Vol 15, No. 3 NOVEMBER, 1960

JOURNAL

OF THE NEW ZEALAND ASSOCIATION OF BACTERIOLOGISTS

CONTENTS

OXOID

The advantages of **Dehydrated Culture Media** in granule or tablet form

GRANULES: Far less hygroscopic than the conventional fine powder and, consequently, can be stored almost indefinitely. 'OXOID' Culture Media are, therefore, particularly suitable for tropical use.

TABLETS: Ideal for making up small quantities of culture media. Rigorous laboratory control ensures uniformity of content.

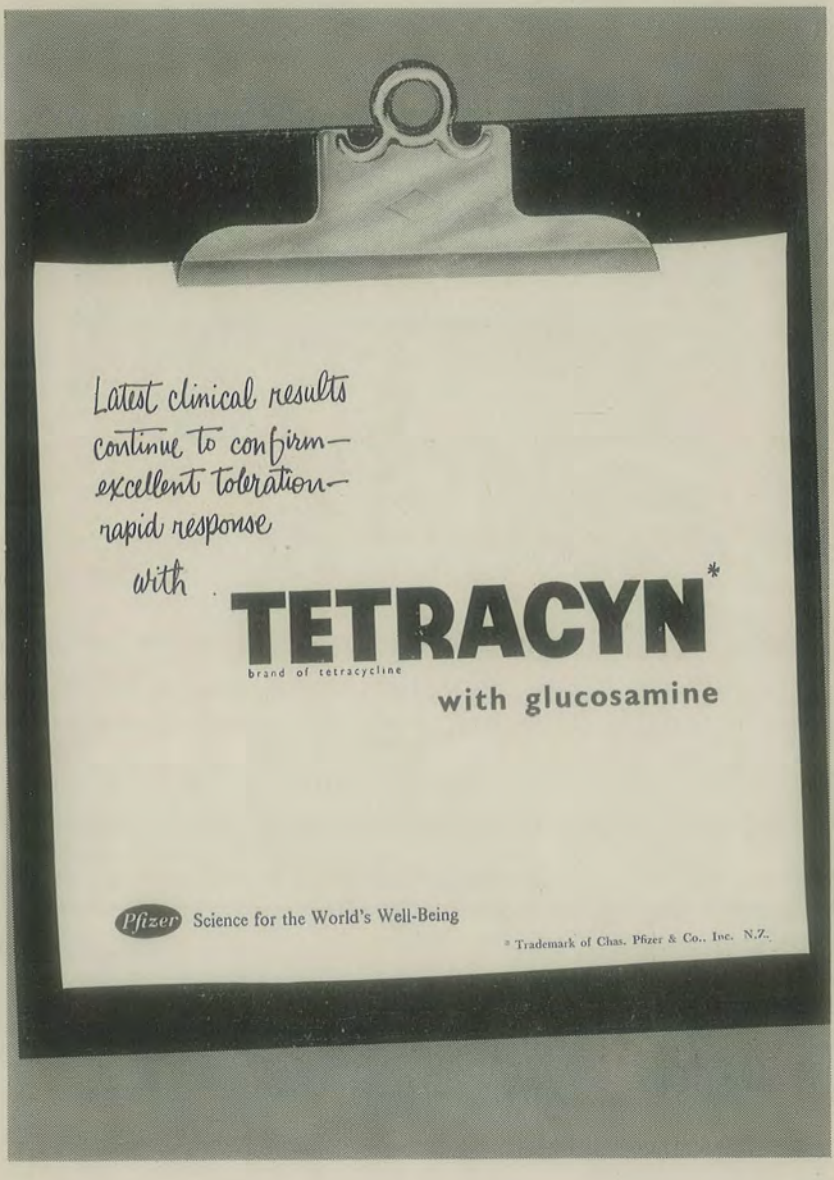
Tablets and granules need only the addition of a fixed quantity of distilled water, drastically reducing the time and effort involved in preparing media.

RECENT ADDITIONS TO THE RANGE

CHARCOAL AGAR — LACTOSE BROTH — WHEY BROTH
PURPLE SERUM AGAR BASE — SALMONELLA SHIGELLA AGAR
TRYPTONE GLUCOSE EXTRACT AGAR — CANDIDA MEDIUM
TRYPTONE SOYA AGAR — POTATO DEXTROSE AGAR
STUARTS TRANSPORT MEDIUM

OXOID **CULTURE MEDIA**

Manufactured by Oxo Ltd., London
Sole New Zealand Agent: EDWIN A. PIPER LTD.
2 DINGLE ROAD, ST. HELIERS BAY, AUCKLAND, E.1


Latest clinical results
continue to confirm—
excellent toleration—
rapid response
with
TETRACYN*
brand of tetracycline
with glucosamine
Pfizer Science for the World's Well-Being
* Trademark of Chas. Pfizer & Co., Inc. N.Y.

JOURNAL OF THE NEW ZEALAND ASSOCIATION OF BACTERIOLOGISTS

Vol 15, No. 3 NOVEMBER, 1960

Communications regarding this JOURNAL should be sent to the Editor, Department of Pathology, Christchurch Public Hospital, Christchurch.

Communications primarily affecting the Association should be addressed to the Secretary, Mr H. E. Hutchings, Pathology Department, Palmerston North Hospital.

All moneys should be paid to the Treasurer of the New Zealand Association of Bacteriologists (Inc.), Mr D. J. Philip, Pathology Department, Middlemore Hospital, Auckland.

Subscription to this JOURNAL is five shillings per year or two shillings per copy, post free.

ADDRESSES

If the address as printed on this envelope is incorrect, **please** notify the Editor as soon as possible of your **correct** address.

ACID-BASE METABOLISM

PRACTICAL DETAILS OF THE ASTRUP MACRO METHOD OF ASSESSMENT

H. G. BLOORE, B.Sc.
(*Clinical Laboratory, Wairau Hospital, Blenheim*)

The Astrup method of assessment of changes in the acid-base state of patients is a quick, reliable procedure, giving positive information of an unequivocal character.

It involves obtaining the following values:—

(1) Actual pH of whole blood, anaerobically collected.

(2) Whole blood pH, determined after equilibrating the sample with a carbon dioxide-oxygen mixture of known CO_2 tension.

From the latter value (equilibrated pH) plus the haemoglobin value, by using a nomogram, may be ascertained the following:—

Base Excess or Deficit, Standard Bicarbonate and Buffer Base.

If in addition, the former value (Actual pH) is obtained, then the following may also be ascertained:—

pCO_2, Total CO_2, Actual Bicarbonate, and CO_2 combining power.

Of these, the important values are—

(1) Base Excess or Deficit—reflecting metabolic changes.

(2) pCO_2 —reflecting respiratory changes.

Since metabolic changes in the acid-base state are by far the commonest in general hospital work, it is found that most requirements are met by simply measuring the pH of an equilibrated sample of blood, estimating its haemoglobin level and referring to a nomogram.

Definitions as given by Astrup et al.

(1) *Standard Bicarbonate* (6, 7).

This is the bicarbonate content of the plasma part of whole blood measured at 38°C. at a pCO_2 of 40mm. of Hg, with the haemoglobin fully oxygenated.

Under these standardised conditions, the pH of the sample of blood is a direct indication of its bicarbonate content. The conversion of pH to standard bicarbonate is obtained either from the calibrated scale of the pH meter, the Henderson-Hasselbach equation, or from the nomogram of Andersen & Engel (1). Expressed in m.Eq/litre.

(2) *Buffer Base* (1, 8) (B.B.).

The sum of the buffer a.nions, mainly bicarbonate and proteinate ions—in m.Eq/litre.

(3) *Base Excess* (1) (B.E.).

The difference between the Buffer Base found and the normal

buffer base. Expressed as plus or minus (the latter indicating a deficit) in m.Eq/litre.

(4) pCO_2.

This is the partial pressure of CO_2 in the blood at the moment of sampling. This is the measure of the respiratory component of the acid-base state, expressed in m.m. of Hg.

(5) *Normal Buffer Base* (1) (NBB).

The Buffer Base of blood with a pH of 7.38 at a pCO_2 of 40 m.m.Hg.

NBB = 40.8 + 0.36 x haemoglobin conc. (in g.per 100 ml.).

As the standard bicarbonate does not give *directly* the amount in m.Eq. per litre of fixed acid or base causing a change in the base content of the blood, the value of Base Excess is employed instead.

PRINCIPLE OF THE ASTRUP METHOD

If a graph is prepared on semi-log paper (logarithmic, 1 cycle x natural), showing the relations between pH and log pCO_2 of a blood sample, the result is a straight line. The *slope* of the line is a function of the buffer capacity of the blood, and the *position* of the line is a function of the base content of the blood.

Once the line is obtained in any given case, if the actual whole-blood pH is found, then the actual pCO_2 can also be found. The nomogram of Andersen and Engel is such a graph (1).

The original Astrup apparatus employs equilibration with one gas mixture and requires a knowledge of the haemoglobin level, while the new Ultra Micro Astrup Method employs equilibration with two samples and two different gas mixtures, and the haemoglobin value is not required.

I propose to outline the actual method of using the original Astrup apparatus, as employed in this laboratory.

APPARATUS

Astrup Macro Apparatus Type E50101—made by Messrs Radiometer, Copenhagen, kept permanently warmed via the water jacket at 38°C.

Water Bath—a plastic tank is suitable and cheap.

Thermostatted circulating pump—The Techne Tempunit is satisfactory.

pH Meter—a reading accuracy of 0.005 pH unit is desirable. The combined electrode of the Radiometer Model 22 pH meter is designed to fit the Astrup apparatus.

The Radiometer extension meter gives the required reading accuracy.

CO_2/O_2 Cylinders—ordered from N.Z. Industrial Gases Ltd., to contain 5.6% CO_2 and 94.4% oxygen. The CO_2 content must be known to within 0.05%, i.e., to two significant figures, and should be within the range of 5.4% to 5.8% CO_2.

These mixtures are analysed by the Dominion Laboratory before being forwarded.

Reducing Valve—British Oxygen Co.'s gas regulator valve is suitable.

Stoppered Tubes—for distilled water and standard buffer, filled and placed in rack in water bath at 38°C., five minutes before use.

Syringes—10ml., oiled and dry-heat sterilized in some sort of container to maintain sterility.

REAGENTS

1. *Heparin Solution*—5000 units/ml.

For adding to syringe

Dissolve 200 mgms (= 10,000 units) of calcium heparin in 2 mls. sterile distilled water, in sterile dropping bottle.

Store in refrigerator.

Use 1 drop per syringe.

2. *Heparin-Fluoride Solution*

For Base Excess Bottle:

Sodium heparin	10,000 units or 200 mgms. powder
Sodium Fluoride A.R.	2.5 grams
Distilled water to	100 mls.

Place 0.5 mls. of this solution (1 mgm. or 50 units of heparin and 12.5 mgms. NaF) in bottle and dry in oven. For 5 mls. blood.

3. *Sodium Fluoride Solution*

For Base Excess Bottles when pH of blood is also being done:

Sodium fluoride A.R.	2.5 grams
Distilled water to	100 mls.

Place 0.5 mls of this solution (12.5 mgms. NaF) in bottles and dry in oven.

For 5 mls blood.

4. *Silicone Defoamer* as purchased from Messrs Radiometer.

5. *Potassium Chloride A.R.* as fine crystals and as saturated solution.

6. *Standard Buffer Solutions*

Stock solutions

A. M/15 primary potassium phosphate

2.2695 grams of dry KH_2PO_4 (Sorenson salt, Merck) are dissolved in 250 mls. of deionized distilled water. Refrigerate.

B. M/15 Secondary Sodium Phosphate—
11.8667 grams of Na_2HPO_4. $2H_2O$ (Sorenson salt, Merck) are dissolved in 1000 mls. of deionized distilled water. Refrigerate.

Working Solution M/15 pH 7.380 ± 0.005 @ 38°C.

Warm stock solutions to room temperature and mix 20 mls. of A with 80 mls of B.

7. *Paraffin Oil/Ether*—equal parts neutralized paraffin oil and anaesthetic ether.

The oil may be neutralized as follows:—

In a 500 ml. separatory funnel place about 200 ml. of oil, an equal volume of distilled water, and a few drops of 0.1% phenol red solution.

Add 0.02 N NaOH a drop at a time with vigorous shaking until the water solution of indicator becomes permanently pink.

Centrifuge the oil to remove suspended water droplets.

Decant the clear oil with care, and store in a stoppered bottle.

8. *Deionised Water*

Distilled water is passed through a column of ion exchange resin. The resin employed is Biodeminrolit supplied by J. J. Niven & Co. Ltd.

This process is quick, and removes CO_2 from the water without the need to boil it.

This water is hereinafter referred to as Water.

PROCEDURE

Immediately prior to performing an estimation, the pH meter is switched on to warm up, and adjusted to read the correct pH when the chamber of the Astrup apparatus is filled with standard buffer solution at 38°C.

After rinsing the chamber three times with water at 38°C, the instrument is left with the chamber filled with water, and is ready for use.

COLLECTION OF BLOOD

(1) *When Base Excess or Standard Bicarbonate* only are required:—

Venous blood is collected with any dry syringe without any particular precautions, except avoiding venous stasis as far as possible, and 4-5 mls. are placed in a specimen bottle containing dried heparin/sodium fluoride mixture, and shaken briefly. This specimen is suitable for analysis for an hour or two.

(2) *When actual pH,* in addition to Base Excess and Standard Bicarbonate is required:—

Venous blood from a preferably warmed arm is collected with preferably no stasis, in a well-fitting, oiled 10 ml. syringe to which has been added just before using one drop of a calcium heparin solution (5,000 I.U. per ml.).

This drop, plus the oil, leaves almost no air space in the syringe, thus assisting the anaerobic collection of the sample.

Immediately on removing the needle from the vein, the syringe is pointed upwards, and any tiny air bubble is ejected into a cotton wool swab held over the needle point. A large drop of mercury is then sucked into the syringe from a small bottle containing 1-2 mls. The needle point is then pushed into an ordinary clean cork and the blood mixed thoroughly.

The actual pH should be determined as soon as possible.

Some 12-13 mls of blood can be collected in a good 10 ml. syringe, and this provides sufficient specimen for actual pH, Base Excess or Standard HCO_3, Hb and P.C.V. and Na, K, Cl and N.P.N. estimations. (For the N.P.N. a small correction must be made, allowing for the nitrogen in the heparin. This may readily be found by experiment.)

Just before the pH determination, the syringe is briefly warmed in hot water.

DETERMINATION OF ACTUAL BLOOD pH

The water is ejected from the chamber of the apparatus and mercury raised up to fill the bottom of the cup, which is then dried with a piece of folded filter paper. The specimen is thoroughly mixed in the syringe, the needle removed and replaced with a 1 inch piece of sphygmomanometer tubing (narrow bore and thick wall) which is immediately filled with blood.

As rapidly as possible, the syringe is inverted and the end of the tubing pressed firmly against the bottom of the cup, under the surface of the mercury.

By manipulating the taps, the mercury in the chamber is run down, drawing blood after it, until the chamber contains 2-3 mls. of blood—enough to cover the electrode. Both top and bottom taps are closed, the syringe removed and the needle and cork replaced on it. The pH meter is switched to the reading position and a reading made after about 1 minute.

The blood may then be raised into the cup and pipetted off for other tests, e.g., Hb and P.C.V., and N.P.N.

After rinsing the chamber 3-4 times with warm water, the pH meter setting is checked again with warm standard buffer. If necessary, the process must be repeated with more blood from the syringe, which may be kept in the water bath at 38°C. in the meantime. The chamber is finally left filled with water.

The remaining blood in the syringe is now distributed into specimen bottles as required, e.g., 3 mls. in Base Excess bottle, and 6-7 mls. in a centrifuge tube for plasma Na, K and Cl. estimations, if these are requested.

DETERMINATION OF EQUILIBRATED pH

The pH meter and apparatus are prepared as described above to the point where the mercury fills the lower part of the cup and the latter is dried.

About 2.5 mls. of blood from the 'Base Excess' bottle is poured into the cup, and a small portion of antifoam emulsion is smeared round the stem of the cup with a piece of glass rod.

Mercury and blood are then run slowly into the chamber, at the same time using the glass rod to help some of the antifoam emulsion to pass into the chamber also. This emulsion is like vaseline in consistency and very little is actually needed to prevent foaming and consequent haemolysis.

When the top of the mercury column is level with the lower edge of the side tube of the chamber, the lower tap is closed.

With the tap of the side arm closed, and the flow indicator tap turned to air, the gas cylinder tap is turned on, and by adjusting the reducing valve control, 3-4 bubbles per second are allowed to flow through the indicator. By turning the above-mentioned taps, the gas is caused to flow through the blood sample, and any necessary adjustment is made to the flow rate. If flow is too fast, blood is splashed about vigorously and haemolysis may occur, while if the rate is too slow, equilibration will not be complete in the desired time.

After about 2 minutes, the main cylinder valve may be closed, the reducing valve containing sufficient gas to last the remaining three minutes. The life of the cylinder may thus be almost doubled (if the cylinder is nearly empty, the reducing valve may hold perhaps only 1 minute's supply of gas, and the final portion is ejected violently. The result is that blood is sprayed all over the room!)

After a total of 5 minutes' bubbling, proceed as follows:—

Turn gas supply to flow to air from flow indicator, raise the blood to cover the electrode, close bottom and top chamber taps, switch pH meter to reading position, turn off gas at cylinder reducing valve, and read the pH after about half a minute.

The extension pH meter made by Radiometer is calibrated also in m. Mols. per litre of standard bicarbonate, and this may be read off directly, provided the pCO_2 of the cylinder gas is 40 mm. Hg. or close to it.

The blood is then pipetted off and the pH meter buffer again checked. If necessary, the equilibrated pH may be determined again on the same blood sample. The chamber is left filled with water and the pH meter switched off. As the whole apparatus is kept constantly at 38°C. it is always ready for use.

CORRECTIONS

1. The pCO_2 of the cylinder gas is found according to the following (3):—

$$pCO_2 = \frac{(B - W) \times \text{per cent } CO_2}{100}$$

where B = barometric pressure in m.m. Hg., and W is the vapour pressure of water at 38°C. (= 50 m.m. Hg.).

2. If the pCO_2 thus found deviates from 40 m.m. Hg., and is between 36 and 44 mm. Hg., it may be convenient, in using the nomogram, to correct the equilibrated pH as follows (6):—

pH_{40} = pH measured + 0.006 x (pCO_2 used — 40)

It is helpful to prepare tables in each case for quick reference. A table should also be prepared relating Normal Buffer Base and haemoglobin concentrations at 0.5 grams per cent intervals.

USING THE NOMOGRAM OF ANDERSEN AND ENGEL

In the procedures described, when equilibration is carried out with a single CO_2/O_2 mixture, the Haemoglobin value is required to obtain the Normal Buffer Base of the sample. We thus have the following items for use with the nomogram:—

Normal Buffer Base, Actual pH, and Equilibrated pH.

The Normal Buffer Base gives the slope of the line to be drawn, the equilibrated pH gives the position of the line, whence the Base Excess may be directly obtained, and the point on the line provided by the Actual pH gives the pCO_2 of the sample.

When the Ultra Micro Astrup method (2) of analysis is performed, equilibration is carried out at two different CO_2 tensions and the position and slope of the line obtained, without the need for knowledge of the haemoglobin value. This method employs capillary blood, is quicker to perform than the original macro method described here and is thus eminently suitable for serial determinations, especially in following the respiratory component of the acid base metabolism.

In practice, using the macro method the whole procedure of obtaining actual pH and Standard Bicarbonate and Base Excess figures takes 12-13 minutes total elapsed time. If Standard Bicar-

bonate or Base Excess only are required, as is usually the case here, only about 5 minutes' work is involved. During equilibration other work may be proceeded with.

The appended references cover the work which has been done in the course of the development of this new method of investigation of the acid-base state up to the present time.

REFERENCES

1. Andersen, O. S. and Engel, K. (1959), Scand. J. Clin. Lab. Invest. (in press).
2. Andersen, O. S., Engel, K., Jorgensen, K., and Astrup, P. (1959), Scand. J. Clin. Lab. Invest. (in press).
3. Astrup, P. (1956), Scand. J. Clin. Lab. Invest. **8**, 33.
4. Astrup, P., Jorgensen, K., Andersen, O. S. and Engel, K. (1960), Lancet **1**, 1035.
5. Astrup, P and Schroder, S. (1956), Scand. J. Clin. Lab. Invest., **8**, 30.
6. Jorgensen, K and Astrup, P. (1957), Scand. J. Clin. Lab. Invest., **9**, 122.
7. Astrup, P. (1955), Danish Med. Bull., **2**, 136.
8. Singer, R. B. and Hastings, A. B. (1948), Medicine **22**, 233.
9. A Symposium on pH and Blood Gas Measurement edited by Ronald F. Woolmer, London, Churchill (1959).

VIRUS DIAGNOSTIC PROCEDURES

MISS ROSEMARY E. ALLEN

(*Senior Bacteriologist, National Health Institute, Wellington*)

Viruses have been described as differing from bacteria in three ways . . . that they were ultramicroscopic, filterable and not able to be cultivated on inanimate substrates. This requirement of a living substrate for the growth and cultivation of viruses is the most important distinction between viruses and bacteria and because of this virus techniques differ greatly from those used in routine bacteriology.

Many of the tests one is familiar with in bacteriology are little used in virus diagnostic work; for example, agglutination and precipitation tests, while other tests have been developed that are used in work with viruses; for example, neutralization tests and haemagglutination tests. Complement fixation tests as used in bacteriology are also widely used in virology.

At the National Health Institute, our virus work might be divided into three sections:

(i) virus isolations;
(ii) the testing of sera by neutralization or haemagglutination-inhibition tests;
(iii) the testing of sera by complement fixation tests.

VIRUS ISOLATIONS

Viruses multiply only within living cells and this determines their cultivation techniques. Virsuses may be grown in—

(A) cultures of living tissue;
(B) embryonated eggs;
(C) experimental animals.

These are the substrates used when virus isolation is attempted. Viruses exhibit a high degree of specificity for different animal tissues and this specificity is thought to be a chemical one.

TISSUE CULTURES

Living cell cultures used in this work are grown in nutrient solutions containing inorganic salts, amino acids and an animal serum. The cell cultures may be of two kinds: (a) continuous cell lines, e.g., HeLa cells, which are a line of cells used in virus laboratories throughout the world (b) primary cell cultures, e.g., cultures of cells made by the trypsinization of human amnions or monkey kidneys. Viruses that grow in tissue culture may produce some visible cytopathogenic effect in the cells, which may become rounded and refractile or granular and degenerate. In some

cases, for example, when influenza virus is grown in tissue culture, no cytopathogenic effect is visible and the virus is only detected by the addition of red blood cells which will adhere to infected cells. Alternatively, virus is detected by the performance of haemagglutination tests on the nutrient fluids.

EMBRYONATED EGGS

Embyronated eggs are widely used and may be inoculated by a number of different routes, e.g., into the amniotic cavity, the allantoic cavity, the yolk sac or on to the chorioallantoic membrane. They may also be inoculated at different stages of development and different temperatures of incubation according to the requirements of the virus to be propagated or the suspected virus to be isolated. In influenza virus isolations, 11-13 day old eggs would be used and these would be inoculated by the amniotic route. Psittacosis virus is best grown by inoculating material into the yolk sac of 5-7 day old embryonated eggs. Evidence of virus growth and multiplication in embryonated eggs may be found by different methods. With Psittacosis-L.G.V. group and Rickettsia where a yolk sac inoculation has been used, smears are usually made from this structure and after staining virus elementary bodies may be clearly visible. The chorioallantoic membrane, which is used for growing the pox viruses, will show visible lesions a few days after inoculation. Amniotic and allantoic fluids from eggs inoculated with a suspected myxovirus may be tested in haemagglutination tests for evidence of virus propagation.

EXPERIMENTAL ANIMALS

Mice, guinea pigs, and rabbits may all be used for the isolation and growth of viruses but, again, the age of the animal may be important. Vaccinia virus may be grown by inoculating the skin of an adult rabbit but, on the other hand, coxsackie B viruses can sometimes only be isolated in suckling mice that are less than 24 hours old. In animals, as in human beings, clinical symptoms may indicate the multiplication of the virus or the only evidence may be histological found after post mortem.

Because of the variety of both the living "cultures" that may be used and the routes of inoculation, the problem of virus isolation is often difficult and time consuming, especially if one has no idea of the clinical symptoms of the patient or the duration of the illness. Unfortunately, whatever the means employed, the techniques of virus isolation available today are not ones where a result can be obtained overnight. It might be necessary for a specimen or an extract of it to undergo at least two blind passages

in either tissue culture, embryonated eggs or animals before any evidence of a virus will be seen. This may take up to fourteen days. The identification of the virus isolated might again take several days and this may be done using specific antisera in one of several ways. Neutralization tests may be carried out either in tissue cultures, in embryonated eggs or in animals. Different antiviral sera might each be mixed with a suspension of the virus and, when these mixtures are inoculated into the living culture medium, the growth of the virus will be inhibited by that serum which contains specific antibody for it. Haemagglutination inhibition tests may be carried out, a specific antiviral serum preventing the haemagglutination of red blood cells by the virus. Tissue culture fluids or extracts of egg or animal tissues may be used as antigens and identified in complement fixation tests employing immune sera.

One further method of virus isolation must be mentioned. If fresh biopsy or post mortem tissue is available, the tissue itself may be cultured in nutrient media and, as the cells reproduce, so also may the virus contained within them. This virus may later cause degeneration of the supporting tissue or the virus may be found free in the nutrient fluids.

To prove that a virus isolated is the cause of the patient's illness, it is necessary to show that the virus has actually produced an antibody response in the patient as demonstrated by a four-fold rise in titre between the acute phase serum and a sample obtained in convalescence.

SEROLOGICAL TESTS

The testing of sera is a large part of virus diagnostic work. Little information can usually be gained from the testing of a single specimen, and the only significant results of aid in diagnosis are those from the testing of a patient's acute and convalescent sera. If the latter shows at least a four-fold rise in titre against a virus isolated from the patient, this would indicate a recent infection with that virus. It is important in all serological tests that both acute and convalescent sera are examined in the same test as they will both have been subjected to the same test conditions. Cultures of live virus are used in neutralization tests but live or inactivated cultures are used in haemagglutination-inhibition tests.

Neutralization tests on sera may be carried out in tissue cultures in embryonated eggs or in animals, the most convenient of these these being tissue culture. Serial dilutions of the test serum are mixed with a quantitative amount of virus suspension. This serum-virus mixture is then inoculated into the living substrate

which, after a further interval, are examined for evidence of viral growth and multiplication. The absence of virus growth indicates the presence of specific antibody in the serum tested. Diseases where neutralization tests may be used in testing sera include poliomyelitis, vaccinia, herpes and coxsackie virus infections. The greatest difficulty, however, is that many of these viruses contain numbers of different serological types, e.g., 25 recognised types of Coxsackie A virus, 6 of Coxsackie B virus. It is, therefore, in most cases not practicable for sera to be routinely tested in this way. This does not mean that where infections of coxsackie virus are suspected that the sending of paired sera in conjunction with isolation specimens is of no avail. Such sera are of value, for when a coxsackie virus has been isolated the isolated strain may then be used in neutralization tests against the paired sera to detect a possible rise in specific antibody.

Haemagglutination-inhibition tests may be used to examine acute and convalescent phase sera from patients that have contracted infections of influenza virus. This haemagglutination of red blood cells by a strain of influenza virus will be inhibited in the presence of sera containing strain specific haemagglutination-inhibiting antibodies. For this test, recently isolated strains of virus should be used as within one type of influenza virus the different strains may show markedly different antigenic properties. Haemagglutination-inhibition tests may also be used in many other virus diseases.

Viral Complement Fixation Tests

These are analogous to those used in bacteriology. Antiviral sera fix complement in the presence of their specific antigens.

An antibody response may be demonstrated by the complement fixation test in such diseases as mumps, influenza, poliomyelitis, variola, herpes simplex, lymphocytic choriomeningitis, psittacosis, Q fever and adenovirus infections.

Viruses, like bacteria, are antigenic. A virus may have more than one antigen in its structure. Some antigens are separate from the virus particle and are referred to as "soluble" antigens. They are usually of small particle size.

Antigens used in viral complement fixation tests may be either extracts of infected tissue, tissue culture fluids or fluids from infected embryonated eggs. Often there is difficulty in preparing concentrated viral complement fixing antigens that are free from non-specific interfering substances.

In some cases, the soluble antigens are of great use in indicating group specificity, e.g., those of influenza are used to detect infections by Influenza A, B or C, each group possessing

a different soluble antigen, whereas with adenovirus there is one soluble antigen that can be used to identify infections by any of the adenovirus types. Similarly with a Coxsackie B mouse brain complement fixing antigen.

The success of the complement fixation test depends on the standardization of the reagents used in relation to each other and the use of adequate controls in all tests performed. These should include the testing of each antigen against its known positive antiserum and also controls for every antigen and serum used in case any should be anti-complementary. A titration of the dilute complement used in each test should always be included.

Complement fixation tests represent a simple and highly satisfactory method for the examination of material provided that they are adequately controlled.

SUMMARY

In this brief paper, I have shown that living tissue is necessary for the growth of viruses. This tissue may be either cell cultures, embryonated eggs or experimental animals. A virus infection produces an antibody response in the patient. This response is demonstrated by testing the acute and convalescent phase sera in neutralization, haemagglutination-inhibition or complement fixation tests.

ACKNOWLEDGMENTS

I am indebted to the Director-General of Health for permission to publish this paper, Dr. J. D. Manning, Director, and Dr. W. Hamilton, Virologist, National Health Institute, for their help and advice in the preparation of this paper.

A BRIEF OUTLINE OF THE NATURE AND DISCOVERY OF BACTERIOPHAGE

K. R. JAMES

(*Pathology Department, Waikato Hospital*)

2nd Prize Junior Essay Competition, 1960

Although work with bacteriophage is not carried out in routine hospital laboratories in this country, the subject is one which appeals to those who are interested in the biology of living matter as it affects human beings. The basis of hospital laboratory technique comprises a small portion of the latter subject. However, apart from our general interest in bacteriophage or phage, as a comparatively recently discovered and novel life form, it has a direct application in the field of bacterial classification. This is found in the phage-typing of Staphylococci and Salmonellae, as carried out by the National Health Institute in Wellington. However it is not within the scope of this article to give an account of the procedures involved.

Matter may be placed in two broad categories—that which is living and that which is non-living. Living matter may be further subdivided into animals and plants. Bacteria exhibit some of the characteristics of animals and some of plants, but are not typical members of either kingdom. A further group, the viruses, lies on the borderline between living and non-living matter. Some of the viruses are large, approaching in size the smaller of the bacteria. Others are little more than complex protein molecules which may be isolated in crystalline chemically pure form. A particular type of virus, parasitic on the bacteria themselves, will now be described.

Between 1910-1915 F. H. Twort carried out a series of experiments with calf vaccinia in his search for artificial culture media on which viruses could be grown. He inoculated the vaccinia lymph, which also contained staphylococci, on to agar slopes. Amongst the staphylococcal colonies he obtained on incubation, he noticed some minute glassy transparent areas, or plaques, which failed to grow when subcultured. However, if a vigorous culture of the same staphylococcus was touched with a minute portion of this glassy material, the bacterial growth on further incubation became transparent at the point touched. This change gradually spread over most of the plate.

The causative agent for this infection of the bacterial colonies could not be detected under the microscope, but it could pass through a fine Chamberland filter. It showed no evidence of independent growth on artificial culture media unless the staphylococcus was also present. On the other hand it retained its vital-

ity under favourable conditions for many months without the presence of its host. It was specific in that it had no lytic effect upon other bacteria. It appeared to belong to the virus group. However, it was found not to be the vaccinia virus. Twort gave it the name of Bacteriolytic Agent.

At about the same time as this, a French bacteriologist, Felix D'Herelle, discovered a similar phenomenon in relation to a coccobacillus which causes septicaemia and diarrhoea in locusts. He then followed a case of Shiga dysentery in a man, from the time of admission to the end of convalescence.

During the first three days Sh. shiga was isolated from the faeces and gave normal growth. On the fourth day D'Herelle filtered an emulsion of the stools through a Chamberland candle. He added a drop of this filtrate to a broth culture of the bacillus isolated on the first day. After overnight incubation the broth culture was found to be perfectly clear, the bacteria having all been lysed. A striking feature was that at the same time the condition of the patient improved greatly. D'Herelle attributed this to the lytic agent acting on the Shiga bacillus within the patient. He coined the term Bacteriophage to describe this agent.

All these researches were carried out before the advent of the electron microscope. Electron micrographs show the smallest phages to be almost spherical particles. Larger phages appear to consist of a "head" and a "tail". It is found that the head contains desoxyribonucleic acid (D.N.A.), a substance which is abundant in the nuclear chromatin of the cells of animals. This is surrounded by a protein envelope.

By correlating the number of phage particles counted by electron microscopy with the number of plaques formed by dilutions of the same specimen, it has been found that one head and tail unit is responsible for the formation of a single plaque.

Much work has been done to elucidate the mechanism whereby the bacteriophage is able to lyse a bacterium. Seven particular phages, of the many which attack B. coli have been most studied. These are among the larger phages and have been arbitrarily named T, T_2, etc., to T_7. Different strains of B. coli (or of any bacterial species) are highly specific with regard to the phages to which they are susceptible. Use is made of this fact in the phage typing of bacteria already mentioned.

When infection takes place, the phage is absorbed on to the surface of the bacterium tail first. The protein of the tail then contracts so that the tail becomes shorter and thicker. The contents of the head (D.N.A.) then move into the bacterial cell. The now empty protein envelope remaining outside the bacterial cell

is known as a ghost phage. It is found that ghost phages produced by artificial removal of D.N.A. from a normal phage are still able to lyse bacteria. However, in the absence of the phage D.N.A., no multiplication of the phage occurs.

After a normal phage has injected its D.N.A. into a bacterial cell, little or no more bacterial material is synthesised. All the available bacterial processes are directed towards the production of bacteriophage constituents. It is as if the bacterium gives its life to the phage it contains. Yet at this point no bacteriophage can be detected within it.

The presence within a normal bacterial cell of a certain specific nucleic acid is required for each specific property which that cell possesses. It would appear that nucleic acids direct the metabolic processes of the cell. When, however, foreign nucleic acid is injected from a phage, metabolism is directed by the new nucleic acid.

It is an interesting observation that nucleic acid extracted from certain bacterial strains, when introduced into closely related strains, causes a transfer of genetic material to the second strain. The new property conveyed by the acid manifests itself in the second strain and all its progeny.

In favourable conditions the bacterial cell usually bursts twenty to thirty minutes after the beginning of infection, liberating several hundred phages each of which may infect a new bacterium. This represents a phenomenal rate of multiplication even when compared with that of bacterial multiplication.

When a phage attacks a bacterial cell, an alternative sequence of events from those described above may ensue. The phage, instead of reorganising the metabolism of its host, may lapse into a non-inefective stage which thereafter reproduces synchronously with its host. Bacteriophage in this condition is known as prophage, and the bacterium is said to be lysogenic, since from time to time members of its progeny may lyse and liberate infective phage.

The lysogenic bacterium represents a resistant variation of the original susceptible strain. The presence of phage material within the bacterial cell cannot be detected by ordinary means. It is the formation of strains resistant to bacteriophage that is probably one of the reasons for the disappointing results obtained with bacteriophage as a therapeutic agent. In the competition for survival, in which the fittest live to multiply, the possession of prophage gives the lysogenic bacterium a distinct advantage over the non-lysogenic but susceptible bacterium. This is because from

(Continued on page 70)

AGAR

MR S. W. WHITE, B.Sc., A.N.Z.I.C.
(*Davis Gelatine (N.Z.) Ltd., Christchurch*)

In this short exposition on Agar I propose to divide the subject matter into four main sections.

1. Definition.
2. Historical.
3. Technological (Manufacture).
4. Applications.

Various definitions of agar have been given but simply it may be described as a gel forming purified extract from certain types of red seaweeds. In technical terms it is the calcium or magnesium salt of a linear polygalactose sulphuric ester obtained from Gelidium, Gracilaria, Pterocladia and other related genera of the Rhodophyceae.

It should be mentioned that not all types of red seaweed contain agar. There is an almost complete gradation of types from non-gel forming through weak gel forming to the strong gel agar type. Of the intermediate types the only one of economic significance is carrageen known to us as Irish Moss.

Incidentally, as we know, not all seaweeds are red. Most of those around the South Island are brown. These contain another colloid, algin, which is of commercial importance but not exploited in New Zealand.

Seaweeds and seaweed jellies have been used for many centuries in the Orient.

The credit for the discovery of the method of purifying the extract is attributed to a 17th century Japanese, Minoya. By chance, he noticed that some seaweed jelly thrown out the previous night, had frozen, and thawed and dried in the sun to produce translucent flakes. His discovery is still the basis of agar manufacture in Japan and elsewhere. The Japanese term "Kanten" meaning cold weather—an allusion to the method of manufacture—was applied to the frozen dried extract.

Chinese immigrants introduced it to the East Indies where the Malayan term agar-agar, already used to denote edible seaweeds and jellies, was applied to it.

Dutch influence in the East Indies carried the product to Europe where it became known as a gelatin substitute.

The idea of using agar as culture medium for bacteria is attributed to a housewife, Frau Hesse. When her husband, Dr. Walther Hesse, was having trouble using gelatin to cultivate pathogenic bacteria she suggested he try agar.

The trial was as we know a success. The result was communicated to the famous Robert Koch who in 1882 formally announced the use of agar as a new culture medium.

Agar thus gained increasing importance in "Western" countries. The demand was satisfied almost entirely by imports from Japan. The entry of Japan into the war in 1941 removed Japanese agar from the world markets and the Allied Powers turned to other sources of this important and indeed strategic colloid. Intensive investigations, and searches around the coastline were conducted in many countries to determine the presence and extent of suitable seaweeds.

Without belittling efforts in other countries it may be fairly stated that New Zealand was particularly successful. Thanks to the Dominion Laboratory and especially to Miss Lucy Moore, Senior Botanist of D.S.I.R., the presence of economic quantities of the seaweed genus Pterocladia was established. It is indeed fortunate that Pterocladia proved to be the source of the highest grade agar. The most prolific areas of Pterocladia growth are around the North and East coasts of the North Island, the only growing area in the South Island being off the Kaikoura Peninsula.

Pterocladia grows on rocky off-shore reefs in turbulent water and is generally collected as drift weed on beaches near these reefs, although in certain areas it can be picked off the rocks themselves.

During the collection of Pterocladia care is taken to avoid foreign seaweeds, some 70 in number. After collection the weed is placed over wire frames for sun drying following which it is further sorted, shaken free of loose sand and coral, and finally baled for despatch. These operations are carried out by some 600 Maori and pakeha collectors as a part-time occupation.

The manufacturing process, although fundamentally similar to the traditional Japanese process, is rather different in practice. Handling is largely mechanical and artificial freezing and drying replace the use of the natural agencies of frost, sun and wind. In addition copious use of water is made to produce the final product.

The seaweed when received at the Christchurch factory from the collectors is first weighed and examined. This examination is not only for impurities but also to ascertain the variety of Pterocladia. In general there are two species, Pterocladia lucida, a coarse weed, and Pterocladia capillacea, a fine weed. Each variety—related broadly to the locality of collection—yields an agar of slightly different characteristics and hence in the production of a standard product blending has to be started at the raw seaweed stage.

After selection, the weed is first washed to remove any sand or coral, etc., and is then loaded into pressure extraction vessels. In these vessels the seaweed is subjected to heating in water under steam pressure to yield a liquor containing agar in the crude form. This process is repeated several times at increasing temperatures, each being dependent upon the seaweed type, until all the agar is extracted.

The crude liquor is run off from the weed residue, or scutch and filtered through presses using diatomaceous earth as a filter aid. This removes any fine weed particles and other debris to give an optimum clarity to the final product—an important factor in bacteriological media. At this stage, however, the agar liquor still contains inorganic salts and colouring matter extracted from the seaweed.

The filtered liquor is run on to a cooling belt where it is chilled and cut into strips. The strips are run into vats where they are washed or dialysed, for 24 hours in running water, thereby removing most of the colouring matter and inorganic salt impurities.

Now agar exhibits a curious phenomenon in that if a gel is frozen, during freezing the agar expels approximately two-thirds of its water which it does not re-absorb in thawing. Along with water, soluble impurities are also expelled.

Use of this phenomenon is made in the manufacturing process. The dialysed strips are frozen in ice cans and then thawed under water showers. This is followed by further dialysis for impurity removal to yield a purified agar in a wet flake form.

The drained flakes are dried on stainless steel containers in a flow of warm filtered air to form a sheet. These sheets are crushed to a powder which is tested, blended and retested before final packing.

APPLICATIONS

Agar is used for many purposes, commercially, in meat canning, confectionery, etc., and technically for salt bridges, electrophoresis experiments, corrosion plate tests as well as in bacteriology.

Briefly agar is used wherever a strong, inert gel is required which is not extensively degraded by heating to high temperatures under neutral or mildly alkaline conditions.

The latter property is of great importance in bacteriology where sterilisation by autoclaving under pressure is the normal practice.

Another advantage of agar in culture media is that it is not attacked or liquified by the organisms usually cultured. Gelatin, for example, being a protein derivative is more akin to body

tissue and is very susceptible to decomposition by pathogenic organisms.

A very important property of agar in bacteriological work is that of hysteresis. This can be explained as follows:—

If we prepare an agar solution by autoclaving the powder in water it remains liquid until cooled below about 35°C. when it sets to a gel. Now on reheating it does not melt again until the temperature has reached about 92°C.—almost boiling point.

A liquid agar culture medium in, say, a test tube, may thus be inoculated with the test organism and kept for a period immersed in a water bath at 37-40°C. until we are ready to pour the plates, without risk of setting or affecting the viability of the organism. Once the medium has gelled it may be incubated at optimum growth temperature without possibility of melting.

The particular properties desired in agar for bacteriological use are high gel strength, so that the surface of the gel will not break on streaking, and that it does not contain any substances toxic to bacteria, or impurities that could react adversely with the many and varied components of culture media.

As may be gathered from the earlier description of the process, considerable care is taken to ensure that New Zealand agar meets the above specifications. J. L. Forsdike found in his survey of available agars that it was not only the strongest agar, a 0.7% concentration gel being equivalent to a 1.0% Japanese gel, but it had also the lowest ash content, under 1.2% as against the B.P. limit of 5.0%.

This quality of New Zealand agar was rapidly appreciated in Great Britain and it is significant today, when agars from various sources are freely available in that country, that a media manufacturer of the calibre of OXO Ltd., firms such as Burroughs Wellcome and B.D.H., and the Public Health Laboratories still rely on New Zealand agar. Closer to home are the well known Commonwealth Serum Laboratories in Victoria.

It should be finally mentioned that agar produced according to the process outlined must, by virtue of its purity, contain a minimum of harmless substances which may well act as positive growth factors for certain organisms. This must of course be overcome by the bacteriologist by the addition of the requisite material to his medium.

To quote a particular instance in our experience. The South African Institute for Medical Research working on the growth of the B.C.G. strain of tubercle bacillus in a horse serum medium found considerable variation in counts according to the sample of agar used. They eventually traced the variable factor to iron.

Although some iron was present in the medium it was below the optimum level and hence the iron content of the agar itself became critical.

This is a particular example of the paradoxical situation where a purer product is regarded as inferior. However, the bacteriologist who relies on a crude agar to supply nutrients is liable to encounter many variations and complications.

Although much work has been done there is obviously plenty of scope for further investigations into the role of trace materials, both organic and inorganic, in the nutrition and growth of micro-organisms.

Regarding the future of agar, it appears because of its unique properties, to be firmly established in bacteriology at least, and is unlikely to be replaced in the near future, if ever, by natural or synthetic substitutes.

REFERENCES

C. K. Tseng (1946), Phycocolloides—Useful Seaweed Polysaccharides in "Colloid Chemistry", Alexander, p. 629, Reinhold Publishing Corp.

Japanese Agar-Agar Industry (1946), Report B.O.I.S./J.A.P./PR/814, London, H.M. Stationery Office.

J. L. Forsdike (1950), J. Pharm & Pharmac, **11**, 796.

P. A. Christensen (1957), Bull. Wld. Hlth. Org., **17**, 307.

J. Lavollay (1953), Revue General des Sciences Tome LXII Nos. 3-4.

A BRIEF OUTLINE OF THE NATURE AND DISCOVERY OF BACTERIOPHAGE

K. R. JAMES (Pathology Department, Waikato Hospital)

(Continued from page 65)

time to time an occasional lysogenic bacterium will lyse liberating hundreds of phages which quickly eliminate the closely related non-lysogenic bacterial strains.

We may conclude by observing that although the hope of the earlier workers as to the value of the bacteriophage in the combatting of disease have not been realised, their work has opened up a whole new field of research, the results of which have provided medicine with useful contributions in other directions.

THE NEW ZEALAND ASSOCIATION OF BACTERIOLOGISTS (INCORPORATED)

Minutes of the council meeting held in Wellington on July 30, 1960. The meeting opened at 10.40 a.m.

Those present were Messrs Olive, Reynolds, Donnell, Bloore, Walker, Philip, Hutchings and Misses Mattingly and Scarf.

Moved: That the rules of the New Zealand Association of Bacteriologist (Inc.) be applicable in the meantime to the New Zealand Institute of Medical Laboratory Technology (Inc.). Bloore, Philip.

Mr Olive expressed appreciation of the work done by past members of the council, in particular the immediate past President, Mr Reynolds. Mr Olive also extended a welcome to the new members of council, pointing out in passing that this was the inaugural meeting of the council of the Association under its new name.

Apologies were received from Messrs Rose and Cameron. Olive, Donnell.

Minutes: Moved that the minutes of the previous meeting be approved and confirmed. Olive/Donnell.

Mr Reynolds signed a copy of the above minutes and at his request was excused from the meeting.

Applications and Resignations:

Moved: That the applications be accepted and that the resignations be received with regret. Donnell, Bloore.

Moved: That the secretary write to Mr Rankin expressing regret at his resignation and extending the good wishes of the Association. Donnell, Bloore.

Correspondence:

The secretary was asked to write to Mr Barrington and refer him to rule 30 of the constitution re the formation of branches to the Association.

Mr Walker was asked to convey to the Christchurch staff that the consideration of cardigans as part of protective uniform could not be the concern of the Association but rather a domestic matter.

Moved: That the secretary be asked to write to the Federal Secretary of the A.I.M.L.T. pointing out that we have received information from Strasbourg, via London, that we have joined with them, and with them have applied for membership of the I.A.I.M.L.T. That the Secretary of the A.I.M.L.T. be asked for clarification, how the situation arose and for further information about the A.I.M.L.T. Bloore, Donnell.

The secretary was asked to write to the Registrar of Incorporate Societies enclosing the Annual Report, the Balance Sheet and advising him of the change of name of the Association and of the resolution of the Council that until suitable rules were compiled and approved the exisiting rules of the N.Z.A.B. (Inc.) shall apply to the N.Z.I.M.L.T. In the event of the above not being acceptable a request for further advice be made.

Moved: That the secretary be asked to write to the General Manager N.A.C. asking him if there is a reduction in air fares for delegates for Conferences.

Moved: That inward correspondence be received and that outward correspondence be approved. Donnell, Philip.

Journal Report:

The Journal for August was reported to be in the hands of the printer and proceeding satisfactorily.

Moved: That a competition be run to suggest a possible design for a common seal. The closing date should be October the 1st and the prize for the accepted design be £2/2/-. Walker, Mattingly.

Treasurer's Report:

The treasurer reported on the state of the subscriptions received and stated that further notices were being sent out.

Moved: That the expenses to the Council meeting be paid. Olive, Mattingly.

Moved: That the treasurer's report be accepted. Philip, Bloore.

General Business:

The secretary was asked to write to the Director-General of Health withdrawing the request for official recognition of the sub-committee to meet the Society of Pathologists.

Moved: That the sub-committee which was to have met the Society of Pathologists with the addition of Mr Bloore be the committee to collect and collate relevant information pertaining to training and examinations. Philip, Mattingly.

Moved: That the Standing Sub-Committee consisting of Messrs McKinley, Whillans and Reynolds with the addition of the President be kept informed. Mattingly, Scarf.

The meeting closed at 4.40 p.m.

LABORATORY HINT

It does not appear to be necessary to take the customary few minutes to perform a routine Gram stain. A quicker modification has been in use at this laboratory for some years. The usual aqueous stains are used with acetone as decolourizer and 1% saffranine as the counterstain of choice. The solutions are applied and tipped off almost immediately, the whole staining process being completed and the slide blotted in about 15 seconds. In practice the time each stain remains in contact is about two seconds which seems to be adequate.

R. W. BARRINGTON,
Public Hospital,
Hawera.

A well aimed blow by Pyrex

A machine called the Turret Chain made these: it automatically makes blownware such as flasks, beakers and other types of laboratory ware, to a very high standard of uniformity. It's the only machine of its kind in Europe. Just an example of how PYREX, the first and most important source of borosilicate glassware in this country, use the latest and best processes available. PYREX are always improving their production methods to attain even higher standards of quality. This is one good reason (among many) why everyone who is looking for quality glassware, looks for PYREX.

Pyrex beakers and flasks

are made in all practical sizes and shapes for students, routine or research work.

Extremely low expansion coefficient virtually eliminates breakage from thermal shock allows more robust construction giving greatly increased mechanical strength

High stability against attack from water and all acids (except hydrofluoric and glacial phosphoric)

Therefore durable, accurate, economical, dependable, most used.

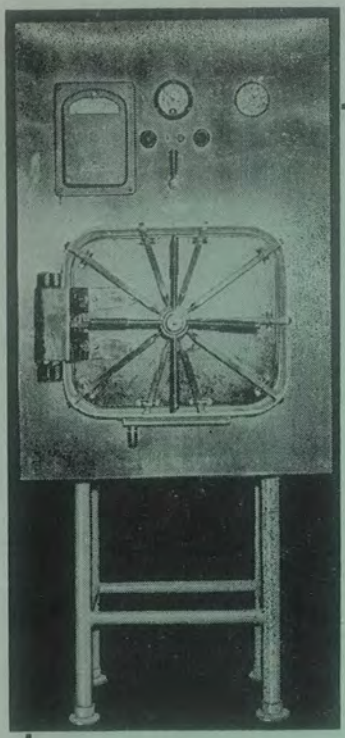
PYREX

Regd. Trade Mark

MADE IN JP PYREX JP ENGLAND

Laboratory and scientific glass

N.Z. Agents: **Messrs. F. O. & H. S. HART, 20 Victoria Street, Wellington, C.1**



MERCER
Bacteriological Autoclave

(Rectangular Type)

These rectangular single jacketted units are ideal for Bacteriological work in that all Chamber space is working space. Type illustrated is fitted with Flush Panel mounting adjustable thermometer and adjustable timer . . . both being integrated to permit presetting by means of keys of both temperature and time, taking guesswork out of all types of media sterilising.
Any temperature may be selected up to the equivalent of 30lbs. pressure for any length of time required up to 60 minutes. Push button control, two light stage indicator.

CRAFTSMEN
Mercer
IN METALS

J. Mercer & Sons Ltd., Christchurch, Head Office: Moorhouse Avenue. Phone 69-679. Auckland, 36 Carr Road, Mt. Roskill. Phone 556-061 or 556-921. Wellington, 10 Vivian Street. Phone 50-400 or 53-047. Otago and Southland, Home Heating Supplies Co. Ltd., 17 St. Andrew Street. Dunedin. Phone 70-052.

Printed by Simpson & Williams Ltd., 169 St. Asaph Street, Christchurch.
33571