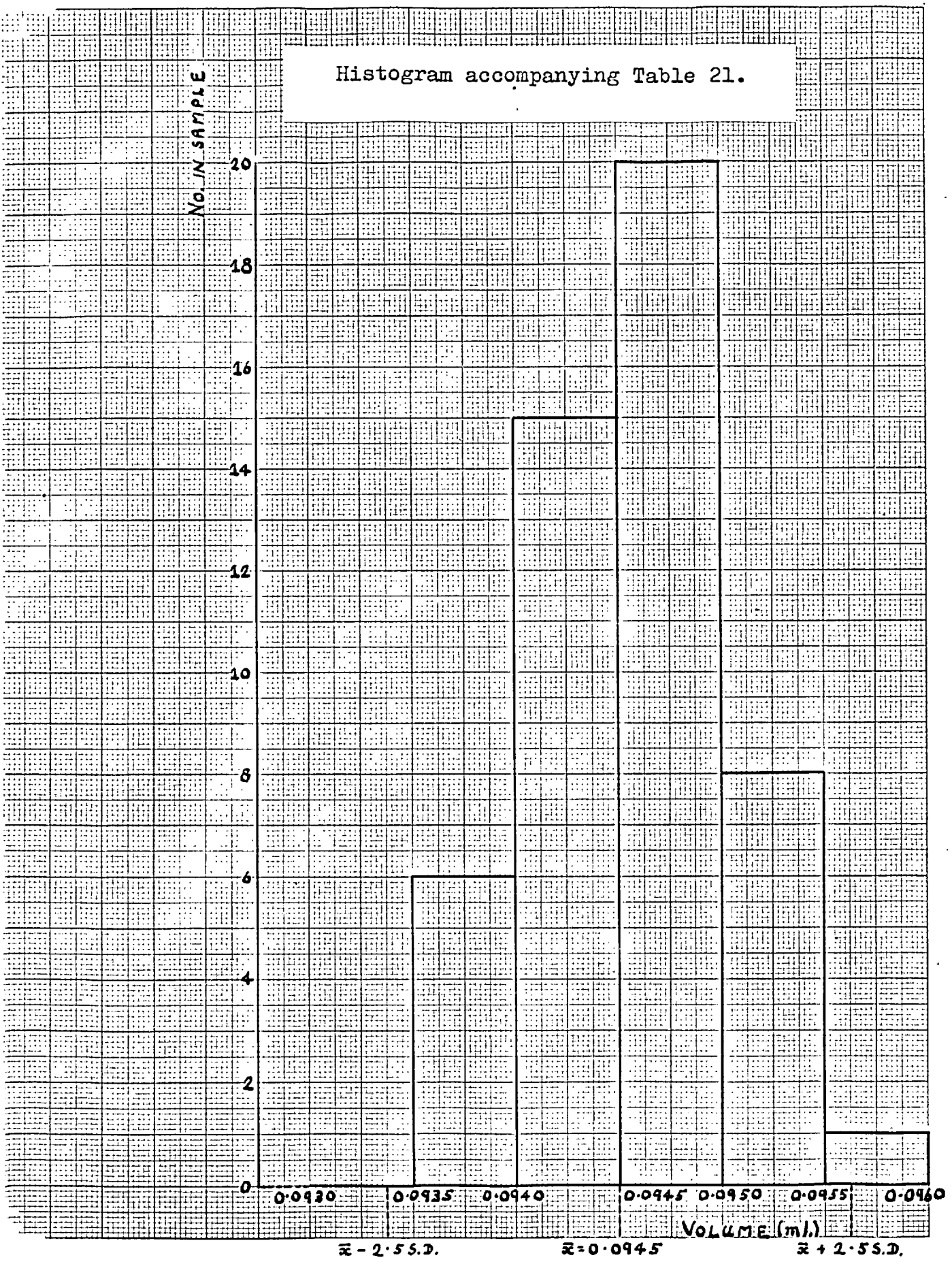
TABLE 21

Accuracy of valve/syringe dispensing mechanism

Tube number	Volume dispensed in ml.	Tube number	Volume dispensed in ml.
1	0.0945	26	0.0938
2	0.0944	27	0.0946
3	0.0942	28	0.0941
4	0.0949	29	0.0937
5	0.0940	30	0.0945
6	0.0943	31	0.0950
7	0.0946	32	0.0947
8	0.0947	33	0.0951
9	0.0947	34	0.0948
10	0.0942	35	0.0941
11	0.0946	36	0.0939
12	0.0947	37	0.0943
13	0.0955	38	0.0949
14	0.0954	39	0.0952
15	0.0949	40	0.0952
16	0.0943	41	0.0947
17	0.0946	42	0.0946
18	0.0952	43	0.0952
19	0.0947	44	0.0946
20	0.0941	45	0.0936
21	0.0952	46	0.0942
22	0.0942	47	0.0939
23	0.0941	48	0.0937
24	0.0942	49	0.0944
25	0.0945	50	0.0947

$\bar{x} = 0.0945$ S.D. = 0.00046

Histogram accompanying Table 21.



We were concerned also with differences between syringes since four mechanisms were to be actuated by a single pneumatic cylinder and therefore the volumes dispensed would not be independently variable. We therefore constructed apparatus in which four valve-syringe systems are mounted together, the four syringe pistons being actuated by a single pneumatic cylinder (Fig. 43), and dispensed four sets of 12 volumes into tubes. The results are shown in Table 22. There are significant absolute differences in the volumes dispensed by these four mechanisms, which were not specially selected, but nevertheless the accuracy, which is approximately $\pm$ 2%, is more than adequate. We therefore adopted this mechanism and the apparatus (Fig. 43) was used in the first prototype.

3.3.3.2 Automatic feeding of reaction tubes

As the sera have to be dispensed manually and as the reaction tubes have to be put in a water bath, we required means for easily removing the tubes from the apparatus: we considered the possibility of building an incubator into the apparatus but did not pursue the idea. Small racks that may be simply attached to the device are the obvious choice, although there are other possibilities such as a continuous flow system operated by a chain. However, similar criteria to those for the serial diluter apply (Sect. 3.1.3.2), and we decided, therefore, to use racks which can be attached to a rotary table, which is permanently attached to the apparatus, and which will stand on a bench for the manual dispensing of sera. A rack is

TABLE 22

Final evaluation of valve/syringe dispensing mechanism

Tube number	Volume dispensed in ml. Valve number			
	1	2	3	4
1	0.1518	0.1517	0.1488	0.1503
2	0.1511	0.1519	0.1496	0.1503
3	0.1514	0.1526	0.1495	0.1514
4	0.1511	0.1523	0.1492	0.1506
5	0.1509	0.1518	0.1488	0.1504
6	0.1516	0.1527	0.1493	0.1504
7	0.1504	0.1528	0.1491	0.1505
8	0.1511	0.1523	0.1494	0.1506
9	0.1515	0.1523	0.1491	0.1509
10	0.1505	0.1520	0.1483	0.1508
11	0.1512	0.1520	0.1475	0.1504
12	0.1512	0.1512	0.1485	0.1501
$\bar{x}$	0.1511	0.1521	0.1489	0.1505
S.D.	0.00041	0.00046	0.00060	0.00035

shown in Fig. 44. The number of racks that one needs to attach to the table at any one time depends on circumstances and we decided to make provision for a maximum of 5 racks: this necessitates a table of approximately 12 in. diameter. The mechanism used for rotating the table is identical to that used in the serial diluting apparatus (Sect. 3.1.3.2, Fig. 22 and Appendix 3.2) except that, as in this case we require the table to stop at 60 positions per revolution, the disc F (Fig. 22) has 60 grooves.

3.3.3.3 The complete apparatus

The dispensers are mounted in two groups, the four 0.1 ml. valves on one support and the other two valves on another support (Fig. 45), and the reagent reservoirs are mounted behind the supports (Fig. 46). The valve outlet nozzles are fixed, in appropriate positions, in a holder (Fig. 47) which is mounted in such a way that the racks move underneath it (Fig. 48) and such that it may be moved radially: the four tubes containing one patient's serum are in a straight line along a radius.

The control unit (Fig. 49) produces the following sequence of events. All three pneumatic cylinders are operated to dispense 4 x 0.1 ml. volumes, 1 x 0.18 ml. volume and 1 x 0.28 ml. volume into the appropriate reaction tubes. The nozzle holder is then moved, radially, so that the 0.18 ml. valve nozzle is over tube 3, and the three cylinders return to their original positions, thus re-filling each valve. 1 x 0.18 ml. volume is dispensed, the

nozzle holder moves back to its original position, the 0.18 ml. volume valve is re-filled, the table rotates and the process is repeated a pre-selected number of times. As each rack is completely filled with reagents it may be removed and placed in the waterbath: a freshly prepared rack of sera may be put in the completed rack's place and the procedure repeated as necessary. The sensitised sheep cells are added independently whilst the racks are in the water bath. The complete apparatus is shown in Fig. 50 and the circuit diagram is given in the accompanying reprint (Pneumatics in the Pathology Laboratory - R.E. Trotman, 1967).

3.3.4 Evaluation of automatic method

The apparatus takes 20 seconds to complete one cycle, which is equivalent to 20 minutes for 60 sets of reaction tubes. It takes approximately 10 minutes to prime and prepare the valve mechanisms and consequently, it is advantageous to use the apparatus even if one has as few as 24 tests to perform, but considerable saving of time is effected if 100 specimens have to be processed. However, the priming volume of a valve mechanism is 6 ml. and this volume of reagent is wasted. Consequently the waste of reagents is more significant when small numbers of sera are processed than when large numbers of sera are processed.

The maximum number of specimens that can be processed depends mainly on how long the diluted complement and the antigens may be left standing at room temperature without deteriorating. We therefore performed some simple experiments to assess this.

Twelve sera were taken, 6 known to be positive and 6 known to be negative to both antigens. The reagents required for the reactions were made up and distributed on the machine and the test was completed in the usual way. The apparatus was left standing for two hours and the procedure repeated using fresh samples of the same 12 specimens. The results were identical even in two cases in which only a few cells remained. The procedure was repeated after a further two hours with identical results.

This demonstrates that the apparatus may be run continuously for at least 3 hours without any deterioration of the reagents and there is a considerable safety margin. In that time, 540 specimens would be processed which is many more than we are likely to have to process on any one occasion. One could, of course, run for a further hour and, if it ever became necessary, the reagents could be packed in ice: the apparatus is designed to permit this.

3.3.5 Comparison of manual method with automatic method

Forty-eight specimens of sera were taken, and the complete diagnostic procedure carried out on each by the manual method as described in Sect. 3.3.1: this was done in the normal course of events. Samples of the same 48 sera were taken and the procedure was carried out on the machine. Complete agreement was obtained even in a few cases when the Wassermann reaction was positive and the Reiter reaction negative. A total of 250 specimens were tested in this way, spread over a period of several weeks, and complete agreement was reached in all cases. We have therefore every

reason to assume that the apparatus performs its function well.

The maximum number of sera that can be processed manually in one day is approximately 120, which number is processed in only 40 minutes by the machine. This speaks for itself, bearing in mind the operator does not spend 40 minutes performing the task but can utilize this time in carrying out other tasks.

3.3.6 Final discussion

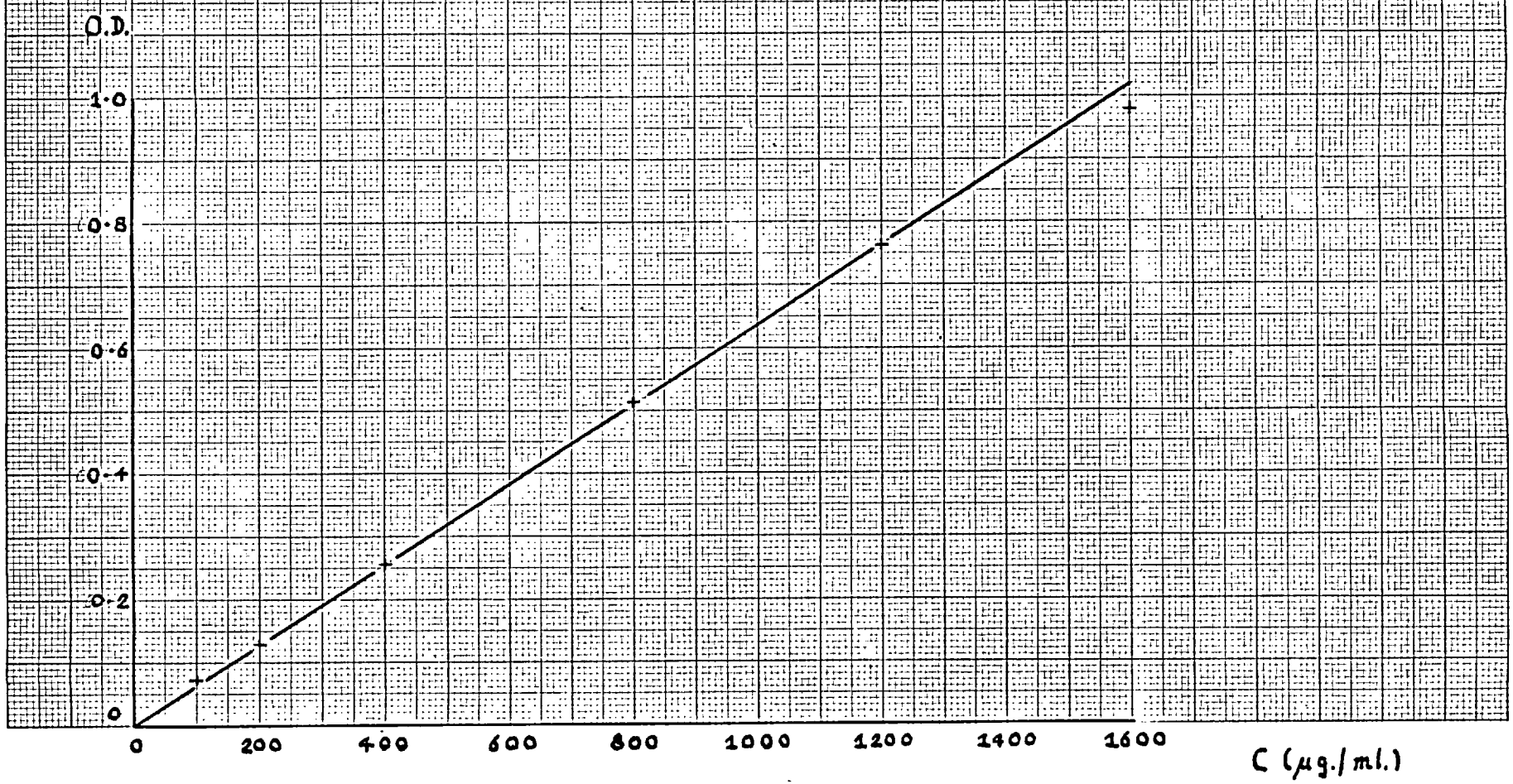
We have demonstrated that this is a satisfactory aid in performing the serological diagnosis of syphilis, which is now carried out by only one junior technician. It requires comparatively cheap equipment and does not suffer from the disadvantages of the auto-analyzer method (Pugh and Gaze, 1965). It is suitable for carrying out the procedure on up to between 500 to 600 specimens per day and in smaller laboratories, for which it is particularly suitable, pathologists might well be encouraged to store samples, this effecting a considerable saving of time.

We might have devised means for automatically detecting lysis, but the time taken to do this manually is very small compared with the time taken to dispense the reagents and we decided, therefore, not to do so.

The basic principles of the apparatus could be used in apparatus designed for use in, say, epidemological studies, but a modified reaction tube feed device may be more suitable (Sect. 3.3.3.2): in this case, more attention would have to be paid to the deterioration of reagents, and more exhaustive experiments than

those reported here would have to be performed before the design was finalized. Similarly, the same basic principles could be used for a method of performing any complement-fixation technique.

Fig. 17 — Calibration curve of the Unicam S.P. 500 spectrophotometer: concentration of standard solution of albumin (C) vs. measured optical density (O.D.).



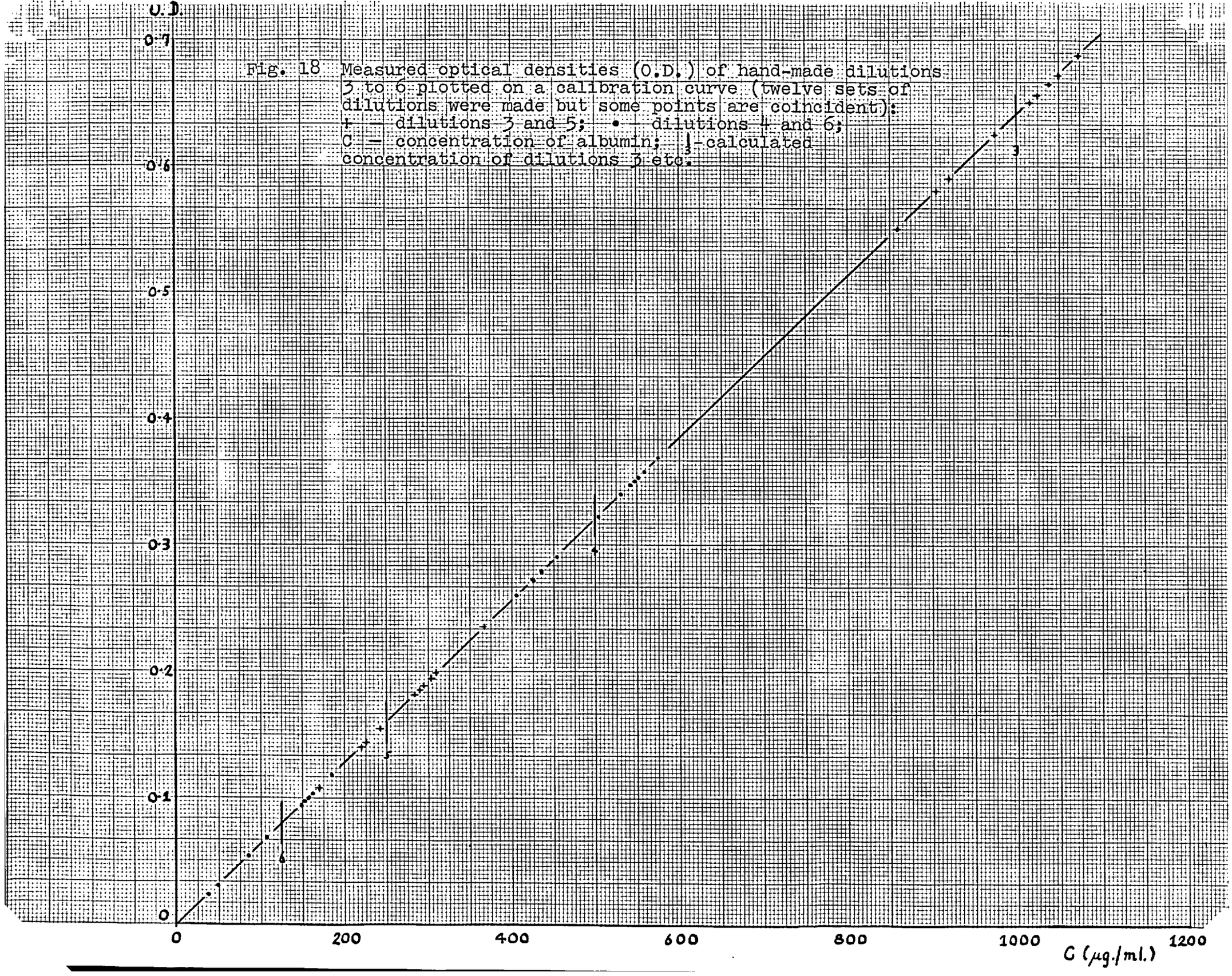


Fig. 18 Measured optical densities (O.D.) of hand-made dilutions 3 to 6 plotted on a calibration curve (twelve sets of dilutions were made but some points are coincident): + — dilutions 3 and 5; • — dilutions 4 and 6; C = concentration of albumin; ↕-calculated concentration of dilutions 3 etc.

Fig. 19. Drawing of a syringe/pipette system as used in the serial diluter: see text for legend.

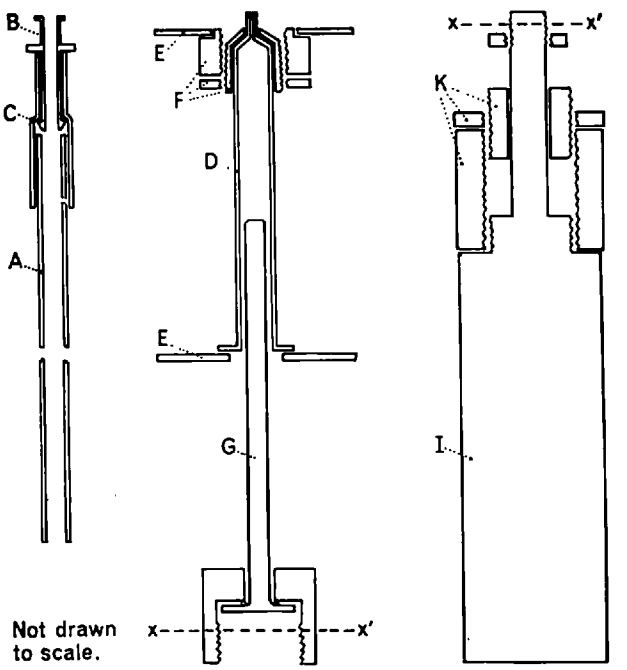


Fig. 20. A mechanically actuated syringe used for evaluating the syringe/pipette device used in the serial diluter: I - pneumatic cylinder, K - screw-thread device for adjusting the stroke length of the pneumatic cylinder, D - Sumit, 1 ml. tuberculin syringe.

Fig. 21. Four syringes, mounted together and actuated by a single mechanism, as used in the serial diluter.

Fig. 22. Drawing illustrating principle used to rotate the rack of tubes in the serial diluter: see text for legend.

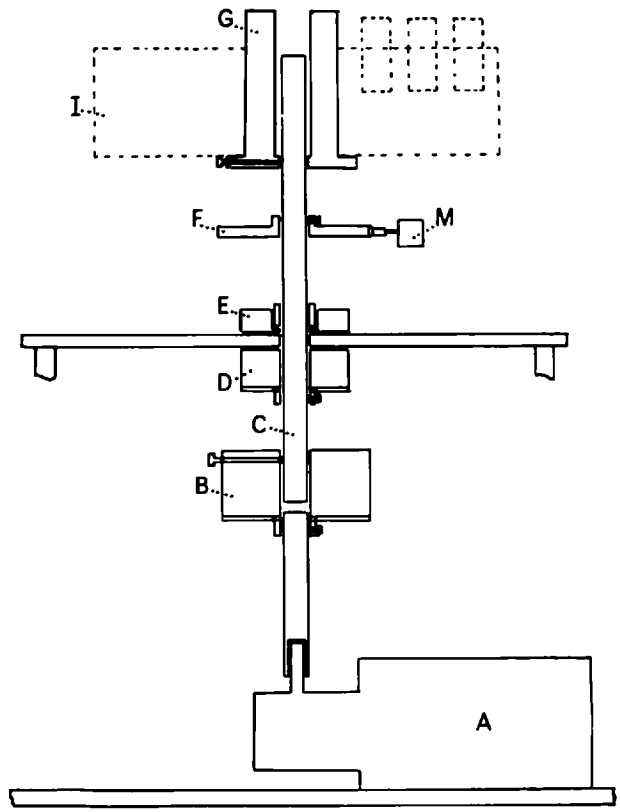


Fig. 23. Drawing showing lay-out of racks used in the serial diluter: rows a and b contain 12 tubes, row c contains 24 tubes.

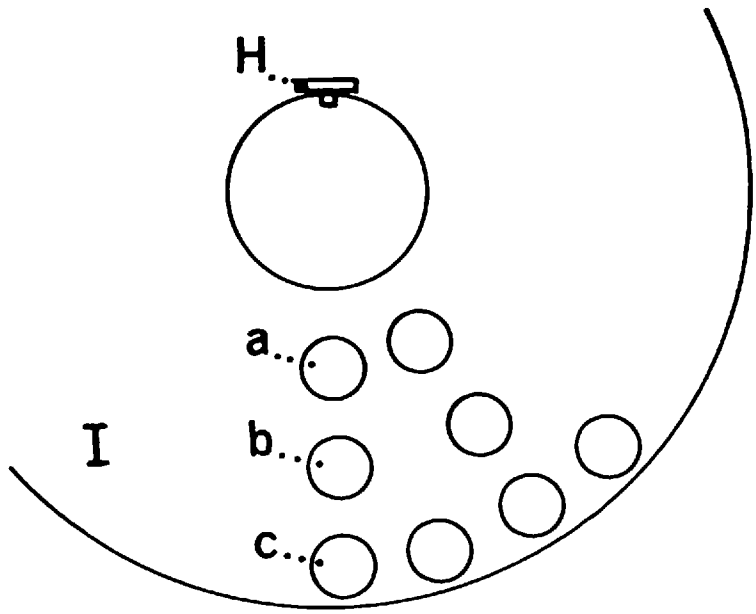


Fig. 24. The pipette holder - the pipettes, each covered by a glass tube, are loaded from the top of the holder which is arranged to accomodate the varying diameters of different parts of the pipette.

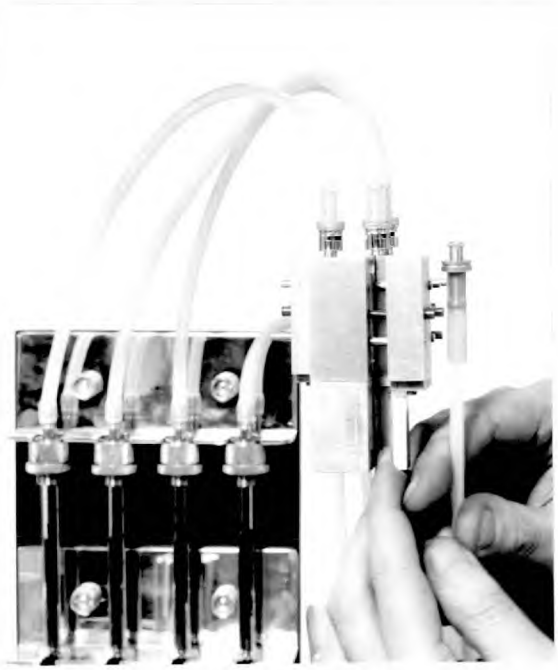


Fig. 25. The serial diluter and the controller.

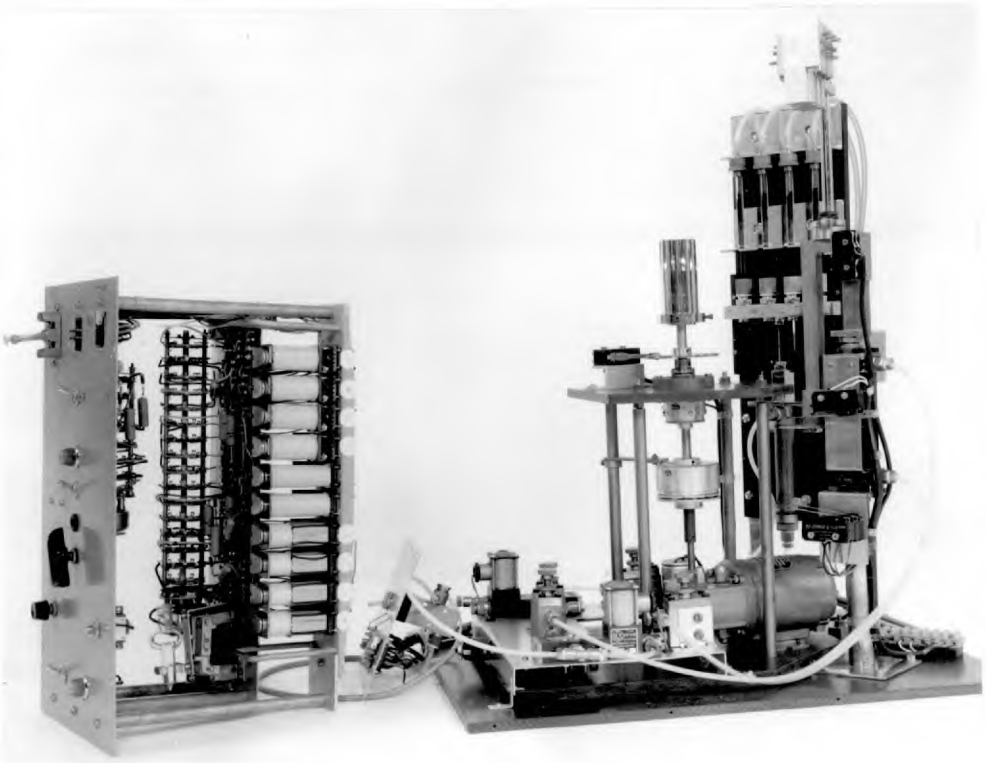


Fig. 26. Circuit diagram of pneumatic cylinder control system.

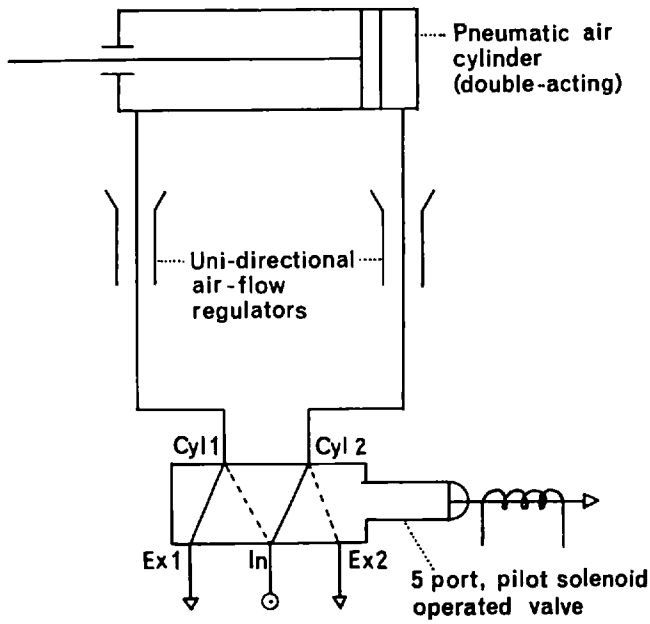


Fig. 27. Close up view of the top of the serial diluter, showing a rack of tubes and four pipettes loaded in the holder and connected to the syringes ready for use.

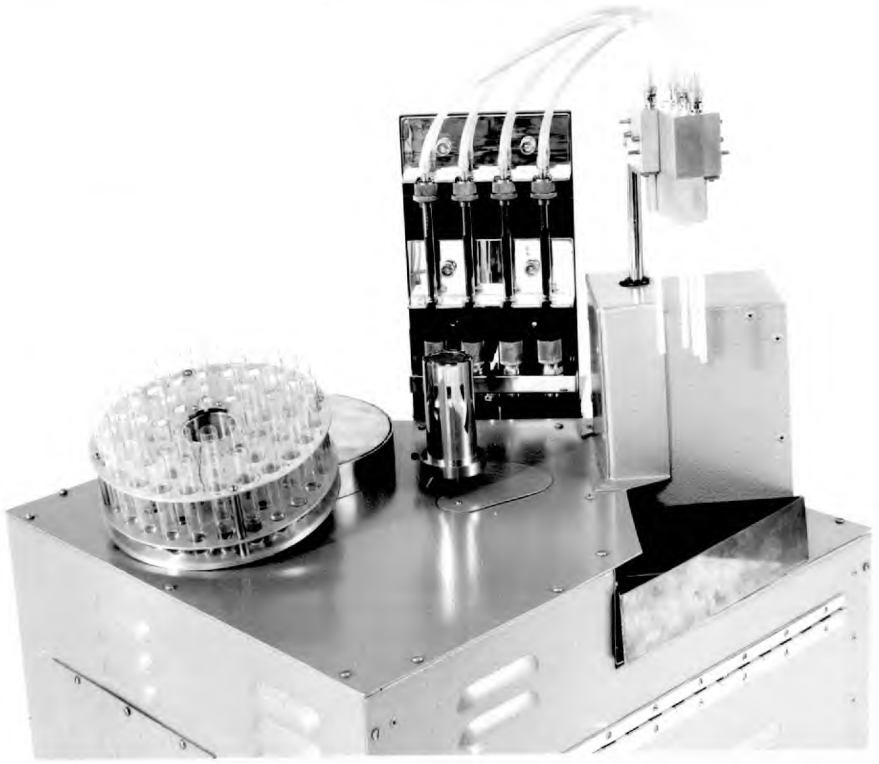
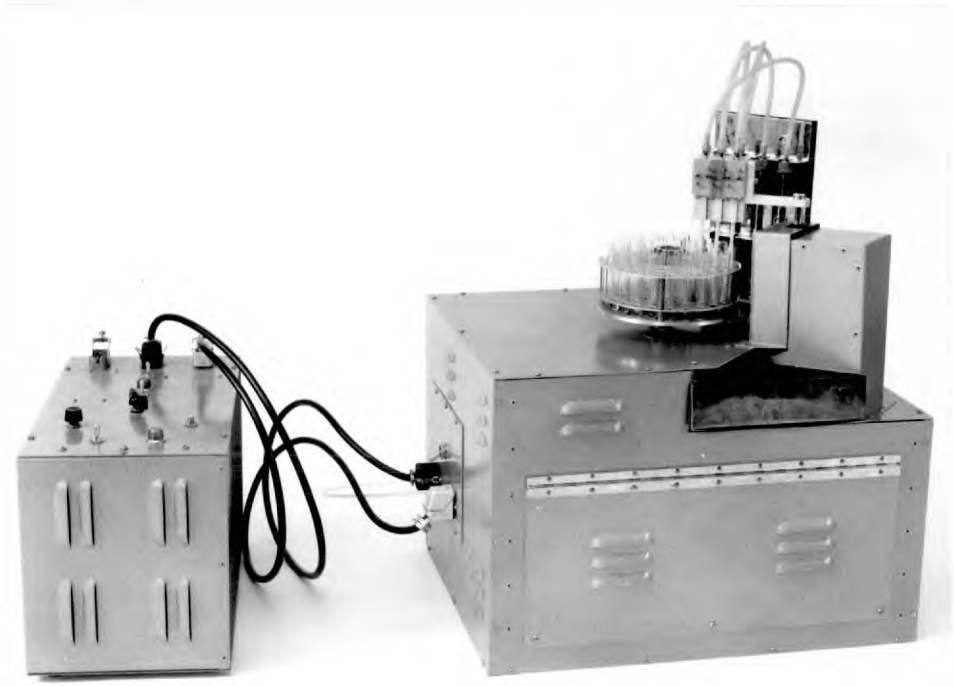


Fig. 28. The serial diluter in use.



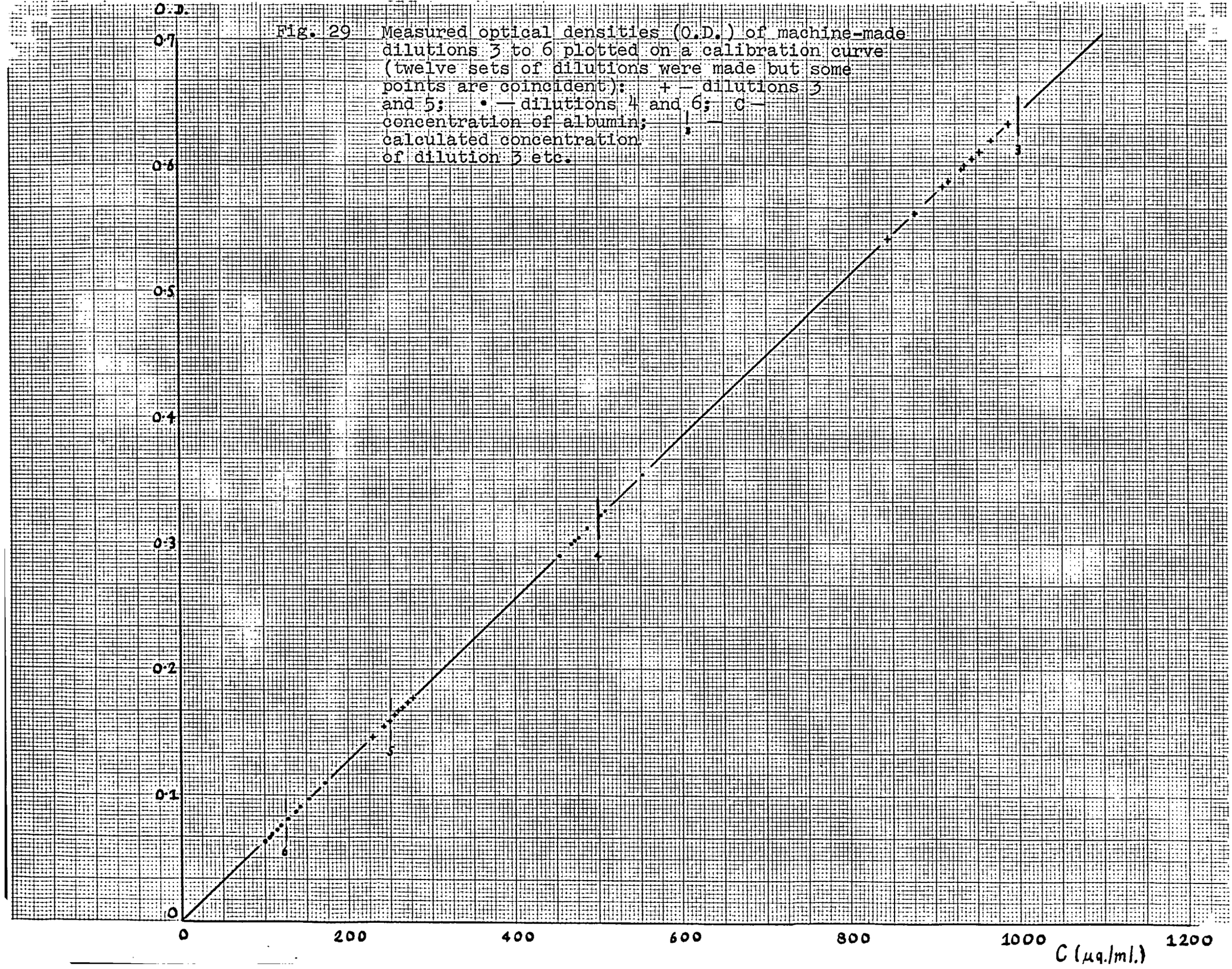


Fig. 29 Measured optical densities (O.D.) of machine-made dilutions 3 to 6 plotted on a calibration curve (twelve sets of dilutions were made but some points are coincident): + — dilutions 3 and 5; • — dilutions 4 and 6; C — concentration of albumin; ! — calculated concentration of dilution 3 etc.

Fig. 30 — Measured optical densities (O.D.) of hand-made and machine-made dilutions 3 to 6 plotted on identical, but vertically displaced, calibration curves (only the highest and lowest values of each of the 8 groups of points are plotted): C — concentration of albumin; H — hand-made dilutions; M — machine-made dilutions; | — calculated concentration of dilution 3 etc.

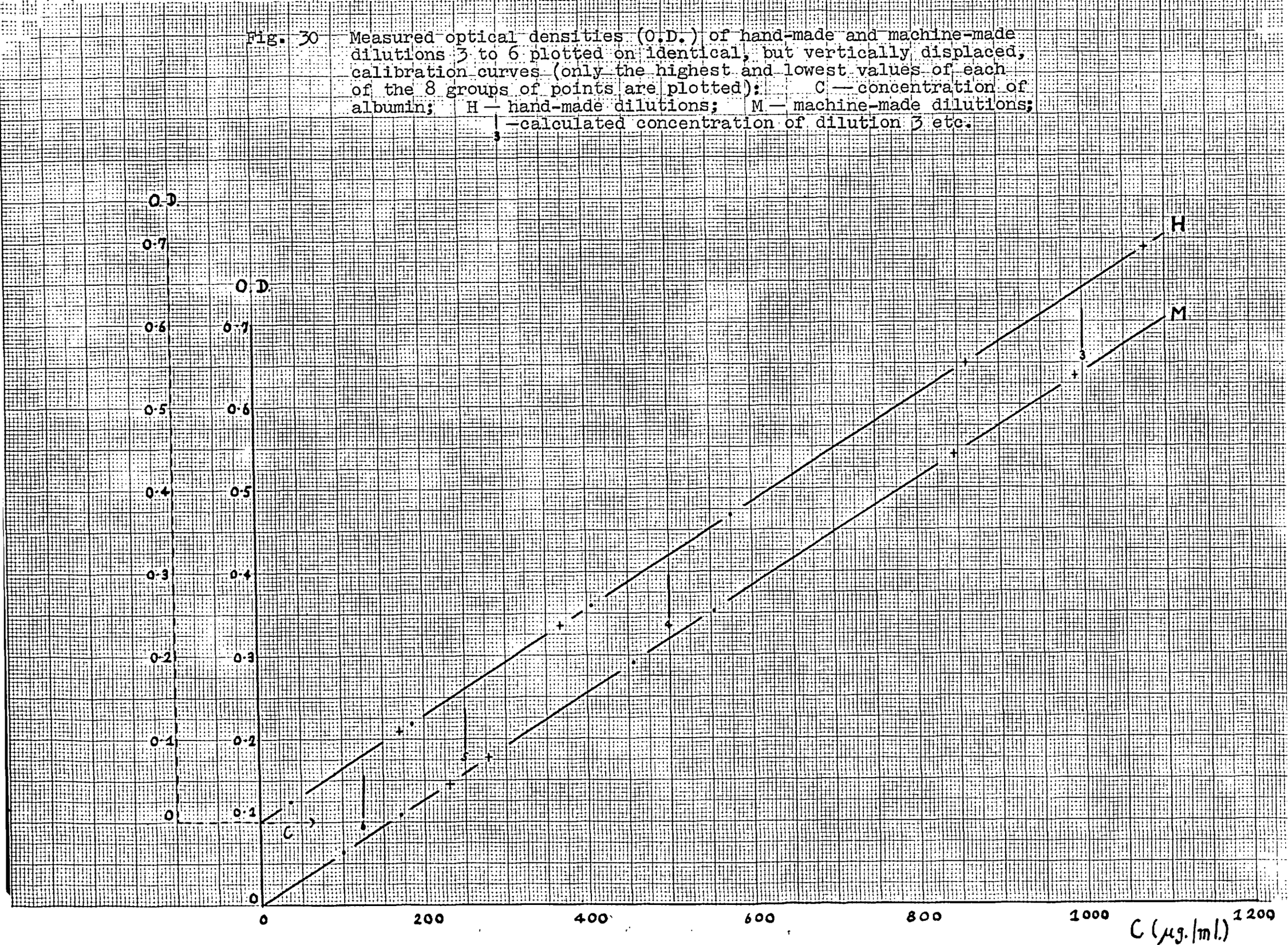


Fig. 31 Diagram showing the mean value ($\bar{x}$) of 12 drops dispensed by each of twelve 50 droppers

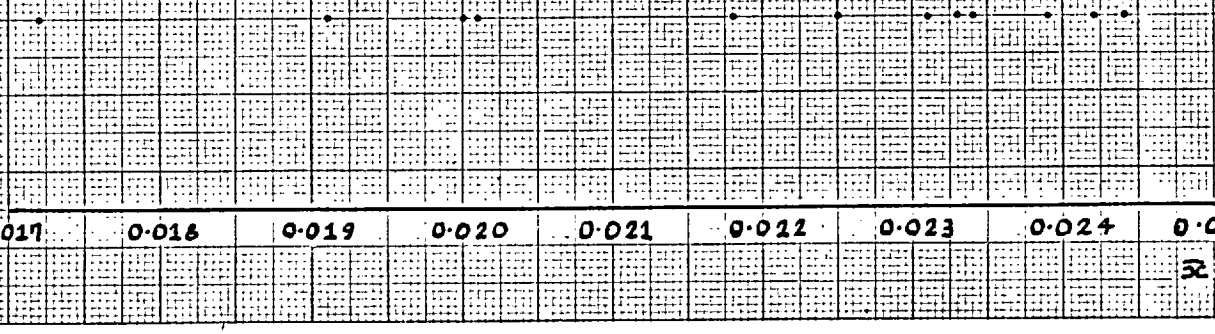


Fig. 32. A Repette.

Fig. 33. Close up view of the Repette mechanism: see text for legend.

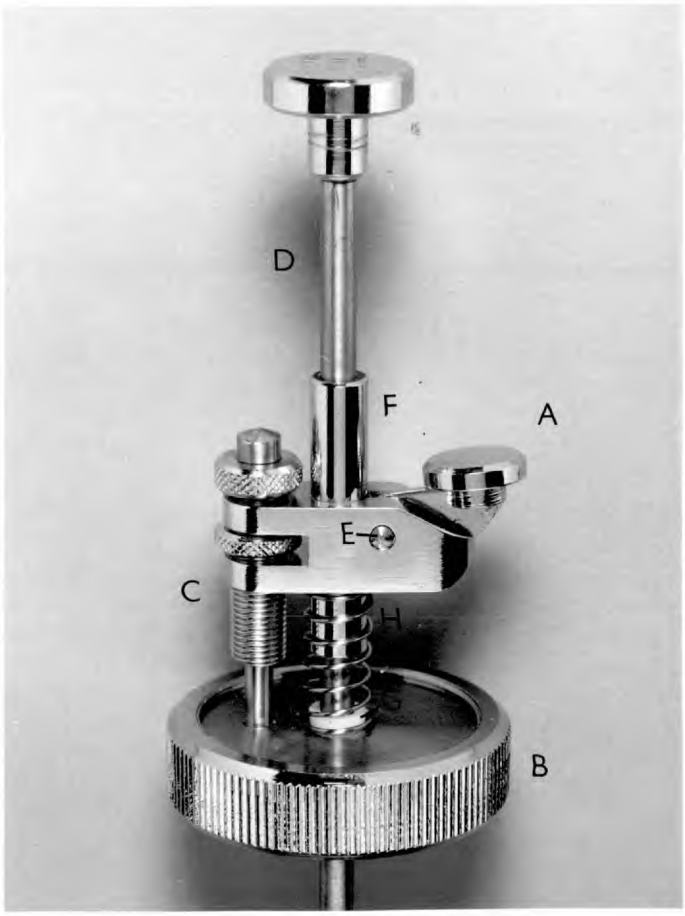


Fig. 34. Drawing of modification to Repette enabling the use of removable disposable syringes instead of the fixed glass syringe (Fig. 32): see text for legend.

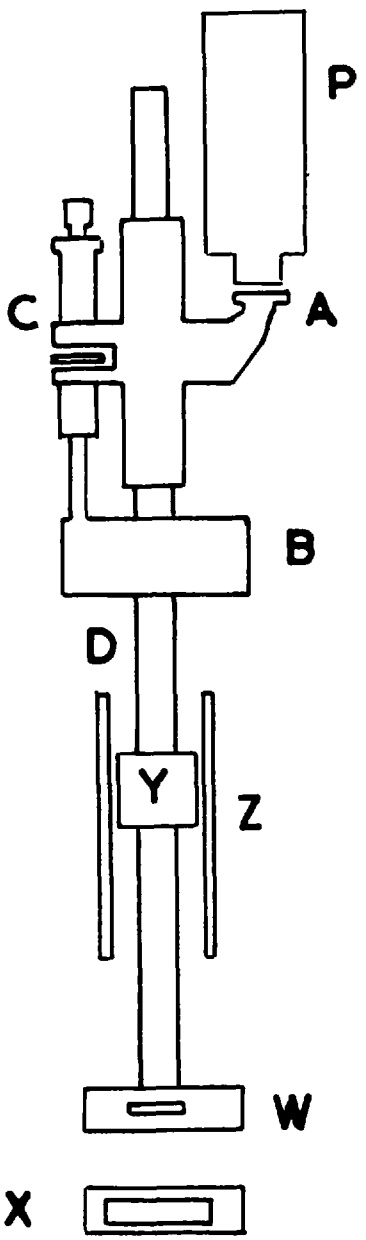


Fig. 35. The dispensing mechanism of the liquid culture dispensing apparatus: A - 2 in. stroke pneumatic cylinder for filling the syringe, R - Repette mechanisms.

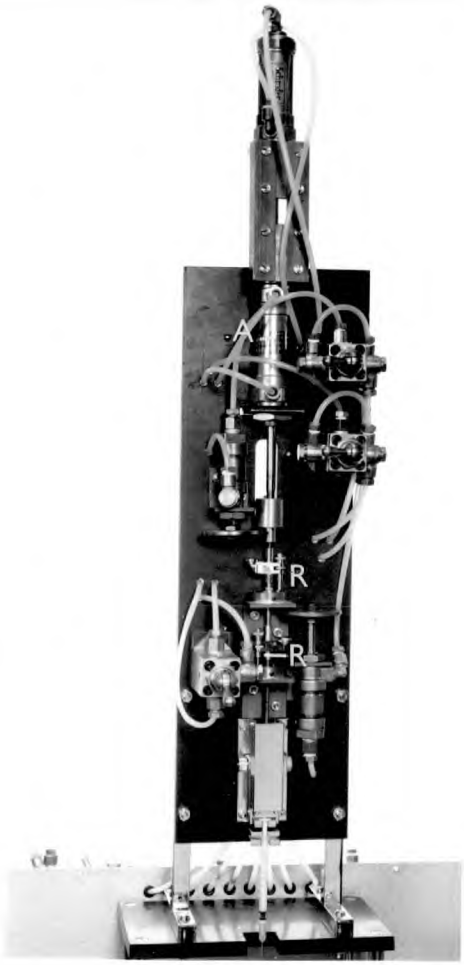


Fig. 36. The pneumatic control unit of the liquid culture dispensing apparatus.

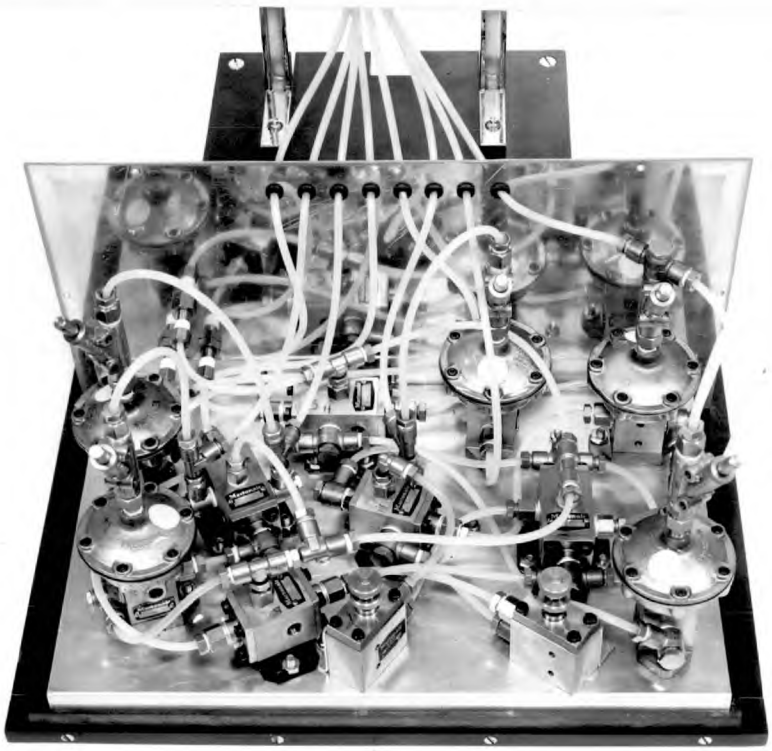


Fig. 37. The liquid culture dispensing apparatus.

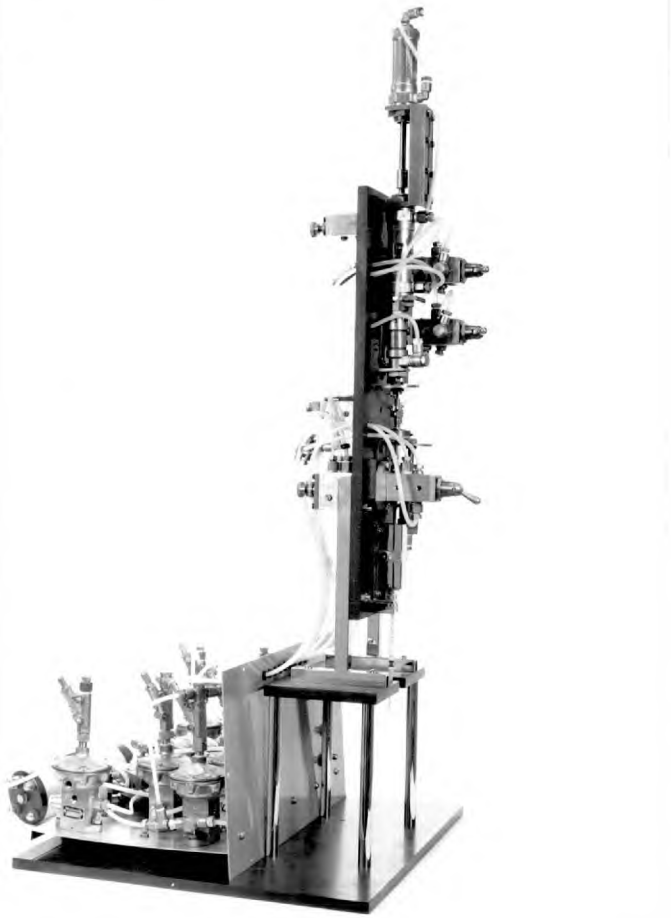


Fig. 38

Reagents required for the serological diagnosis of syphilis

Tube number	Reagents
1 (Control)	0.02 ml. of serum 0.08 ml. of saline 0.1 ml. of saline 0.1 ml. of 1 minimal haemolytic dose (M.H.D.) complement
2	0.02 ml. of serum 0.08 ml of saline 0.1 ml. of Wassermann antigen 0.1 ml. of 1.25 M.H.D. complement
3	0.02 ml. of serum 0.08 ml. of saline 0.1 ml. of Reiter antigen 0.1 ml. of 1.25 M.H.D. complement
4	0.1 ml. of serum 0.1 ml. of Reiter antigen 0.1 ml. of 1.5 M.H.D. complement

Fig. 39

Reagents required for determination of the minimal haemolytic dose (M.H.D.) of complement

Tube number	Reagents
1 (Control)	0.1 ml. of diluted complement 0.1 ml. of saline
2	0.1 ml. of diluted complement 0.1 ml. of Wassermann antigen
3	0.1 ml. of diluted complement 0.1 ml. of Reiter antigen

Fig. 40

Combination of reagents, for the serological diagnosis of syphilis, that may be conveniently dispensed automatically

Tube number	Combination of reagents
1 (Control)	0.08 ml. saline + 0.1 ml. saline + 0.1 ml. 1 M.H.D. complement
2	0.08 ml. saline + 0.1 ml. 1.25 M.H.D. complement 0.1 ml. Wassermannantigen
3	0.08 ml. saline + 0.1 ml. 1.25 M.H.D. complement 0.1 ml. Reiter antigen
4	0.1 ml. Reiter antigen 0.1 ml. 1.5 M.H.D. complement

Fig. 41. A simple valve/syringe mechanism suitable for dispensing small, measured volumes of liquids: I - tube to reservoir of liquid, O - outlet, V - valve.

Fig. 42. A valve/syringe mechanism and its actuating mechanism: C - pneumatic cylinder, K - screw-thread device for varying the stroke length of the pneumatic cylinder and hence the volume dispensed.

Fig. 43. Mounting for four valve/syringe mechanisms - all four syringes are actuated by a single pneumatic cylinder C.

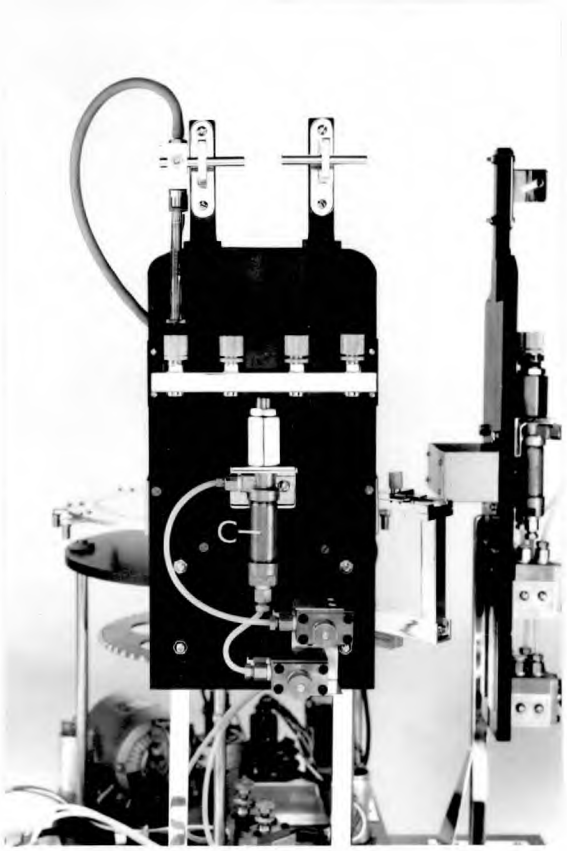


Fig. 44. A rack into which the reaction tubes for the Wassermann reaction are placed.

Fig. 45. Photograph showing mountings for the six valve mechanisms in the apparatus for dispensing the reagents for the Wassermann reaction - the four valves on the left-hand panel each deliver 0.1 ml., and the other two valves deliver 0.18 ml. and 0.28 ml. respectively.

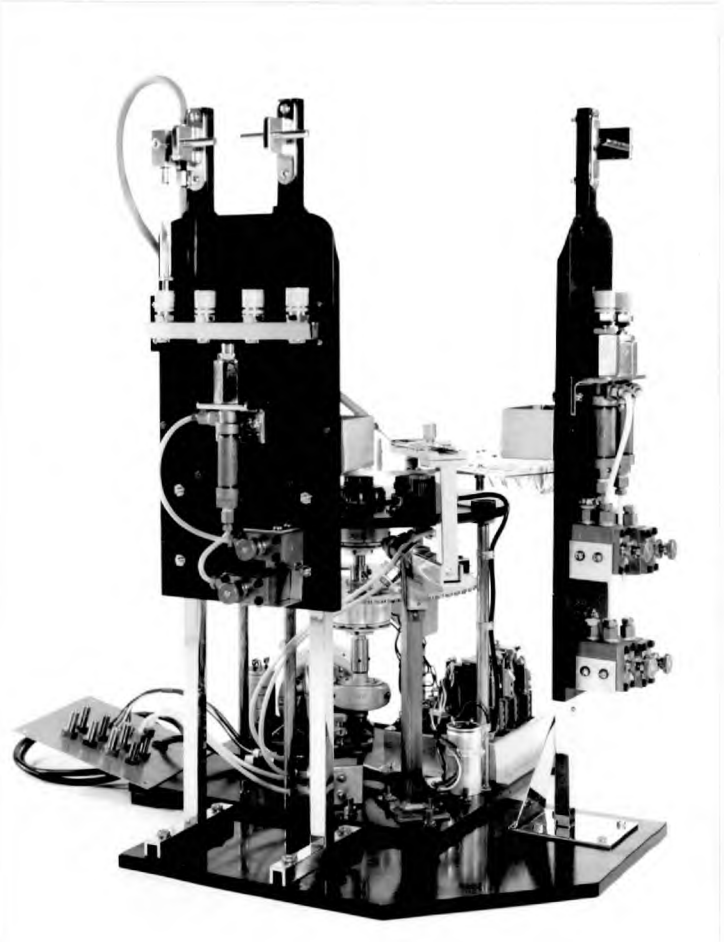


Fig. 46. Photograph showing positions of the reagent reservoirs in the apparatus for dispensing the reagents for the Wassermann reaction.

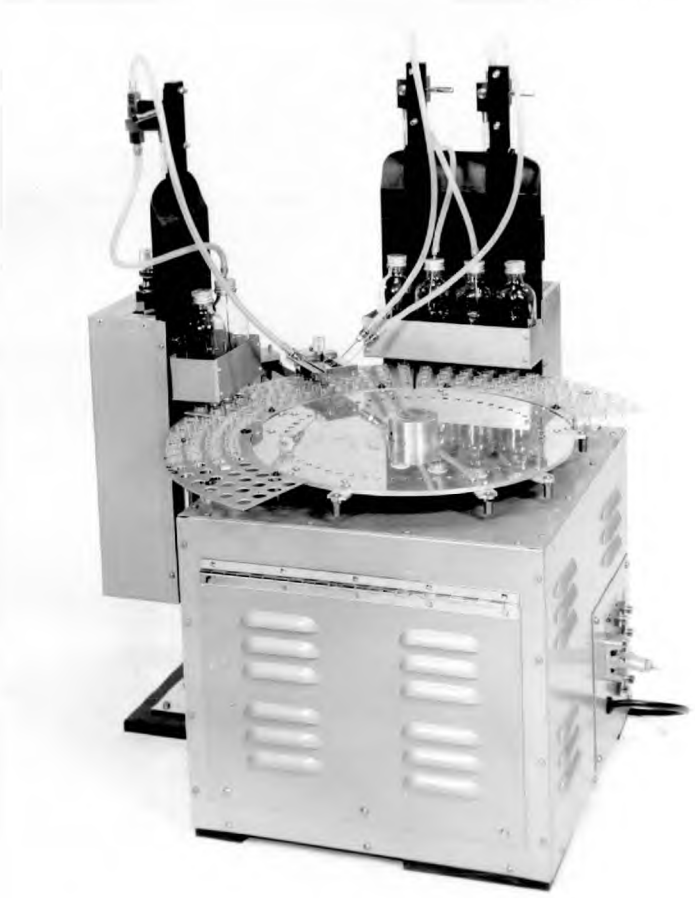
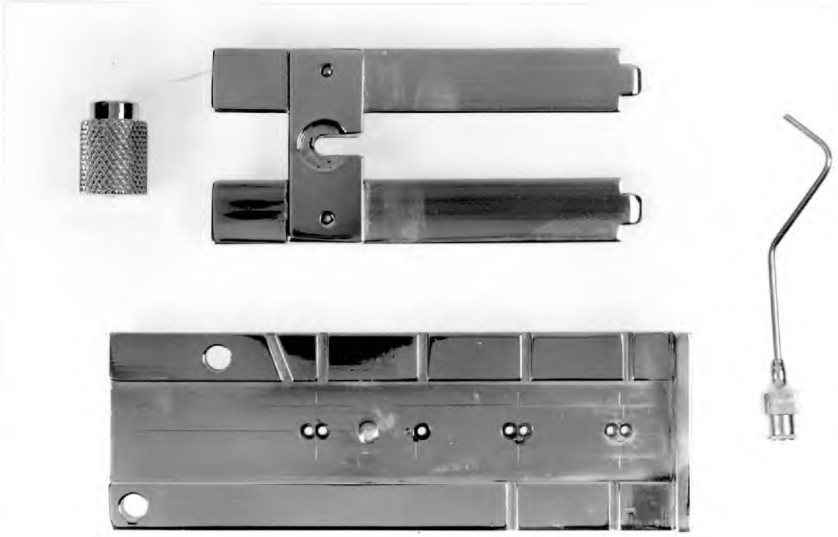
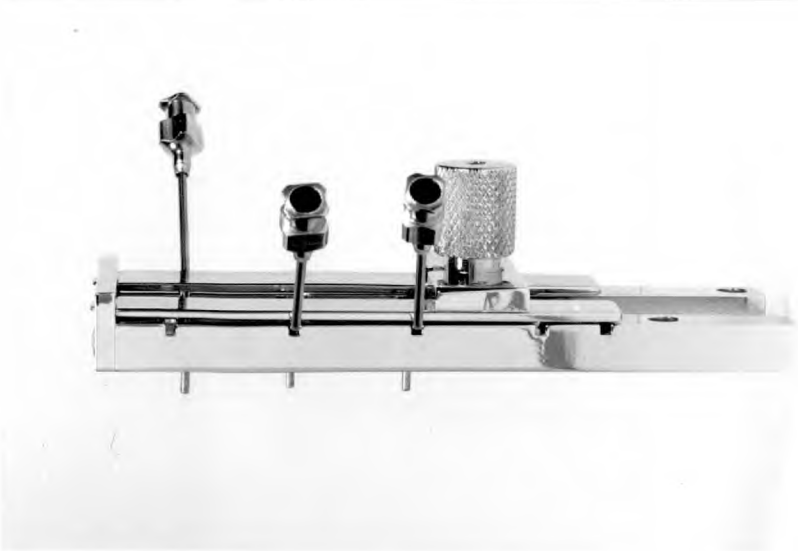


Fig. 47. The holder for the valve outlet nozzles in the apparatus for dispensing the reagents for the Wassermann reaction: a - exploded view, b - assembled holder.



a.



b.

Fig. 48. Photograph showing a rack of reaction tubes passing underneath the valve outlet nozzle, mounted in the holder, in the apparatus for dispensing the reagents for the Wassermann reaction.

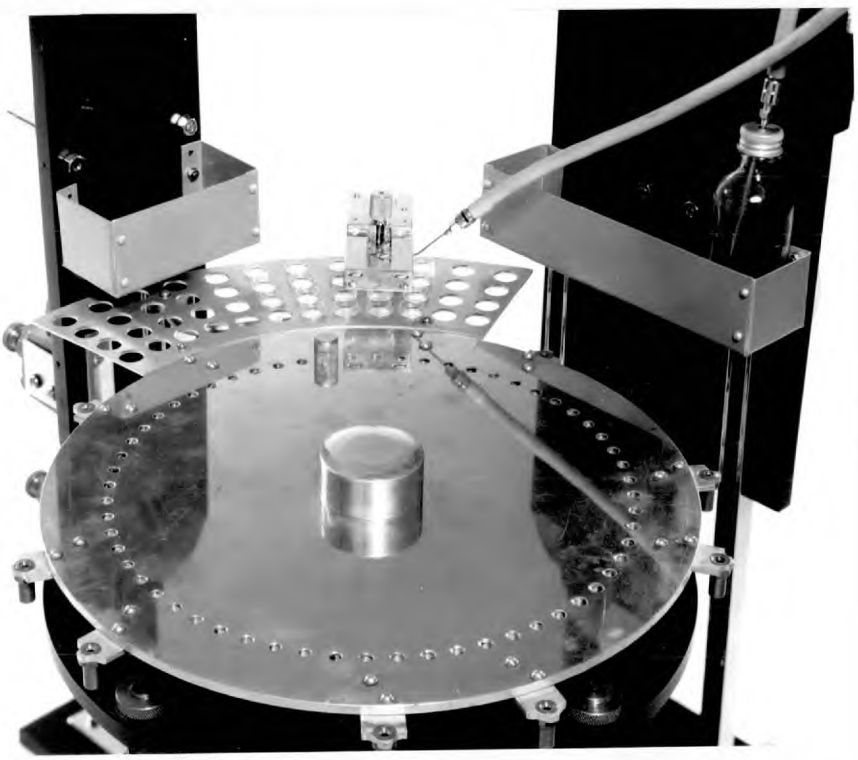


Fig. 49. The control unit of the apparatus for dispensing the reagents for the Wassermannreaction.

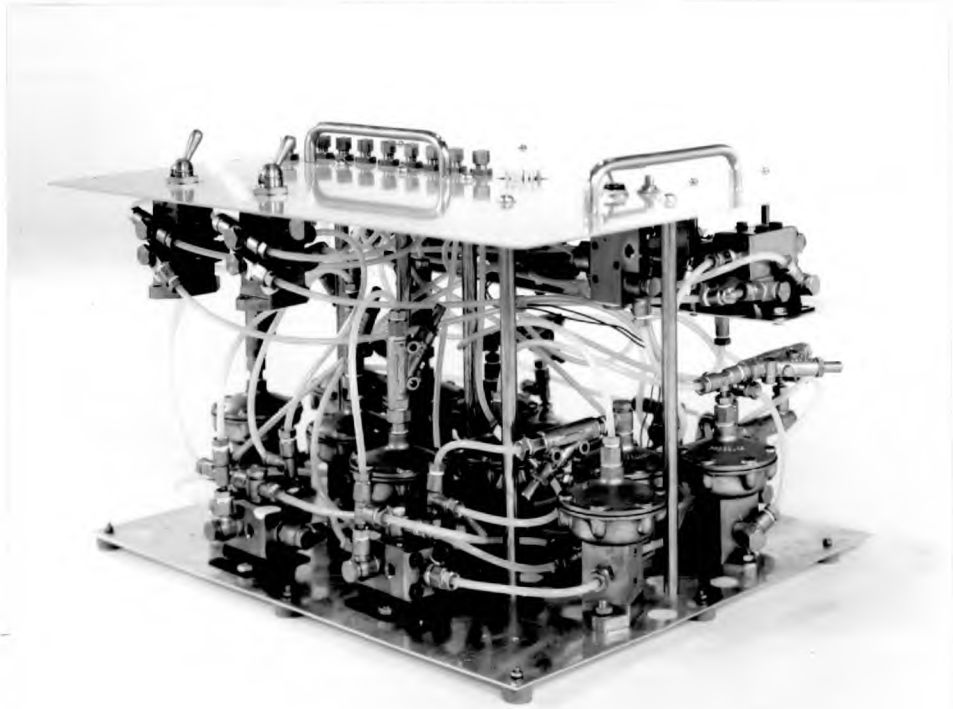
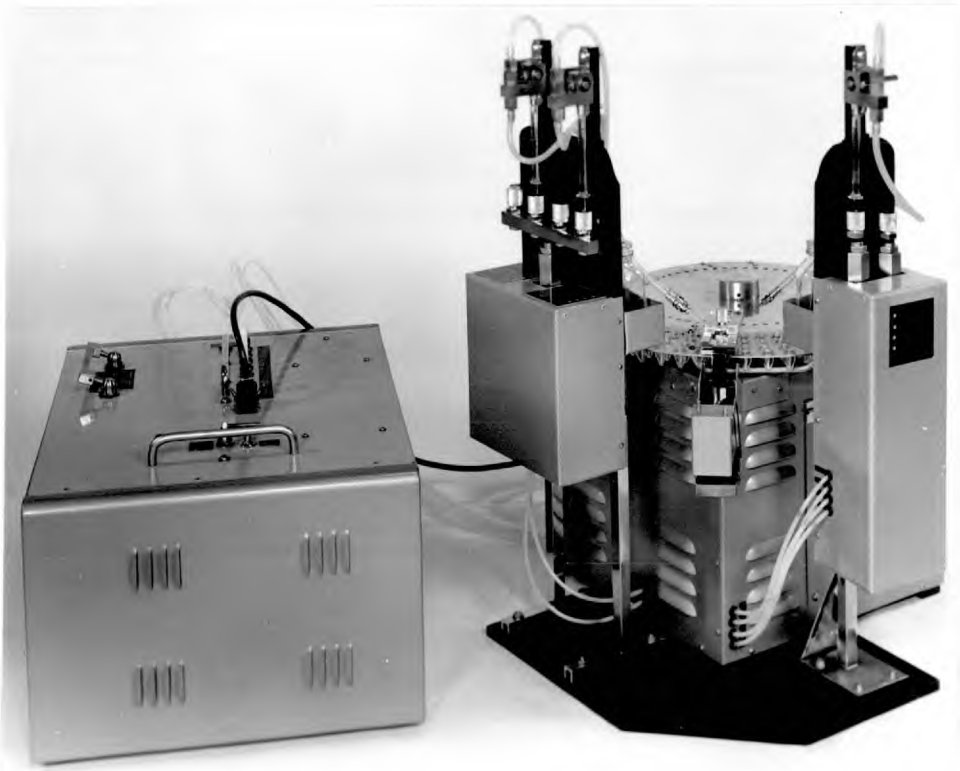


Fig. 50. The apparatus for dispensing the reagents for the Wassermann reaction.



Chapter 4

Experimental Methods

4.1 Spreading Bacterial Culture over a Solid Agar Plate

An automatic device for spreading bacterial culture over a solid agar plate would be an extremely useful piece of apparatus because the procedure is an essential part of many diagnostic test procedures (Sect. 1.3.2).

We have investigated the possibilities of producing such a device and, although we do not have a definitive machine in routine use, considerable progress has been made.

4.1.1 Evaluation of the manual method

The object of spreading a culture over an agar plate is to cover the whole plate with successive dilutions of inoculum so that, eventually, discrete colonies of the organism are isolated. There are a variety of techniques for doing this and one of the commonest is illustrated in Fig. 51. The plate is streaked at A with the charged loop. The loop is then sterilized and streaked at B: note that it partly overlaps A. This procedure is repeated at C and D and it is hoped that sufficient dilution of the inoculum is obtained so that single colonies appear in D. The advantage of the method is that one requires nothing more than a simple bacteriological loop and an agar plate, but some skill is required.

A simple experiment to measure the time taken to spread a culture gave the following results:-

Plate No.	1	2	3	4	5	6
Time taken to spread culture, in seconds.	30	32	38	30	28	31

We conclude that it takes approximately 30 seconds to spread a culture by the above method.

In routine work, the complete procedure is not always adopted. We therefore observed some members of the routine laboratory staff spreading a culture during their normal work. The results were:-

Plate No.	1	2	3	4	5	6	7	8
Time taken to spread culture, in seconds	10	10	10	8	6	8	8	8

We conclude that it takes approximately 8 seconds to spread a culture over an agar plate by the methods normally used in routine bacteriology.

Similarly, we found that it takes approximately 15 seconds to inoculate a plate from a swab and plate-out the inoculum: the plate is inoculated by rolling the swab over part of the plate (A in Fig. 51) and the inoculating loop is used to spread the inoculum, as before.

In 1967, approximately 110,000 agar plates were supplied to our routine laboratory and a substantial proportion had cultures spread over them. A rough calculation shows, that if one technician spread all cultures, it would take him 5 to 6 working weeks each year, which is approximately 10% of his time.

Nevertheless, the method is very flexible in that all types of culture can be spread with the bacteriological loop, and it is unlikely that a cheaper method of spreading a culture will be found.

4.1.2 Criteria for an automatic method

It is unlikely that one can devise an automatic method for spreading a culture over a plate which is quicker than the manual method usually adopted in routine work. It is essential, therefore, to develop a method that is automated to at least such an extent that whilst the operator is marking an agar plate, for specimen identification, the machine is spreading the previous plate.

Two other important criteria are, that the total path traversed by the loop be long enough to give sufficient dilution of the culture to isolate single colonies, and that the pressure of the loop on the agar be sufficient to maintain contact between the loop and the agar but not so great as to damage the surface of the agar: it is desirable for the loop to leave a permanent mark to assist in identifying contaminants.

One method for spreading a culture automatically is to move the loop in a straight line across a rotating agar plate: the loop will describe a spiral curve on the agar, and the parameters of the curve

can be adjusted by altering the relative speed between the loop and the plate. If it is so arranged that the space between any two adjacent parts of the curve is as small as practicable, maximum use of the available space will be made.

The ease with which one can sterilize the electrically sterilizable loop (Sect. 2.1.2) in situ makes it particularly suitable for use in an automatic spreading machine based on the principles outlined above. However, it is desirable to avoid the need to sterilize the loop during the spreading procedure, as is recommended in the manual method.

4.1.3 The first experimental apparatus

The rotary table, on which the agar plate was placed, and the moving arm to which the loop was attached were driven by the same motor through a variety of gears (Fig. 52): the ratios of the gears determined the space between adjacent turns of the spiral curve. The loop was hinged to the moving arm and the tip of the loop rested, under its own weight, on the agar. The copper braid connecting leads were permanently attached to the loop, as in Fig. 6, and the power supply shown in Fig. 4 was used. For preliminary tests, the agar plate was inoculated from a single colony on an agar culture by touching the colony with an inoculating loop and then streaking the loop across the centre of the plate. The plate was then placed on the rotating table, the loop on the apparatus, having been heated and allowed to cool, was lowered carefully until it rested on the inoculum and the motor was started and left running

until the loop had traversed the plate: the loop was then lifted off the plate and sterilized, and the plate was removed and incubated.

Typical results are shown in Fig. 53. Lengths of agar were gouged out by the loop because it was too heavy and because the flexible copper braid connecting leads applied a variable, uncontrollable constraint to the loop: in any case, insufficient dilution of the culture was obtained. Nevertheless, some single colonies were isolated, and had we started with a smaller inoculum we might have isolated more single colonies.

The results were encouraging, so it was decided to pursue the method in principle, but to construct more refined apparatus: the first experimental apparatus was constructed with very cheap components.

4.1.4 More refined apparatus

4.1.4.1 The first design

The apparatus is shown in Fig. 54. The essential differences between this and the first experimental apparatus were that the loop was a different shape (Fig. 55), it was mounted horizontally and it had a variable counterbalance: the force the loop applied to the agar could, therefore, be varied and was more consistent than the force applied by the loop when mounted as in Fig. 52. We also used more precise components.

The apparatus was driven by a continuously running variable speed motor, and an electro-magnetic clutch was operated to rotate the table and move the arm supporting the loop: the gears used gave a space between adjacent turns of the spiral curve of approximately $\frac{1}{4}$ in.

Figs. 54 and 55 show that the electrical connection to the loop was made through a pair of plugs and sockets and that the loop pivoted about the plugs. This was unsatisfactory, because friction between the plugs and the bearing surfaces was such that the loop did not rotate as freely as desired. We therefore modified this part of the apparatus.

4.1.4.2 The final design

The loop was mounted on miniature ball races, and the electrical power was supplied through copper braid leads permanently connected to the loop (Fig. 56). We found that these modifications reduced the friction between the loop and its bearing and that this force remained constant. The power supply used was that shown in Fig. 4: no further modifications to the first design were made.

The counter-balance was adjusted by trial and error. We simply set it and ran the loop across a variety of different agar plates to ensure that the pressure on the agar was within limits: it was unnecessary to adjust the counter-balance for use with different agar plates.

4.1.5 Evaluation of the automatic method

It was necessary to develop inoculation techniques, and since there are three main types of culture that have to be spread, namely solid culture, liquid culture and swabs, we discuss the work in three sections.

4.1.5.1 Spreading solid culture

To inoculate the plate from a solid culture, we took a portable inoculating loop, touched a colony and streaked the loop along a

radius for approximately ½ in., commencing just off the centre of the plate: by inoculating the plate this way, one avoids having to specify too precisely the part of the plate to be inoculated, because the loop on the apparatus will pass through the inoculum at least once, regardless of its exact position.

The plate was then placed between the locating springs on the rotary table, the loop was lowered and the inoculum was spread across the whole plate without sterilizing the loop during the process: the parameters of the apparatus were such that it took approximately 10 seconds for the loop to traverse the plate.

Typical results are shown in Fig. 57. The path described by the loop is clear and many single colonies were isolated. Many similar experiments were conducted with very consistent results.

The methods for inoculating an agar plate from a solid culture and spreading the inoculum are satisfactory. The only forseeable difficulty is the possibility of not obtaining sufficient dilution of the inoculum and care has to be taken to ensure that the inoculum is not too heavy: a single stroke of the inoculating loop is adequate.

4.1.5.2 Spreading liquid culture

We took an inoculating loop full of a broth culture of Staph. albus (approximately 10^6 organisms/ml.) and streaked it along a radius of an agar plate for approximately ½ in., as for inoculating a plate from a solid culture, and the culture was spread with the un-modified machine. The results were very poor. The inoculum appeared to be adequate, but the loop did not pick up sufficient culture.

We repeated the procedure, but streaked the inoculating loop along the whole radius of the agar plate: the loop on the machine crossed the inoculum more times than hitherto, so there was a greater chance of the loop picking up and spreading culture. The results were marginally better, but still very poor.

We then inoculated the agar plates by placing 0.02 ml. of urine just off the centre of the plate: samples of urine taken for diagnosis were used. The result of spreading this inoculum was a vast improvement on previous methods, in most cases many single colonies were isolated and there was no indication that insufficient dilution would be obtained. Occasionally, however, only a few single colonies were isolated.

Consequently we inoculated some plates with two 0.02 ml. drops of urine spread along a radius to approximately half way across the plate, and some plates with three 0.02 ml. drops of urine spread across the whole radius. The results showed that the optimum inoculum was two 0.02 ml. drops of culture spread along a radius to approximately half way across the plate: there was no sign that insufficient dilution would be obtained but many single colonies were invariably isolated. Typical results are shown in Fig. 58. Similarly, we have often isolated two or more organisms in a mixed culture (Fig. 59).

In all the above experiments, the inoculum was spread before it had completely dried. We performed some further experiments, therefore, and compared the results obtained when the inoculum was

spread immediately after it had been placed on the plate, with the results obtained when the inoculum was spread after it had completely dried. The difference was not significant.

Additional experiments were performed to discover if the agar plate was rotating at the optimum speed. We compared the results obtained when the loop on the machine took approximately 3 seconds, 6 seconds and 13 seconds to traverse the agar plate with the results obtained hitherto, when the loop took approximately 10 seconds to traverse the plate.

The results obtained when the process time was 3 seconds were very poor: hardly any spreading of culture was achieved. The results obtained when the process time was 6 seconds were not quite as good as those obtained when the process time was 10 seconds, and the results obtained when the process time was 13 seconds were no better than those obtained when the process time was 10 seconds. The results suggest that the speed of rotation of the agar plate is not critical but that a process time of approximately 10 seconds is the optimum.

The results obtained in each series of experiments in which the plates were inoculated with one or more 0.02 ml. drops of urine were remarkably consistent (there were, of course, differences due to the wide variations in the number of organisms in the inocula). A total of 106 plates were spread in these experiments and, with the exception of 5 plates spread in 3 seconds, all were perfectly adequate for diagnostic purposes. The technique is adequate and practical

for inoculating agar plates with liquid culture and spreading the culture to isolate single colonies.

However, although 50 droppers, which were used for producing the 0.02 ml. drops of culture, are cheap ($\frac{3}{4}$d each), it was desirable to find an even cheaper and quicker inoculation technique. We used wax drinking straws for inoculating the plates. The tip of a sterilized straw was dipped into the urine to a depth of $\frac{1}{8}$ in., approximately 0.04 ml. of urine was taken up and this was spread half way along a radius of the agar plate: it was not necessary to hold a finger over the end of the straw, the surface tension being adequate to hold the culture in the straw. The straws are very cheap (2/- for 500), and for this application there was no need to plug them: one merely had to sterilize them.

To test the efficacy of the sterilization of the straws, we took 12 and autoclaved them at 10 lbs./sq. in. for 20 minutes. Each straw was immersed in sterile nutrient broth which was incubated overnight. No growth was obtained in any of the 12 tubes of broth. Similarly, we contaminated 12 straws, by sucking a Staph. albus broth culture into each, and then repeated the above procedure. No growth was obtained in any of the 12 tubes of broth and we conclude, therefore, that sterilization of wax drinking straws by autoclaving is satisfactory. Physically, sterilization had very little observable effect on the straws. They were slightly softer after the sterilization process than they were before, but their condition was perfectly satisfactory for inoculating agar plates by the method described above, and we have used straws in many

experiments. We see no reason why straws should not be used for transferring small volumes of culture, if the volume to be transferred is not critical.

4.1.5.3 Spreading swab culture

We inoculated a blood agar plate by streaking a swab across a small square just off the centre of the plate, and the inoculum was spread on the machine as before. The plate is shown in Fig. 60. Further similar experiments gave very inconsistent results and indicated that a larger inoculum was required.

Plates were inoculated, therefore, by streaking the swab across a radius of the plate: two such plates are shown in Fig. 61. Some plates were adequate but far from perfect, and we next performed some experiments in which the inoculum was spread over a larger area than hitherto. In general, the results were slightly better but we felt that the inocula were not sticking to the loop, probably because the inocula were dry.

We therefore conducted a series of experiments with wet agar plates. The results were an improvement on results obtained hitherto, and the best plates were perfectly adequate. However, we found that when the initial inoculum was light, the spreading was poor and decided to attempt to obtain more consistent results.

We replaced the loop with one that had a flat platinum tip approximately 3/16 in. long and 3/32 in. wide (Fig. 62). The replacement loop was mounted in exactly the same way as the previous loop and no other modifications to the apparatus were made.

The first experiments with this loop were carried out with the counter balance in the same position as before: the pressure of the loop on the agar was, therefore, significantly less than with the other loop because the area of contact was much larger. The results were a significant improvement (Fig. 63). We conducted a further series of experiments in which the pressure of the loop on the agar was gradually increased until the loop made a permanent mark on the agar. In this series of experiments, the plates were inoculated immediately after the technician in the routine laboratory had inoculated the plates required for diagnostic purposes. A typical result obtained with the machine is shown in Fig. 64.

A total of 108 plates, inoculated from 108 swabs taken for diagnostic purposes, were spread with the flat platinum tipped loop. Each culture was more than adequate for diagnostic purposes and each compared very favourably with the corresponding manually spread culture: there were no discrepancies and approximately 20 automatically spread cultures were significantly better than the corresponding manually spread cultures. The automatically spread cultures are as consistent as one would expect with the wide variations in the initial inocula (Figs. 65 and 66).

4.1.6 Discussion

The methods for inoculating agar plates and spreading the inoculum described in Sect. 4.1.5 are perfectly adequate for diagnostic bacteriology. Further experiments might well be performed; for example, we have not investigated spreading solid

and liquid cultures with the flat ended loop. Before a definitive piece of equipment for use in the routine laboratory can be designed, many refinements will have to be developed, but we feel that any further experimental work should be carried out with a particular application in mind. The possibilities are discussed in Chapter 5.

4.2 Routine Examination of Urine

4.2.1 The manual method

Many methods for examining urines are used in different establishments. Our method is as follows. An approximate count of bacteria present in the urine is obtained by the blotting paper method (Leigh and Williams, 1964). In this method, one dips a $\frac{1}{4}$ in. x $\frac{1}{2}$ in. area of fluffless blotting paper into the specimen and then holds this area on the surface of an agar plate, thus transferring those organisms which are absorbed by the blotting paper and which remain on the surface of the paper to the agar plate. The number of colonies on the inoculated area is, within certain limitations, directly proportional to the number of organisms in the urine.

Then, 10 ml. of the specimen is centrifuged at 2,500 rev./min. for 5 minutes and the supernatant fluid disposed of. An inoculating loop full of the residue is examined under a microscope, and if more than a pre-determined number of white blood cells is present, the specimen is assumed to be infected (more than 10^4 to 10^5 organisms/ml. are present) and further examination of the specimen is carried out.

This further examination consists of spreading some of the centrifuged residue on both McConkey and blood agar plates, for identification of the organism, and of spreading five 0.02 ml. drops of undiluted sample on an agar plate, for a more reliable quantitative surface viable count than the blotting paper method.

On average, 50 specimens a day are subjected to the initial screening procedures and approximately 10 - 20% are found to be infected, and thus require further examination. The total time taken to process 50 specimens is approximately 2 hours.

The method is extremely simple, moderately accurate and quick. It is also very economic in agar plates since counts of bacteria in six specimens are estimated using only one agar plate, and only two additional plates are used for each specimen that is processed in depth.

4.2.2. Discussion of possible automatic methods

Since the number of specimens examined in depth is so small (5 to 10 per day), it is more important to devise screening methods. However, the blotting paper method for estimating the number of organisms present and the preparation and examination of the microscope slides for the white blood cell count are so very simple, that it is difficult to devise equally simple automatic methods that require less time to perform than the manual methods take: the capital cost of automatic apparatus is likely to be relatively very high indeed.

The initial approximate count of bacteria present in the specimen could be found by placing 0.02 ml. of the 1/100th dilution of the specimen on to an agar plate by the method described in Sect. 3.2.4. We suggest the 1/100th dilution because 10^5 organisms/ml. of specimen is the critical level, and therefore, in 0.02 ml. of the 1/100th dilution of such a specimen there are 20 organisms, which number is easily counted and gives a statistically valid result. We have repeatedly shown that when one inoculates plates in this way, the urine spreads over a sufficiently large area to enable one to count 20 organisms easily (Table 23).

Similar drops of many different specimens could be placed on one plate, or, one could put one drop of the 1/100th dilution of specimen in the centre of the plate, or slightly displaced from the centre, and spread a drop of undiluted urine, planted approximately half-way along a radius, with the automatic spreading apparatus (Sect. 4.1.4). Typical cultures are shown in Fig. 67: the organism is E.coli on McConkey agar. In this method, all specimens would be spread on agar plates which is unnecessary, but methods for performing the manipulations have been devised and could easily be used in a definitive apparatus for routine use. Also, if subsequent examination in depth were necessary, one would merely have to obtain a more precise quantitative count which could easily be done with the same apparatus.

It is possible that if one put a small measured quantity of the specimen on a plate and spread it with the machine (Sect. 4.1.4), the

TABLE 23

Area a 0.02 ml. drop of urine spreads after it has been placed on an agar plate and allowed to dry

Drop number	Dimensions of rectangle enclosing area covered by drop in mm.
1	14 x 14
2	17 x 15
3	18 x 15
4	17 x 14
5	19 x 15
6	19 x 15
7	17 x 15
8	18 x 15
9	18 x 15
10	23 x 14
11	19 x 14
12	19 x 15
13	19 x 15
14	17 x 15
15	16 x 16
16	16 x 14
17	16 x 14
18	16 x 15
19	17 x 14
20	18 x 13
21	20 x 15
22	17 x 16
23	18 x 16
24	19 x 16
25	19 x 14

length of that part of the spiral curve that contained growth would be a measure of the number of organisms in the specimen. This idea clearly needs further investigation.

It is difficult to visualize an automatic method to replace the microscopic examination of the centrifuged residue, for counting white blood cells. The preparation of the slides could be mechanized and one could easily feed the slides on to the microscope stage, but manual intervention would be necessary at that point: in any case, automatically centrifuging the specimens presents problems. As an alternative, it might be possible to use a cell-counter for counting white blood cells, but the problems are considerable (Sect. 1.2.1).

Whilst these are some possible methods for processing urines that might be investigated more fully, it is unlikely that any could be developed into a practical and economic automatic method unless one can sterilize the vessels to be used for handling the specimens in situ. We found (Sect. 3.2.4) that the apparatus for dispensing drops of culture, which is similar to part of the apparatus required for processing urines by the method outlined above, is of limited use due to the fact that one has to replace the syringe each time a different specimen is handled. In processing urines, the need to change a syringe or any other vessel repeatedly, whether by hand or mechanically, would lead to an unwieldy method.

Further work on this investigation was, therefore, postponed and we concentrated on devising methods for sterilizing vessels in situ.

4.3 Sterilizing Vessels in Situ

Many pieces of equipment, such as pressure transducers and automatic syringes, are sterilized by ethylene oxide or alcohol, but the method takes many hours and is, therefore, unsuitable for sterilizing vessels in automatic apparatus: the time taken to sterilize the electrically heated inoculating loop is approximately 6 seconds (Sect. 2.1.2) and this is very convenient.

Steam sterilization is also unsuitable. The apparatus required is very cumbersome and it is inconvenient to have high pressure steam apparatus in a routine laboratory.

Wright and Colebrook (1921) sterilized syringes by means of hot oil, but an M.R.C. Working Party (1962) found the method to be ineffective. Fleming and Ogilvie (1951) sterilized hypodermic needles by immersion for 5 to 10 seconds in liquid paraffin B.P. at 140°C, or in boiling water, but this was also found to be ineffective with some organisms, including *E.coli* and *Staph.aureus* (M.R.C. Memorandum, No. 41, 1962).

Ideally, one requires a method for generating thermal energy in such a way that it can be applied to a variety of different vessels and so that it can be applied only to the required area. It is also necessary to generate the heat very quickly. Passing an electric current through a vessel, as in the electrically

sterilizable inoculating loop, is possible only in a very few types of vessel. One might use radiant heat from an electric resistive generator, but this is inefficient and requires a cumbersome piece of equipment very near to the vessel to be sterilized, which severely limits its usefulness. Also, the time taken for such a device to generate maximum thermal power is too long, and one would, therefore, have to keep the device permanently switched on and remove the vessel to be sterilized from the heater. The heat would then be directed at other parts of the apparatus and, in view of the temperatures reached, this would cause many design difficulties.

A possible method for generating thermal energy is by radio-frequency induction. We have experimented with an induction heater, and the work is discussed in Sect. 4.3.2. We first, however, discuss some attempts to sterilize syringes with boiling water: this is such a simple sterilizing technique that we felt we should first evaluate its possible application to our problem, despite doubts about its suitability.

4.3.1 Using boiling water

A sterile 5 ml. all glass syringe was contaminated by drawing infected urine into it through a needle: it was so arranged that the piston of the syringe at no time contacted that part of the barrel that had contained liquid. The urine was ejected to waste, the needle was removed and boiling water was drawn into the syringe, allowed to stand for a fixed time,

and ejected to waste. Finally, the syringe was filled with nutrient broth which was subsequently ejected into a sterilized bottle and incubated overnight. The experiment was repeated, with a freshly sterilized syringe, many times and the results are given in Table 24: not one syringe was sterilized.

In a second set of experiments the above procedure was repeated, except that, after the boiling water had been ejected to waste the syringe was filled with boiling water a second time. This was allowed to stand, the same length of time as the first filling, and ejected to waste: the syringe was then filled with nutrient broth and the procedure completed as before. This procedure was repeated many times and the results are given in Table 25: as before, not one syringe was sterilized.

The results show that it is not possible to sterilize a syringe in a reasonable time simply by drawing boiling water into it: they also show that rinsing a vessel through with water does not remove bacteria from surfaces.

On being drawn into the syringe, the water quickly cools down. Consequently, the next step would be to devise a method for keeping the water boiling whilst it remains in the syringe. To do this, one would have to supply thermal energy to the water, and any method suitable for doing this would probably be equally suitable for heating the syringe directly. There is, therefore, no point in developing a method specifically for keeping the water boiling, particularly as the method would be suitable for sterilizing only

TABLE 24

Sterilization of glass syringe using boiling water

Time boiling water left in syringe in seconds	Number of failures in three tests
15	3
30	3
45	3
60	3
75	3
90	3
105	3
120	3
150	3
180	3

TABLE 25

Sterilization of glass syringe using boiling water

Time boiling water left in syringe in seconds	Number of failures in three tests
15 + 15	3
30 + 30	3
45 + 45	3
60 + 60	3
75 + 75	3
90 + 90	3
105 + 105	3
120 + 120	3
150 + 150	3
180 + 180	3

vessels in which water can be contained: the syringe needle would have to be immersed in the water and this would be very inconvenient in many circumstances.

4.3.2 Using r.f. induction heating

4.3.2.1 Induction heating

Radio-frequency induction heating is commonly used in industrial processes, particularly for melting, hardening and tempering ferrous and non-ferrous metals: soldering and annealing are other applications.

The principle of the technique is, briefly, that one produces an r-f magnetic field in the work piece and the field causes eddy-current losses in a non-magnetic conductor, or hysteresis and eddy-current losses in a magnetic conductor (Fig. 68). The magnetic field is produced by an oscillator, the output of which is connected to a hollow, copper coil which surrounds the work piece (Fig. 69). The closer the coil is to the work piece the higher the efficiency of the system. It is also possible to have an interior heating coil (Fig. 70), but this is less efficient than an equally close external coil.

The method is very flexible because one can make a coil to suit a particular work piece: for example, one can have a multi-turn coil (Fig. 71), a hair-pin coil (Fig. 72) or a rotary skid type coil for use with a rotary table (Fig. 73). Induction heating provides a very high concentration of heat and the r.f. power is simply switched on and off.

The r.f. power required and the optimum frequency of the field depend on the size, configuration and material of the work piece, the maximum temperature required and the time in which this temperature has to be produced. In general, the higher the resistivity of the work piece the higher the efficiency of the system, and the higher the frequency of the field the higher the surface current density.

For heating poor or non-conducing materials, one places a conducting susceptor between the coil and work piece.

4.3.2.2 Experimental work

Fig. 74 shows the apparatus which, in principle, consisted of a vertical $\frac{1}{8}$ in. i.d., 22 gauge stainless steel pipette connected by flexible silicone tubing to a 1 ml. syringe. The syringe was operated mechanically to suck up and expel fluid, which occupied approximately 3 in. of the pipette. The lower 4 in. of the pipette was surrounded by a fixed copper coil, which consisted of 8 turns each of $1\frac{1}{4}$ in. diameter, which was connected to the r.f. generator (Fig. 75): the pipette was lowered, mechanically, into vessels placed below the heating coil. A tube from a reservoir of distilled water was connected to a three way tap in the silicone tubing connecting the pipette to the syringe, so that the pipette could be rinsed through with distilled water: the system was arranged so that infective material could not go above the level to which the syringe draws fluid.

To heat the pipette, r.f. power was coupled to it through

the coil. There were two independent variables, the magnitude of the power and the time it was switched on. The magnitude of the power was fixed by two dependent variables, the current passing through the r.f. oscillator valve and the efficiency of the coupling between the coil and the pipette (Sect. 4.3.2.1). We chose, therefore, to specify the heating process by the current passing through the oscillator valve and the time the power was on: both parameters were easily varied.

We found that the most efficient coil (in which there was very little space between the coil and the pipette) heated the pipette to red heat in a few seconds, even at the lowest possible oscillator current. In order to obtain a set of variables such that the heat generated in the pipette was insufficient to sterilize it in a reasonable time, we used comparatively inefficient coupling: the use of a $1\frac{1}{4}$ in. diameter coil reduced the efficiency of the coupling by approximately 40%.

To test the efficacy of this method of sterilization, we adopted the following technique. With a given oscillator current, the r.f. power was switched on until the pipette became red hot and was left on for an additional 5 seconds to ensure the lower 4 in. of the pipette was sterile. The pipette was lowered into a broth culture of Staph.aureus which was drawn up into the pipette and expelled. Distilled water was passed through the pipette for 5 seconds, the pipette was raised so that the infected portion was between the heating coil, and the r.f. power was switched on for

a given period. After the pipette had cooled, it was lowered into a tube containing sterile broth which was sucked up into the pipette and expelled twice: the broth was incubated overnight. The pipette was then washed through with distilled water, raised to the sterilizing position and heated to red heat. The experiment was repeated 20 times for each of a variety of combinations of the two variables. A similar series of experiments was carried out using E. coli and another series using B. Stearothermophilus.

The results (Table 26) show that the optimum conditions for sterilizing the pipette were 350 mA oscillator valve current for 8 seconds. However, those figures apply only to the particular coil, pipette and generator used and cannot be used to formulate general criteria.

4.3.2.3 Discussion

We have shown that this is a practical method for sterilizing vessels in situ. It is very flexible because of the ease with which one can make a coil to suit a particular work piece and because the generator can be remote from the workpiece. The generator used in the experiments discussed in Sect. 4.3.2.2, was a 1 kW unit with an output frequency of 2 Mc/s. This is much more powerful than is likely to be necessary for many applications that we can foresee, and it is necessary therefore to acquire a more suitable generator for these applications. Other problems remain, in particular, the time the pipette took to cool down was longer than desirable, but this problem should

TABLE 26

Sterilization of stainless steel pipette by r.f. induction heating

r.f. oscillator valve current in milliamperes	Time power on in seconds	Number of failures in 20 tests		
		E. coli	Staph. aureus	B. stearothermophilus
300	4	9	19	20
	8	4	1	8
	12	2	0	0
	14	0	0	0
325	4	4	17	20
	8	0	0	4
	12	0	0	0
	14	0	0	0
350	4	6	14	5
	8	0	0	0
	12	0	0	0
	14	0	0	0

not be difficult to solve.

Possibly the biggest disadvantage with the method is that one cannot heat non-conductors directly. In this case one uses a susceptor, a conducting component that is heated by eddy-current and hysteresis effects, and the work piece is heated indirectly. We do not anticipate this being a major problem, however, because we see no reason why the majority of vessels we are likely to use cannot be made from suitable conducting materials.

Another possible application of r.f. heating to bacteriology is general purpose sterilization which at present is usually carried out by autoclave in a central supply department. The components to be sterilized could be packed in a suitable container which would be placed in a boat-shaped susceptor: alternatively, the container could be the susceptor. The boats would automatically pass through a horizontally mounted multi-coil (Fig. 76), by means of a conveyer-belt type device, and a hot zone would move along the component. It is probable that more power than 1 kW would be required and there are many problems one can foresee, but this is another application of r.f. induction heating that might well be investigated.

Further experimental work on sterilization by r.f. induction heating must be carried out, but this is better done in connection with the development of a definitive automatic method, such as the analysis of urine, and further discussion is postponed to Chapter 5.

Fig. 51. Drawing illustrating a manual technique for spreading a culture over an agar plate: see text for legend.

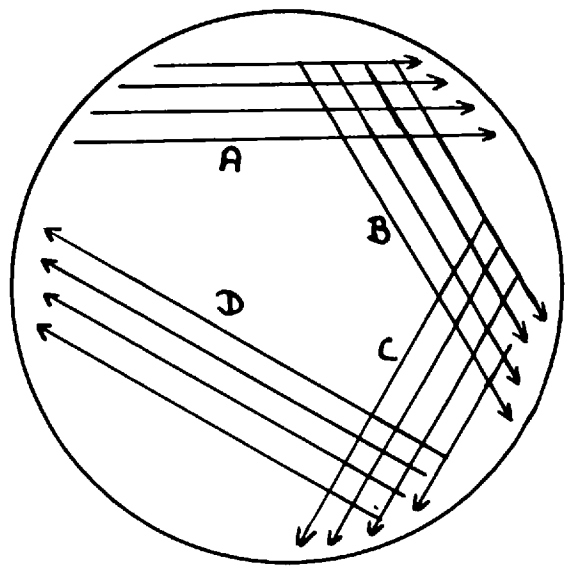


Fig. 52. Schematic diagram of first experimental apparatus for automatically spreading culture over an agar plate: T - agar plate on rotating table, L - electrically heated inoculating loop attached, by a hinge, to an arm moved by rack R and pinion P, M - motor, G_1, G_2 - gears.

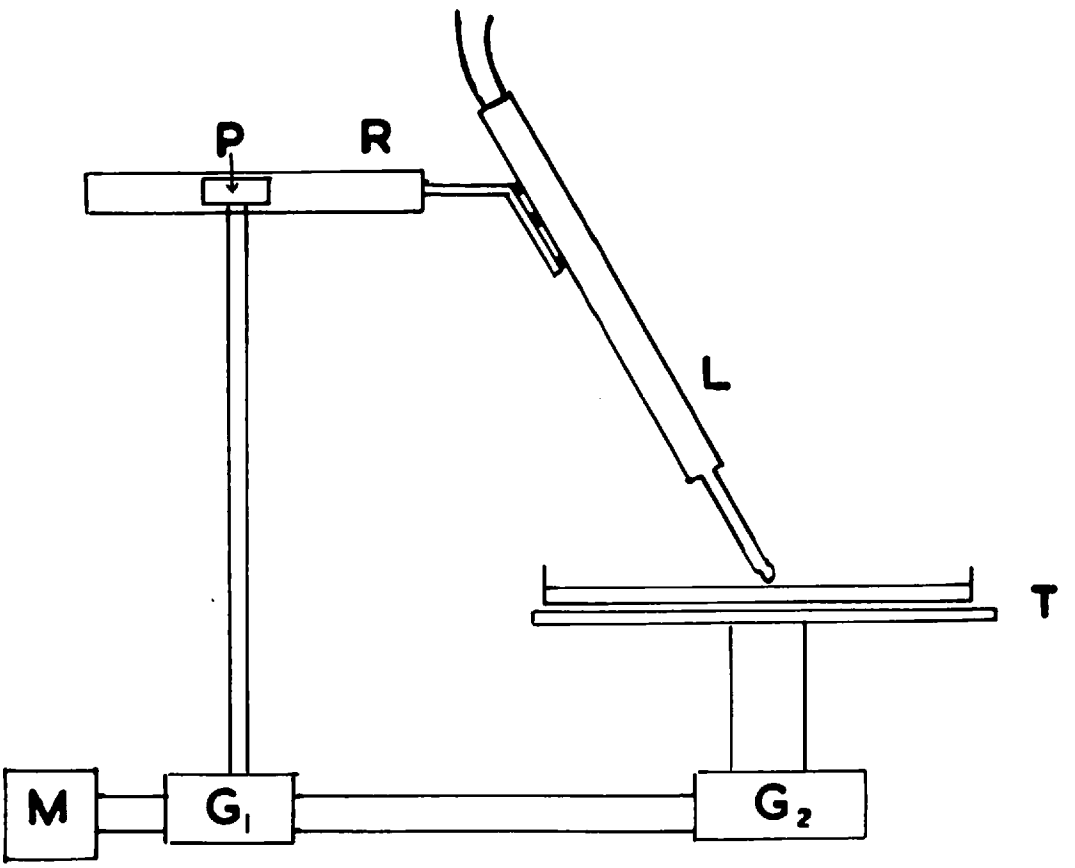


Fig. 53. Typical results obtained by spreading a culture on the first experimental apparatus: E.coli on McConkey agar.

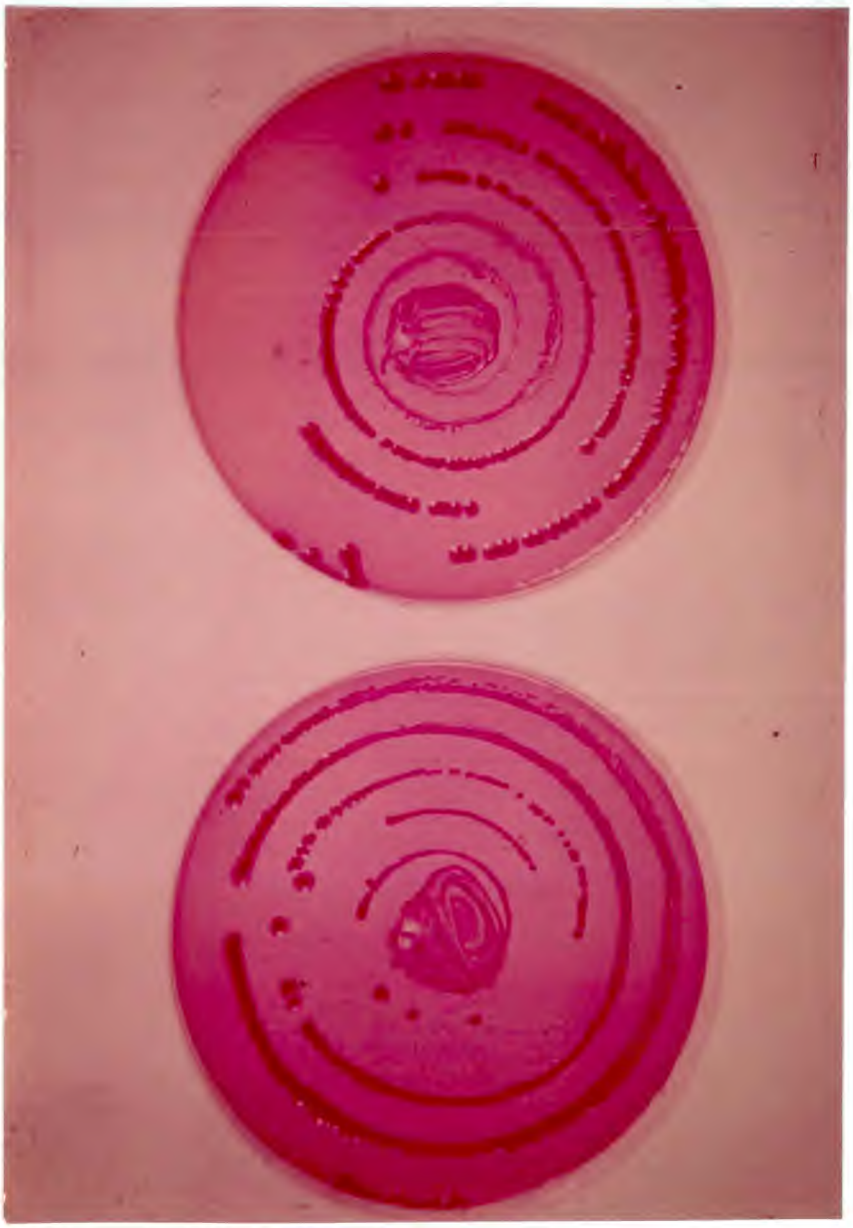


Fig. 54. More refined apparatus for automatically spreading a culture over an agar plate.

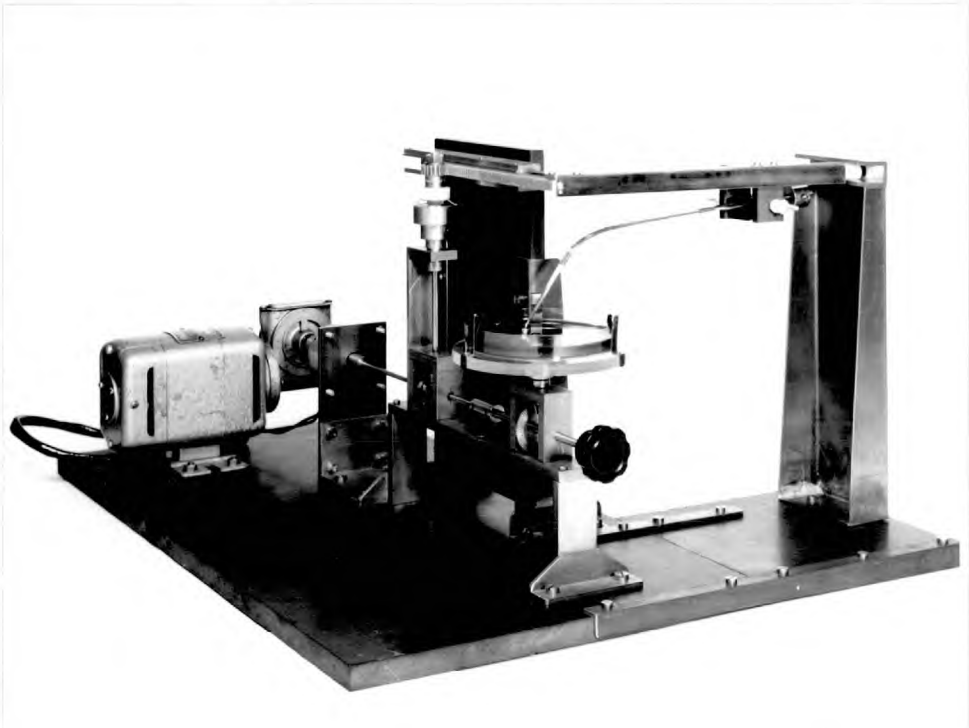


Fig. 55. The electrically heated inoculating loop used in the more refined apparatus for automatically spreading a culture over an agar plate.

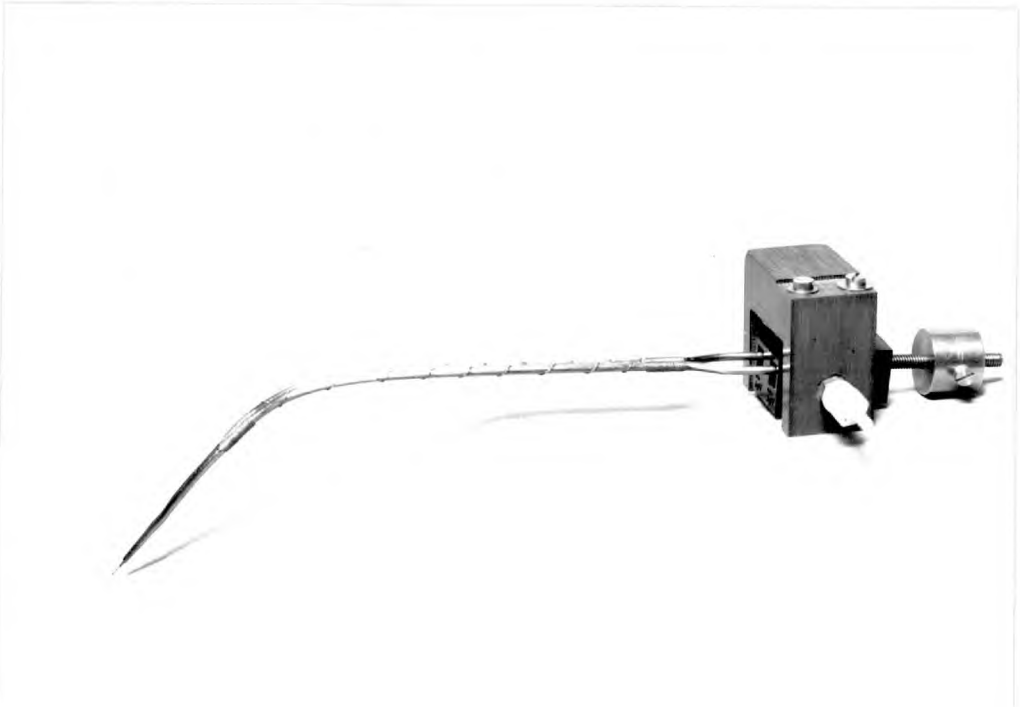


Fig. 56. Photograph showing final form of the mounting of the inoculating loop used in the apparatus for automatically spreading a culture over an agar plate.

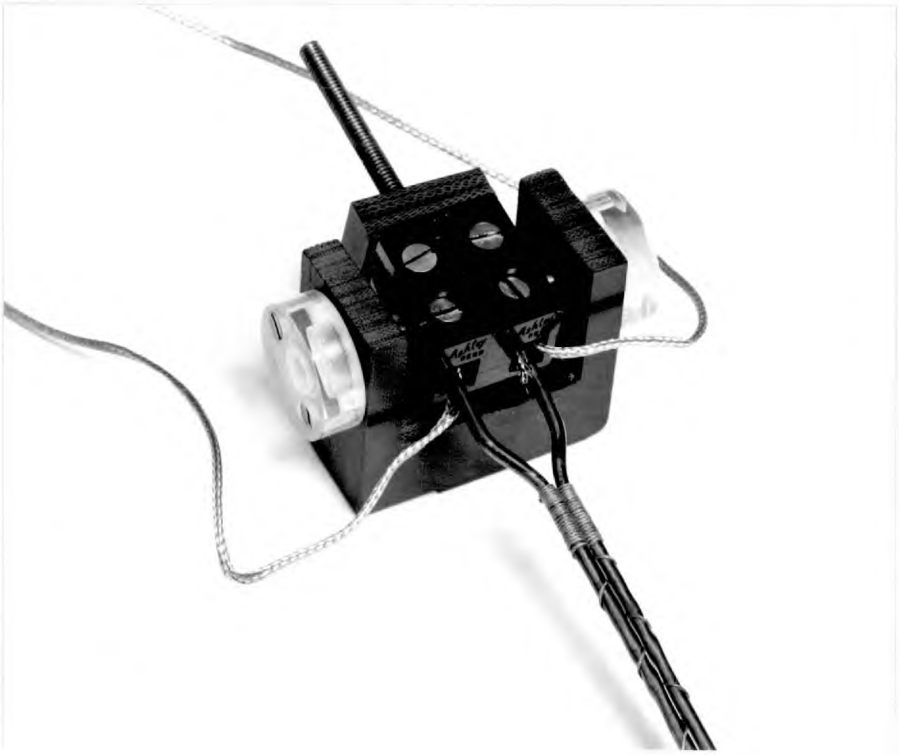


Fig. 57. Typical results obtained by spreading a culture on the more refined automatic apparatus - plates inoculated from a solid culture: staphylococcus on blood agar.

Fig. 58. Typical results obtained by spreading a culture on the more refined automatic apparatus - plates inoculated with two 0.02 ml. drops of urine: staphylococcus on blood agar.

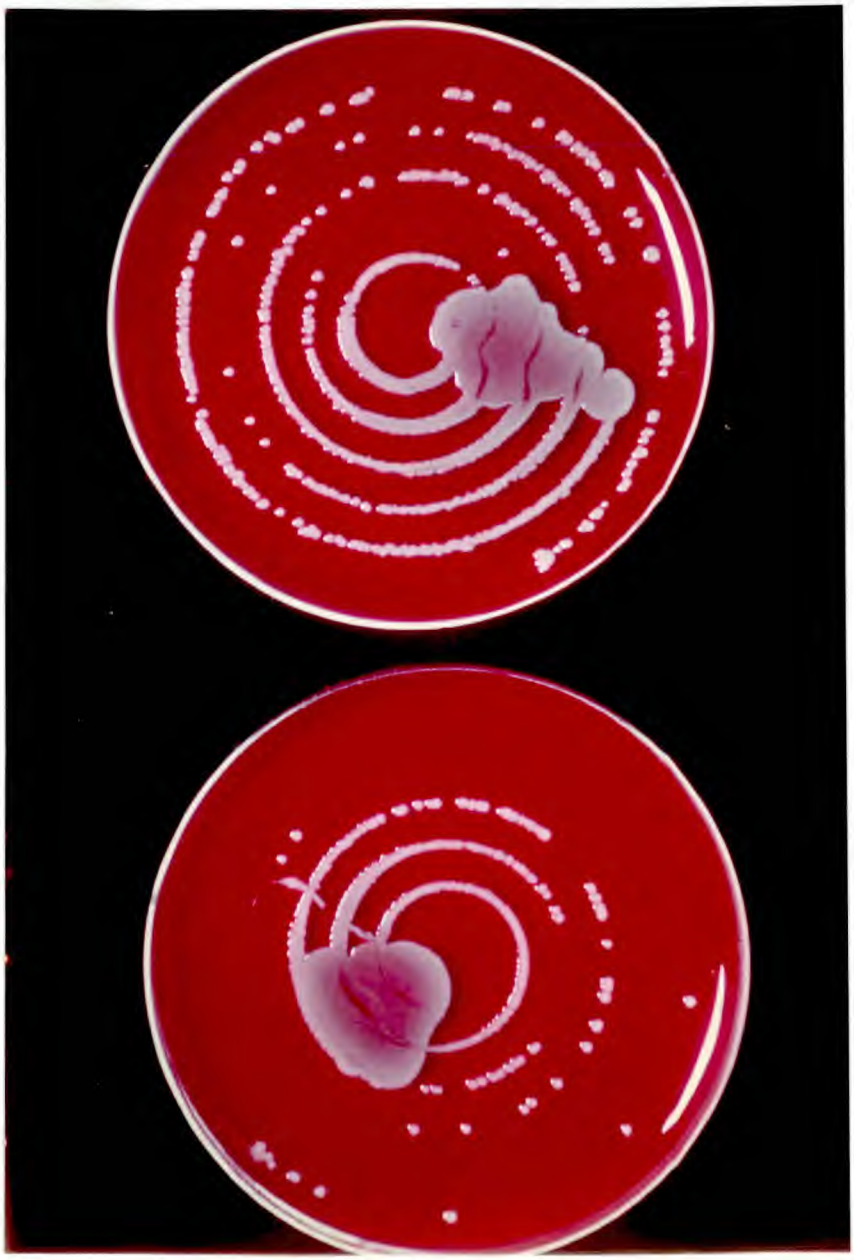


Fig. 59. Typical results obtained by spreading a culture on the more refined automatic apparatus - plates inoculated with two 0.02 ml. drops of urine: E.coli and staphylococcus on McConkey agar.

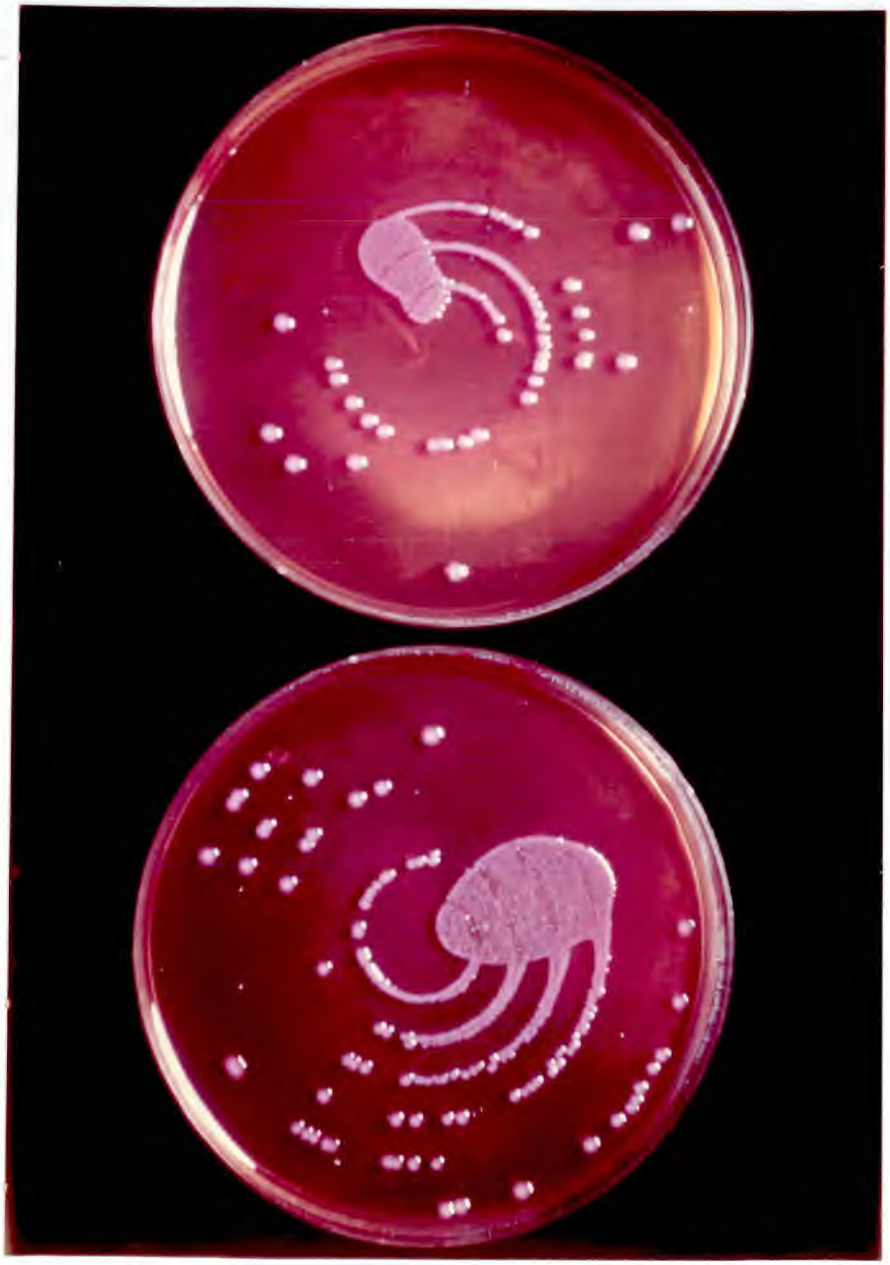


Fig. 60. Result obtained by spreading a culture on automatic apparatus-plate inoculated from a swab: staphylococcus and staphylococcus on blood agar.

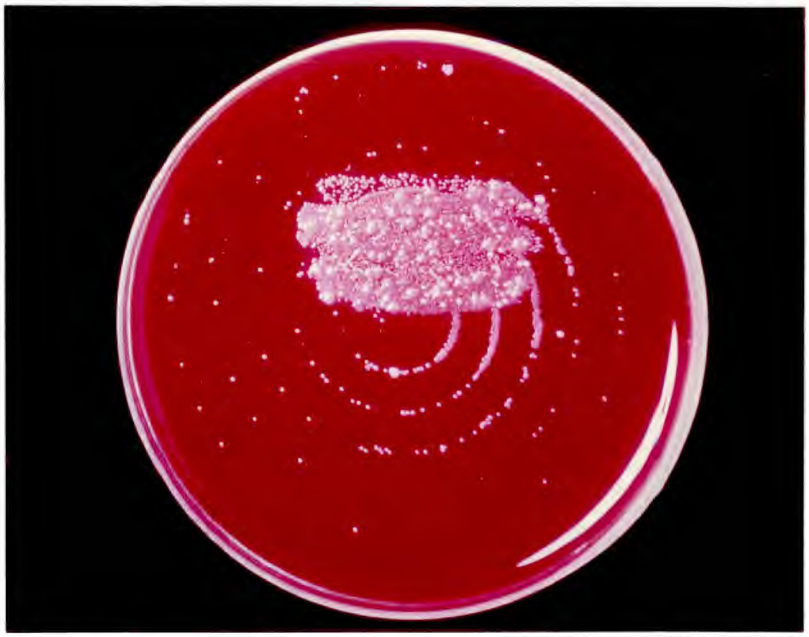
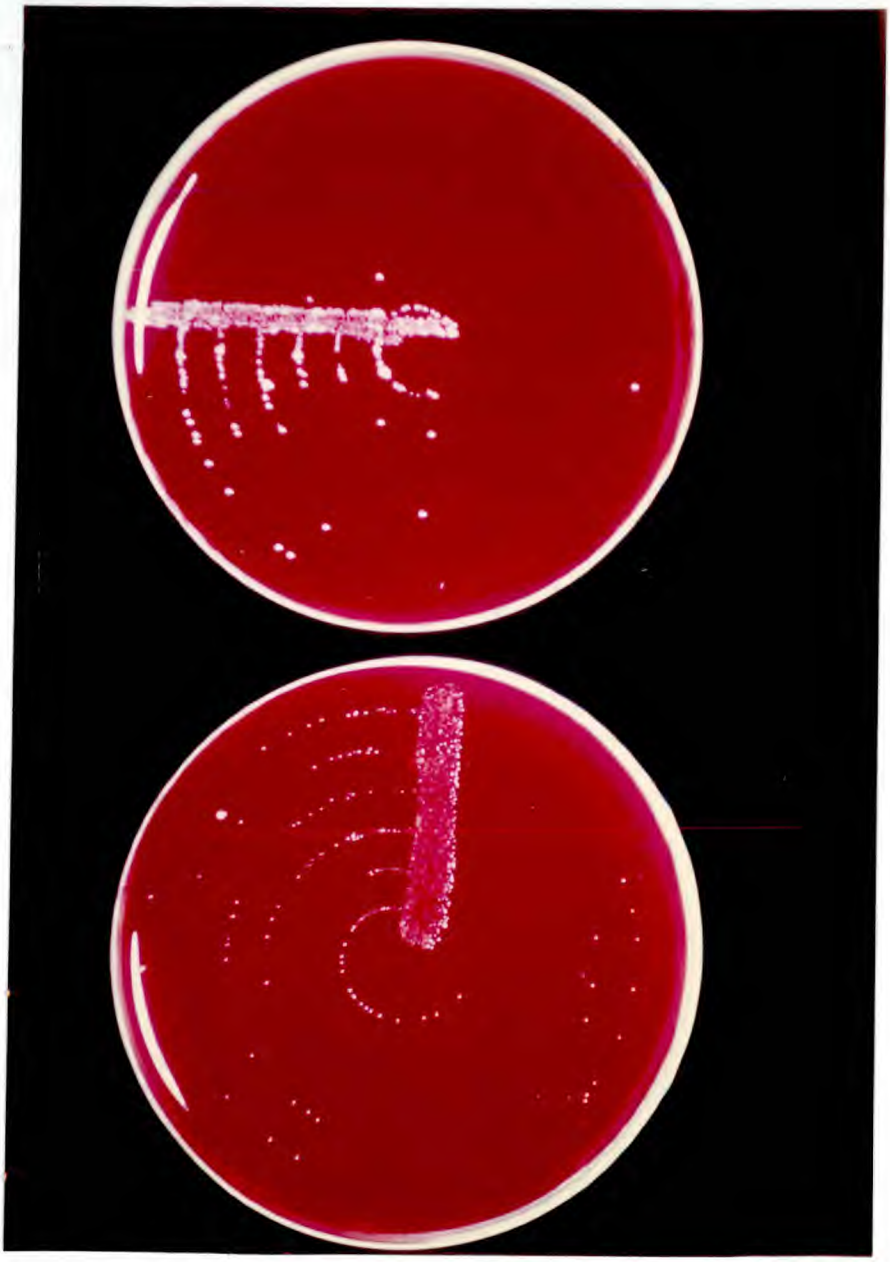


Fig. 61. Results obtained by spreading a culture on automatic apparatus - plates inoculated from a swab: a - staphylococcus on blood agar, b - staphylococcus and streptococcus on blood agar.



a.

b.

Fig. 62. Flat-tipped electrically heated inoculating loop as used in automatic culture spreading apparatus.

Fig. 63. Result obtained by spreading a culture on automatic apparatus with flat-tipped inoculating loop - plate inoculated from a swab: staphylococcus on blood agar.

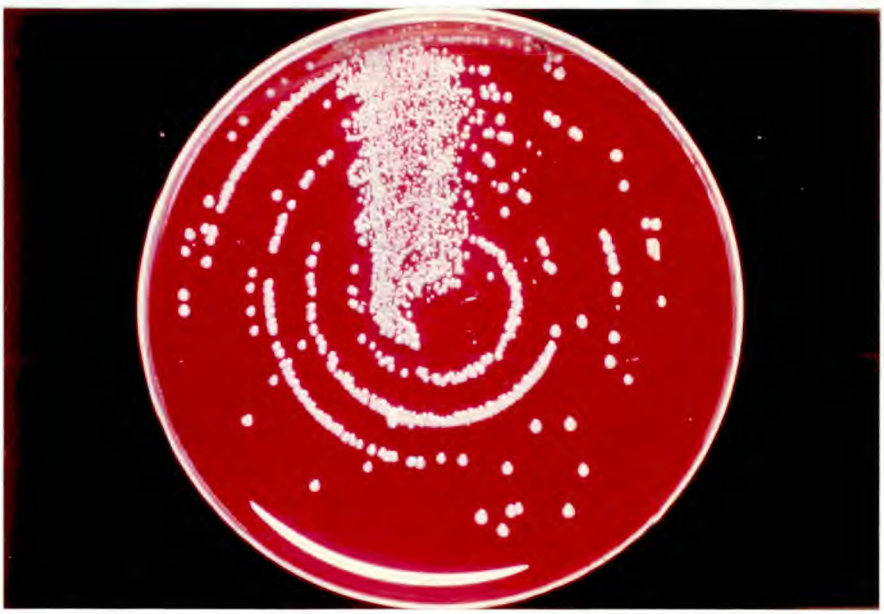


Fig. 64. Typical result obtained by spreading a culture on automatic apparatus with flat-tipped inoculating loop - plate inoculated from a swab: staphylococcus and streptococcus on blood agar.

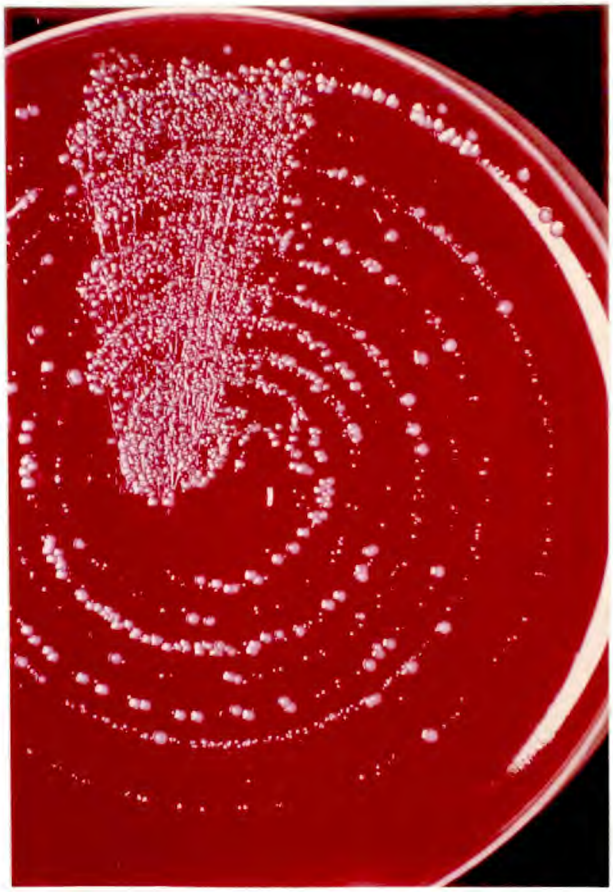


Fig. 65. Typical result obtained by spreading a culture on automatic apparatus with flat-tipped inoculating loop - plate inoculated from a swab - heavy inoculum: E.coli on blood agar.

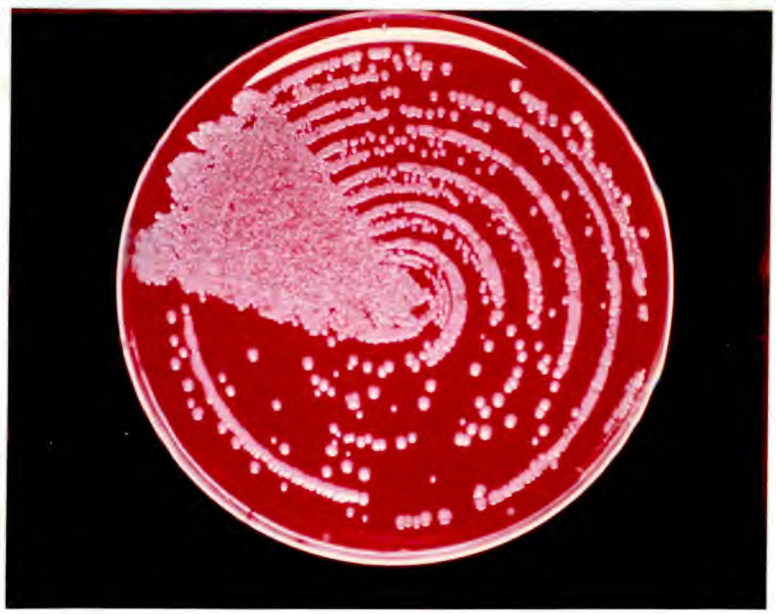


Fig. 66. Typical result obtained by spreading a culture on automatic apparatus with flat-tipped inoculating loop - plate inoculated from a swab - light inoculum: staphylococcus and staphylococcus on blood agar.

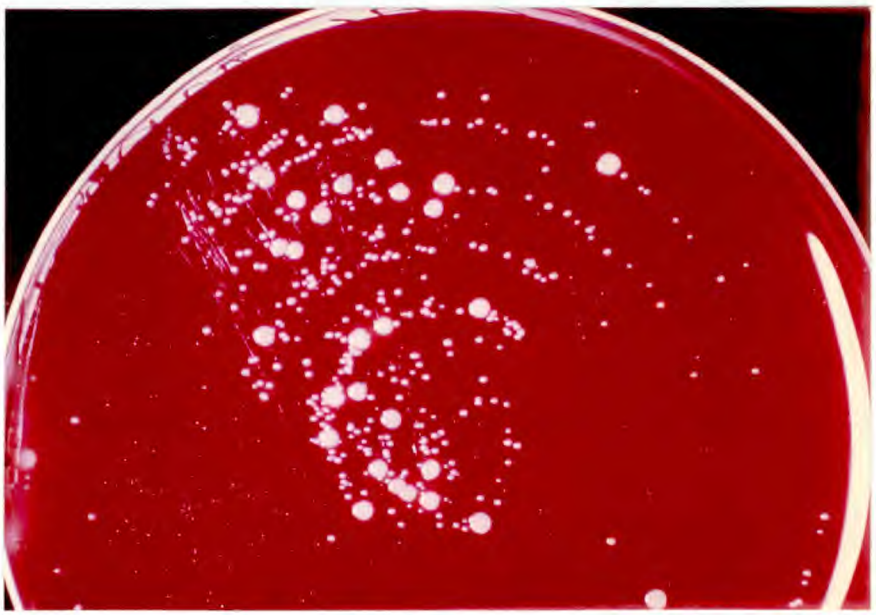


Fig. 67. A culture with a 0.02 ml. drop of the $^{1}/_{100}$th dilution of urine placed in the centre, for estimating the surface viable count, and with one 0.02 ml. drop of undiluted urine spread round the circumference: E.coli on McConkey agar.

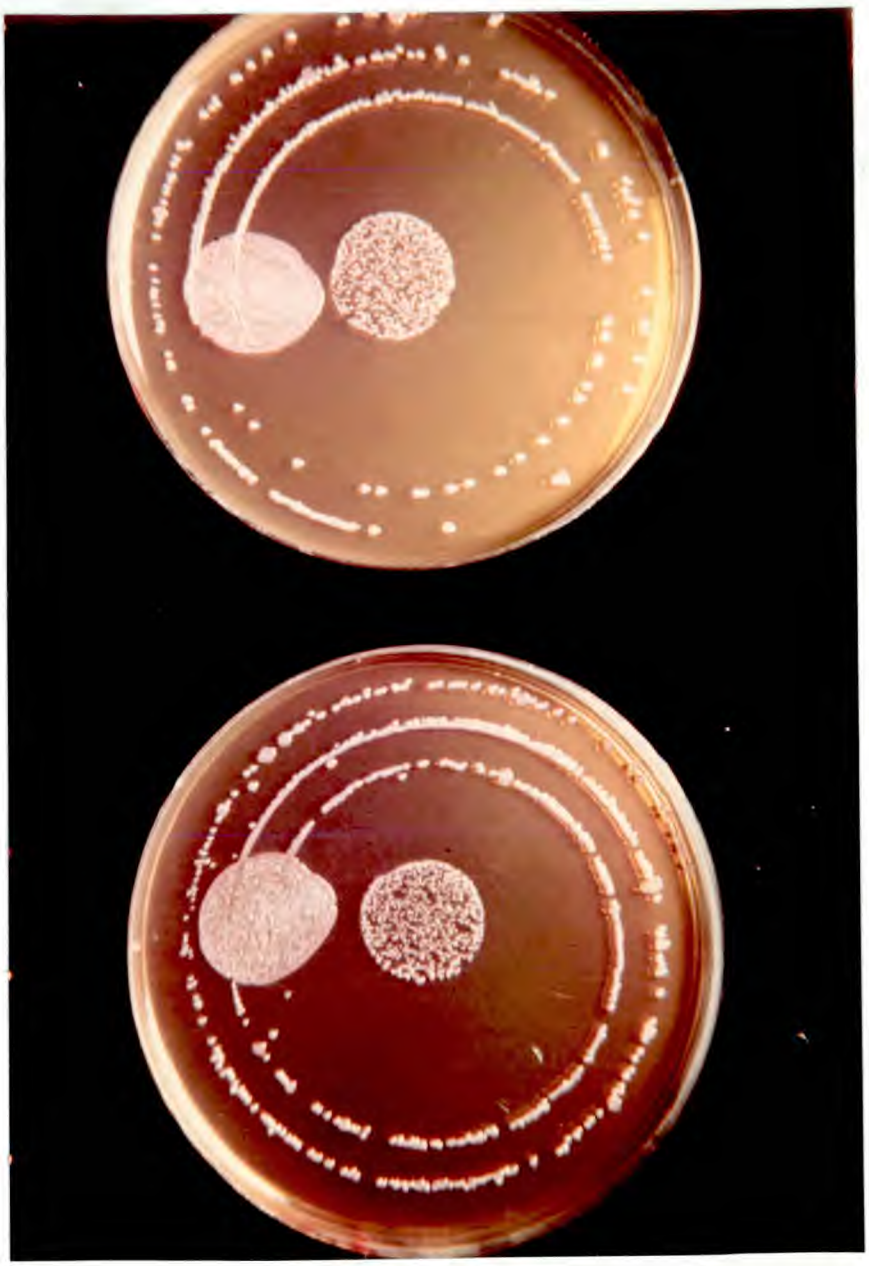


Fig. 68. Drawing illustrating the principle of r.f. induction heating: G - r.f. generator, C - coil which induces an r.f. magnetic field M in the work piece W.

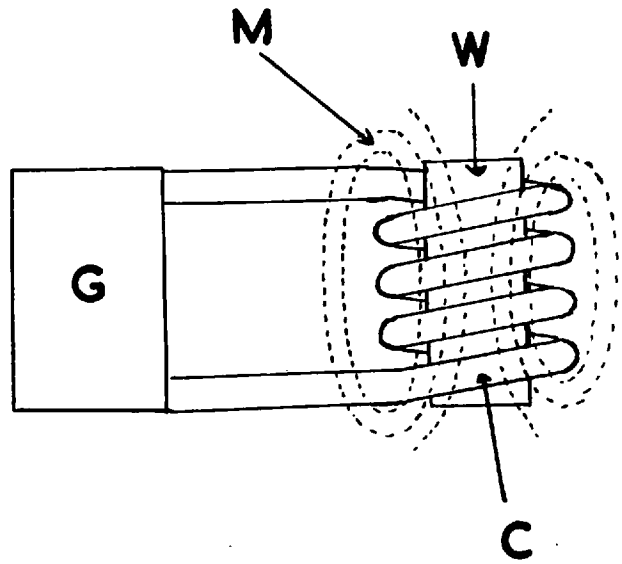


Fig. 69. Drawing of a hollow copper coil C surrounding a work piece W - an external coil.

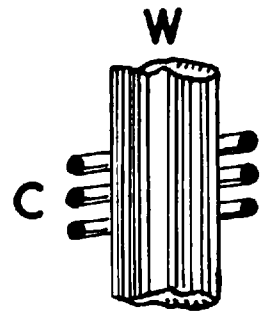


Fig. 70. Drawing of a hollow copper coil C surrounded by a work piece W - an internal coil.

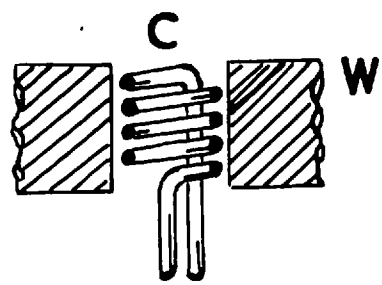


Fig. 71. A hollow, copper, multi-turn r.f. induction coil.

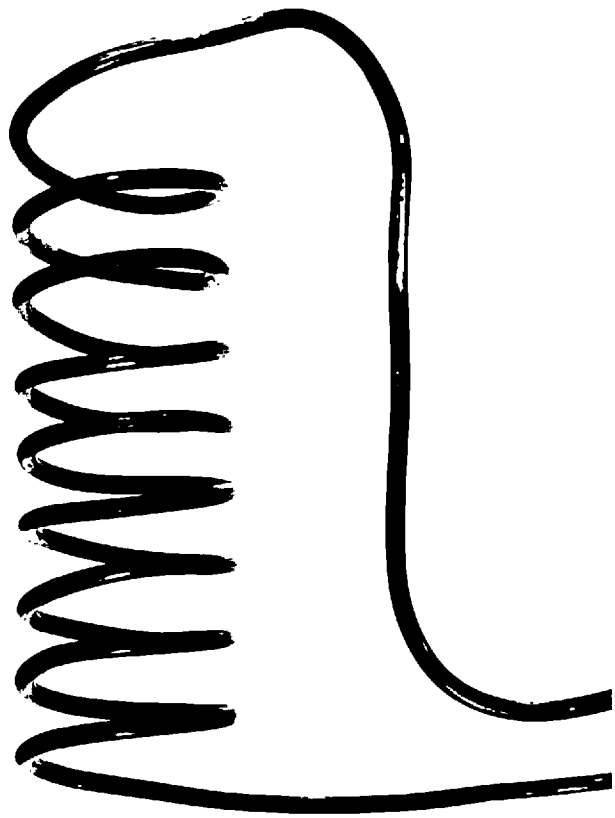


Fig. 72. A hollow, copper, hair-pin r.f. induction coil in glass fibre insulated sleeving.

Fig. 73. A rotary-skid type r.f. induction coil in glass fibre insulated sleeving.

Fig. 74. The apparatus used to test the efficacy of sterilization by r.f. induction heating: G - r.f. generator, M - mechanically operated syringe, R - water reservoir connected to pipette, A - mechanism for raising and lowering pipette, C - r.f. coil.

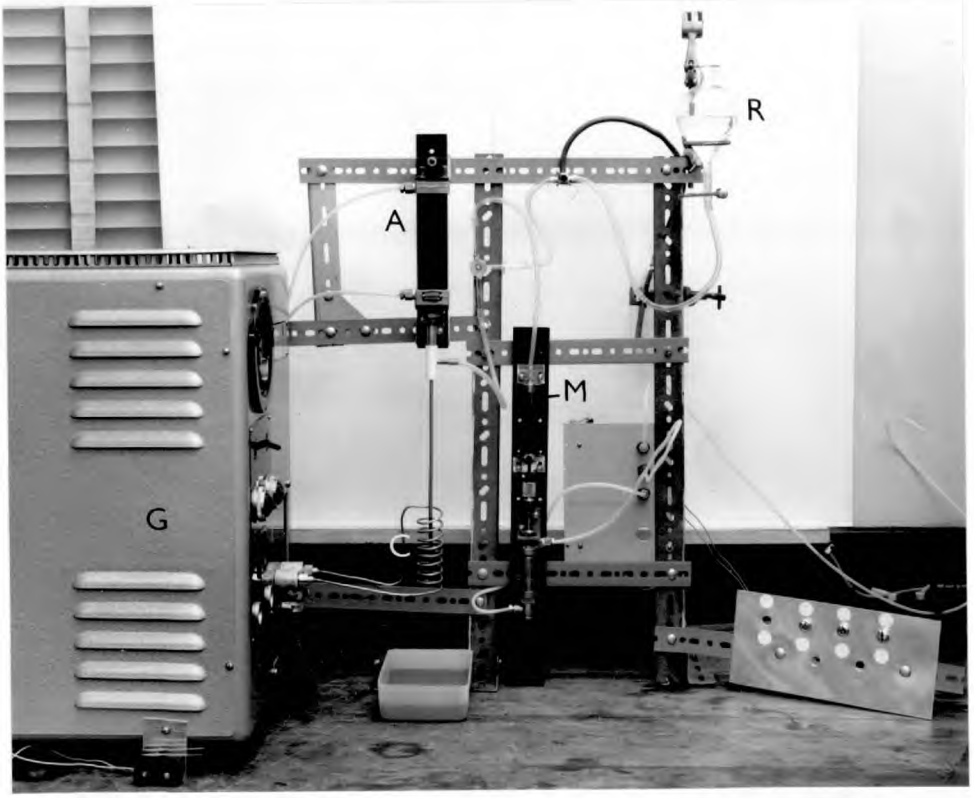


Fig. 75. Close view of the r.f. coil C, the pipette P and the r.f. generator G, in the apparatus used to test the efficacy of sterilization by r.f. induction heating.

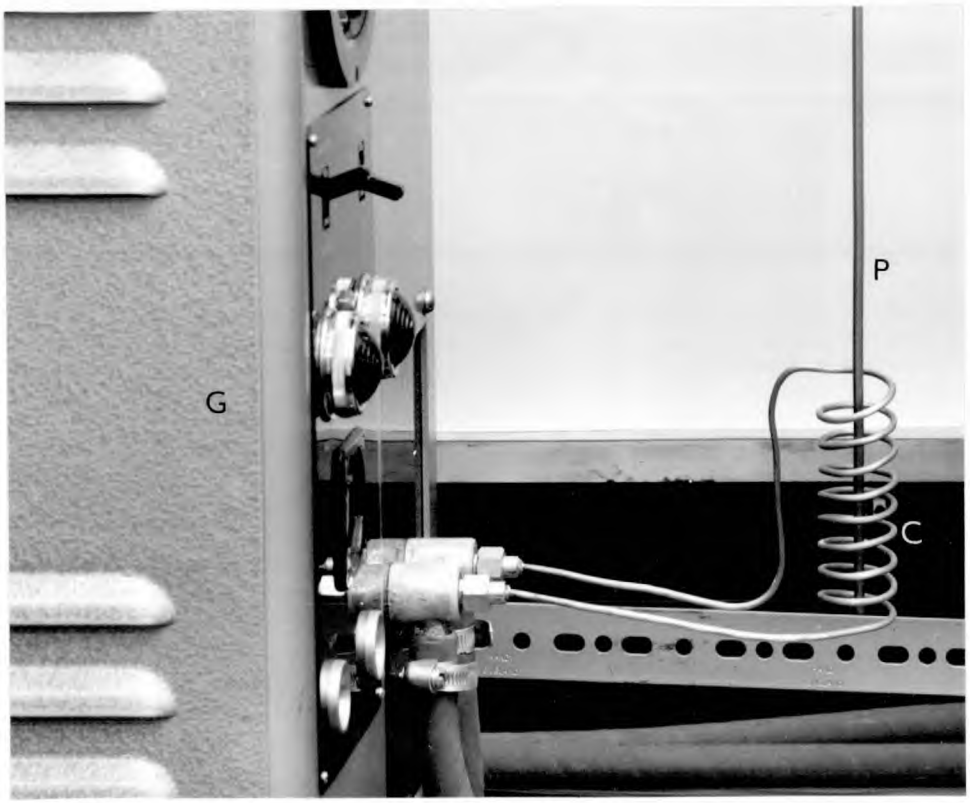
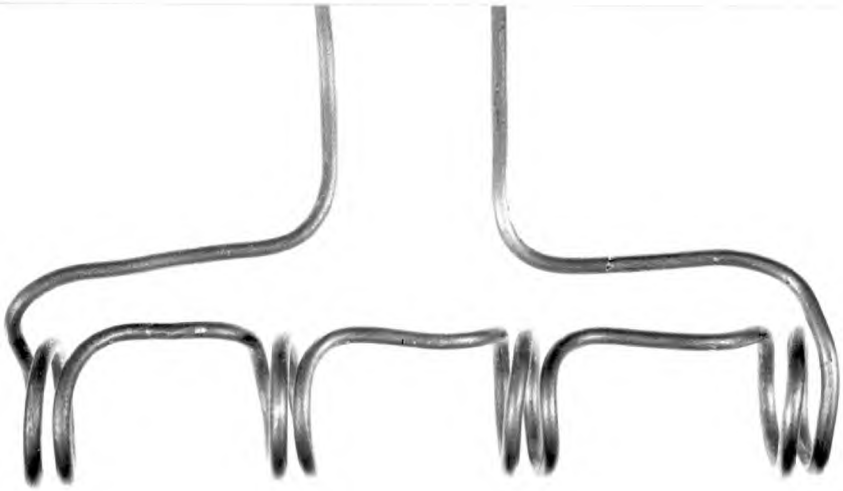


Fig. 76. An r.f. induction multi-coil - the work piece may be moved through the coil and four hot zones will move along the work piece.



Chapter 5

Conclusions

The possibilities of mechanically manipulating specimens and other materials have been investigated, and definitive automatic equipment for performing some stages of a variety of routine test procedures has been designed and evaluated: the apparatus is now in routine use. Whilst it is not very sophisticated by automation standards, the apparatus works satisfactorily, and it satisfies our criteria for automatic diagnostic test equipment: it is simple to operate, suitable for processing relatively small numbers of specimens and all parts that come into contact with infected material can be removed and discarded or sterilized by heat or both. The apparatus cannot perform complete diagnostic test procedures without manual intervention, but within this limitation, the methods serve a useful purpose although one may argue that one should have introduced more automation: for example, one might have included means for automatically scanning the sample cups of the apparatus for dispensing the reagents for the Wassermann reaction, to detect lysis.

It is difficult to determine the optimum balance between fully automatic methods and mechanical aids to manual methods, but we feel we have achieved a reasonable balance between these two extremes, except in the method for distributing liquid culture (Sect. 3.2),

particularly in view of the number of specimens processed in the average routine laboratory, the need to gain experience in a field very few other workers have tackled, the need to show potential users of automatic methods the possibilities and the need to alleviate quickly the difficulties created by the ever increasing work load and the shortage of technical staff. The number of times one needs to dispense 3 or more drops of a single liquid culture is limited, and the inconvenience of having to replace the syringe in the method for distributing culture (Sect. 3.2.6) is more significant than forecast, and we must re-consider this method in the light of our more recent experience.

A major obstacle to the development of automation in diagnostic bacteriology has been our inability to sterilize many components with which infected material comes into contact, in situ and in a reasonable time. An effort to circumvent this difficulty, by independently mechanising those stages of a test procedure that do not involve handling infective material (Sect. 3.1), has been successful. However, our attempt to use pre-sterilized disposable vessels, that are loaded in the apparatus and unloaded manually, when handling infected material (Sect. 3.2), has met with very limited success, and it is now evident that the difficulties in mechanically handling infective material cannot be circumvented but must be overcome. Some very promising preliminary experiments on an r.f. induction heating method for sterilizing vessels in situ have been carried out (Sect. 4.3): to our knowledge this method has

hitherto not been used. Whilst further experiments have to be conducted, we have reason to believe that we will be able to develop this method of sterilization to such an extent that, in the near future, it will be possible to sterilize most types of component likely to be required for mechanically handling infective material in automatic apparatus, in situ and in a reasonable time.

If we succeed, and the r.f. induction heating method is being actively explored, many possibilities will be opened up. In the method for distributing liquid cultures (Sect. 3.2), one could use a fixed syringe (or other suitable vessel) which would be sterilized after each specimen had been processed. The apparatus would then be suitable for dispensing single drops of culture, and would be a much more useful device than one which utilized pre-sterilized disposable syringes that are manually replaced before a second culture is distributed. One could also develop methods for automatically feeding the tubes containing the cultures and the tubes to be inoculated into the apparatus, thus making the culture distributing technique fully automatic. By combining this apparatus with the serial diluter (Sect. 3.1), which in turn incorporated a device for distributing diluent, a fully automatic method for measuring the minimal inhibitory concentration of antibiotic and a serum antibiotic level would be produced.

The principles used in the serial diluter could be used in a method for serially diluting bacteria: one would have to sterilize the pipettes between each stage of the procedure and many precautions

against spilling infective material would have to be taken.

One could also devise a method for inoculating an agar plate from a broth culture and spreading a lawn of the culture, for testing antibiotic sensitivities by the disc method. A pipette would be used to draw up an appropriate volume of culture and to place it on the agar plate which in turn would be rotated and rocked until the culture had spread over the surface of the agar: the surplus culture would be removed by the pipette and discharged to waste. Apparatus for dispensing discs is commercially available, and it could be incorporated in a fully automatic device for measuring antibiotic sensitivities by the disc method.

The methods for processing urines discussed in Sect. 4.3 would become practical. We decided that, since the number of specimens examined in depth is so small, the greatest need is for a method for screening the specimens: this would be an alternative to the blotting paper technique for measuring surface viable counts of bacteria and for the microscopic examination of the specimen for counting white blood cells. To be an improvement on the manual method an automatic method has to be as near fully automatic as possible and ideally, one should merely have to put the specimen jar into the apparatus which would then perform the complete procedure. We consider a fully automatic method for processing urines would be most useful, and we are considering methods for screening the specimens for white blood cells: one possible method is to use a device similar to the slide centrifuge (Watson, 1966) in which the centrifuged cells in suspension are concentrated

directly on to a microscope slide. The development of a method for screening the specimens for white blood cells is likely to take some time however, and, on the assumption that vessels with which infected material comes into contact can be sterilized in situ by r.f. induction heating, we have decided to develop a prototype device for determining surface viable counts and for performing the remainder of the urine test procedure as an intermediate step.

Experimental work automatically spreading a culture over an agar plate for identification (Sect. 4.1) was very encouraging, and we are now conducting further research leading to the design of definitive apparatus for the routine laboratory.

Various possibilities present themselves. We could devise a general purpose machine suitable for spreading inocula from all types of specimen. In its simplest form, the operator would inoculate the plates, put them on the apparatus and remove them after the inoculum had been spread. A more complex possibility is one in which the operator inoculates the plates and puts them on a device that feeds them on to the spreading machine (which automatically spreads the inocula): a similar device would remove the plates from the spreading machine and transport them to the incubator. Even more complex equipment would incorporate automatic means of inoculating the agar plates.

In view of the relative numbers of specimens to be processed, we have decided to concentrate primarily on developing a method for processing swabs, and we have concluded that the minimum of

automation acceptable is a device which performs all operations except inoculating the plates. However, apparatus in which the agar plates are automatically inoculated would be most useful.

Two methods for inoculating the plates from swabs are under investigation; inoculating the plate directly from the swab by mechanically streaking it across a radius of the plate, as in the manual method, and inoculating the plate from a broth culture which was previously inoculated from the swab: the swab is immersed in broth and it is rotated vigorously. The former method, whilst probably not impossible, will almost certainly require a very intricate mechanism, but the latter method lends itself to mechanisation and the results of our preliminary experiments are encouraging (Fig. 77). We intend to pursue the experiments further before deciding which type of device for processing swabs we will develop.

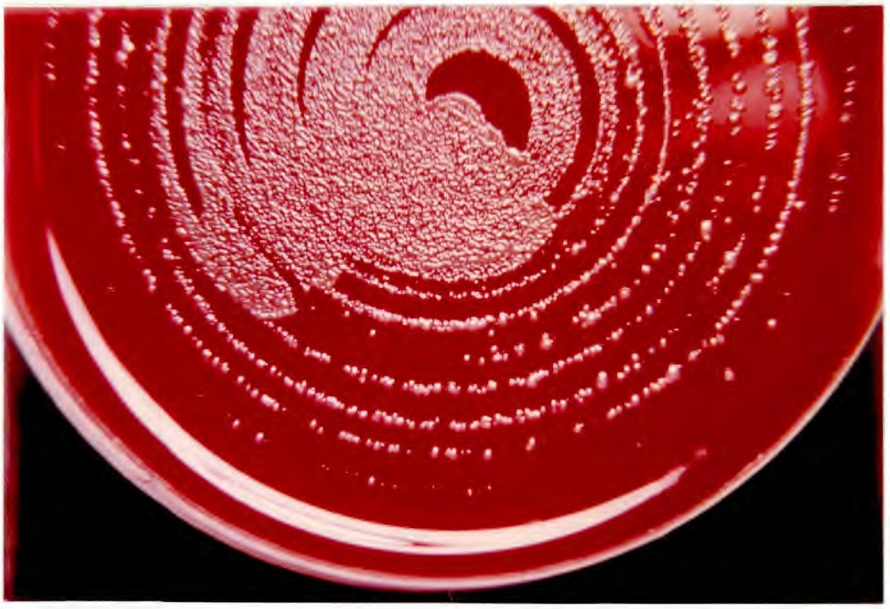
It is now almost certain that fully automatic apparatus for use in diagnostic bacteriology will be much more complex than apparatus hitherto used in bacteriology laboratories, and therefore, such apparatus will be uneconomic if used in the average routine laboratory. It may well be advantageous, therefore, to concentrate the specimens to be processed in a few relatively large laboratories, and early consideration of this is desirable.

If we continue to make progress in the mechanical handling of infective material, we may soon be able to turn to other fields of study, particularly to the measurement and presentation of test results (Sect. 1.4.1): we hope soon to evaluate a prototype 'Bowman'

apparatus (Sect. 1.2.1), but we do not expect this development to have a direct effect on our immediate future programme.

Automatic methods in diagnostic bacteriology is a field that hardly anybody else has tackled. We believe the hesitancy of others to enter this field is unjustified, because, whilst we have only just scratched the surface of the problem, we have made some progress towards solving the major obstacle to further developments, and have shown that there are many unexplored but potentially very fruitful avenues.

Fig. 77. Typical result obtained by spreading a culture on automatic apparatus with the flat-tipped inoculating loop - the plate was inoculated with two 0.02 ml. drops of nutrient broth which, in turn, was inoculated by dipping a swab into the broth and spinning the swab: staphylococcus and staphylococcus on blood agar.



Appendix 1.1 A Modification to the "Spekker" Absorptiometer

The principle of the absorptiometer is as follows. A beam of light is passed through a heat filter, an iris diaphragm, a lens and on to a photo-cell. A second beam of light, from the same lamp, is passed through a heat filter, a variable shutter controlled by a graduated drum (calibrated in optical density and percentage transmission), a lens and on to a photo-cell. The outputs from the photo-cells are connected, in opposition, to a centre-zero galvanometer which has a variable resistor in parallel with it to vary the sensitivity. Shutters close to keep the light from the photo-cells when not in use.

The calibrated drum is set to zero on the density scale and the iris is closed - the galvanometer should be balanced. The sample is placed in the cell, the shutters are opened and the sensitivity control varied until the light spot of the galvanometer moves to full scale deflection (f.s.d.). The spot is then brought back to zero by opening the iris diaphragm, the shutters are closed and the apparatus is ready for use. On opening the shutters, the light-spot deflection is reduced to zero with the calibrated drum and the optical density is read on the appropriate scale of the drum: the procedure is repeated at frequent intervals to obtain a growth curve.

In our modification, the centre-zero galvanometer is replaced by a direct writing potentiometric recorder (0 - 500 μV f.s.d.) and the shutters are operated by a 2 in. double-acting pneumatic cylinder which in turn is actuated by a timing device. This produces a pulse that opens the shutters for 10 seconds in every minute or for 10 seconds in every five minutes: the frequency is pre-selected to suit the anticipated growth rate of the organism under test.

The apparatus is prepared for use as before. When the shutters are opened however, we do not reduce the deflection of the pen to zero with the calibrated drum but simply record the deflection of the pen. Consequently, the pattern obtained on the recording paper is a series of equidistant rectangles, and the height of each peak is related to the optical density of the specimen. By joining the peaks of the rectangles one obtains a growth curve.

The circuit diagram of the control system is shown in fig. A1. The pneumatic cylinder C (Martonair, type S778) is operated through a 5 port, pilot operated solenoid valve V (R.C.S., type E.P. 125/8): the uni-directional air-flow regulators (Martonair, S577) restrict the exhaust air flow, thus controlling the stroke rate of the cylinder. The timer is based on the motor M which has two cams, one with five ramps and one with one ramp, which operate micro-switches M.S.1 and M.S.2. respectively. The motor speed is 1 revolution in 5 minutes, so that M.S.1 operates every minute and M.S.2 operates every five minutes. Switch S.W.3 selects either

M.S.1, to operate the valve V every minute, or M.S.1 and M.S.2 in series, to operate the valve V every 5 minutes: the lengths of the ramps are such that the valve V is energised for approximately 10 seconds. The shutters are manually operated by S.W.4 and S.W.2 switches the timing device on and off: S.W.1 is the mains switch.

This is a very simple modification to the "Spekker" which relieves an operator of much tedious and time consuming work, in that the apparatus will work unattended for as long as required.

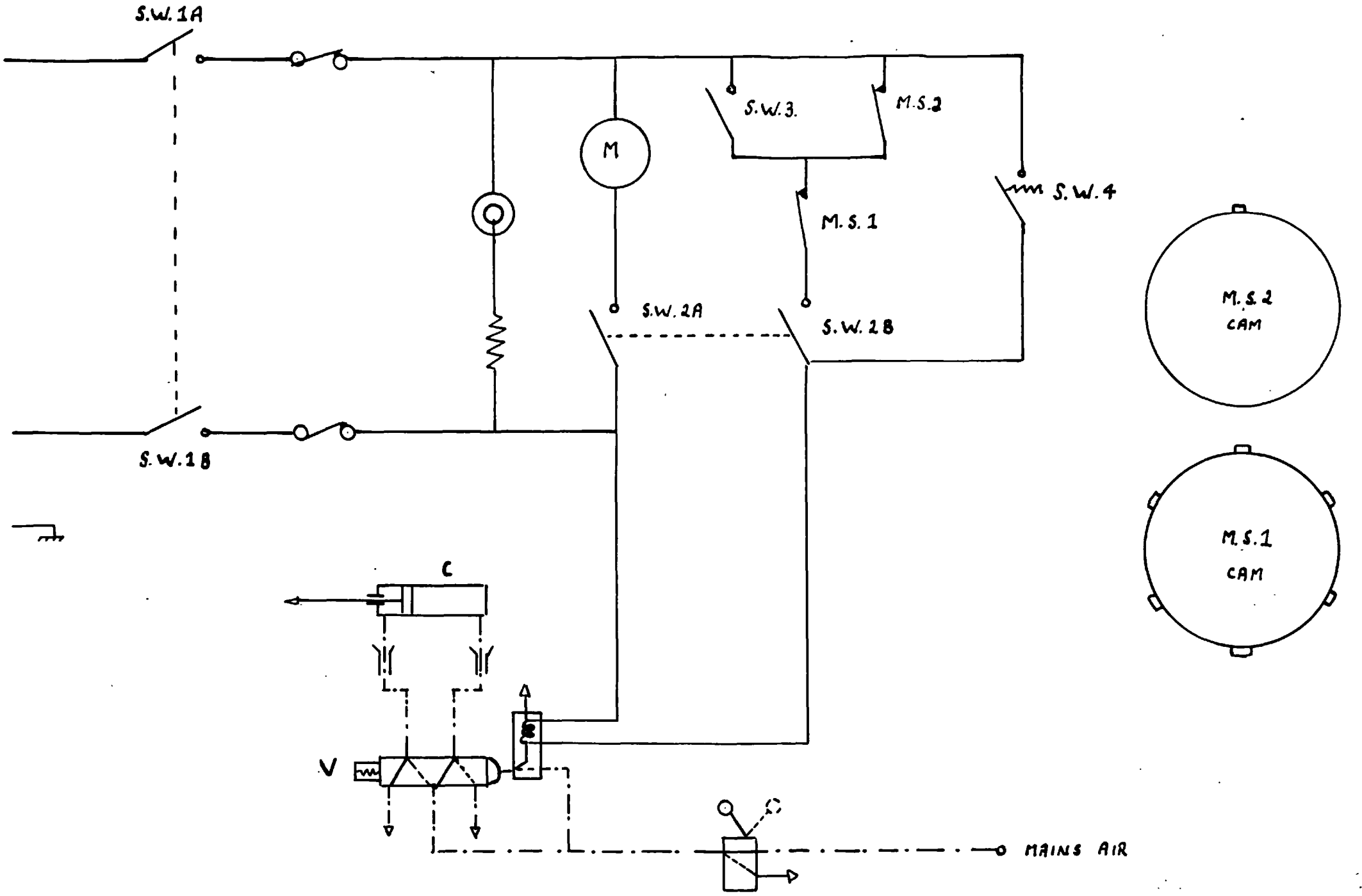


Fig. A1. CIRCUIT DIAGRAM OF MODIFICATION TO "SPEKKER" ABSORPTIOMETER.

Appendix 2.1 The Control Unit of the Peristaltic Pump Dispenser

The peristaltic pump (Watson Marlow, type M.H.R.E.) is operated remotely by closing the contacts J (fig. A2) which are in the pump.

The controller is based on a commercially available timer (Crouzet, type 207) which has four sets of change-over contacts. To operate the timer, the timer motor T and the clutch C′ are energised. Contacts C and D change over immediately the clutch is energised and the timed period, which is easily set on a dial, commences. At the end of the timed period, contact B changes over and after a short interval, contact A changes over. When the clutch is de-energised, the four sets of contacts revert to their original positions and the timer re-sets itself.

To repeatedly dispense volumes of broth, the selector switch S.W.2 is turned to position 1 and S.W.1 is closed. Power is then supplied to motor M through contact D and S.W.1A. Two cams, which operate micro-switches M.S.1 and M.S.2 respectively, are attached to the motor (speed 12 rev./min.) and when it has turned almost a complete revolution, M.S.2 is actuated by ramp d_2 and, immediately afterwards, M.S.1 is actuated by the groove d_1. M.S.1 operates the timer T and clutch C′ through S.W.1B, and contacts C and D change over thus closing the contacts J and starting the pump. Power to the timer is maintained through contact D and S.W.1A, and power to motor M is maintained by M.S.2 until its actuator passes ramp d_2:

M.S.1 changes to its original position just before M.S.2 opens to stop motor M. At the end of the timed period, contact B changes over thus opening the circuit to contacts J and stopping the pump. Soon after contact B has changed over, contact A changes over, thus de-energising the timer and clutch so that contacts A, B, C and D revert to their original positions (as shown) and the timer stops and re-sets itself. The motor M is now energised through contact D and S.W.1A and the process is repeated so long as S.W.1 is closed.

To run the pump continuously, one turns selector switch S.W.2 to position 2. The timer is not in circuit now and one merely operates S.W.4 to close contacts J: the contact Y.1 of relay Y/1 is an interlock which is opened when S.W.2 is in positions 1 and 3, to ensure that S.W.4 is inoperative.

To dispense a single volume, S.W.2 is turned to position 3 and S.W.3 is operated. Relay X/3 is energised through S.W.3A, and locked on by contact X.1: contacts X.2 and X.3 are closed. Timer T and clutch C' are energised, and contacts C and D change over to start the pump and to maintain power to the timer and clutch. At the end of the timed period, contact B opens followed by contact A, thus stopping the pump and de-energising the timer and clutch so that contacts A, B, C and D revert to the positions shown, and the timer stops and re-sets itself. The motor M is thus energised through contacts X.2 and D. When the micro-switch actuator L1 falls into groove d_2 relay X/3 is de-energised and contact X.2 opens thus stopping motor M: contact X.3 and relay Z/1 constitute an interlock

so that S.W.3 is ineffective once the timing cycle has commenced. The motor M stops with the micro-switch actuators L1 and L2 in groove d_1 and on ramp d_2, respectively, and not as shown. If S.W.3 is actuated to dispense another single volume, the pump will start and motor M will rotate until the micro-switch actuators L1 and L2 are in the positions shown when the motor will stop: it takes approximately 1 second for the micro-switches to revert to the positions shown and as this is less than the minimum timed period, the micro-switches will be in the required positions at the end of the timed period. Similarly, if after dispensing a single volume, S.W.2 is turned to position 1, the motor M will rotate until the micro-switches are in the position shown.

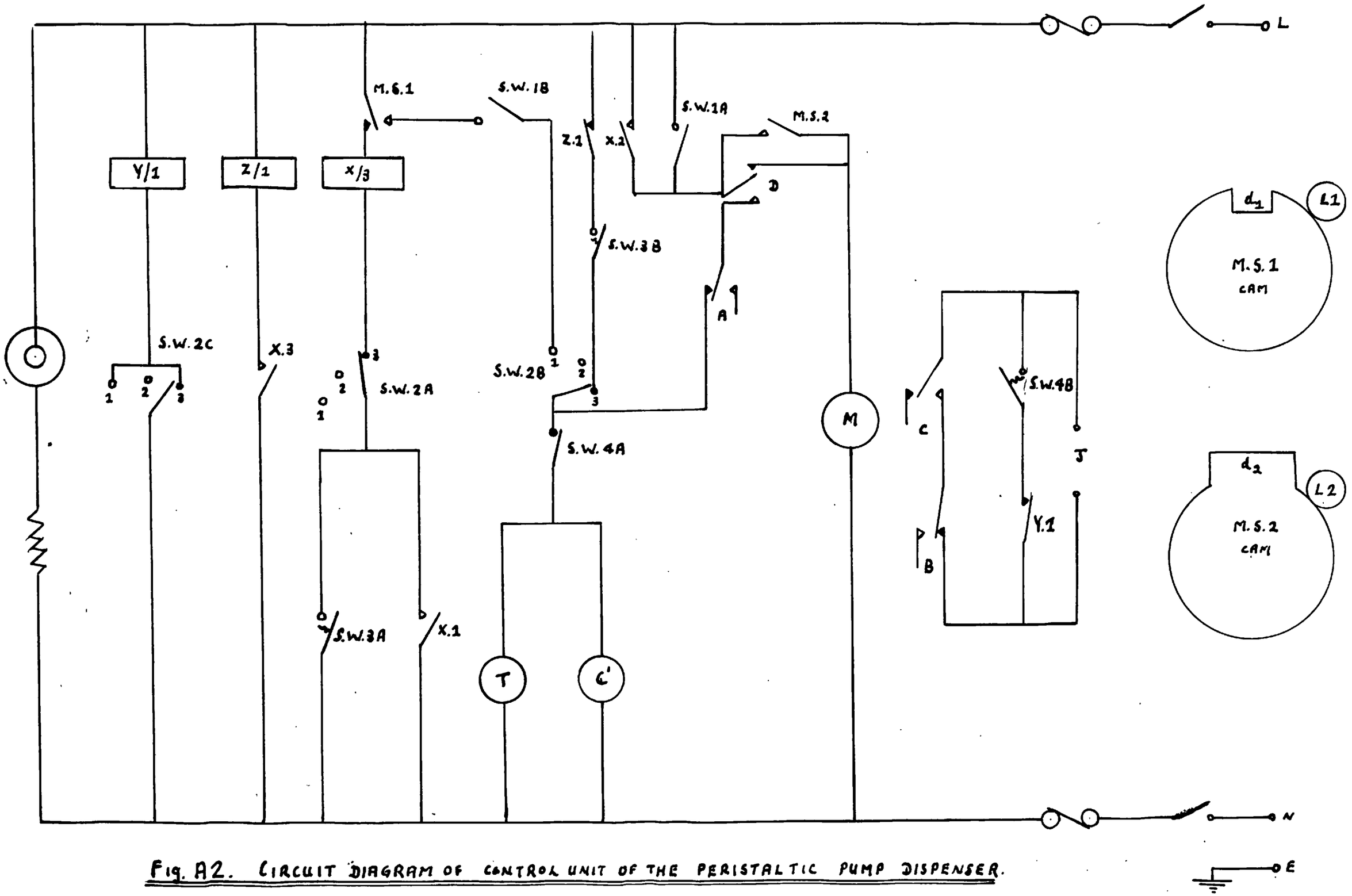


Fig. A2. CIRCUIT DIAGRAM OF CONTROL UNIT OF THE PERISTALTIC PUMP DISPENSER.

Appendix 3.1 The Control Unit of the Apparatus for Distributing Liquid Culture

The 2 in. double-acting pneumatic cylinder A (figs. 35 and A3) is a through-rod type, and one end of the piston is permanently connected to a 2 in. standard double-acting pneumatic cylinder B (fig. A3): the other end of the through-rod piston is connected to rod D (fig. 35). The two cylinders that actuate the Repette mechanisms are switched into the position of cylinder C as required: the switches and second cylinder are not shown.

To fill the syringe, valve F is operated (manually) and, provided the interlock diaphragm valve 1 is relaxed (cylinder C is fully negative), mains air passes to the appropriate pilot port of pilot operated valve S and mains air is passed to line I: line II air is exhausted. Line I air changes pilot operated valve T, to ensure that no air is in cylinder B, and valve U to allow mains air to drive cylinder A (and therefore cylinder B) positive: the speed is controlled by restricting the exhaust air of cylinder A by means of uni-directional air-flow regulator R1. When cylinder A is fully positive, the pilot air in diaphragm valve 2 exhausts through regulator R2, and the valve relaxes thus passing line I air to line Id. Line Id air operates pilot operated valve V, thus exhausting air from cylinder A so that its piston is free to be moved in a negative direction by the Repette

mechanisms.

Cylinder C reciprocates if foot operated valve G is closed, provided, valves 3 and 4 are relaxed (cylinders A and B are exhausted), valve M is open (cylinder A piston is not fully negative) and valve 1 is relaxed (cylinder C is fully negative). On operating G, mains air changes the pilot operated valve W and mains air drives C positive: the speed is controlled by regulator R3. When C is fully positive, valve 5 exhausts through regulator R4 and relaxes, mains air changes valve W and mains air drives C negative. When C is fully negative, valve 1 relaxes thus changing W to drive C positive. This sequence continues so long as G remains operated: when G is closed the sequence continues until C is fully negative.

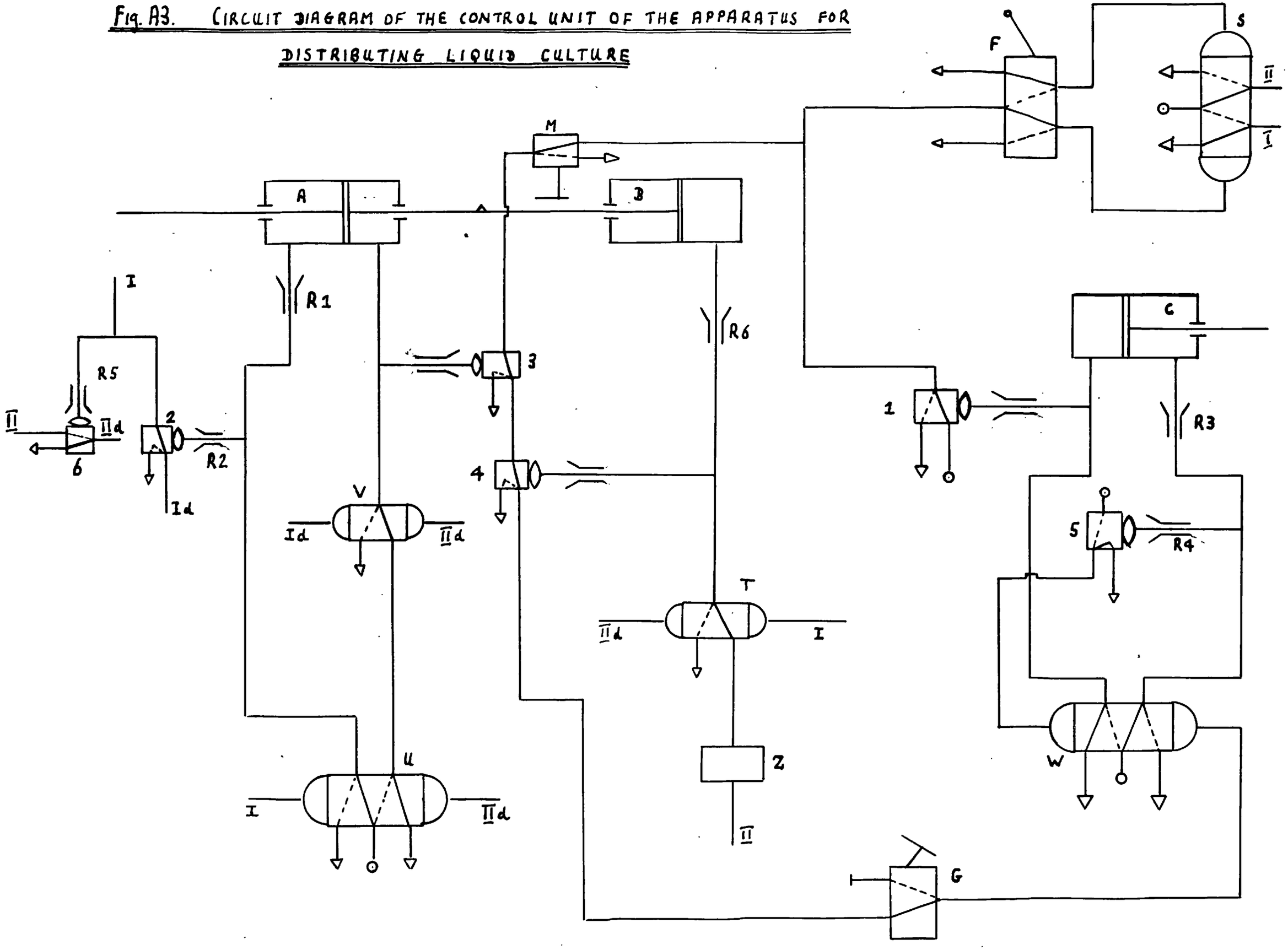
To discharge the remaining culture to waste, valve F is returned to its original position thus exhausting line I air and passing mains air to line II (assuming valve 1 is relaxed - C fully negative). Line II air fills cylinder B through reservoir Z, so that cylinder B (and therefore A) moves slowly: the movement is not as slow, or as smooth, as when restricting exhaust air flow (which is not possible here), but is smooth enough since one is simply moving culture already in the syringe further into the syringe. Whilst B is going positive, pilot air in valve 6 is exhausting through regulator R5. When the valve relaxes, which is some time after B is fully positive, line II air passes to line IId, line IId air changes valves U and T and mains air drives A (and therefore B) negative: the air in valve B

exhausts through regulator R6, and valve T, to control the speed of pistons A and B.

Components

Cylinders	Sohrader,	type 491
Valves S, U, W	Martonair,	type S.556/3
Valves V, T	Martonair,	type S.560/3
Valves 1 - 6	Martonair,	type S.560/42
Valve F	Martonair,	type S.556/7
Valve G	Martonair,	type S.256
Regulators R1, R3, R6	Martonair,	type S.577
Regulators R2, R4, R5	Martonair,	type S.836
Valve M	Davis Pneumatics,	type MV-10 (Mead)

Fig. A3. CIRCUIT DIAGRAM OF THE CONTROL UNIT OF THE APPARATUS FOR DISTRIBUTING LIQUID CULTURE



Appendix 3.2 The Control System of the Serial Diluting Apparatus

The control system is based on an 18 way, 12 position, rotary-solenoid operated switch, Ledex II (N.S.F. Ltd.) This is energised, through two electro-magnetic relays, by micro-switches M.S.I_1 and M.S.I_{2a}, situated at either end of the stroke of the pneumatic cylinder driving the diluting syringes respectively, and by micro-switches M.S.II_1 and M.S.II_{2a}, situated at either end of the stroke of the pneumatic cylinder which raises and lowers the pipette holder respectively: the cylinders are operated by pilot-operated air-solenoid valves I and II (R.G.S., type EP.125/8) respectively. The contact arrangements of the switches on Ledex II are shown in fig. A4.

To prepare the apparatus the power supply is switched on and relay M/5 (fig. A5) is energised and locked on by contact M.2: contacts M.1 and M.3 open, contact M.5 closes and contact M.4 changes over: relay Y/2 is also energised. S.W.6 is closed, delay relay LQAT3/1 is energised and, after a delay of approximately 5 seconds, contact LQAT3.1 closes, thus energising relay L/5 which is locked on by contact L.4: contacts L.1 and L.3 open and contacts L.2 and L.5 close.

The pipettes are loaded into the pipette holder which is then turned to the operative position so that M.S.IV is switched to the position shown. S.W.1 is operated to energise Ledex II (through

closed interlock L.I.2 - see below) which turns from position 6 (in which position it had stopped when the apparatus was last used) to position 7, and to de-energise Y/2. Contact L.II.2 energises air-solenoid II thus lowering the pipette holder. When the holder reaches the end of its stroke, M.S.II_{2b} closes to energise air-solenoid I which operates the 'diluting' cylinder so that the broth/antibiotic mixture is sucked up into the pipettes: the machine stops so that the operator may check that the pipette/syringe systems are not leaking. On depressing S.W.5, relay M/5 is de-energised, contacts M.1 to M.5 revert to the positions shown and the motor starts to rotate: M.S.I_{2b} is an interlock which ensures that S.W.5 is ineffective unless the 'diluting' cylinder has reached the end of its stroke. Since M.S.II_{2a} and L.II 10 are closed, relay A/1 is energised and contact A.1 de-energises relay B/1 (which has been energised throughout). After a short interval, contact B1 closes to actuate Ledex II which turns to position 8. L.II.1 is opened to de-energise air-solonoid I so that the fluid in the pipettes is ejected into the tube, and L.II.10 is opened to de-energise A/1, energise B/1 and open B.1. When the diluting cylinder reaches the upper end of its stroke, M.S.I_1 is closed and, since L.II.7 is closed, A/1 is energised to turn Ledex II to position 9. L.II.1 is closed to operate the 'diluting' cylinder thus sucking fluid into the pipette. When the cylinder reaches the end of its stroke, M.S.I_{2a} is operated and, since L.II.8 is closed, A/1 is energised to turn Ledex II to position 10 from which

it automatically 'homes' to position 1 (the 'homing' device circuit diagram is in fig. A4). When Ledex II is in position 1, the contacts are in the same state as they are in when Ledex II is in position 8 (except in the case of contact L.II.14 - see below), and the sequence from position 8, with the exception of the 'homing' process, is repeated two more times until Ledex II is in position 5: the broth is ejected and sucked up two more times. In position 5, L.II.1 is closed, thus keeping the fluid in the pipettes, but L.II.2 is operated to raise the pipette holder. When the holder reaches the end of its stroke, M.S.II_1 is closed and, since L.II.9 is closed, A/1 is de-energised to turn Ledex II to position 6. L.II.3 is closed to operate relays X/1 and Z/1. Contact X.1 changes to de-energise the brake and energise the clutch, thus rotating the rack of tubes, and consequently, the actuator of M.S.III (M in fig. 22) is raised out of its groove on disc F (fig. 22): M.S.III changes over from the position shown to maintain power to Z/1 and X/1. When Z/1 is energised, contact Z.1 opens and, after a short interval, relay D/2 (which has been energised throughout) is de-energised to open contact D.1 and close contact D.2. When the micro-switch actuator drops into the next groove, M.S.III changes to the position shown, thus energising Ledex II, through L.II.11 and D.2, which turns to position 7: when M.S.III changes to the position shown, X/1 and Z/1 are de-energised to stop the rack of tubes rotating and to energise D/2 so that contact D.2 closes and contact D.2 opens. In position 7, L.II.2 is closed to energise air-solenoid II.

The pipettes are lowered and M.S.II_{2a} is closed: since L.II 10 is closed, A/1 is de-energised to turn Ledex II to position 8.

The sequence of events that has taken place since Ledex II was last in position 8 repeats until stopped, either manually or automatically. The mechanism for stopping the sequence is based on another rotary-solenoid operated switch, Ledex I, and relay Y/2. To stop the machine automatically, S.W.3 is turned to select the number of dilutions required. Each time Ledex II turns to position 1, contact L.II.14 operates relay R/1 which in turn actuates Ledex I. When contact L.I.1 reaches the position corresponding to the position S.W.3 is in, Y/2 is energised and locked on by contact Y.2: Y/2 may also be operated manually, by means of S.W.4, at any time except when Ledex II is in position 6. Contact Y.1 opens when Y/2 is energised and consequently, the machine will stop when Ledex II next turns to position 6, because Y.1 prevents X/1 and Z/1 from operating. The machine stops, therefore, with the pipettes raised and with the pipettes containing 1 ml. of the final dilution of antibiotic: the machine will stop at no other point in the sequence, but in an emergency the pipettes may be raised, by means of S.W.2, whilst the sequence to position 6 is completed. When the pipette holder is turned to the loading position, M.S.IV is changed from the position shown, thus, de-energising air-solenoid I, to eject the final 1 ml. to waste, energising M/5 and stopping the motor.

The apparatus is now in its original state and ready for

further use. Ledex I is returned to position 18, through L.II. 16 and M.5, when S.W.1 is operated to turn Ledex II from position 6 to position 7, at the beginning of the complete sequence of events: L.I.2 is an interlock which ensures that S.W.1 is ineffective when the machine is operating.

Further details of the mechanical design of the serial diluter than those given in the text are in figs. A6 and A7.

References.

ALEXANDER, N.E. and GLICK, D.P. (1958), I.R.E. Trans. Med. Electron., P.G.M.E. 12, 89.
Automatic counting of bacterial cultures - a new machine.

ALPER, T. and STERNE, M. (1933), J. Hyg., 33, 497.
The Measurement of the opacity of bacterial cultures with a photo-electric cell.

BARCLAY, G.A. (1965), J. appl. Bact., 28 (2), 283.
An instrument for continuous viewing of roll tube cultures under a stereoscopic microscope.

BARON, S., BURCH, B.L. and UHLENDORF, B.W. (1961), Am. J. clin. Path., 36, 555.
An automatic multiple diluting machine.

le BOUFFANT, L. and SOULE, J.L. (1954), Brit. J. appl. Phys., Supplement No. 3, S143.
The automatic size analysis of dust deposits by means of an illuminated slit.

BOWMAN, R.L., BLUME, P. and VUREK, G.G. (1967), Science, 158, 78.
Capillary tube scanner for mechanized microbiology.

BRANCH, A., STARKEY, D.H. and POWER, E.E. (1965), Appl. Microbiol., 13, 469.
Diversifications in the tube dilution test for antibiotic sensitivity of micro-organisms.

BRECHER, G., SCHNEIDERMAN, M. and WILLIAMS, G.Z. (1956), Am. J. clin. Path., 26, 1439.
Evaluation of electronic red blood cell counter.

BROUGHTON, P.M.G. (1965), Association of Clinical Biochemists, Technical Bulletin No. 6.
A guide to automatic pipettes.

CHEESEMAN, G.C. and SILVA, M.T. (1959), J. appl. Bact., 22 (2), 294.
Differentiation of bacterial species by paper chromatography, VI An examination of the heterofermentative lactobacilli.

COULTER, W.H. (1953). U.S. Patent No. 2, 656, 508.

CRUICKSHANK, R. (1965). Medical Microbiology. E. & S. Livingstone Ltd.

CULLING, C.F.A. (1957). Handbook of Histopathological Technique. Butterworth.

CUMMINS, C.S. and HARRIS, H. (1956), J. gen. Microbiol., 14, 583.
The Chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character.

CURBY, W.A., SWANTON, E.M. and LIND, H.E. (1963), J. gen. Microbiol., 32, 33.
Electrical counting characteristics of several equivolume micro-organisms.

DACIE, J.V. (1956). Practical Haematology. J. & A. Churchill Ltd.

DONALD, R. (1913), Lancet, 1, 1447.
A method of counting bacteria in water.

DONALD, R. (1915), Lancet, 2, 1243.
A method of drop-measuring liquids and suspensions.

DRASAR, B.S. (1967), J. Path. and Bact., 94, 417.
Cultivation of anaerobic intestinal bacteria.

FLEMING, A. and OGILVIE, A.C. (1951), Brit. med. J., 1, 543.
Syringe needles and mass inoculation technique.

FORREST, W.W. and STEPHEN, V.A. (1965), J. sci. Instrumn., 42, 664.
A simple recording nephelometer for studies of bacterial growth.

GLYNN, A.A. and MILNE, C.M. (1967), Immunology, 12, 639.
A kinetic study of the bacteriolytic and bactericidal action of human serum.

HALE, L.J. and INKLEY, G.W. (1965), Lab. Pract., 14, 452.
A semi-automatic device for multiple inoculation of agar plates.

HANEY, T.A., GERKE, J.R. and PAGANO, J.F. (1963), Ch.5, Analytical Microbiology, Ed. F. KAVANAGH
Automation of microbiological assays.

HENIS, Y., GOULD, J.R. and ALEXANDER, M. (1966), Appl. Microbiol., 14, 513.
Detection and identification of bacteria by gas chromatography.

HEWITT, L.F. (1950). Oxidation-Reduction Potentials in Bacteriology and Biochemistry. E. & S. Livingstone Ltd.

KAVANAGH, F. (1963). Analytical Microbiology. Academic Press.

KUBITSCHEK, H.E. (1958), Nature (Lond.), 182, 234.
Electronic counting and sizing of bacteria.

LEIGH, D.A. and WILLIAMS, J.D. (1964), J. clin. Path., 17, 498.
Method for the detection of significant bacteruria in large groups of patients.

LIDWELL, O.M. (1959), Bull. Hyg., 34, 818.
Apparatus for phage typing of Staphylococcus aureus.

LOEBL, H. (1966), Lab. Equip. Dig., 4, No. 8, 48.
A new approach to the automation of clinical laboratories.

MACKERETH, F.J.H. (1964), J. sci. Instrumn., 41, 38.
An improved galvanic cell for determination of oxygen concentration in fluids.

MALLIGO, J.E. (1965), Appl. Microbiol., 13, 931.
Evaluation of an automatic electronic device for counting bacterial colonies.

MANOR, H. and HASELKORN, R. (1967), Nature (Lond.), 214, 983.
Size fractionation of exponentially growing Escherichia coli.

MARSH, W.H. (1963). Automation in Clinical Chemistry. C.C. Thomas.

MARYMONT, J.H. and WENTZ, R.M. (1966), Am. J. clin. Path., 45, 548.
Serial dilution antibiotic sensitivity testing with the microtitrator system.

MATTICK, A.T.R., CHEESEMAN, G.C., BERRIDGE, N.J. and BOTTAZZI, V. (1956), J. appl. Bact., 19 (2), 310.
The differentiation of species of lactobacilli and streptococci by means of paper partition chromatography.

MEDICAL RESEARCH COUNCIL (1962). Memorandum No. 41.
The sterilization, use and care of syringes.

MEYNELL, G.G. and MEYNELL, E. (1965). Theory and Practice in Experimental Bacteriology. Cambridge University Press.

MILES, A.A. and MISRA, S.S. (1938), J. Hyg., 38, 733.
The estimation of the bactericidal power of the blood.

NORRIS, K.P. (1959), J. Hyg., 57, 326.
Infra-red spectroscopy and its application to microbiology.

PEGG, D.E., TROTMAN, R.E. and PIERCE, N.H. (1963), Brit. med. J., 1, 1207.
Apparatus for continuous infusion chemotherapy.

PHIPPS, L.W. (1966), J. sci. Instrumn., 43, 485.
A pneumatic device for scanning control of a microscope slide.

PROOM, H. and WOIWOD, A.J. (1949), J. gen. Microbiol., 3, 319.
The examination, by partition paper chromatography, of the nitrogen metabolism of bacteria.

PUGH, V.W. and GAZE, R.W.T. (1965), Brit. J. vener. Dis., 41, 221.
Automated screening method for the Wassermann reaction.

PUGH, V.W. and GAZE, R.W.T. (1966), J. med. Lab. Tech., 23, 126.
Modifications to the automated Wassermann reaction.

PULVERTAFT, R.J.V. and LEMON, C.G. (1933), J. Hyg., 33, 245.
Application of photo-electricity to the determination of bacterial growth rate.

QUADLING, C. and COLWELL, R.R. (1964), Can. J. Microbiol., 10, 87.
Apparatus for simultaneous inoculation of a set of culture tubes.

REINER, E. (1965), Nature (Lond.), 206, 1272.
Identification of bacterial strains by pyrolysis - gas - liquid chromatography.

REINER, E. and EWING, W.H. (1968), Nature (Lond.), 217, 191.
Chemotaxonomic studies of some Gram negative bacteria by means of pyrolysis - gas - liquid chromatography.

RICHAR, W.J. and BREAKELL, E.S. (1959), Am. J. clin. Path., 31, 384.
Evaluation of an electronic particle counter for the counting of white blood cells.

SEQUEIRA, P.J.L. (1964). Personal communication.

SELLERS, W., MITCHELL, R.B. and DAVIS, I.(1956), Bact. Proc., p.89.
Chromatography as a method for the identification of bacteria.

SEVER, J.L. (1962), J. Immunol., 88, 320.
Application of a microtechnique to viral serological investigations.

SKEGGS, L.T. (1957), Am. J. clin. Path., 28, 311.
An automatic method for colorimetric analysis.

SIMON, H.J. and UNDSETH, S. (1963), J. Bact., 85, 1447.
Simple method for phage typing of staphylococci.

SNELL, F.D. and SNELL, C.T. (1948). Colorimetric Methods of Analysis. D. Van. Nostrand Co., Inc.

TAKATSY, Gy. (1955), Acta. Microbiol. Hung., 3, 191.
The use of spiral loops in serological and viral micro-methods.

TARR, H.A. (1958), Bull. Hyg., 33, 698.
Mechanical aids for the phage typing of Staphylococcus aureus.

TATTAM, F. (1966). Personal communication.

TAYLOR, C.E.D. (1968). Personal communication.

TOENNIES, G., ISZARD, L., ROGERS, N.B. and SHOCKMAN, G.D. (1961), J. Bact., 82, 857.
Cell multiplication studied with an electronic particle counter.

VARGUES, R. (1965), Ann. N.Y. Acad. Sci., 130, 819.
The use of the autoanalyzer for the automatic titration of antigenic preparations by means of complement-fixation.

WEITZ, B. (1957), J. clin. Path., 10, 200.
An automatic dispenser for multiple serological titrations.

WILSON, G.S. (1922), J. Bact., 7, 405.
The proportion of viable bacteria in young cultures with especial reference to the technique employed in counting.

WILSON, G.S. (1935), Spec. Rep. Ser. med. Res. Coun., Lond., No. 206.
The bacteriological grading of milk.

WRIGHT, A.E. and COLEBROOKE, L. (1921). The Technique of the Teat and Capillary Glass Tube. Constable.

ZIERDT, C.H., FOX, F.A. and NORRIS, G.F. (1960), Am. J. clin. Path., 33, 233.
A multiple-syringe bacteriophage applicator.

List of reprints of the author's published work, submitted in support of the thesis

(1) TROTMAN, R.E. (1967), J. appl. Pneumatics, 6, No. 48, 9.

Pneumatics in the pathology laboratory.

(2) TROTMAN, R.E. (1967), Digest 7th Int. Conf. Med. and Biol. Engng., Stockholm, p.191.

Automatic methods in diagnostic bacteriology.

(3) TROTMAN, R.E. (1967), J. clin. Path., 20, 770.

Automatic serial diluting: an instrument for use in bacteriological laboratories.

Pneumatics in the Pathology Laboratory

R. E. Trotman, M.Sc., C.Eng.
Wright-Fleming Institute,
St. Mary's Hospital Medical School,
London, W.2.

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PNEUMATIC APPARATUS HAS hitherto been very little used in medical and biological engineering but, in the course of an investigation of the possibilities of automation and mechanization in diagnostic bacteriology, we have demonstrated the practicability of utilizing pneumatic components in apparatus used in pathological laboratories. In this article we describe one such piece of equipment.

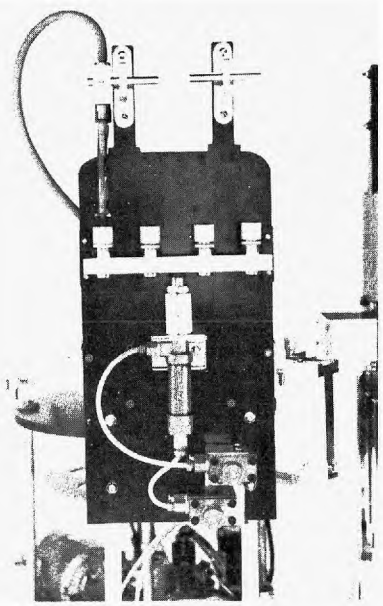
As a result of infection, a substance known as antibody is found in blood and tissue fluids: it counteracts infection. Consequently, the presence of antibodies in human serum is often sought as an aid to diagnosis, a common method being to add an antigen, a substance which stimulates the formation of antibodies, and to study the reaction: the test is highly specific because antibodies often react only with the antigens which stimulate their production, and therefore a positive reaction not only indicates the presence of antibodies but greatly assists in identifying the microbe associated with the antibody.

There are many antigen-antibody reactions but we are particularly interested in those involving a class of antibodies known as complement-fixing, complement being a substance present in fresh normal serum. In this type of reaction, complement added to the serum-antigen mixture is removed if antibodies are present in the serum but otherwise it remains. Thus, if one can detect complement in such a mixture, the presence of antibodies in the serum, and thus of infection, can be determined: some complement-fixation reactions produce an invisible effect and in these cases complement is detected by means of a second, similar, reaction which produces a visible effect.

In principle, therefore, the procedure involves dispensing a variety of different reagents into a set of receptacles containing serum, and, in order to obtain a quantitative result, the quantity of each reagent has to be controlled.

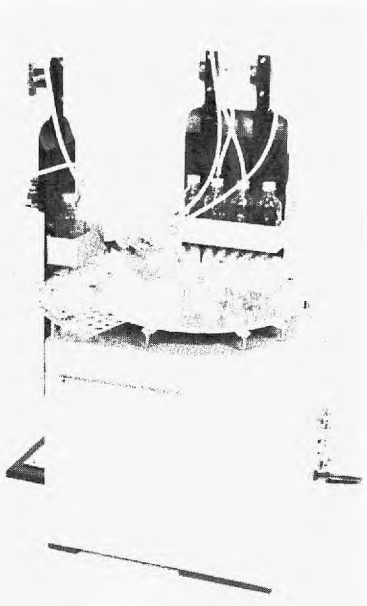
A common complement-fixation reaction is that used to diagnose syphilis, the antigen used being known as the Wassermann antigen. In our laboratory we also test the serum against another antigen and the complete procedure involves placing a quantity of serum into each of four tubes and dispensing different volumes of six different reagents. With many sera to be so tested, this is a tedious and time consuming procedure and one that lends itself to mechanization.

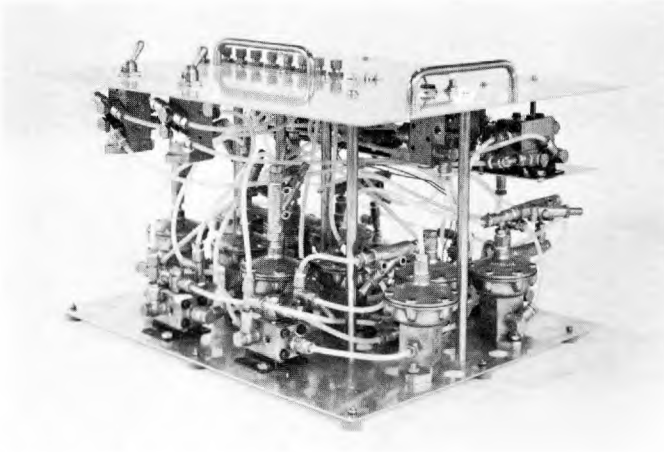
The mechanism for dispensing each reagent consists of a syringe attached to a simple pin valve assembly (Fig. 1): the syringe piston is moved to suck up and expel the fluid. 0.1 ml. of each of four reagents is dispensed and therefore four syringe pistons are actuated by the same mechanism: since a variable speed, reciprocating linear motion is required, the obvious choice of mechanism is the pneumatic cylinder, the stroke length, and hence the volume dispensed, being adjusted by a simple screw-thread device. 0.18 ml. and 0.28 ml. of the fifth and sixth reagent respectively are dispensed by independent mechanisms.



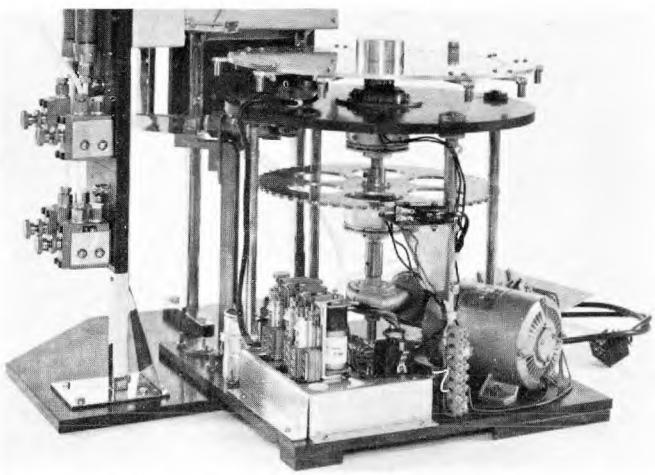
▲ *Fig. 1. A valve-syringe-cylinder system.*

▼ *Fig. 2. A view of the apparatus showing tube racks and valve outlet holder.*





▲ *Showing the control unit of the apparatus.*



▲ *The mechanism for turning the rotary table.*

The tubes containing the sera are placed in a removable rack which is attached to a rotary table (Fig. 2); the four tubes containing one patient's serum are in a straight line along a radius. Each set of tubes moves into position under the valve outlets which are fixed, over appropriate tubes, in a holder. This is attached to a cylinder which moves the valve outlets radially: one reagent is required in each of two adjacent tubes. If we designate the three dispensing cylinders A (0.28 ml.), B (0.18 ml.) and C (4 x 0.1 ml.) and the cylinder attached to the valve outlet holder D, the required sequence of events is:

A+, B+, C+, D+, B–, A–, C–, B+, D–, B–, rotate table, continued; or when grouped, as shown in the circuit diagram.

A sequentially operated controller is preferred, and the simplest approach is to have each cylinder piston actuate a trip valve at each end of its stroke. However, the stroke length of three cylinders is approximately $\frac{1}{4}$ in. so mechanically operated valves are unsuitable. Diaphragm valves are a suitable alternative. To control the stroke rate of the cylinders, uni-directional flow regulators are used: with S778 miniature cylinders, only S577 regulators give the degree of control required. However, if the pilot air from the diaphragm valves is exhausted through these regulators the delay in actuating the valves is considerable. Consequently they are provided with independent S836 uni-directional flow regulators which give adequate control of the delay.

The circuit diagram excludes the electro-pneumatic system for rotating the table and delaying the initiation of the cycle until the next set of tubes is in position: this is essentially an interlocking device with pressure indicators, in Group IV and Group I air lines, actuating electrical microswitches and a solenoid operated valve.

When the S.256C/3 valve is operated, a Group I pilot air signal reverses the control valves of cylinders A, B and C: these cylinders outstroke. The pilot air in valves 1, 2 and 3 slowly exhausts to atmosphere, the valves operate and a Group I pilot air signal passes through them to reverse the control valve of cylinder D: this cylinder outstrokes. The pilot air in valve 4 slowly exhausts, the valve operates and a mains air signal passes through it to select Group II air.

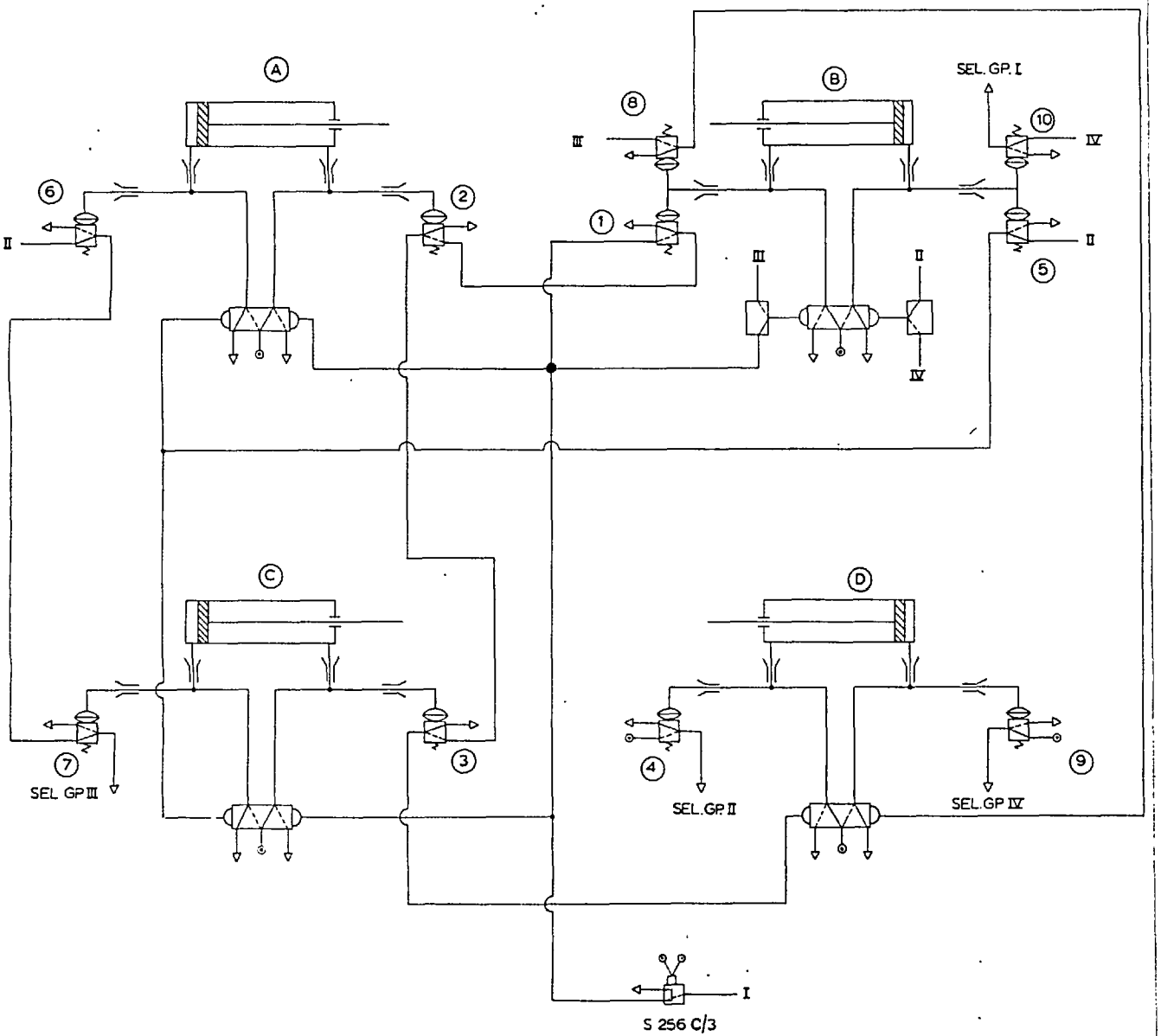
A Group II pilot air signal reverses the control valve of cylinder B: the cylinder instrokes. The pilot air in valve 5 exhausts, the valve operates and a Group II pilot air signal passes through it to reverse the control valves of cylinders A and C: the cylinders instroke. Valves 6 and 7 operate and a Group II air signal passes through them to select Group III air.

Group III air reverses the control valve of cylinder B: the cylinder outstrokes. Valve 8 operates and the control valve of cylinder D is reversed: the cylinder instrokes. Valve 9 operates and a mains air signal selects Group IV air.

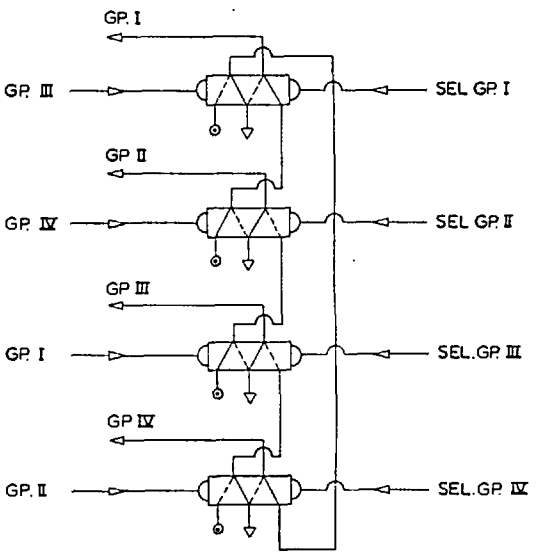
Group IV air reverses the control valve of cylinder B: the cylinder instrokes. Valve 10 operates and a Group IV air signal selects Group I air: the sequence is repeated until the start valve is reversed.

Whilst our embodiment performs only the procedure adopted in our laboratory for the diagnosis of syphilis, very similar apparatus could be used to perform any complement-fixation or similar reaction.

The machine is not very sophisticated but it demonstrates that pneumatic apparatus has a place in pathological laboratories and that designers of medical laboratory equipment might well make more use of the range of components now available: it is clear there is no reason why similar apparatus should not be used in any hospital department.



SEQUENCE	
A+, B+ C+ D+	GP I
B− A−C−	GP II
B+ D−	GP III
B−	GP IV
ROTATE TABLE	
REPEAT	



▲ *The pneumatic circuit diagram.*

11–15 Automatic Methods in Diagnostic Bacteriology

R. E. Trotman
Wright-Fleming Institute of Microbiology
St. Mary's Hospital Medical School
London, W.2. England

The work load in many bacteriological departments has been increasing rapidly, but automatic methods have hitherto been very little used. This seems surprising, but there are two important complicating factors pertaining, exclusively, to bacteriology. First, relatively few specimens are subjected to any given test procedure and second, the specimens are infective and the spread of infection and contamination of the specimens must be avoided.

Most test procedures consist of a series of discrete manipulations and some of these are common to at least two procedures. If, therefore, one constructs certain basic units, each of which may be utilised in many different procedures, it may be possible to produce an economic, automatic diagnostic system.

We have embarked on a broad study of the problems of automation and mechanisation in diagnostic bacteriology, and in this paper describe some of the apparatus developed: since the most time consuming part of the work, and therefore the most urgent aspect of the problem, is manipulating the specimens, we have concentrated on this rather than methods for measuring and displaying results.

Two oft used procedures are the measurement of the minimal inhibitory concentration of an antibiotic (M.I.C.) and of the serum antibiotic level. The method used for determination of the M.I.C. in our laboratory is as follows. 2 ml. of appropriately concentrated antibiotic solution in broth is placed in a test tube and 1 ml. of broth is placed in each of a series of 10-12 tubes. 1 ml. of the antibiotic containing broth is transferred from the first tube to the 1 ml. of broth in the second tube and mixed thoroughly. This transfer is repeated 9 or 10 times to give a series of two-fold falling dilutions. Subsequently a standard suspension of the bacterium under test is added to each tube. After incubation one observes the lowest concentration of antibiotic that inhibits growth. For a serum antibiotic level, serum is similarly diluted and each tube is inoculated with a standard organism: a control M.I.C., in human serum, is also performed. After incubation, one observes which dilution of serum contains sufficient antibiotic to inhibit growth of the organism and the corresponding concentration of antibiotic is calculable from the M.I.C.

We have developed equipment for carrying out, mechanically, three stages: the distribution of initial volumes of diluent, the successive dilution steps and the addition of culture. Three separate machines are used so that each may be utilised for other tasks.

The diluent dispenser is based on the peristaltic pump.

In the serial diluter, the test tubes are mounted in a circular rack which revolves under four P.T.F.E. pipettes, so arranged that there are, in effect, four sets of 12 tubes each. The pipettes are connected by silicone rubber tubes to 1 ml. syringes: all parts may be sterilised by heat and are removed and replaced simply without affecting the calibration of the system. The four syringes are operated by a single pneumatic cylinder to suck up and expel the fluid.

The rack of tubes is mounted on a spindle, the pipettes are lowered, by a pneumatic cylinder, into the first tube of each series and 1 ml. of liquid is sucked up into the pipettes. This 1 ml. is now put back into the tube, sucked up and replaced twice and sucked up again. The pipettes are raised, the rack rotated and the pipettes lowered into the second tube of each series: this procedure is repeated a pre-selected number of times.

In the apparatus for dispensing drops of culture, the barrel of a pre-sterilised disposable syringe is fixed vertically. The piston is attached to a rod, the upper end of which is attached to a pneumatic cylinder to facilitate filling and emptying the syringe. The rod passes through two intermittent linear motion mechanisms each of which moves the rod a predetermined distance: these are actuated by separate pneumatic cylinders and dispense 0.02 ml. and 0.1 ml. respectively.

The syringe, with a needle, is placed in situ and approximately 1 ml. of culture is drawn in. On depressing the foot switch, the preselected dispensing mechanism reciprocates thus repeatedly dispensing drops of culture. On releasing the switch, the process stops: the remaining culture is ejected to waste and the syringe and needle are discarded.

Both machines are constructed almost entirely of commercially available components and are controlled by electro-pneumatic logic circuits. By any automation standards they are not very sophisticated, but they save considerable time and perform their tasks far more accurately than these can be performed by hand. We have shown that diagnostic bacteriology technology can be modernised to considerable advantage.

Automatic serial diluting: an instrument for use in bacteriological laboratories

R. E. TROTMAN

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Automatic serial diluting: an instrument for use in bacteriological laboratories[1]

R. E. TROTMAN

From the Wright-Fleming Institute, St. Mary's Hospital Medical School, London

SYNOPSIS A machine for automatic serial diluting in bacteriological laboratories is described. It is simple to operate, not too expensive, and results are sufficiently accurate for use in a diagnostic laboratory.

Automatic methods have hitherto been very little used in diagnostic bacteriology, despite the fact that the work load in many departments has been increasing at a rate of about 10% per annum. However, the serious shortage of technical staff justifies the design of automatic apparatus that is suitable for processing small numbers of specimens even if it seems, at first, rather expensive. If such apparatus can be assembled from a few basic units, each of which may, in principle, be utilized in a variety of different procedures, it may be possible to produce an economic automatic diagnostic system.

In any equipment of this nature simplicity of operation is essential, even at the cost of complicating the control mechanism, but it is rarely necessary for automatic apparatus to perform its function more quickly than it can be performed by hand; what is important is that the total time the operator spends performing the test be minimal.

For many diagnostic test procedures a very high order of accuracy is not essential, and one should aim at producing as high an order of accuracy as is consistent with the other criteria; in fact, it is likely that automatic equipment can easily be made to give a higher degree of accuracy and reproducibility than is generally achieved in routine diagnostic laboratories.

A broad study of the possibilities of mechanization and automation has been embarked upon, and in the present paper a machine constructed to make serial dilutions, as used in the determination of the minimal inhibitory concentration of an antibiotic or a serum antibiotic level, is described.

[1]The invention is covered by patent application no. 32470/66 and full patent rights have been assigned to the National Research and Development Corporation.

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METHOD FOR DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (M.I.C.) OF ANTIBIOTICS

In this laboratory the M.I.C. is determined by the tube dilution method: by successively diluting 1 ml. of antibiotic solution in broth, we produce a series of two-fold falling dilutions and subsequently add a standard inoculum of the bacterium under test to each tube.

There are three stages that could be carried out mechanically; the distribution of the initial 1 ml. volumes of diluent into the tubes, the successive dilution steps, and the final addition of culture. In this laboratory, the first stage is performed by an automatic dispenser, based on the peristaltic pump. The apparatus described here performs the second stage and apparatus to perform the third stage is under development.

METHODS FOR TRANSFERRING LIQUIDS

The efficacy of any apparatus for performing serial dilution depends on the device for transferring the liquid from one tube to another. Two important requirements are that it must be a simple matter to change the pipettes in order that dilutions of a number of different antibiotics can be made in quick succession, and that all parts that come into contact with the liquid can be dismantled, sterilized by heat and re-assembled quickly, without affecting either the calibration or the reproducibility of the volume transferred.

Some manually operated and automatic devices for pipetting liquids are commercially available (Broughton, 1965), and Weitz (1957) published details of a manually operated multi-volume pipetting device. Sequeira (1964) has also produced a similar hand-operated machine. However, it is neither convenient nor economic to incorporate these devices in a multi-volume automatic pipetting device and many of them do not satisfy our requirements. It was decided, therefore, to produce a different system. In principle, this consists of a pipette

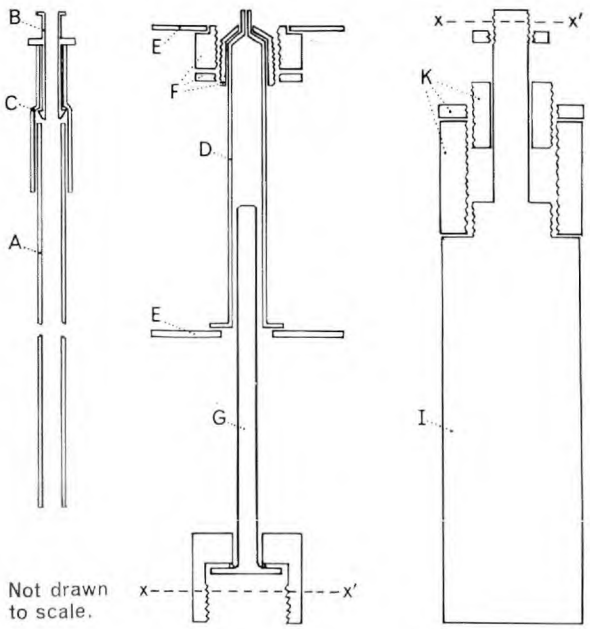


FIG. 1. *Drawing of syringe-pipette system. A Polytetrafluorethylene tubing; B Luer lock connector; C silicone tubing; D syringe barrel; E fixed brackets; F adjustable device; G syringe piston; I pneumatic cylinder; K adjustable device.*

made of a straight length of polytetrafluorethylene (p.t.f.e.) tubing connected by a flexible tube to a 1 ml. syringe which is operated mechanically to draw up and expel the fluid. The pipette-syringe unit is mounted in such a way that the pipette can be lowered automatically into the tubes for the dilution and mixing steps.

CONSTRUCTION OF THE DILUTING MACHINE

THE LIQUID TRANSFER ASSEMBLY (Fig. 1) A syringe is connected, by a Luer connector, to a length of silicone tubing and this in turn is connected to a piece of p.t.f.e. tubing A by a Luer lock connector B. The female part of the Luer lock connector is linked to the p.t.f.e. tube by a short length of silicone tubing C. The syringe barrel D is mounted between two fixed brackets E, with the aid of device F which is adjustable to allow for tolerances in the external dimensions. The syringe piston G is connected to a 1 in. diameter double-acting pneumatic cylinder I, the inward stroke length being varied by device K.

The p.t.f.e. tube, which has a $\frac{1}{8}$ in. internal diameter and a $\frac{3}{16}$ in. outside diameter, is approximately $6\frac{1}{2}$ in. long, so that 1 ml. occupies approximately $\frac{3}{4}$ of its length; there is therefore no risk of the fluid being drawn into the flexible connecting tube. By disconnecting the pipette at the Luer lock connector a replacement can be introduced very quickly. Using a Sumit tuberculin syringe, the apparatus repeatedly transfers 1 ml. within a tolerance of $\pm 1\%$.

Four pipette-syringe units have been mounted together (Fig. 2) so that up to four separate series of dilutions can be performed at one time. The four syringes are operated by a single pneumatic cylinder and con-

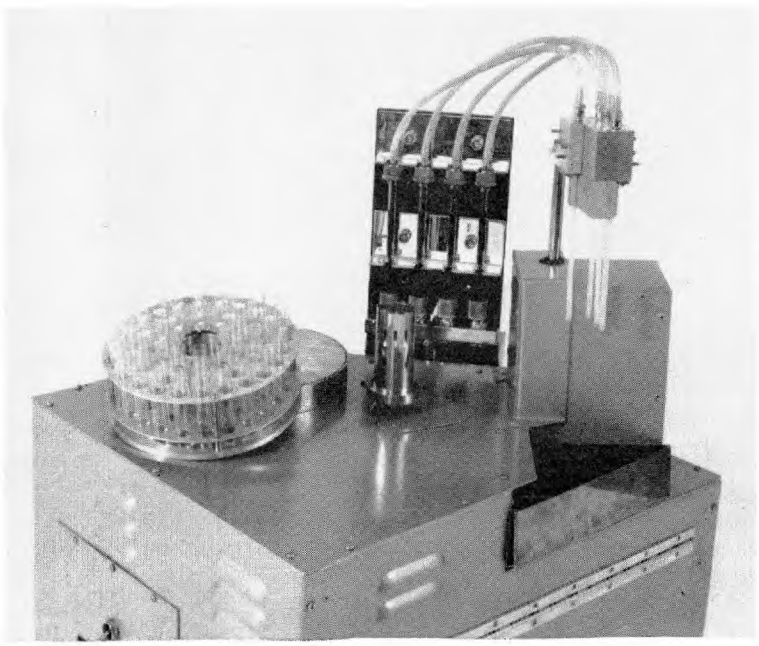


FIG. 2. *Photograph of diluting machine showing four syringe-pipette units mounted together and a rack of dilution tubes.*

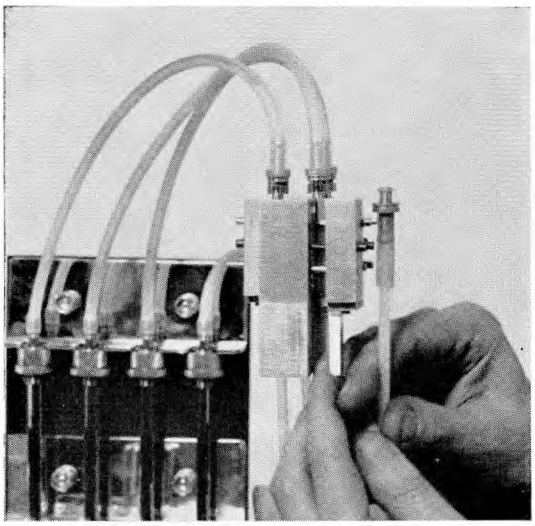


FIG. 3. *Close-up view of pipette holder.*

sequently the stroke lengths are not independently variable. However, we have found that the syringes in use, which were not especially selected, have a spread in volume transferred of $\pm$ 0·5% and so one can quote an overall accuracy of $\pm$ 2%.

The pipette holder (Fig. 3) is comparatively complicated, but it enables the pipettes to be aligned properly without the operator having to spend a great deal of time checking and altering their positions. It is raised and lowered by means of a second double-acting pneumatic cylinder.

One advantage of a pneumatic cylinder over an electrically driven reciprocating mechanism is that the stroke rate in either direction is independently variable; fine speed control is obtained simply by the turn of a screw thread in a unidirectional air-flow regulator placed in each exhaust air line. This facility is very desirable because one needs to draw up the mixture into the pipette very slowly, in order not to create too much turbulence, to prevent air being sucked up, and to ensure that there is no 'overshoot'. On the other hand, the liquid should be ejected at a much greater rate in order to provide efficient mixing and to ensure that all the liquid is ejected from the tip of the pipette: in some devices the drop left in the tip can be as much as 20% of the volume transferred.

This is a simple, flexible method of transferring liquids; any small volume may be transferred merely by using an appropriately sized syringe, although if used for volumes smaller than 0·5 ml. the accuracy would probably be reduced. The mechanism is so arranged that the meniscus always rises to the same level in the pipette, thus eliminating one source of error often present in manual methods.

The device can simply be made into a heat sterilizable liquid dispenser by attaching a suitable valve mechanism to the nozzle of the syringe. There are many applications for both devices.

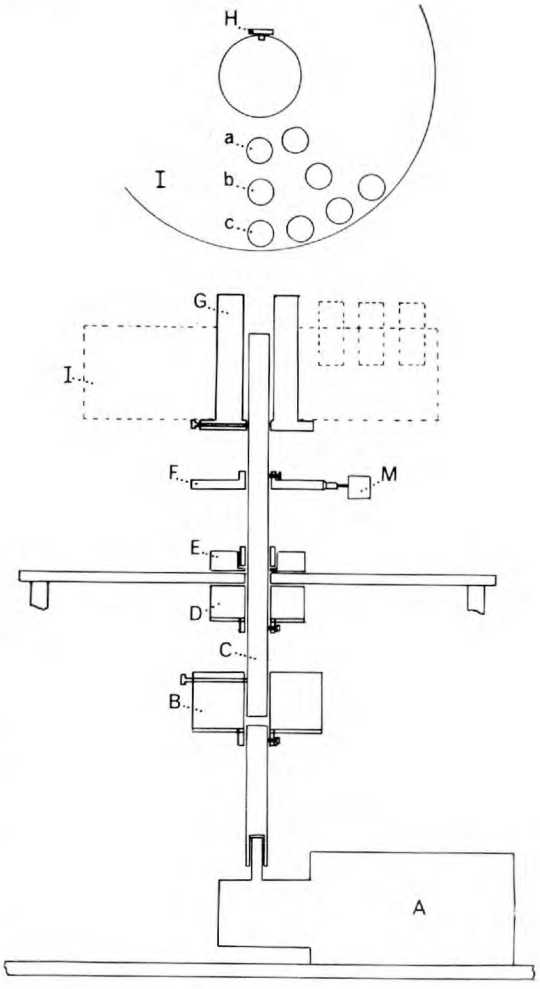


FIG. 4. *Drawing of mechanism for automatically feeding the dilution tubes (not to scale). A Induction motor; B electromagnetic clutch; C shaft; D electromagnetic brake; E thrust-race; F grooved disc; G splined rod; H lug; I rack; M microswitch.*

AUTOMATIC FEEDING OF DILUTION TUBES There are a number of 'fraction collector' type devices available today but it is surprisingly difficult to find a cheap, compact system that will accommodate only a small number of tubes in a rack that is both small and removable. Consequently it was decided to build one especially.

Figure 4 is a drawing showing the principle used, which is, briefly, as follows. An induction motor A runs continuously, and its shaft is connected to the armature of an electromagnetic clutch B. The rotor of the clutch is connected to another shaft C which runs through an electromagnetic brake D and thrust-race E. The armature of the brake is attached to this shaft and the fields

of both clutch and brake are fixed.

A disc with 12 grooves round the circumference, F, is mounted on this shaft and actuates a microswitch M which in turn operates the clutch-brake system in such a way that the shaft will stop at 12 positions per revolution.

Attached to the top of the shaft is a splined rod G. There are 12 splines and a lug H fixed on the racks. I will fit any of the splines, thus enabling the rack to be mounted in any position.

The brake is necessary to prevent the rack overshooting its correct position and to ensure that the splined rod is not rotated when removing or replacing the racks.

Figure 4 shows the layout of the racks; the inner two rows *a* and *b* each contain 12 tubes and the outer row *c* contains 24 tubes. The layout of the pipettes is such that, in effect, we have four series of 12 tubes (or, say, eight series of six tubes). The overall diameter of the rack is 7 in.

This mechanism has a simple basic principle and by varying the number of grooves on disc F, the shaft C can be made to stop at any number of positions per revolution; consequently, any number of tubes can be accommodated, facilitating its use in a variety of applications. With a device for moving a dispensing head it makes a versatile fraction collector.

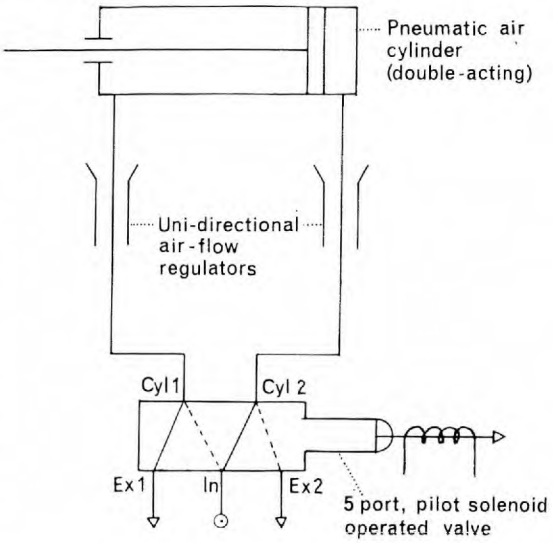


FIG. 6. *Circuit diagram of pneumatic air cylinder control system.*

THE COMPLETE APPARATUS Figure 5 shows the complete apparatus. The mechanism for feeding the dilution tubes is mounted in the centre of the right-hand unit and the liquid transferring mechanism is in the right background. On the left-hand side of the motor is the chassis containing the solenoid valves and the unidirectional air-flow regulators that control the pneumatic cylinders; Fig. 6 is a drawing of the air control circuit for each of the cylinders. In the right foreground (Fig. 5) is the mechanism for raising and lowering the pipette holder.

The left-hand unit is the control system which produces the following sequence of events. On starting, after having placed the prepared rack and pipettes into

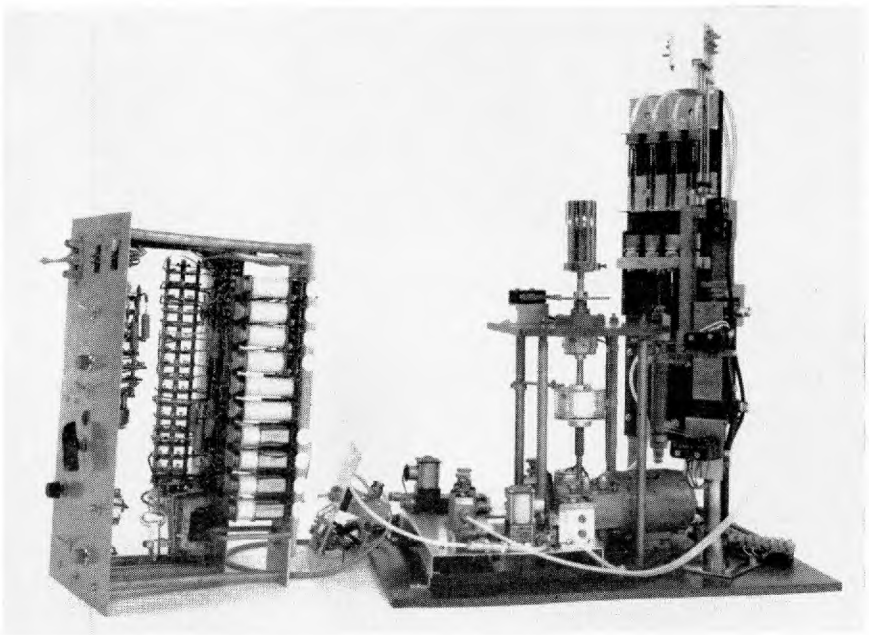


FIG. 5. *Photograph of apparatus.*

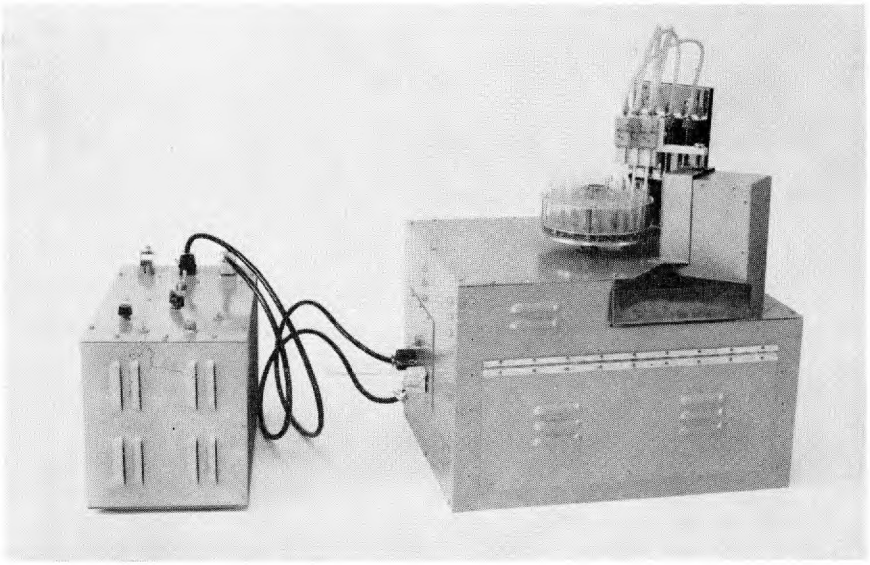


FIG. 7. *Photograph of apparatus in use.*

position, the pipettes are lowered into the first tube of each series and 1 ml. of broth and antibiotic is sucked up into each pipette. After a short delay, this 1 ml. is put back into the tube, sucked up and replaced twice and sucked up again. The pipettes are now raised, the rack rotated, the pipettes lowered into the second tube of each series and the procedure is repeated: as no bacteria are present, the same pipettes are used throughout. The number of times that the cycle is repeated is pre-selected by a 12-position switch and the machine stops with the pipettes raised and containing 1 ml. of the final dilution. When the pipette holder is rotated in an anticlockwise direction to the position shown in Fig. 2 the final 1 ml. is automatically ejected into a waste tray. Figure 7 shows the machine in use.

The instrument is controlled by a sequentially operated electrical system. Post Office electromagnetic relays and solenoid-actuated rotary switches are utilized, together with microswitches which operate when the pistons of the air cylinders reach the end of their strokes. The advantage of the sequentially operated system over a timed sequence is that if one step in not completed properly, the machine will stop. The operator will thus know that something is wrong, which is most important.

It was decided not to use pneumatic control throughout because, first, a counting mechanism is required; secondly, one wanted to keep the demand of air down to a minimum; and, thirdly, this particular sequence of events is more simply achieved by electrical techniques. However, the balance is fairly evenly divided and a pneumatically controlled system has its advantages, particularly if an unlimited supply of compressed air is available.

THE USE OF COMPRESSED AIR

It may be argued that pneumatic equipment, as used in this apparatus, requires a supply of lubricated compressed air that is often not readily available in pathological laboratories, whereas an electrically actuated system would require only the a.c. mains which is invariably available. At first this may appear to be an overriding consideration, but modern compressors are clean, compact and reasonably quiet and it is a simple matter to have one (cost of the order of £150) suitable for operating many pieces of equipment and to pipe the air to various points in the laboratory, as is commonly done in industrial and university laboratories today: if this practice becomes widespread in pathological laboratories, designers of medical laboratory equipment will be able to use, to considerable advantage to all concerned, a vast range of equipment rarely considered hitherto because of lack of air supplies.

We have shown that a practical alternative source of compressed air is the 110 cu. ft. cylinder: the machine is used exclusively in the laboratory and there is little difficulty in obtaining and handling the number of cylinders required.

COMPARISON OF PERFORMANCE OF MACHINE WITH MANUAL METHOD

It is a simple matter to test the accuracy with which 1 ml. of liquid is transferred from one dilution tube to the next, but this operation is only one of many possible sources of inaccuracy in the serial dilution technique; poor mixing of the solution and diluent is another obvious source. In order to make a useful comparison between hand-made and machine-made series of dilutions, it was decided to obtain a measure of the concentration of the solution left in each dilution tube at the end of the procedure. We diluted a solution of Armour bovine albumin powder fraction V in distilled water and measured the optical

density of each dilution at 2,800Å on a Unicam S.P.500 spectrophotometer (using 0·5 ml. silica cells, a 12 × 4 mm. slit and collimating lens).

To ensure that any differences in the results were due primarily to those parts of the technique performed by the machine, namely, transferring the solution and mixing it with diluent, very great care was taken to eliminate errors due to inaccurate dispensing of the initial volumes of both solution and diluent. Also, the same initial solution of albumin was used for all tests: inaccuracies in preparing this solution, which are important when an absolute determination of concentration is to be made, are comparatively unimportant when comparing results as here.

It was found to be impractical to measure more than four dilutions of a series because, first, of doubts about the linearity of the calibration curve at intermediate concentrations; secondly, its slope is very small at high concentrations; and, thirdly, the initial solution (dilution 1) would have to be much more highly concentrated and would, therefore, be far too viscous. However, if one obtains satisfactory results for dilutions 3 to 6, it is reasonable to assume that dilutions 7 to 12 would also be satisfactory, provided the volume of the solution transferred remains within tolerance, as is the case with the machine.

A series of standard solutions, in the range 100 to 1,500 μg./ml., was prepared and the optical densities were measured. With a solution of 4,000 μg./ml. in the first tube (dilution 1), a series of six serial dilutions was produced and then the optical densities of dilutions 3 to 6 were measured. Twelve such series of dilutions were made on the machine and 12 were made manually by members of staff using the technique they normally adopt in their routine work. Thus, we have two sets of 12 optical density measurements for each of dilutions 3 to 6. In Fig. 8, the highest and lowest values of each set are plotted on the calibration curves. The short vertical lines indicate the calculated concentrations of each dilution. The 12 points for each dilution are not plotted but are fairly evenly distributed between the limiting points.

The points obtained from the machine-made dilutions are more closely bunched together than those obtained from the hand-made dilutions, indicating that the machine produces more consistent results. In the case of dilutions 4 to 6, the machine-made dilutions are closer to the calculated concentration than those made by hand, indicating that the machine produces more accurate dilutions than those produced manually. Machine-made dilutions 3 appear to be systematically displaced from their calculated positions. However, if the machine introduces a systematic error, dilutions 4 to 6 would be similarly displaced. This is not the case and it is reasonable to conclude, therefore, that these dilutions are more consistent than hand-made dilutions 3 but not significantly more accurate: the displacement is presumably due to a sampling error.

Independent tests to measure the speed and accuracy with which manual operators transfer the solutions from one tube to another showed that the accuracy varied considerably, being as bad as ± 7·5% in one case and as good as approximately

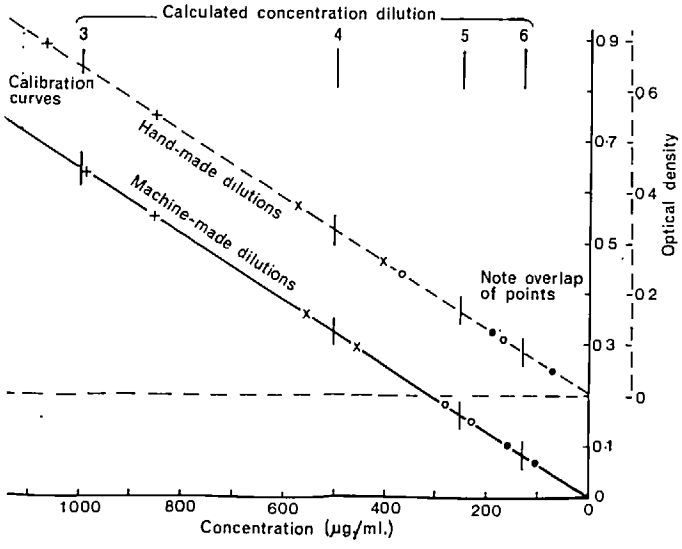


FIG. 8. *Graphs showing measured optical densities of hand-made and machine-made dilutions 3 to 6 plotted on identical, but vertically displaced, calibration curves.* +, ×, ○, ●—*measured concentrations* (μg./ml.) *dilutions 3 to 6 respectively* (*limiting points only*).

± 1% in another (Table I), whereas the machine is consistently within ± 2%. This could explain the fact that in the comparative tests described above (Fig. 8), machine-made dilutions 3 were no more accurate than hand-made dilutions 3: hand operators can perform the transfer as accurately as the machine but, being far less consistent, soon make a very inaccurate transfer, thus introducing a large error which may not be nullified.

TABLE I

SPEED AND ACCURACY OF MANUAL AND MACHINE OPERATIONS COMPARED

Operator No.	*Percentage Error in Volume Transferred*		*Time Taken to Perform One Series of 12 Dilutions*	
	Maximum Positive	*Maximum Negative*	*Min.*	*Sec.*
1	6·9	2·3	1	15
2	2·5	1·9	1	17
3	6·0	3·2	1	17
4	6·5	1·1	1	35
5	7·7	2·1	2	0
6	8·0	7·4	2	0
7	5·1	1·7	2	0
8	6·0	4·1	2	5
9	7·6	1·0	2	5
10	3·3	1·3	2	30
11	5·0	1·1	2	30
12	8·7	4·5	3	0
13	4·7	2·7	3	0
14	3·2	1·6	3	0
15	4·2	4·7	3	5
16	6·4	5·2	4	0
17	0·7	1·1	4	40
18	4·8	1·1	5	0
19	4·3	5·5	7	30

Eleven out of 19 people performed one series of 12 dilutions in under 2½ minutes (see Table I), the time taken by the machine. The operators knew why they were asked to perform the task and were doubtless trying very hard, and also they performed only one series of dilutions so that the task did not become tedious. It is reasonable, therefore, to assume that this is the best performance they are likely to achieve in the normal course of events. Since the machine performs four series of dilutions simultaneously, it will perform three or four series more quickly than they can be performed manually, assuming the preparative work takes the same time. Considerable saving of time is achieved, however, if five or more series have to be performed, because while the machine is diluting four series more dilution tubes can be prepared.

FINAL COMMENTS

Although the machine is not very sophisticated, it satisfies the criteria against which we estimate its suitability for use in a diagnostic bacteriology laboratory: it is suitable for processing small numbers of specimens, all parts that come into contact with the liquid can simply be dismantled and sterilized by heat, and it performs a task which is a necessary part of at least two routine diagnostic procedures. Although our embodiment is arranged to transfer 1 ml. of solution and to accommodate four series of 12 tubes, it is, in principle, very flexible and it may well find applications in other disciplines.

One may ask if the overall accuracy of the method is adequate. It is possible, of course, to design apparatus that performs each part of the procedure more accurately, or to design apparatus that produces a series of dilutions in such a way that errors are not cumulative. However, in either case, the apparatus would be much more complex and costly, and would probably not be economic in view of the comparatively small numbers of specimens to be processed. In the case of M.I.C.s, it is necessary to decide, therefore, whether the tube dilution method or one of the alternative methods is the most suitable in any given circumstance. If one decides to use the tube dilution method, it is necessary, as Branch, Starkey, and Power (1965) have pointed out, to standardize the procedure. A machine, such as the one described here, could well be the nucleus of such a procedure.

We are greatly indebted to the Trustees of the Nuffield Foundation who are financing the project.

The technical assistance of S. Haskey, G. Dickerson, and J. R. Robinson is gratefully acknowledged.

REFERENCES

Branch, A., Starkey, D. H., and Power, Edna E. (1965), *Appl. Microbiol.*, 13, 469.

Broughton, P. M. G. (1965). Association of Clinical Biochemists, Technical Bulletin No. 6.

Sequeira, P. J. L. (1964). Personal communication.

Weitz, B. (1957). *J. clin. Path.*, 10, 200.

ACKNOWLEDGMENTS

I am greatly indebted to the Trustees of the Nuffield Foundation, who are financing the project.

It is a pleasure to acknowledge the enthusiastic encouragement of Professor R. E. O. Williams, whose assistance has been invaluable.

The technical assistance of G. Dickerson and S. Haskey is gratefully acknowledged.

COMMUTATING SWITCH - LEDEX II.

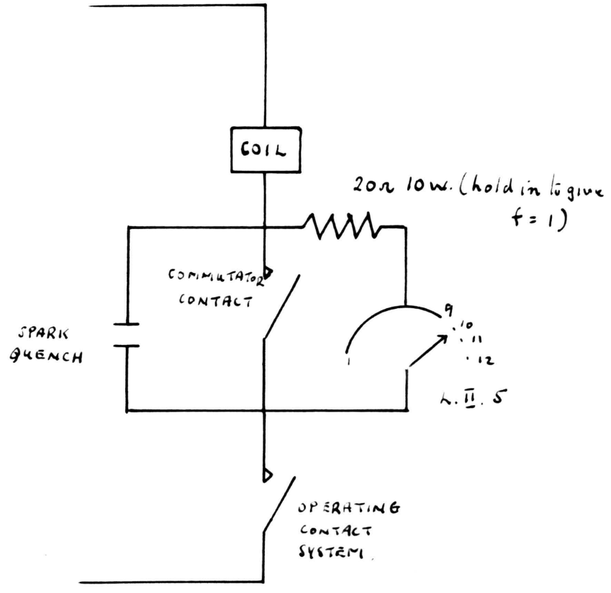


Fig. A4. LEDEX II SWITCH

POSITION	OPERATION	L. II. 1-S	2-S	3-NS	4	5-S	6	7-S	8-S	9-S	10-S	11-S	12-S	13-NS	14-NS	15-NS	16-NS
1	EJECT		X					X						X	X		
2	SUCK UP	X	X						X					X			
3	EJECT		X					X						X			
4	SUCK UP	X	X						X					X			
5	RAISE PIPETTES.	X								X				X			
6	ROTATE RACK	X		X								X	X				
7	LOWER PIPETTES	X	X								X	X	X	X		X	X
8	EJECT		X					X						X			
9	SUCK UP	X	X						X					X			
10		X	X						X					X			
11		X	X						X					X			
12		X	X						X					X			

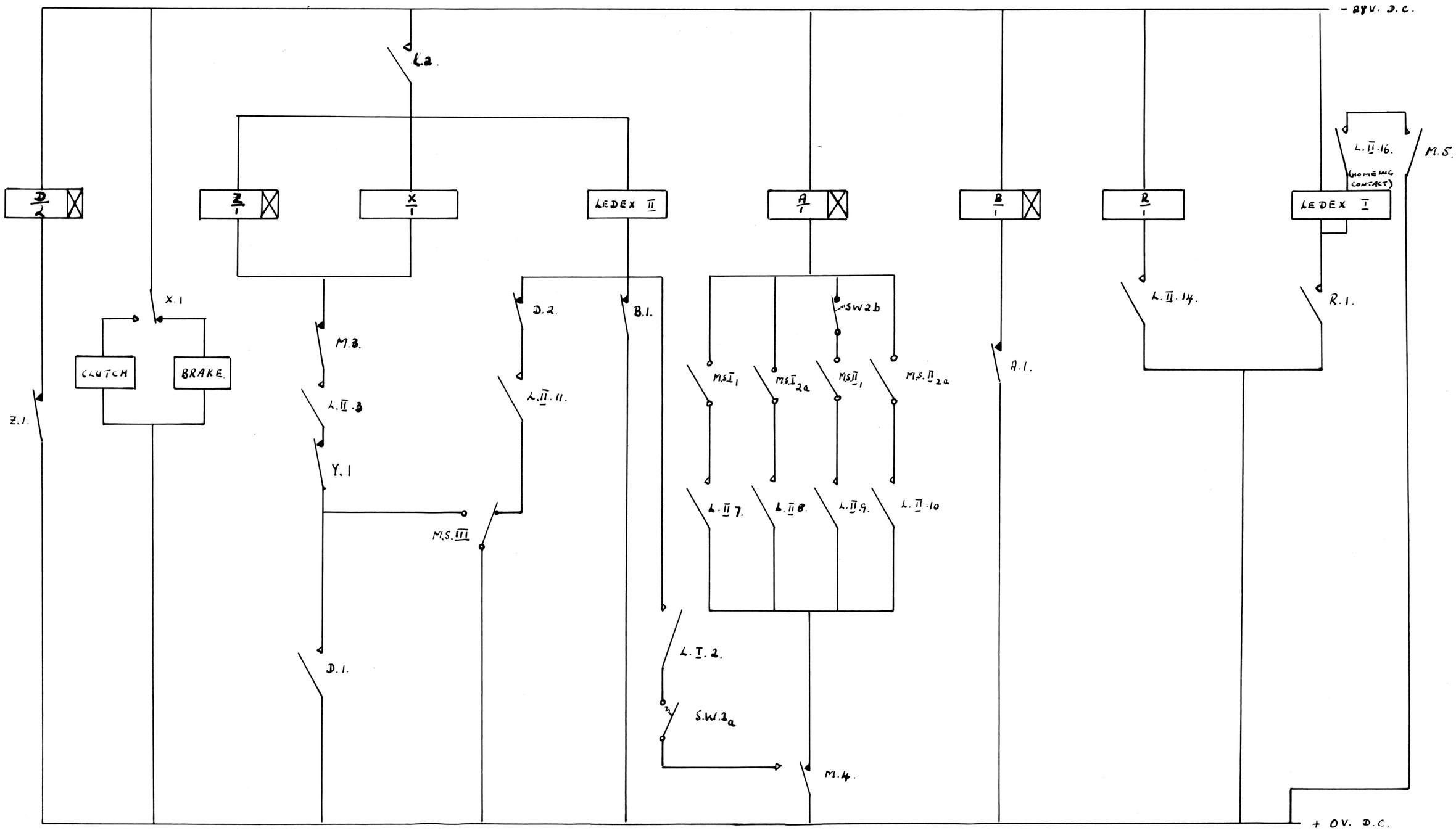
6 - STOP
7 - START

10 - HOME TO POSITION 1

X = CONTACT CLOSED

S. = SHORTING CONTACT
NS. = NON-SHORTING CONTACT.

SERIAL DILUTER CONTROL CIRCUIT.



MICRO-SWITCHES.

M.S.$\underline{I}_1$ ≡ on "diluting" cylinder - upper end

M.S.$\underline{I}_{2a,b}$ ≡ " " " - lower end.

M.S.$\underline{II}_1$ ≡ " pipette holder cylinder - upper end.

M.S.$\underline{II}_{2a,b}$ ≡ " " " " - lower end.

M.S.$\overline{III}$ ≡ " grooved disc.

M.S.$\overline{IV}$ ≡ " pipette holder.

SWITCHES.

S.W.1 ≡ prepare

S.W.2 ≡ raise pipette holder

S.W.3 ≡ Select no. of dilutions

S.W.4 ≡ Stop.

S.W.5 ≡ Start.

S.W.6 ≡ MAINS (PRESS UNTIL NEON LIGHTS)

LEDEX $\underline{I}$ ≡ 18 Positions - 8 WAY (NOS. 6 to 8 NON-SHORTING)

LEDEX $\underline{II}$ ≡ 12 " - 18 WAY (NOS. 13 to 17 " ")

AIR SOLENOID I ≡ "DILUTING" cylinder control.

AIR SOLENOID $\underline{II}$ ≡ Pipette raise/lower cylinder control

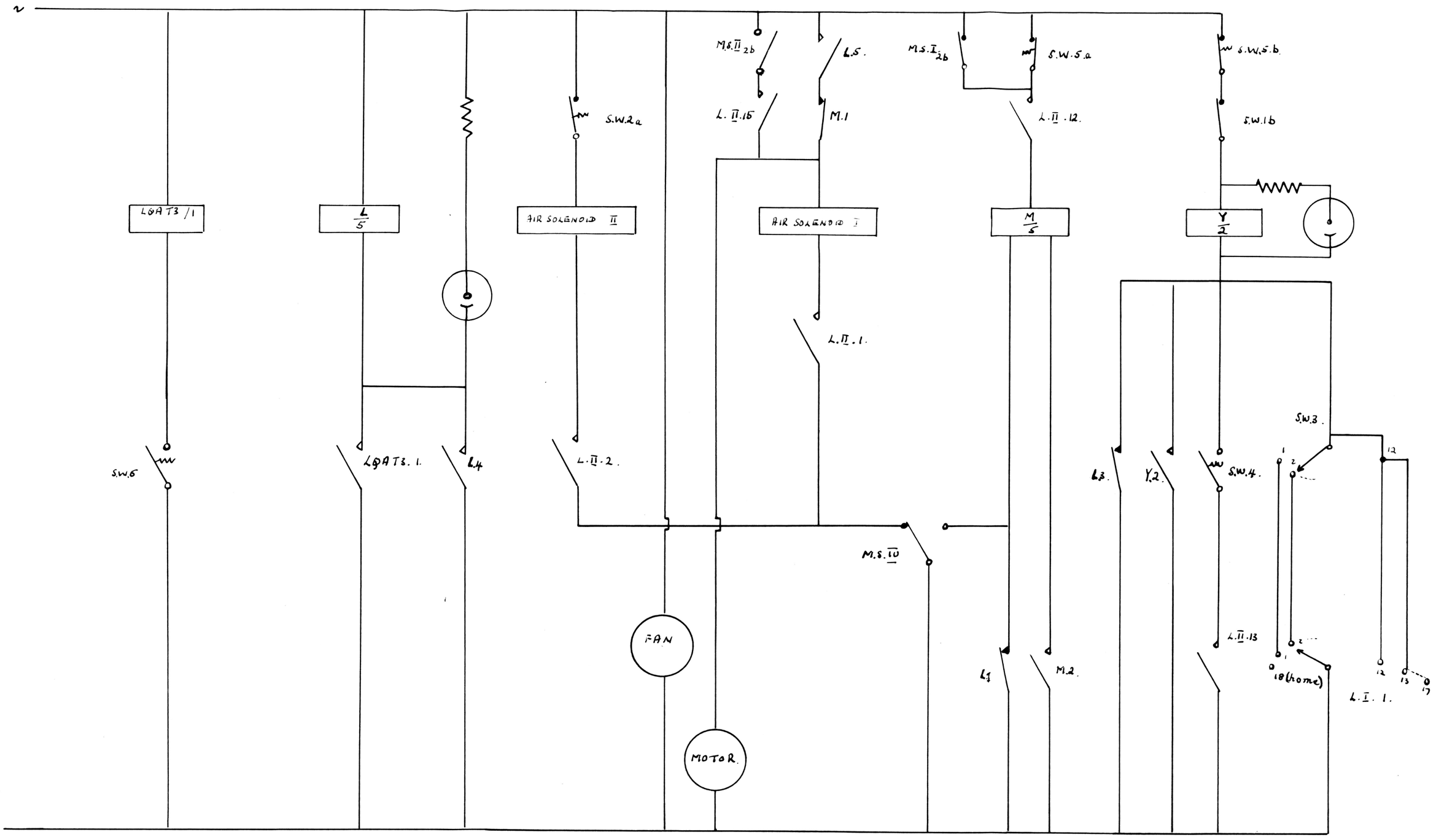
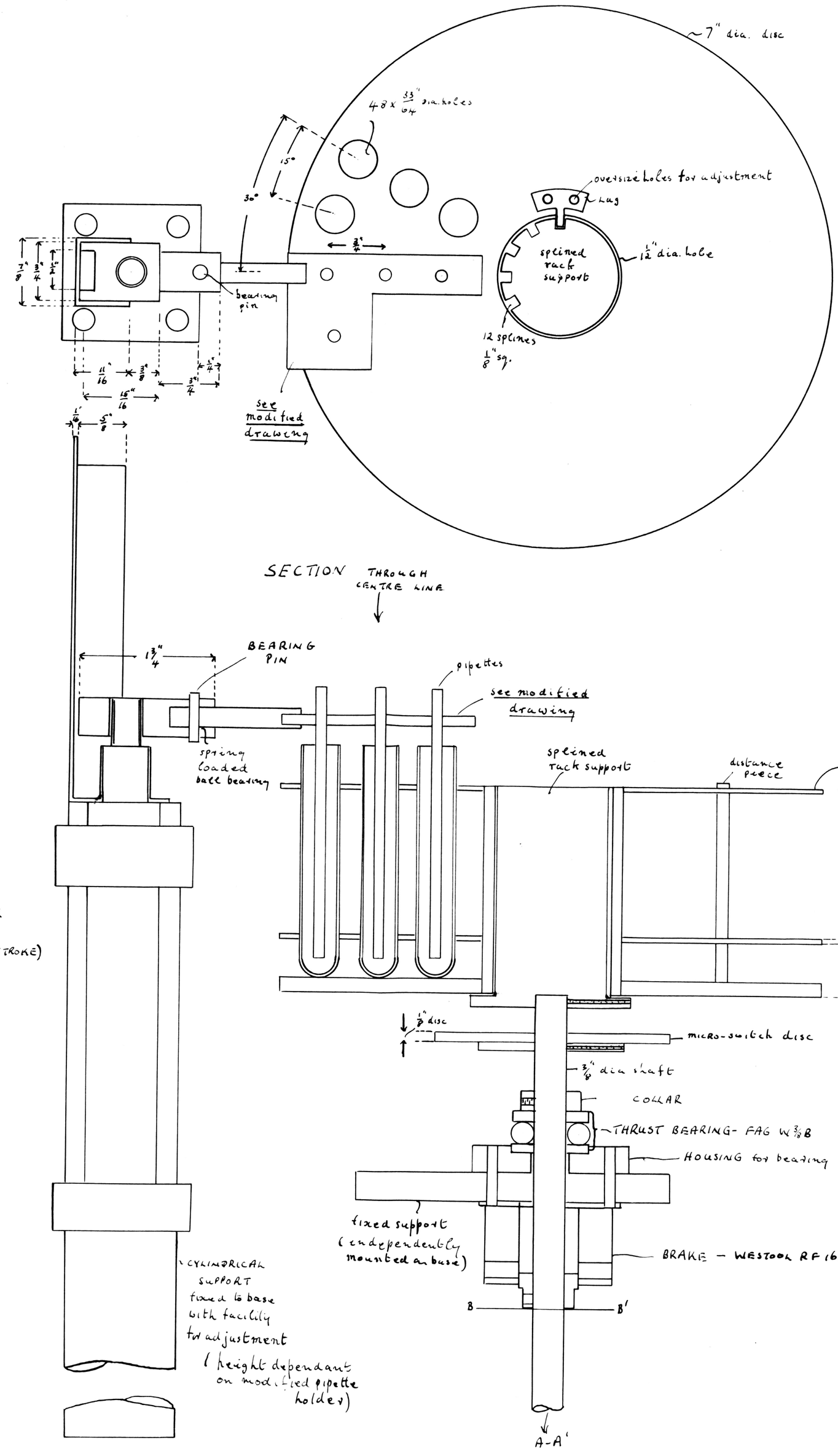


Fig. A5 SERIAL DILUTER CONTROL CIRCUIT.

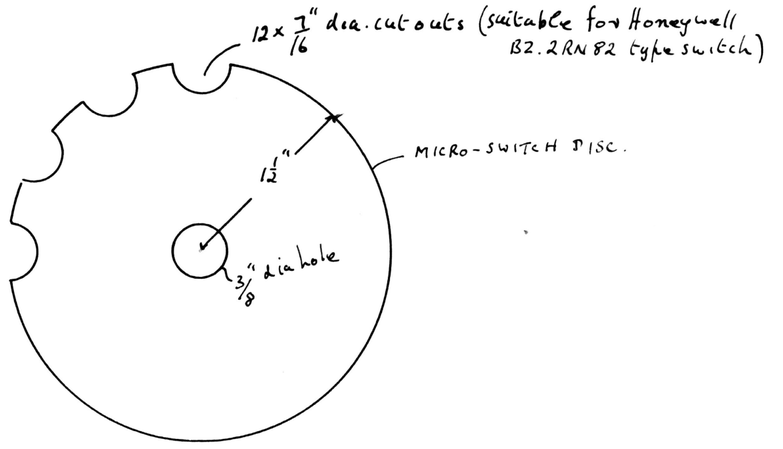
R E. TROTMAN PH. D.

SERIAL DILUTING APPARATUS

R.E.T. 4/5/67.



PIPETTE RAISE/LOWER MECHANISM.



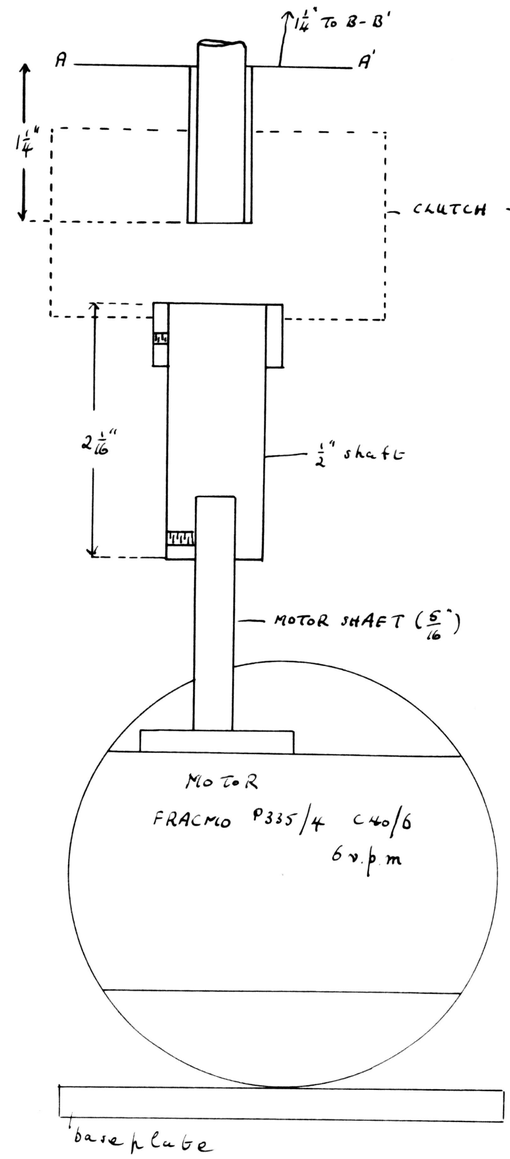
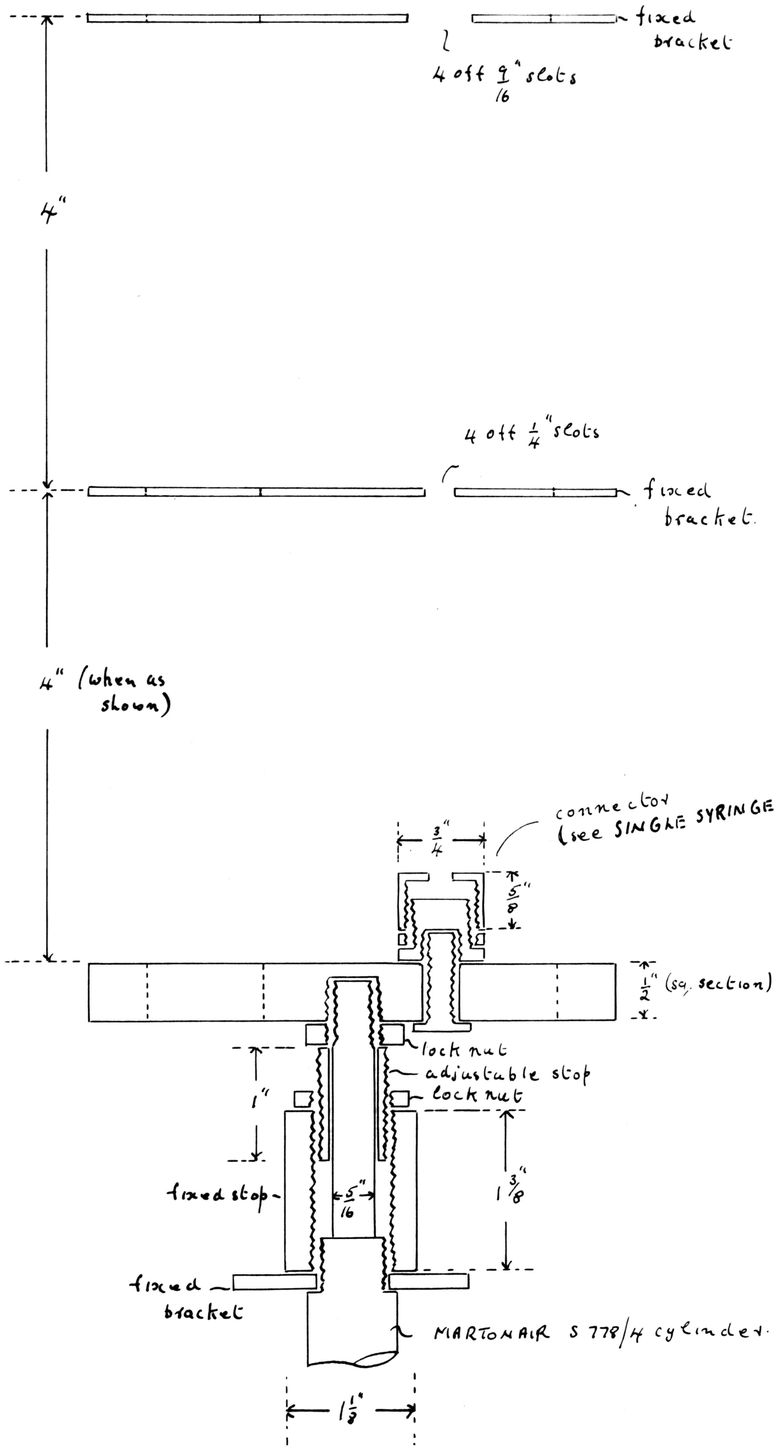
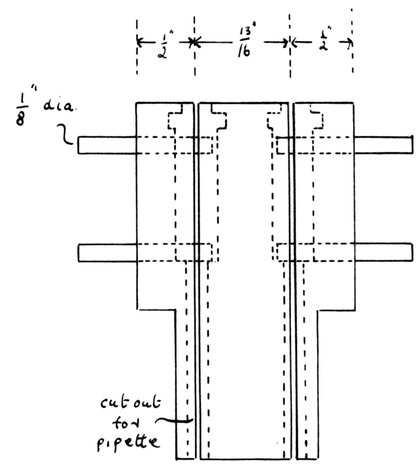
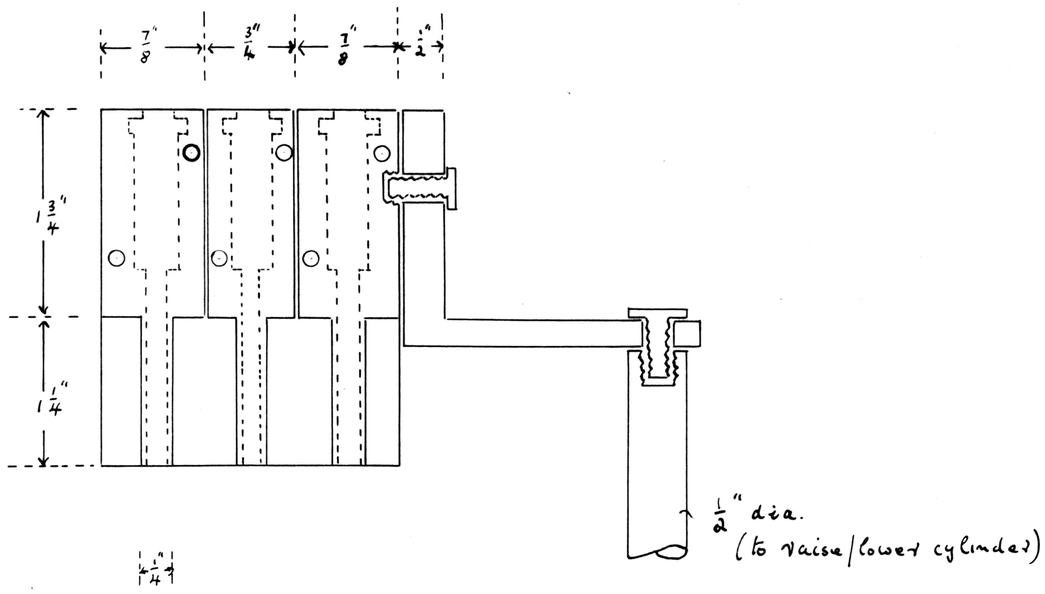


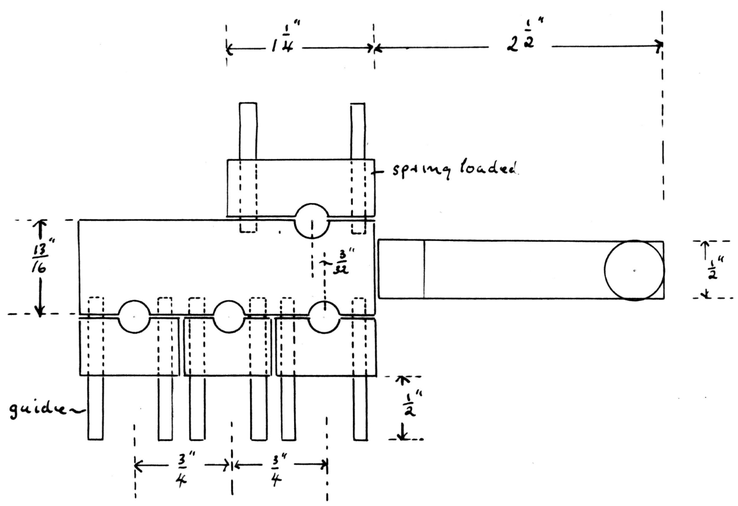
Fig. A6. ROTATING TABLE MECHANISM



SYRINGE SUPPORT and DRIVE MECHANISM







PIPETTE HOLDER
(MODIFIED DRG.)

SERIAL DILUTING

APPARATUS

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Fig. A7.