VOL. 5 - NO. 2 APRIL, 1950

JOURNAL OF THE NEW ZEALAND ASSOCIATION OF BACTERIOLOGISTS

EDITED, PRINTED AND PUBLISHED FOR THE ASSOCIATION

BY

DOUGLAS WHILLANS

CONTENTS.

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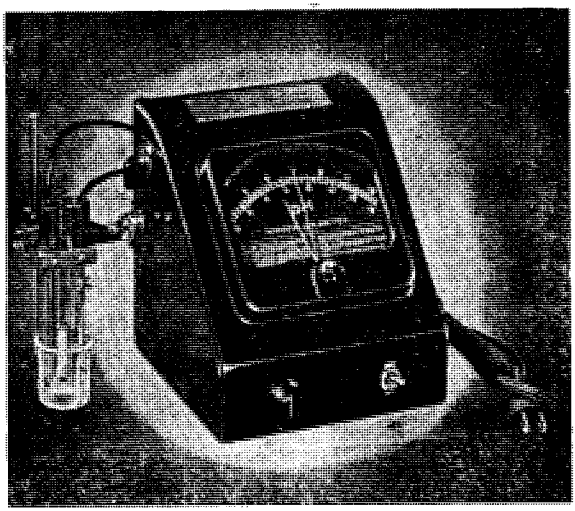

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JOURNAL
of the
NEW ZEALAND ASSOCIATION OF BACTERIOLOGISTS

Vol. 5., No. 2. APRIL, 1950

THE ELUSIVE TUBERCLE BACILLUS

A. J. Samuel (*Medical School, Dunedin*).

Introduction.

It should be possible to attain an enormous improvement over direct smear examination—by a factor of, say, 10,000 times —if the utmost use is made of the unique properties of the Tubercle Bacillus—acid-fast staining and resistance to acid or alkali.

Pooling of specimens, efficient homogenisation, concentration, a fluid enrichment medium, and fluorescence microscopy—either direct examination of the concentrate or of the micro-colonies after 3 to 10 days—would be the ideal.

Valuable methods have been published for most of these procedures, and we have to remember when deciding to adopt any method, that a saving of time will enable us to do more work and thus obtain a two-fold advantage. In practice, some of the procedures are inter-related with others, and it is not always practicable to adopt what appear to be the best methods at every stage. In Dunedin, we have been concentrating in recent months on fluorescence microscopy and a fluid enrichment medium. Trials of the medium have not yet been completed, so a report on this will be held over for a later issue.

Pooling of Specimens.

Pottenger (1) has stated that, if specimens of sputum taken on three successive days are pooled, the chance of finding Tubercle Bacilli is double that of finding them in one specimen. Note that the volumes of sputum examined in each case are the same. That is, it is not necessary to examine the whole pooled specimen to obtain this gain. Naturally, if the entire pooled specimen can be examined, this will lead to a further gain. A great deal of time can be saved by examining a pooled specimen rather than a series of three specimens individually.

Homogenisation.

"Good mechanical agitation cannot be over-emphasised, since it minimises drastic chemical treatment." This is from a report on evaluation of laboratory procedures to the American Tuberculosis Association. (2) Some American Laboratories have used a paint conditioning machine for this purpose. (3) This is a shaker with much the same speed and throw as a Kahn shaker, fitted with spring-loaded end-pieces so that the containers of various shapes may be quickly slipped in and out without clamping.

In Dunedin, we have used with some success, continuous, slow, end-over-end rotation of the bottles so as to get a slopping action. In the over-night incubation with tri-sodium phosphate method we use, this mixing is sufficient to liquefy the most tenacious specimens, but the speed of rotation is critical. A slower or faster rotation does not produce the slopping action. We came to the conclusion that violent shaking for short periods at intervals during the treatment would be the best. Continuous violent shaking would, of course, destroy the acid-fastness of many of the Tubercle Bacilli, and would probably kill a high proportion. Recently this intermittent method has been reported on favourably by Keningale, (4) who was using brief treatment with sodium hydroxide. He found that contaminants were markedly reduced by this method. However, the real purpose of homogenisation is to ensure that there will be an even distribution of Tubercle Bacilli in the specimen, for whether the final process be microscopic examination or culture, the whole of the deposit may not be used, and uneven distribution of bacilli will then result in fewer positive results than would be obtained if the bacilli were distributed according to the laws of chance.

The tendency of Tubercle Bacilli to form clumps and pellicles is well known, and these characteristics are of profound importance in diagnostic tests, as well as in quantitative biochemical work. Firstly, a clump of bacilli is no easier to find in general than a single organism, therefore a significant increase in the possibility of finding Tubercle Bacilli in a specimen can be obtained if the clumps can be broken up into single bacilli, and this gain will be in direct proportion to the size of the clumps, and will be effective both in culture and microscopic methods. The gain to be expected in sputa would seldom be less than a factor of two, and might be much higher. Hunter (5), in comparing strains of acid-fast organisms suspended in distilled water, and inoculated in serial dilution, found that a strongly clumping strain required 10,000 times the wet weight of organisms in the smallest inoculum giving growth, over the wet weight required in the case of a strain exhibiting no clumping. The gasoline emulsion method is reported (6) to be most effective in breaking up clumps, but no mention is made of the viability or otherwise of the organisms so treated. Surface-active agents might also be effective in breaking up clumps without killing the organisms.

Concentration.

Methods of pooling specimens, homogenisation, and concentration are closely inter-related, for if drastic chemical or physical methods are used, then a careful watch must be kept on the various steps, and timing may be vital. A method which requires accurate timing is a nuisance in a laboratory doing a variety of work, as neutralisation or washing and centrifuging must be carried out on time, so interrupting other work. Since the Christ-

church conference in 1947, the Dunedin laboratory has been using the tri-sodium phosphate method of Corper and Stoner (7). The main advantage of this method is that specimens can be treated overnight, and neutralised, examined microscopically and/or cultured in the morning.

It may be of interest to describe the set-up used in Dunedin. The 23% tri-sodium phosphate which is a saturated solution, and the 25% hydrochloric acid used in neutralisation are kept in Winchesters fitted with siphon tubes. The air inlet to each bottle is bent downwards and plugged with cotton wool, while the siphon tube has a drawn-out glass nozzle at the lower end. We have not found it necessary to use a hooded pipette here. The incrustation of tri-sodium phosphate and the creeping of hydrochloric acid at the tips keeps them sterile. The indicator solution is kept in a bottle with the dropping pipette sealed through a loose-fitting metal cap which enshrouds the neck of the bottle. The containers in which tuberculous specimens are concentrated are very important: they should hold the whole specimen and an equal volume of alkali, and should have a reliable seal—preferably a screw cap with a rubber insert, as a leaking tube can be very dangerous when being shaken or centrifuged, We have found that 1oz McCartney bottles are very convenient for this purpose: they are on the small side, and it is difficult to pour a tenacious specimen through the rather narrow neck, but against this, they have a good screw cap, and withstand centrifuging without breaking. Thus the specimen has only to be transferred once to the bottle, and no other transfers need be made until the deposit is inoculated. This reduces the chance of contamination and risk to the worker. A full 25 cc. McCartney bottle is the same weight as a full 50 cc. centrifuge tube, so the maximum speed allowed on the International Centrifuge for the 8-place head with 50 cc. tubes holds also for the McCartney bottles, which fit the 50 cc. cups.

Centrifuging.

Little is known of the fate of Tubercle Bacilli in the centrifuge, but this is an essential part of almost all concentration methods, and the sooner we know with certainty how to retrieve a small number of Tubercle Bacilli from a large volume of fluid the better. Silverstolpes (8) has reported that young, actively growing T.B. in cerebro-spinal fluid are to be found in the surface layer after centrifuging, whereas the older bacilli are to be found in the deposit. He uses a special centrifuge tube with a narrow neck closed by a rubber stopper at both top and bottom of the tube, and examines the surface layer and deposit separately. He claims that the ratio of bacilli found in top and bottom is an index of the success of the treatment; an excess of bacilli in the upper layer indicates ineffective treatment, while an excess of bacilli in the deposit indicates that treatment is successful in

preventing growth of bacilli.

A bulky flocculent deposit is much more effective in carrying down the bacilli than is a fine granular one, and this leads to a compromise where microscopic examination or culture on solid media follow. However, a bulky deposit is no advantage in a fluid medium, especially if, as in the alum flocculation method, the deposit may be redissolved after growth has taken place, by making the fluid alkaline.

The need for a method of detecting small numbers of Tubercle Bacilli in cerebro-spinal fluid rapidly, and with certainty, is desperate, whereas a few years ago the question was almost academic. Perhaps the most dramatic way of expressing this problem is this: If someone handed us several cubic centimetres of Cerebro-spinal fluid in which he had put, with the aid of a micro-manipulator, a single Tubercle Bacillus, could we guarantee to find that bacillus? If the bacillus was viable, could we guarantee to produce a diagnostic result, and how long would it take?

If we made a smear and failed to find the bacillus in fifteen minutes' search with the 1/12 objective, the organism could well be on the slide, but it would take about 15 hours to search the whole area of the smear. Since this cannot be done, the organism may remain on the slide, undiscovered, while we attempt to culture it from the remainder of the deposit. While we are attempting to transfer the deposit to one or more culture tubes, the risks of leaving the organism stranded on the wall of a pipette or burning it off on a loop are unknown, but could be formidable. The "spider-web" clot can result in the loss of many bacilli, for, once the clot is entangled in a wire loop, it is almost impossible to get the entire clot free from the loop, except, perhaps, in making a smear on a heated slide.

Fluorescence Microscopy.

As mentioned above, search of an entire smear with an oil immersion lens would take about 15 hours, so that if we ordinarily search a slide for five minutes, we could at once obtain a gain of 180 times in the chance of finding Tubercle Bacilli if we searched the whole smear. If the Tubercle Bacilli could be rendered self-luminous and bright enough, we could see them at much lower magnification, and, at this lower magnification, we could search, in the same time, a larger area of the smear. The area in one microscope field varies indirectly as the square of the magnification, so that, by using a magnification of 100 instead of 1,000, we could search 100 times the area in a given time, and it would become feasible to search the whole of a smear. Fluorescence Microscopy (F.M.) attempts to solve this problem. The theoretical advantages are so great, that it must be made to work in practice. Unfortunately, many papers on this method have not analysed the exact requirements in light

sources, filters, contrast, and optimum magnification, and some authors have made the method appear delightfully simple.

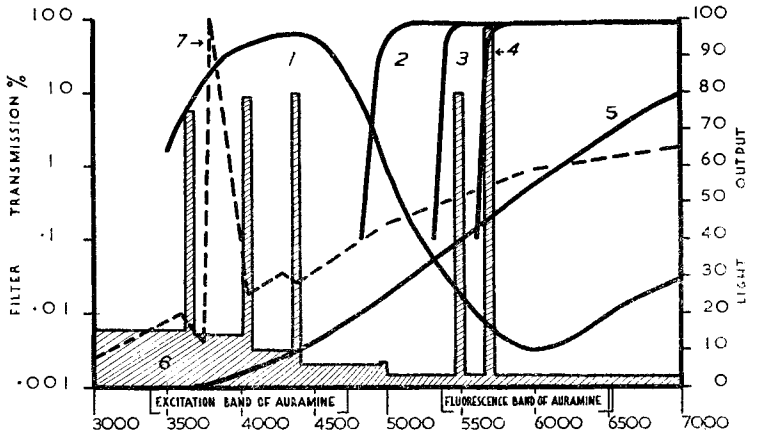
The principle of F.M. as applied to T.B. is this: The slide is stained with Auramine (9) cold, or with Auramine and Rhodamine (10) in which case the stain must be warmed to 70° C., it is decolourised in weak acid alcohol, and a "counter-stain" of Potassium Permanganate is applied for 20 secs., to destroy the fluorescence of the non acid-fast background. The slide is then examined with a powerful illuminant filtered to give only blue and violet light, through an eye-piece filter designed to remove all the blue light and let through to the eye only the yellow or orange light from the fluorescing organisms. The ideal picture would be one in which the organisms were seen sparkling like stars in the sky, but seen as definite rods, against a completely black background. Even with high brightness of the organisms and good contrast against the background, a magnification of 100 is hardly sufficient. At this magnification the bacilli are seen as definite rods, but are just on the limit of resolution of the human eye. They are easily recognisable in a known positive smear if they are present in large numbers, but when the number is reduced to one organism per field, or if the specimen is an unknown, one becomes uncertain, as there are always a few artifacts which stain brightly and may simulate a short bacillus or small clump. Due to the curious custom of supplying microscopes for medical work with 2/3in., 1/6in. and 1/12in. objectives only, we have not been able to try a high aperture 1/3in. objective and a 10 x eye-piece which would appear to be the ideal combination, giving a magnification of 200, passing more light than the other systems at the same magnification, and giving a sharp image with a good depth of focus. There is one point to watch with fluorite or apochromatic objectives, and that is the fluorescence of the objective itself in the intense blue illumination. This must be avoided, even if it means using a lower aperture objective of the achromat type. In searching a slide for fluorescent organisms the optimum magnification depends on instant recognition of the organism. If a higher power must be swung on for confirmation, time is wasted. If too high a power is used in the first place, then the time to search a given area is needlessly increased. Having fixed the magnification, we can calculate the power of the illuminant required if the efficiency of the stain in converting violet light to yellow light is known. Graham (11) found that the conversion efficiency of Auramine was of the order of .02%, and that the excitation band of Auramine was from 3,400 A to 4,700 A with a broad minimum at 4,500 A. Only the most intense light sources will give sufficient illumination in this band. In Dunedin we have compared a 5 ampere A.C. carbon arc, 750 watt movie projection lamp run at its full voltage, 125 watt Black Mercra, and 250 watt mercury vapour

lamp as used by Clegg and Foster-Carter (12). The A.C. arc is poor, although a high intensity D.C. arc would be the best illuminant possible for this work (13). The 750 watt filament lamp is good, but the life is short, and it needs forced cooling. The Black Mercra lamp gives 95% of its output in the 3,600 A line which is on the edge of the excitation band of Auramine, but if a filter of ammoniacal copper sulphate is used to remove the excess red, the 3,600 line is also greatly reduced in intensity. The best cheap source is a Mercra or Osira lamp with the outer bulb removed. The lamp costs over £2, and the associated choke over £3. The next larger lamp, the 250-watt mercury arc used by Clegg and Foster-Carter, costs about £13, and its associated choke more than half as much. This is the best source tried so far.

Filters.

Graham (11) also determined qualitatively the transmission band of ammoniacal copper sulphate. However, it is of value to know the transmission of this solution over a wide range of intensities, and throughout the near ultra-violet and visible spectrum. Due to the very low efficiency of Auramine as a fluorescent stain, the blue light passing up the microscope will be at least 5,000 times as intense as the yellow light from the organisms. It is in the cross-over region of the substage and eye-piece filters that the transmission of the combination is most important. Adoption of dark-ground illumination or vertical illumination would make the problem simpler, by reducing the intensity of the background to negligible proportions, and enabling us to increase the transmission of the substage filter, and so to obtain more intense illumination of the organisms. Note that in the diagram the scale for the transmission of the cuprammonium and yellow filters is a five-cycle logarithmic one: i.e. the range covered is from 100% down to 0.001% transmission. The other graphs are on an arbitrary scale. The most convenient way of obtaining a Cuprammonium filter of known transmission is to make a filter cell of perspex, using strips of ⅜in. material for the edges, and thin sheet—say, 1/16in. for the two faces. It is preferable to seal the filter completely except for two small holes in the top, to cut down evaporation. Taking ⅜in. = 1 cm., and remembering the logarithm of the transmission at any one wavelength is inversely proportional to concentration, a filter of any required characteristics may be made up. (Note.—No filter solution or commercially made filter has been found to equal the Cuprammonium.) A perspex filter cell will not stand the heat of a 750 watt incandescent lamp without buckling, but is quite satisfactory with mercury lamps up to 250 watts. If the solution becomes murky with a deposit of Copper Hydroxide which may cling to the walls, this may be removed with dilute HCl, rinsing with water before and after.

Light Sources and Filters for Fluorescence Microscopy of Tubercle Bacilli.



1. Ammoniacal Copper Sulphate containing 5% of $CuSO_4 \cdot 5H_2O$.
2. Ilford Delta filter.
3. Tartrazine filter. = Wratten Flavazine filter 16.
4. Ilford Micro 5 filter.
5. Spectral distribution of light from Tungsten Filament at 2850 deg. K.
6. Spectral distribution of light from B.T.H. M.E. 250 compact source mercury vapour lamp.
7. Spectral distribution of light from arc with pure graphite electrodes.

Notes.

Filter transmission is on a five-cycle logarithmic scale.

Light output is on a linear scale in arbitrary units.

A tungsten source at photoflood temperature—3400 deg. K. gives a much greater proportion of light in the excitation band of Auramine.

Excitation band of Auramine has a maximum at 4500 A., although it extends as shown from 3400 to 4700 A.

Fluorescence band of Auramine has a maximum at 6000 A.

Rhodamine is unique in that fluorescence is excited in it by light of any wavelength.

Eye-piece Filters.

The requirement here is that the filter should be entirely opaque to blue and green, but should pass the yellow and orange without appreciable change. Fortunately, there is a range of yellow and orange filters which are, on the whole, satisfactory. Ilford Delta has been advised by several authors. This filter passes too much of the yellow green. The best filter tried so far, is a tartrazine filter, which corresponds to the Wratten 16 Flavazine recommended by Mackie and McCartney in their latest edition. This filter does not alter the colour of the fluorescing organisms, whereas Ilford Micro 5 cuts the yellow and reduces the brightness of the Auramine stained organisms. If used in conjunction with the Rhodamine-Auramine stain of Hughes the Micro 5 does not alter the yellow-orange fluorescence of T.B.

But it alters the pale yellow fluorescence of the background to orange, thus depriving the Hughes stain of its main advantage —that of differentiating between T.B. which stain a bright golden orange, and other organisms which may retain a pale yellow-green tint. It can readily be imagined that a yellow filter which is expected to absorb enormous quantities of blue light, might very well fluoresce of its own accord, and this effect is one to guard against. The Delta and Tartrazine filters are very good in this respect, as their fluorescence is negligible if the filter is placed on the diaphragm of the eye-piece. The Micro 5 fluoresces strongly, and requires a light yellow such as the Delta beneath it to prevent this. Any filter shou'd not be mounted with balsam, as this fluoresces. It is much better to use the plain gelatine. The Tartrazine filter we have used was made by soaking a piece of unexposed photographic plate in a 4% solution of the crude dve in water after the plate had been fixed out in hypo and cleared with ferricyanide and plain hypo. With high-power eye-pieces, the surface of the gelatine filter may give rise to an imperfect image, if the gelatine surface is in focus. This may be obviated if the gelatine is put below the eye-piece diaphragm either in the eye-piece or in the draw-tube.

Having covered the choice of magnification, light source, filters and stains, we now have to consider the optical arrangement of the illumination. Due to the inefficiency of the system as a whole, it is essential that no pains be spared in obtaining the maximum light on the slide, on one hand, and the minimum blue light to be filtered out on the other. Most authors have recommended that fluorescence microscopy be carried out in a darkened room. I would say that, if you have a convenient dark room, by all means use it if possible: if a dark room is not used, two precautions must be taken. The first is to prevent room light from falling on the slide or the mirror, as this light will come up the microscope, and markedly reduce the visibility of the organisms. The second is to reduce the stray room light at the eye-piece so that the eye is not trying to see a dim image while receiving bright light from outside the microscope. A hood built round the microscope will obviate both these difficulties. The light source should be enclosed, with its condenser so that only a beam of blue light emerges. The Kohler system of illumination is the best for normal work. In this system, a lens near the illuminant produces an image of the lamp on the substage condenser. The condenser is so focussed that the surface of the lens is in focus in the microscope. When correctly adjusted this system gives an even illumination over the whole low power field. It is advisable to use a microscope whose mirror can be slid up and down so that the rest of the optical system can remain fixed, and the image of the light source can then be made to hit the centre of the mirror. Inexact centering of the mirror can give

rise to loss of a great deal of light.

Possible Further Improvements.

Cruikshank, (14), who was frank enough to title his paper "Towards Fluorescence Microscopy," used a slide culture technique, and examined the micro-colonies by low-power F.M. He stated that the brightness of the colonies had to be seen to be believed. I can bear this out. The clumps after seven days can even be seen with the unaided eye through a yellow filter, when in place on the microscope. This effect, which seems to be due to greater absorption of light by a clump than by the same number of bacilli scattered singly, as well as to the greater apparent brightness of a large object than of a small object of the same actual brightness, is the most valuable gain in the whole system, and I would go so far as to say that the real future of F.M. lies in the examination of micro-colonies either on slide culture, or of smears made from fluid cultures. However, this does not solve the problem of finding small numbers of T.B. in C.S.F. without delay. We must still attempt to perfect F.M., and concentration to the point where we can be certain of finding single bacilli when they are present.

Dark-ground illumination, either vertical or transmitted, as can be obtained with the Cooke microscope, would be a big step forward, but the vertical illumination would not allow ready changing of objectives. Perhaps the simplest all-round method would be to use a normal substage condenser with a central patch stop, and to use as an illuminant four of the Mercra capillary tubes in a hollow square, so that the image fell on the annular ring of the patch stop. This would give at least partial dark ground illumination, and this system could be used also with the Cooke vertical illuminator. Small elliptical or parabolic reflectors behind the mercury lamps could double the illumination. Next year we hope to develop the methods outlined still further. In the meantime, we hope to have stimulated interest in a variety of problems.

Acknowledgments.

I am indebted to Sir Charles Hercus for encouragement in this work, to Mr. S. O. Hughes, of the Physics Department, for the loan of a number of dyes, filters and books, and for much valuable advice on dyes; to Mr. J. G. Howard, of the Anatomy Department, for the loan of filters and dyes; to Mr. R. Malthus, M.Sc., of the Nutrition Research Department, for taking the transmission curve of Cuprammonium; and to many of the staff of the Bacteriology Department for assistance.

Summary of Methods.

Auramine.

Auramine "O" Gurr.	0.3 gm.
3% Phenol	100 ccs.

Warm to 40° C., shake to dissolve, and filter. Use

stain cold for at least five minutes.

Auramine-Rhodamine.

Auramine O.S.	0.4 gm.
Rhodamine B.S.	0.2 gm.
Liq. Carbolic	4 ccs.
Glycerin	30 ccs.
Water	66 ccs.

Add dyes to liquids, bring to boil and cool. To use, warm stain to 70° C., not more; pour on to slide and allow to cool.

Acid Alcohol.

Alcohol	75 ccs.
Water	25 ccs.
Concentrated HCl	0.5 cc.
NaCl	0.5 gms.

Use two changes for 1½ minutes each or less.

"Counter Stain."

Potassium Permanganate 0.1%

After decolourising and rinsing the smear, pour on the Permanganate—leave for not more than 20 seconds, rinse and dry.

Cuprammonium Filter.

1 cm. of an ammoniacal solution containing 5% of $CuSO_4$. $5H_20$.

Eye-piece Filter.

Wratten 16 gelatine or cleared photographic film or plate soaked in 4% Tartrazine.

Illuminant.

B.T.H. 250 watt Box-type lamp M.E. 250, or "Mercra" or "Osira" 125 watt lamp with outer bulb removed.

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RAISED SERUM ACID PHOSPHATASE DUE TO HAEMOLYSED RED CELLS IN SERUM

H. E. Hutchings.
(Pathology Department, Palmerston North Hospital.
Prize technical essay, 1949.)

Contained in the Journal of Clinical Pathology, January, 1948, is an article by Abdul-Fadl and E. J. King on the inhibition of acid phosphatase by the use of formaldehyde, and its use in the determination of serum phosphatase. They suggested that the use of formaldehyde be adopted in routine methods. The following experiments were done to confirm that acid phosphatase, derived from haemolysed red cells, was eliminated by including formaldehyde in the buffer substrate. A commonly used method was slightly modified to include the buffer substrate suggested by Abdul Fadl and King. Reagents required:—

Phenol reagent of Folin and Ciocalteau:

Dissolve sodium tungstate (50 gm.) and sodium molybdate (12.5 gm.) in water (350 cc.), add 85% Phosphoric acid (25 cc.) and concentrated hydrochloric acid (50 cc.) and reflux gently in an all-glass apparatus with a porcelain chip for 10 hours. Add lithium sulphate (75 gm.), water (25 cc.), and a few drops of bromine to decolourise. Boil without condenser for 15 minutes to remove the excess bromine, cool and dilute to 500 cc., filter and store in a tightly stoppered bottle and keep free from dust. There should be no greenish tint. The solution is diluted 1:3 just prior to use.

Stock Standard Phenol Solution.

Dissolve 1 gm. crystalline phenol in 0.1 N. HCl, and dilute to a litre with 0.1 N. HCl. To 25 cc. of this solution in a 250 cc. flask add 50 cc. 0.1 N. NaOH. Heat to 50° C. then add 25 cc. 0.1 N. Iodine solution. Stopper and allow to stand 30-40 minutes at room temperature. Add 5 cc. conc. HCl and titrate excess iodine with 0.1 N sodium thiosulphate. Each cc. of 0.1 N iodine solution removed = 1.567 mgm. phenol. Calculate the strength of the phenol and dilute the solution so that 1 cc. contains 1 mg. phenol. (This solution will keep indefinitely.)

Diluted Stock Phenol Solution.

Dilute 1 part to 10 with distilled water (this will keep 3 months only).

Working Solution Standard.

Take one part of diluted stock Phenol solution, 3 parts diluted phenol reagent and make up to 10 parts of distilled water. (This solution should be made fresh daily.)

Acid Buffer.

26 gms. tri-sodium citrate and 8.5 gms. citric acid made up to 1 litre. (pH 4.9.)

Substrate.

M/50 disodium Phenyl phosphate 4.35 gms. in 100 cc. distilled water.

Sodium Carbonate.

20% solution.

Formaldehyde Solution.

2% solution. 5 cc. 40% formaldehyde made up to 100 cc. in volumetric flask.

Buffer Substrate (as for use, made fresh daily).

2 parts acid buffer
1 part substrate
1 part formaldehyde solution
(Substrate diluted 1 in 4 becomes a M/200 solution).

Method.

Into each of two marked centrifuge tubes put 4 ml. of Buffer substrate and bring to a constant temperature in 37° C., water bath (3-5 minutes). Add to the test tube 0.2 ml. serum and allow both tubes to remain in bath for exactly one hour.

At the end of one hour immediately add to the control tube 0.2 ml. of serum and then to both add 1.8 ml. Folin Ciocalteau (diluted) reagent. Mix and centrifuge.

Colour Development.

Put 4 ml. of filtrate from "Test," "control," and Standard Phenol solution and reagent, into similarly marked tubes. Add to each 1 ml. 20% sodium carbonate and reimmerse in water bath for ten minutes.

Let X be the reading of the standard matched against the test, set at 15 mm. on the colorimeter, and Y be the reading of the standard matched against the control, also set at 15 mm. Then 2 (X—Y) = the serum acid phosphatase present as King-Armstrong units.

A unit is defined as that degree of phosphatase activity which at pH 4.9 and 37° will liberate from the specified buffer-monophenylphosphate substrate dilution 1 mgm. of phenol in one hour.

Experiment 1.

A sample of citrated human blood was obtained and centrifuged. The plasma was removed and the packed cells washed three times with saline. A sample of red cells free from plasma

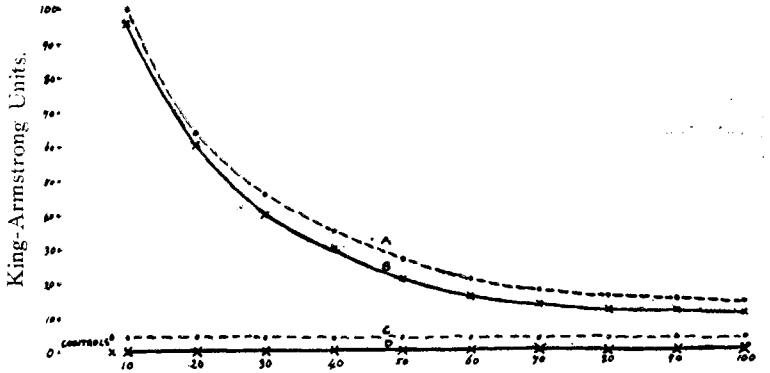
and therefore free from " plasma" phosphatase was thus obtained.

(a) Using the packed cells, above, a series of ten dilutions in saline was made by volume ranging from 1 in 10 to 1 in 100. On each of the ten dilutions an acid phosphatase content was estimated substituting distilled water in the buffer substrate for 2% formaldehyde, i.e., estimating the acid phosphatase due to red cells. The red cells were immediately haemolysed on the addition of the suspension to the buffer substrate, freeing the phosphatase in the cells.

(b) On each of the ten dilutions used in (a) an acid phosphatase estimation was repeated including, this time, 2% formaldehyde in the buffer substrate.

A control estimation was performed on saline alone.

The results are charted on the graph. It can be seen from this that the inclusion of 2% formaldehyde in the buffer substrate of the method eliminates the total acid phosphatase due to red cells.



Solid lines are red cells in saline, and dashes red cells in human serum, being 1 part in 10, 1 part in 20, etc.
A and B without formaldehyde. C and D with formaldehyde.

Experiment 2.

Using human group "O" red cells, washed and packed as in Experiment 1, a series of ten dilutions was prepared by using compatible serum as a diluent. Every care was taken to ensure that this serum contained no free or haemolysed red cells.

Two sets of estimation were done on each series of ten dilutions. One, omitting 2% formaldehyde in the buffer substrate of the method, and one including the formaldehyde, as in Experiment 1 (a) and (b).

The result of each estimation was charted and can be seen on the graph. A control estimation, omitting the formaldehyde from the method was done on the unhaemolysed diluting serum.

From graph 2. by comparing the curves with the result of

the control it can be seen that by inclusion of 2% formaldehyde in the buffer substrate of a routine method the "false" acid phosphatase due to red cells would be eliminated and the acid phosphatase found in serum would be unaffected, thus giving a constant true result.

Experiment 3.

A minute amount of an emulsion of prostate tissue was added to the serum used in Experiment 2. The estimation of acid phosphatase on this gave the result of 64 King-Armstrong units. The presence of 2 % formaldehyde in the method also gave a result of 64 King-Armstrong units. Despite further addition of red cells the result remained at 64 King-Armstrong units, when formaldehyde was *included* in the method.

This seemed to suggest that formaldehyde affects only the acid phosphatase found in red cells.

Summary.

The inclusion of 2% formaldehyde has been tried out in a routine method for the estimation of serum acid phosphatase in order to eliminate false high results due to acid phosphatase derived from haemolysed red cells. The results are tabulated in graph form.

By comparing the results with and without formaldehyde it would seem that Abdul Fadl's and King's suggestion of its inclusion in routine estimations is worthy of adoption.

Acknowledgments are due to Dr. Pullar for his permission to carry out these tests.

References.

Abdul Fadl, M.A.M., and King, E. J. (1948). J. Clin. Path. (1948) 1.80.

THE ELECTRON MICROSCOPE

K. I. Williamson

(From the Dominion Laboratory, Lower Hutt).

A good visible light microscope can just resolve points in objects which are 2500 Angstroms apart, i.e., this is the smallest distance two points can be separated so that they are revealed as two distinct points. If they are closer, they appear as a single point. It is useless to magnify this image further than the size which makes these same points just resolvable by the human eye at a comfortable viewing distance. Increasing the magnification beyond this limit, although of course possible, adds no further useful information, but makes the image appear indistinct. Thus light microscopes have a useful magnification up to 1500 dia-

meters.

The reason for this limit was first given by Abbe late last century, who showed that the resolving power of a microscope depended on the wavelength of the light (and certain physical characteristics of the microscope of no concern here). Thus the shorter the wavelength of the light used, the smaller the distance between two points which can be resolved, and the higher the useful magnification. It is obvious that the first improvement in light microscopes came by using blue light and then the ultra-violet wavelengths. The ultra-violet microscope increased the useful magnification to over 2000 diameters.

Following the discovery that the paths of electrons could be altered by magnetic and electric fields, it was shown that there was almost a complete analogy between light rays in their passage through glass lenses, and electron paths in electrostatic or electromagnetic fields of certain configurations. The discovery was also made that any material particle in motion had associated with it a characteristic wavelength. Electrons moving with a velocity acquired by falling through a potential of 60 Kv have an effective wavelength of 0.05 Angstroms or only about 1/100,000 that of light. It would appear that a suitable combination of electrostatic or magnetic fields employing such high speed electrons should be capable of an extremely high resolving power.

The electron microscope is the instrument resulting from this development. The analogy of the transmission type electron microscope to the light microscope is almost complete. The light source is replaced by an electron source and condenser, objective and projection lenses perform the same functions as the analogous elements in the light microscope. The lenses are usually iron clad axially-symmetric magnetic lenses, although some microscopes have electrostatic lenses or combinations of both types.

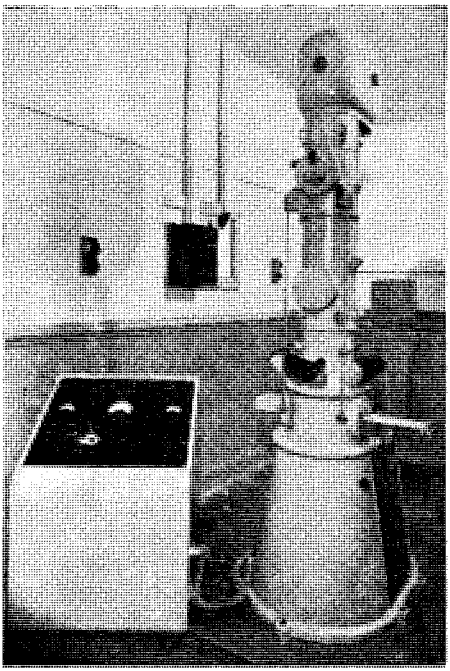
It would appear from the above that the resolving power of the electron microscope should be 100,000 times the resolving power of the best light microscope. Unfortunately, aberrations in the lenses and other considerations reduce this figure to about 100, which thus gives a limit to the useful magnification at 150,000 diameters. (This figure has been exceeded in some cases.)

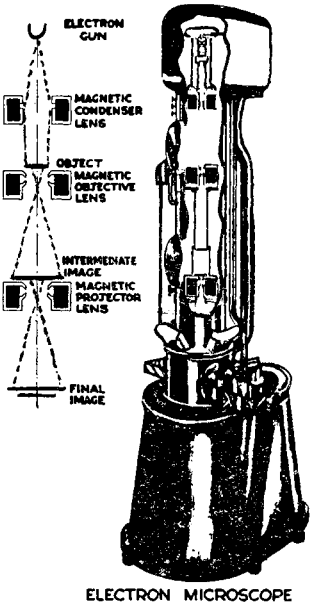
The Instrument at Dominion Physical Laboratory.

The microscope is the only type made in England. It is manufactured by Metropolitan Vickers Electrical Co. Ltd. and the type designation is EM2/1 (see fig.).

A complete description by M. E. Haine, the designer, appears in the Journal of the Institute of Electrical Engineers—Part I., Vol. 94, Oct. 1947, pg. 447.

Space does not permit of more than just a brief outline of





ELECTRON MICROSCOPE

the microscope. The column itself which makes up the microscope is about 5ft. long and stands vertically on a pedestal on the floor. The overall height is thus 7ft. approximately. The electron gun is at the top. Parts which are at a high voltage (60 Kv.) in respect of the remainder of the equipment are enclosed in earthed screens, for the safety of the operators, while the high voltage itself is supplied to the microscope by insulated cable from the D.C. supply in an oil insulated container nearby.

The lenses are supported at their requisite intervals down the column and the image is viewed on a fluorescent screen at the bottom or recorded on a photographic plate. The whole of the equivalent of the optical system is enclosed in a high vacuum, for only under such conditions can the stream of electrons be controlled and used to produce the enormous magnifications. Air locks permit the insertion of the specimen to be viewed and the plates for recording, through the vacuum wall without the necessity for returning the complete interior to atmospheric pressure each time. Following such an operation the microscope is ready for use in about two minutes.

Applications of the Electron Microscope.

Electron microscopes have been used as research or routine tools for about a decade. The initial work was biological, but

since that time an almost unlimited variety of work has been done. That a particular type of problem has not yet been studied does not mean that an electron microscope cannot be used to assist in the work, but probably the limited number of microscopes existing today has prevented it.

It could be said that almost any problem for which the ordinary light microscope has not sufficient resolution can be handled in some way by the electron microscope. To make specimens which may be viewed in the microscope sometimes taxes the ingenuity of the research worker, but usually the standard techniques suffice and these are quite simple.

Particles may be supported directly on the small grids which are the usual specimen support, or may be dispersed on a very thin film of collodion (about 100 Angstroms thick) stretched across such a grid. Bacteria, viruses and similar small entities, triturated organic tissue, etc., are viewed by supporting on similar collodion films on the grids. Complete sections of organic materials can be viewed directly, provided thin sections can be cut. Surfaces of solids are usually examined by the replica technique, since the solids themselves are opaque to electrons. Thin films of collodion, formivar, or silica are prepared which have surface contours the replica of the surface to be viewed. Collodion is the simplest and most frequently used. A 1% solution in amyl acetate is flowed over the surface and allowed to dry. A special technique enables this film to be stripped off, yielding a perfect replica.

It is possible here only to enumerate the fields of work in which electron microscopes have been used and the results, although intensely interesting, will have to be omitted.

Beginning with the biological work, the studies range from investigations of animal tissue, muscle fibres, nerves, bone structures, dental studies, through bacteriology, bacterial morphology, to studies of viruses and action of sera, bacteriophage, and the remainder of the small entities beyond the limits of the light microscope.

Much work has been done in the fields of scientific and industrial physics and chemistry. The microscope is standard equipment for particle size determination of pigments, carbon black used in the rubber industry, and polishing powders. Colloidal particles, crystals and crystal surfaces have all been studied, while the initial investigation on plastics was greatly assisted by the development of the electron microscope. The replica techniques have enabled a vast range of topics to come within the scope of the instrument and with which it would have been otherwise impossible to deal. Metallurgical problems, especially, fall into this category, while it has been used also in wool fibre research, study of large biological organisms too massive to be viewed directly, and countless other places.

DEPARTMENT OF HEALTH
CERTIFICATE OF PROFICIENCY IN HOSPITAL LABORATORY PRACTICE
Medical School, Dunedin, February, 1950.

Examiners:
Dr. J. O. Mercer, Dr. T. H. Pullar, Dr. E. F. D'Ath.

Paper (*3 hours.*)

1. What methods are at your disposal for the preparation and storage of the following:—
   (a) Broth.
   (b) Blood for media.
   (c) Complement.
   (d) Blood grouping sera. (A.B.O. and Rh.)
2. How would you determine the sensitivity to penicillin of the causative organism, in a case of Pyogenic Infection?
3. What types of Bacterial filters are in laboratory use? Discuss the composition, porosity and method of use of any two types. How would you test, clean and sterilize any two of these?
4. Discuss methods of Anaerobic Culture.
5. Discuss active and passive immunity. What preparations are available for the production of these types of immunity in the case of:—
   (a) Whooping cough.
   (b) Scarlet fever.
   (c) Diphtheria.
   (d) Smallpox.
   (e) Gas gangrene.
   (f) Yellow fever.

Bacteriology—Practical Examination.
(*3 hours.*)

Write your name on each sheet of paper used. Write your answers as briefly and clearly as possible.

1. Report on the hairs provided. They have been taken from a suspicious lesion on the head of a child. Describe your technique. Discuss culture methods in such a case.
2. Smear from a centrifuged deposit from a urine in a suspected case of T.B. kidney. Report on it. What specimens may a laboratory be called upon to examine for T.B.? *Briefly* describe methods in use for:—
   (a) Direct examination.
   (b) Concentration.
   (c) Culture.
   (d) Typing.
   (e) Streptomycin sensitivity.
3. Smear 'B' is from a culture from a case of suspected tetanus.

Report on the smear. Discuss methods for obtaining a pure culture of the organism, and further steps necessary in its identification.

4. Tubes 'C' contain the cells (in saline) and serum of a donor 'X'; and tubes 'E' ditto donor 'Y.' Tubes 'D' contain the cells and serum of the recipient who is a Group A. Are the donors suitable? What Groups do 'X' and 'Y' belong to? What are the requirements of a suitable donor?
5. You will be allowed 15 minutes to examine exhibits F, G, H. I. J. Use a separate sheet of paper for this question.

F. Ovum of Enterobius—measurement also required. G. Two dark ground condensers. H. Spirochaetes of Relapsing fever. I. Anthrax colony. J. Leishman-Donovan bodies.

6. Before examining an object (say slide 'B') what adjustments must you make to your microscope? Leave your microscope so adjusted with the slide 'B' in focus.

Clinical Pathology—Practical Examination.

(3 hours.)

1. The serum provided is anti D. Determine from the cell suspension whether the patient to whom the cells belong is Rh. negative or positive.
2. Examine the blood films and report on each one. In addition, make a differential count of Films A and B.

A. Infectious mononucleosis. B. Chronic myelogenous leucaemia, X. Trophozoite forms of Benign tertian malaria.

3. Examine and report on the deposits in the specimens of urine provided.
4. Carry out a cell count on the cerebro spinal fluid provided, determining, as far as possible, the types of cells seen under the counting chamber.
5. How would you prepare Leishman's Stain, and how would you proceed to stain a blood film with it?
6. Outline the method of preparation of Nessler's Reagent, and state the purposes for which it is used.
7. How would you perform a test for occult blood in faeces? What are the chemical principles which underlie this test?
8. A solution of streptomycin for intrathecal use, containing 100 mgms. in 5 ml. is ordered for a patient. Give details of your method of preparation from the original container before you.

Oral Examinations.

(The following are stated by the candidates to have been the questions asked in oral examinations.)

Dr. D'Ath.

pH meters, buffer solutions, molar solutions, decalcification of bone (methods), autoclaves and general sterilisation, blood sugars, bacteriocide in penicillin I.V. containers, serum proteins

and the specific gravity method, sterilisation of oil, antigen-antibody formation, Benedict's qualitative solution, Leishman stain and buffer, care and diseases of laboratory animals, filters, blood sugar standard and preservative, the use of 10% sodium tungstate, sections, preparation of plasma, kaolin, diluent for penicillin, colorimeters, B.S.R. estimation, principle of angle head centrifuge, May Grumwald stain, Folin-Ciocalteau reagent, T.N.P.N., penicillinase, use of Tween 80, general laboratory experience.

Dr. Mercer.

Preparation of Loeffler's medium and inspissation, tellurite medium, types of C. diphtheriae and occurrence in New Zealand, desensitising vaccine and extracting fluids, preservative in typing sera, isolation of S. paratyphi A with reference to Selenite F, Mantoux and its significance, Wintrobe's mixture and tube, maximum and minimum thermometers and their standardisation, platelet count, red cell fragility, virus agglutination, influenza and polio virus, animals used for virus isolation, sterilisation of glassware, Wassermann and Kahn reactions, best place to start a trainee in a laboratory, Loewenstein's medium, streptomycin sensitivities for T.B., use of Tween 80, comparison of bleeding, coagulation and prothrombin times and their significance with reference to associated diseases, technique of prothrombin time, use of liquid media for T.B., sulphonamide crystals in urine, procain with reference to pH, fluorescence microscopy, spectrophotometer, general laboratory experience.

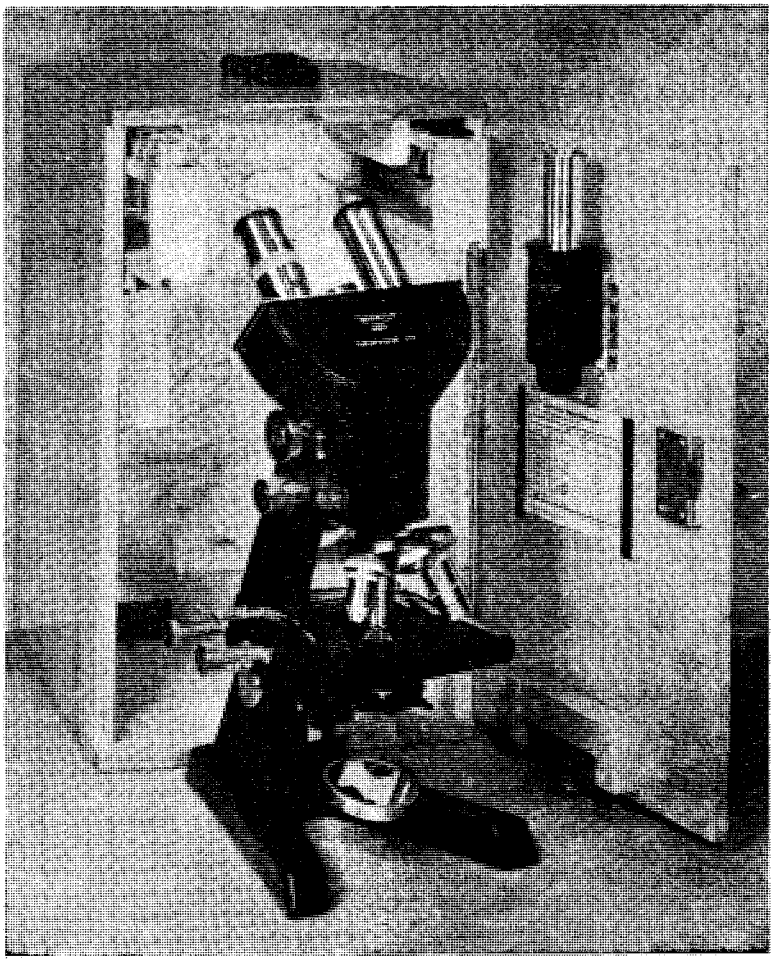
Dr. Pullar.

Tween 80, technique and principle of estimation of the phosphatases, principle of Gregerson's test and comparison with peroxidase staining, prevalence of intestinal parasites in New Zealand, media for inhibiting B. proteus, natural active immunity, fluorescence microscopy with reference to filter, MacIntosh and Fildes' jar and catalyst, relationship between pressure and temperature in an autoclave, principle of estimation of serum calcium and normal range, reticulocyte count, use of beef extract, tape worms, platelet count, Casoni fluid and test, principle of the Wassermann reaction, N.I.H. swabs, anticomplementary sera and how to counteract, virus vaccination, general laboratory experience, urease medium, Klein's iron medium, osmotic pressure.

POSITION VACANT

Would any fully-trained bacteriologist who is interested in a senior post in a private laboratory, please get in touch with Editor.

Printed and Published for the New Zealand Association of Bacteriologists (Inc.) at their registered office, 139 Kohimarama Rd., Kohimarama, Auckland, E.1., by Douglas Whillans.



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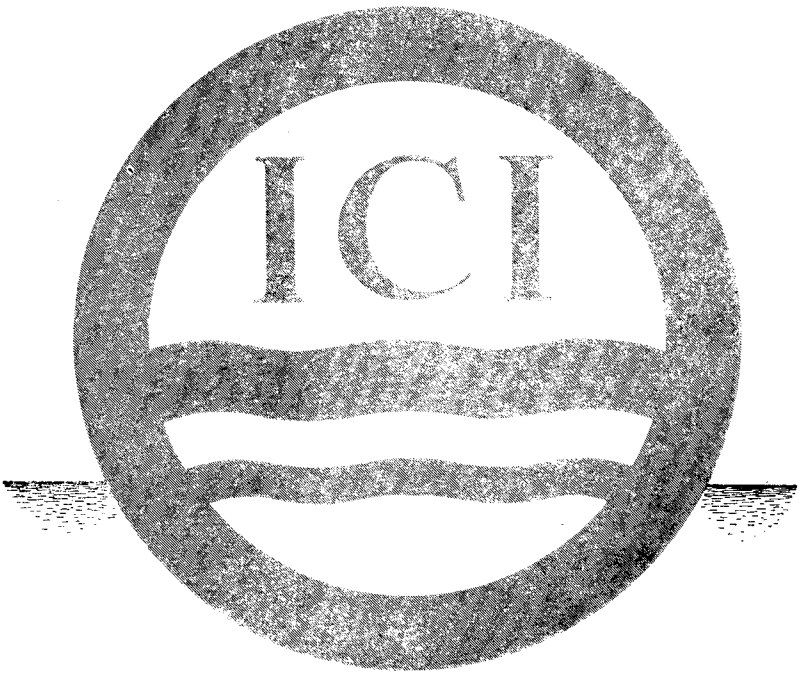

Pharmaceuticals and Fine Chemicals