Bacteriological techniques for dairy purposes.

MINISTRY OF AGRICULTURE, FISHERIES AND FOOD

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Bacteriological Techniques for Dairy Purposes

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BACTERIOLOGICAL TECHNIQUES FOR DAIRY PURPOSES

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Foreword

THE techniques included in this Bulletin were compiled during the past six years by the Publications Committee* of the Conference of Bacteriologists of the National Agricultural Advisory Service in England and Wales. Bacteriologists from the National Institute for Research in Dairying, Shinfield, Reading gave valuable assistance.

Some of the basic bacteriological techniques are modifications of those originally used during the Clean Milk Competitions of 1924—35, others are based on new methods introduced during 1942—46 in connection with the National Milk Testing and Advisory Scheme, while some are advisory or statutory tests developed by advisory bacteriologists during the past 20 years. They were originally issued as provisional techniques and after continuous use in National Agricultural Advisory Service and Milk Service laboratories for several years were eventually approved by the Publications Committee as standard methods.

The various sections give the technical details necessary for carrying out a process by a standard method so as to enable consistent results to be obtained at different laboratories.

It has not been considered possible, within the scope of this Bulletin, to enter into any discussion of the principles on which the techniques are based, or to make recommedations regarding the interpretation of results. Nevertheless, subjective notes on the advisory approach, as well as on sampling and investigational procedures, have been included.

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Senior Science Adviser
National Agricultural Advisory Service

Ministry of Agriculture, Fisheries and Food
March, 1968

*The Publications Committee was composed of the following: S. B. Thomas, O.B.E. (Chairman), J. W. Egdell, J. Harrison, C. A. Scarlett, and Phyllis M. Hobson, M.B.E. and J. J. Panes (Secretaries), as well as the late W. A. Cuthbert, and Christina M. Cousins of the National Institute for Research in Dairying, Shinfield, Reading.

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NOTE

All temperature references in this publication are in centigrade, except where otherwise stated.

Historical Introduction

THE bacteriological examination of milk was being undertaken during the last decade of the 19th century at several university laboratories in Britain. Probably the best known account of the techniques used during the pioneer period is that given by Swithinbank and Newman (1903) in *Bacteriology of Milk* published by John Murray of London. In the course of their work carried out at a private laboratory in Denham, Buckinghamshire, they determined colony counts on nutrient gelatine at 20° or lower and on nutrient agar incubated at 37°. They also estimated the coliform content of the milk by inoculation into phenol bouillon incubated at 37°.

Early Advisory Tests

The introduction of bacteriological grading of milk in England and Wales was mainly brought about by the pioneer work of Professor Sheridan Delépine at Manchester University during 1891—1918 and of Dr. R. Stenhouse Williams at the University College, Reading and at the National Institute for Research in Dairying at Shinfield (N.I.R.D.) during 1913—32. An account of some of the early bacteriological work carried out at the N.I.R.D. is given in four editions of *Studies Concerning the Handling of Milk* published between 1924 and 1931 by H.M.S.O.

The first publication of *standard* routine techniques for the bacteriological examination of milk in England and Wales was issued by the Ministry of Agriculture and Fisheries in connection with Clean Milk Competitions organised on a county basis during 1924—35. The first edition of the *Guide to the Conduct of Clean Milk Competitions* was published by H.M.S.O. in 1924, and four subsequent editions were printed during the next 10 years. These recommendations were drawn up by members of the staff of the N.I.R.D., and advisory dairy bacteriologists who were at the time on the staffs of the agricultural departments of certain universities and agricultural colleges. Methods for taking milk samples, bacteriological examination by means of colony count on standard nutrient agar incubated at 37° for 48 hr, and the coliform test by the inoculation of MacConkey's broth incubated at 37° for 72 hr were included. Keeping quality was determined by smell, taste and a clot-on-boiling test applied daily at 9 a.m. and 5 p.m. to milk samples held at 15·5° (60°F).

A practical guide for media preparation and milk testing entitled *Bacteriological Control of Milk*, prepared by A. G. House (1931) of the N.I.R.D., also formed the basis of the techniques used for the examination of milk during this period.

Mattick (1929–30) discussed various factors which influenced the colony count at 37° as a method of estimating the number of bacteria in milk, and the value of a test for coliform organisms (MacConkey's broth incubated at 37° for 72 hr) as an index of the care taken in the production of milk and of its probable keeping quality. He pointed out that the keeping quality of milk, determined by testing at intervals milk samples held at

15·5° until the first indication of any taint or undesirable flavour, depends upon the kind as well as upon the numbers of bacteria present.

Investigations by Hiscox, Hoy, Lomax and Mattick (1932—33) demonstrated the inadequacy of standard nutrient agar as a medium for colony counts of milk, and showed that the addition of milk to the medium often had a marked influence on the colony counts. Their experiments with the methylene blue reductase test at 15·5° indicated a close correlation between the appearance of taint as indicated by taste and the reduction of methylene blue at this temperature.

Statistical examination of the interrelationship and variability of the colony count, presumptive coliform content and keeping quality of raw milk were carried out by advisory dairy bacteriologists during 1930—40, and were reported by Barkworth (1935), and Barkworth, Irwin and Mattick (1941).

Early Statutory Tests

The statutory grading of milk in England and Wales dates from the Milk (Special Designations) Order of 1922. The bacteriological standards for the various grades of milk were as follows:

Grade	Colony count/ml Nutrient agar 48 hr 37° Not to exceed:	Coliform organisms MacConkey's broth 72 hr 37° Not to be found in:
Certified	30,000	10^{-1}ml
Grade A (T.T.) Grade A	200,000	10^{-2}ml
Grade A (Pasteurized)	30,000	10^{-1}ml
Pasteurized	100,000	No standard

Details of techniques and media were not prescribed in the Order, but were later described in Memo. 139/Foods, *Bacteriological Tests for Graded Milk* published for the Ministry of Health by H.M.S.O. in 1929 and 1937. The colony count on nutrient agar and the coliform test in MacConkey's broth, both at 37°, were prescribed for the examination of raw and pasteurized milk in the 1929 edition. As a result of the classical work of Sir Graham Wilson and his colleagues at the London School of Hygiene and Tropical Medicine, reported in *Bacteriological Grading of Milk* published by H.M.S.O. in 1935, the 1937 edition of Memo, 139/Foods prescribed a $4\frac{1}{2}/5\frac{1}{2}$ hr methylene blue test at 37° in conjunction with the coliform test at 37° for the examination of designated, raw milk. The colony count at 37° was retained for the examination of pasteurized milk, but Yeastrel milk agar was used instead of nutrient agar.

National Milk Testing and Advisory Scheme

Modifications of the resazurin test were developed in England and Wales during 1938–43 at the N.I.R.D., and at the University College of Wales, Aberystwyth. During the period 1942–46 several new bacteriological techniques were issued by the Ministry of Agriculture and Fisheries in connection with the National Milk Testing and Advisory Scheme operating in England and Wales. These were drawn up by a Technical Committee composed of advisory dairy bacteriologists, bacteriologists from the trade and the N.I.R.D.

A ten minute (rejection) resazurin test at 37° was introduced for the examination of farm milk supplies suspected to be of poor quality on arrival at the collecting creamery. Milk showing rapid reduction of resazurin (to Lovibond discs 3½ or less) was rejected and returned to the producer. The ten minute resazurin test proved a most valuable method for appraisal of producer's milk and it continues to be employed for this purpose at all creameries in England and Wales.

A routine temperature-compensated resazurin test at 37° was introduced for the fortnightly examination of all farm milk supplies at creamery and advisory laboratories in England and Wales during 1942–49. Milk supplies were graded into three categories: those giving disc readings of 4 or over were placed in Category A and were considered to have a satisfactory keeping quality; those giving disc readings of 3½ to 1 (both inclusive) were placed in Category B and considered to be of doubtful keeping quality; and those giving disc readings of ½ or 0 were placed in Category C and were considered to be of unsatisfactory keeping quality.

Standard rinse techniques for the examination of washed milk cans and of milk bottles, together with a swab method for examination of milk plant were also issued by the Ministry during 1944–45. These rinse techniques were based on work by Mattick and Hoy (1937) and Hobbs and Wilson (1943) in connection with their investigations on bottle washing machines. The swab technique had been employed in various ways at advisory and creamery laboratories since about 1930 and the standard technique now recommended was a direct development of this pioneer work.

The rinse and swab methods were adopted by the advisory bacteriologists for the examination of farm dairy equipment, and they have been used extensively for this purpose since about 1945.

Modification of Incubation Temperature

The use of Yeastrel milk agar incubated at 30° for 72 hr instead of at 37° for 48 hr was introduced for routine work about 1947, as it had been shown that colony counts of milk, rinses and swabs at 30° were often much higher than those at 37° due to the failure of certain types of thermoduric and of psychrotophic bacteria to form colonies at 37°.

Following the isolation by several investigators of strains of coli-aerogenes organisms from milk, butter and cheese which failed to form gas at 37° in MacConkey's broth, the need arose for amplification of the method of testing for these organisms in milk, and this was considered by Mattick and Hiscox (1935) who questioned the wisdom of the use of an incubation temperature of 37°. Consequently, the presence of coli-aerogenes organisms

in milk, milk products and in rinse and swab solutions has, since 1950, been determined at the laboratories of the National Agricultural Advisory Service (N.A.A.S.) in England and Wales by incubation of MacConkey's broth at 30° for 72 hr, or of violet red bile agar at 30° for 20–24 hr.

Thermoduric Bacteria

Following a series of investigations by N.A.A.S. advisory bacteriologists during 1950–56, a laboratory pasteurization test was introduced for the examination of advisory samples of farm milk supplies, 10 ml amounts of milk being heated at 63·5° for 30 min in completely immersed, thin walled, stoppered test-tubes and the cooled milk plated on Yeastrel milk agar incubated at 30° for 72 hr for the determination of the thermoduric colony count.

Psychrotrophic Organisms

With the increasing use of refrigeration, the importance of psychrotrophic organisms in raw and pasteurized milk in causing defects such as fishiness, rancidity, fruitiness and ropiness has become apparent. The psychrotrophic colony count, determined by plating serial tenfold dilutions of rinses and swabs of dairy equipment and of milk samples on Yeastrel milk agar incubated at 5–7° for 10 days, was therefore introduced in 1960 for the examination of rinses and swabs of farm bulk milk tanks and samples of refrigerated milk from bulk milk tanks, vending and dispensing machines.

Lecithinase Producing Organisms

During recent years there has been an increasing incidence of the defect known as 'bitty' or 'broken' cream, particularly in pasteurized milk which has not been held at satisfactory cold store temperatures during warm weather. A standard technique devised by Billing and Cuthbert (1958) for the enumeration of the most probable number of lecithinase-forming strains of *Bacillus cereus*, is used for the examination of advisory rinses, swabs and milk samples. As a measure of the effect produced by *B. cereus* in milk, the floc count technique is also used.

Lactobacilli and Pediococci

N.A.A.S. advisory bacteriologists have surveyed the incidence of lactobacilli and pediococci in bulk raw and heat-treated milk, starter and Cheddar, Cheshire and Caerphilly cheese sampled at cheese factories, using the modified Rogosa acetate agar of Mabbit and Zielinska (1956) incubated anaerobically in an atmosphere of CO_2 for 5 days at 30°.

Coagulase-Positive Staphylococci

Surveys of the incidence of coagulase-positive staphylococci in raw milk, heat-treated milk and cheese have recently been made, in conjunction with the N.I.R.D., by N.A.A.S. advisory bacteriologists, using dried plates of the selective medium recommended by Baird-Parker (1962), characteristic colonies being confirmed by means of the coagulase test.

Later Statutory Tests

A comprehensive series of investigations on keeping quality and raw milk grading, carried out conjointly by workers at the N.I.R.D., Rothamsted Experimental Station and bacteriologists of the N.A.A.S. during 1944–48, showed a high correlation between methylene blue and resazurin tests, but both were found to be relatively unsatisfactory as measures of keeping quality. Instead, the clot-on-boiling (C.O.B.) test at 22° was recommended for this purpose, compensation being applied for variations in atmospheric shade temperatures during storage of the samples before testing. This temperature-compensated C.O.B. test was introduced for the bacteriological control of Tuberculin Tested, farm milk supplies in 1960, the milk being sampled at the farm and examined at Milk Service Laboratories of the Ministry of Agriculture, Fisheries and Food.

A further series of joint investigations by bacteriologists of the N.A.A.S., the National Milk Testing Service and Public Health Laboratory Service on the suitability of various tests for both raw and pasteurized milk sampled during distribution to the consumer, was carried out during 1949–50. These investigations resulted in the introduction, during 1960, of a statutory half hour methylene blue test at 37° for the examination of Tuberculin Tested and Pasteurized *retail* milk supplies. The milk sample, taken during distribution, is pre-incubated overnight at atmospheric shade temperature during summer and at 18·5° (65°F) during winter.

Since October, 1964 this half hour methylene blue test has also been prescribed for the statutory control of producer-retailer milk supplies (Untreated), sampled and examined by the Milk Service and Local Authorities.

More Recent Advisory Tests

A re-appraisal of the advisory approach to bacteriological problems became necessary when in October, 1964 the 2 hr resazurin test at 37° was introduced at creameries for the payment of milk on a hygienic quality basis. Consequently, advisory milk samples are now being examined within 3 hr of milking, or 3 hr of collection in the case of farm bulk tank milk, by means of an extended 4 hr resazurin test at 37°, the udder cell count and the Whiteside test, in addition to colony count and coliform test at 30°. In order to detect unsatisfactory production methods which may be masked by the refrigeration of the milk on the farm, the milk sample is again examined for colony count and resazurin reduction after pre-incubation for 18 hr at 18·5°.

Tests For Detergents and Disinfectants

Control of the concentration of chemicals used for cleansing dairy equipment is important in determining the efficiency of the process. In 1945, prior to the widespread use of chemical disinfection, a Memorandum on Detergents was issued by the Ministry of Agriculture and Fisheries. It gave information on the different types of detergents and their properties and included tests for detergent strength.

Chemical disinfection of all types of dairy equipment was first permitted on a provisional basis in England and Wales during 1943, and in 1949 was

given equal status with steam and boiling water. Prior to 1957, sodium hypochlorite, conforming to specifications laid down by the Ministry of Agriculture and Fisheries and the Ministry of Health and sold under different trade names, was the only chemical approved for this purpose. Subesquently other chemical agents, mainly combined detergent-disinfectants containing quaternary ammonium compounds, 'chlorine releasing' organic compounds or iodophor were approved. The use of chemical disinfectants has necessitated tests for detecting such chemicals in milk. In addition, tests for the routine determination of alkali, acid and disinfectant concentrations in cleansing solutions have been included in this Bulletin.

Antibiotics in Milk

Extensive surveys of the incidence of penicillin in farm milk supplies were carried out in Britain during 1954 and 1961, the T.T.C. (2–3–5 triphenyltetrazolium) test devised by Wright and Tramer (1961) being used during the latter survey. A modification of this test, involving incubation at 37° instead of 44–45°, now forms the basis of a scheme for the regular monthly testing of all incoming farm milk supplies at creameries in England and Wales.

Microflora of Milk and Farm Dairy Equipment

Recent technological developments have emphasized the need for more information on the composition of the microflora of dairy equipment and milk. Investigations in Britain of the composition and development of bacterial populations in milk have been reported by Gibson and Abd-El-Malek (1957) and Thomas *et al.* (1962). Standard methods for determining the incidence and activity in milk of the main groups of bacteria found on dairy equipment and in milk have been included in this Bulletin, and they have been used by the advisory bacteriologists in microflora studies of milking equipment cleansed by chemical methods, pipeline milking plants, farm bulk milk tanks, washed milk bottles and washed milk cans.

Assessment of Suitability of Dairy Farm Water Supplies

The importance of the provision of an adequate supply of clean water in the farm dairy and cowhouse or milking parlour is emphasized in the Milk and Dairies (General) Regulations, 1959, which state that all registered dairy premises shall be provided with a supply of water suitable and sufficient for the requirements of the regulations.

Since 1949, the advisory bacteriologists of the Ministry have been responsible for the assessment of the suitability for dairy purposes of all farm water supplies, except those taken from a public main. The water supplies are assessed on the basis of detailed topographical examination and the bacteriological examination of a series of samples taken over a period of several months. The tests used are similar to those prescribed by the Ministry of Health (1964) in Report No. 71, *The Bacteriological Examination of Water Supplies*. Water supplies which have been accepted as

suitable for dairy purposes are re-examined at regular intervals in order to detect any deterioration.

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Equipment and Glassware

1. CLEANING AND STERILIZATION OF EQUIPMENT

Cleaning

1. *New Glassware*

(*a*) *Soft Glass* should be soaked overnight in a 1 per cent v/v solution of hydrochloric acid (10 ml of conc. acid per 1 litre). After the acid soak, rinse in running tap water and then in distilled water. Autoclave in distilled water at 121° for 15 min, rinse in acid and then in distilled water.

(*b*) *Hard Glass* should be treated in the same manner, except that the overnight acid soaking may be omitted.

2. *Used Equipment*

Where necessary, used equipment should be autoclaved to render it safe from pathogenic organisms. Petri dishes and test-tubes should be autoclaved at 121° for 15 min, the melted agar or broth poured away, and then washed as follows:

(*a*) *Test Tubes, Petri Dishes and Sampling Bottles.* Rinse glassware in cold or warm water and boil in a suitable alkaline detergent solution (e.g., 0·125 per cent Na_2CO_3) and brush in this solution (cotton wool is more successful than brushes for cleaning Petri dishes). Transfer the glassware to a 0·4 per cent v/v solution of hydrochloric acid (4 ml of conc. acid per litre), checking the strength periodically. Rinse the glassware in running tap water and finally in distilled water. It is convenient to dry glassware in a hot-air oven. Polish Petri dishes with a glass cloth.

(*b*) *Pipettes.* Immediately after use, submerge pipettes in a jar containing a suitable disinfectant (e.g., 500 p/m of chloramine T) together with 1 per cent alkaline detergent. This facilitates subsequent washing and kills any pathogens in a disinfecting solution which is readily rinsed away. Remove the cotton-wool plugs by means of a jet of water and boil the pipettes in an alkaline detergent solution (0·125 per cent Na_2CO_3). Rinse thoroughly in water and then rinse in a bath of 0·4 per cent v/v hydrochloric acid. Rinse again under running water and rinse finally in distilled water. They may be drained and then dried in a hot-air oven.

(*c*) *Rubber Closures.* Rinse in cold water, then boil in a 2 per cent solution of Na_2CO_3 or a 1 per cent solution of NaOH for 10 min. Wash immediately in boiling water and finally rinse in clean cold water.

(*d*) *Sampling Dippers.* Wash as described in para. 2 (*a*).

(*e*) *Chromic Acid Cleaning.* For special purposes, glassware should be soaked overnight in a cleaning mixture prepared as follows: dissolve 90 g of sodium dichromate in 200 ml of water. Add to this solution, *with great care*, 2 litres of concentrated sulphuric acid.

A weaker solution may be found satisfactory, prepared by dissolving 60 g potassium dichromate in 940 ml of water, and *cautiously* adding 60 ml of concentrated sulphuric acid, with constant mixing.

Following either of these treatments, rinse glassware five times under running tap water and twice in distilled water.

Sterilization

3. Before sterilization, test-tubes and bottles should be plugged with cotton wool or protected with a suitable loose fitting closure. Pipettes should be plugged with cotton wool and the ends of the plugs singed. Sampling dippers should be sterilized in metal containers or wrapped separately in greasproof paper before sterilization.

All equipment should be sterilized by one of the following methods:

(*a*) *Hot-Air-Oven.* Heat the pipettes and Petri dishes contained in closed containers in a thermostatically controlled oven for 2 hr at 160–170°. At the end of this period allow the oven to cool before opening the door. Wrap pipettes in suitable paper if containers are not available.

(*b*) *Autoclave.* Steam glassware, rubber closures and other rubberware in an autoclave at 121° for not less than 15 min. (This treatment time may be exceeded for some equipment without adverse results). Take great care to expel air thoroughly from the autoclave by venting before the temperature is raised above 100° and allow a slight bleed of steam during autoclaving. Equipment should not be packed too tightly in the baskets and there should be ample provision for circulation of steam within the autoclave. Do not close containers in which equipment is sterilized prior to treatment in the autoclave. Glassware sterilized in this manner should be dried off in the autoclave or in a hot-air oven at 110°.

2. TESTING BACTERIOLOGICAL TUBES FOR FREE ALKALI

Test-tubes and Durham fermentation tubes for bacteriological use are made of clear glass tubing in two grades (see B.S.625:1959). A test which detects the presence of free alkali is used to distinguish tubes of 'Special Grade' quality from the 'Ordinary Grade'.

Traces of soda leached from the glass may increase the alkalinity of samples during storage or change the pH of media during their sterilization or storage. As these changes can affect the results of bacteriological tests it is essential to use only glassware of the 'Special Grade' quality.

All batches of bacteriological tubes should be tested to ascertain whether the requirements for 'Special Grade' are being satisfied.

Agglutination tubes may be of neutral or soda glass and the provisions regarding 'Special Grade' do not apply.

Method

1. *Solutions Required*

(*a*) *Carbon Dioxide-Free Water.* Boil distilled water, or water of equivalent purity, in a flask of borosilicate glass. After boiling for 10 min fit a closure carrying an absorption tube of soda-lime granules and allow to cool. The carbon dioxide-free water should be freshly prepared each day.

(*b*) *Concentrated Solution of Methyl Red.* Dissolve 35 mg of finely powdered methyl red (free acid, pH indicator grade) in 75 ml ethanol and add CO_2—

free distilled water to make up the volume to about 95 ml. Filter, mix well and adjust the pH to 5·2 by adding N/20 NaOH solution (about 1·5 ml). Make up the volume to 100 ml with carbon dioxide-free distilled water.

Note

A pH meter should be used in the pH adjustment to ensure addition of the correct amount of NaOH solution. If a meter is not available 1·5 ml of N/20 NaOH solution may be added and the pH adjusted to 5·2 with a suitable buffer solution. When correctly prepared a 10 ml sample of the undiluted solution should require the addition of not less than 0·4 ml and not more than 0·45 ml of N/50 NaOH solution to give a colour similar to that of a 10 ml sample of N/1 potassium dichromate.

(*c*) *Acid Solution of Methyl Red.* This solution should be freshly prepared for each day's test. Transfer about 750 ml of carbon dioxide-free distilled water to a clean 1,000 ml measuring flask (B.S. 1792). Using a 10 ml graduated pipette (B.S. 700), add 8·3 ml of N/50 hydrochloric acid. Add 20 ml of strong solution of methyl red by means of a bulb pipette (B.S. 1583). Make up to 1,000 ml with carbon dioxide-free distilled water and mix well.
(*d*) *Comparison Solution of Methyl Red.* Add 0·1 ml of N/20 NaOH solution to 10 ml of the acid solution of methyl red.
(*e*) *Other Solutions and Chemicals*

N/1 Potassium dichromate	(49·04 g $K_2Cr_2O_7$ in 1,000 ml)
N/20 Sodium hydroxide	(2·00 g NaOH in 1,000 ml)
N/50 Sodium hydroxide	(0·80 g NaOH in 1,000 ml)
N/50 Hydrochloric acid	(0·7293 g HCl in 1,000 ml)
Methyl red	(4—dimethylaminoazobenzene—2—carbolic acid, indicator grade)
Ethanol	(95 per cent alcohol).

Procedure

To represent the batch of tubes under investigation select at random at least six tubes from among all the tubes of one size made to one order, and test each one as described below. For a batch to comply with the requirements for 'Special Grade,' each of the selected tubes shall pass the test.

As the tubes may have been stored for short, unequal periods, rinse each one first with tap water, then with 5 per cent v/v aqueous solution of glacial acetic acid, followed by three thorough rinsings inside and out with tap water. Drain for 1 min, then rinse inside and out with distilled water three times, draining for 1 min between rinsings.

Immediately after the rinsing procedure, fill the tubes to nine-tenths of their overflow capacity with the acid solution of methyl red and close each with a free-fitting cap of inert metal foil (e.g., tin or platinum). Place the capped tubes in a rack or holder of chemically inert material so that they are held upright, and place this in an autoclave with the tubes completely above water-level. Close the autoclave, leaving the valve open, and heat for 3 min with steam issuing freely from the valve to ensure that no air remains inside. Then close the valve and allow the temperature to rise to to 121°. Maintain this temperature for half an hour, then discontinue heating and open the valve slightly to allow the temperature and pressure to fall until it is possible to remove the tubes from the autoclave.

Compare the colour of the test solution with that of the comparison solution, either in the tube or by placing a drop of each on a thoroughly washed white glazed tile. The tube passes the test if comparison shows that the colour of the test solution has not changed completely to the yellow of the comparison solution.

Reconditioning after storage. Batches of special grade tubes which have satisfied the requirements of this test may fail to do so after storage. They can be reconditioned by rinsing internally with a 5 per cent v/v aqueous solution of glacial acetic acid. It should be ascertained whether the tubes pass the test after this reconditioning process.

Application to other Glassware

The above test has been devised for test-tubes and for ampoules (B.S. 795: 1961). But it can be applied to examining other and larger pieces of glassware. It must be borne in mind that the ratio of area to volume decreases with the increasing size of vessel and the risk to the contents are therefore reduced. Thus a high grade glass which is imperative for tubes may not be so necessary for large vessels.

Arranged in order of increasing alkalinity the grades of glass tubing are borosilicate, neutral and soda. Bottle glass possesses certain properties peculiar to bottle manufacture and the alkalinity of medical and laboratory bottleware is roughly equivalent to neutral glass, the amount of soda leached out being about half that of soda glass.

Effect of Laboratory Procedures

Tubes which have satisfied 'Special Grade' requirements should not deteriorate in these respects during laboratory use provided they are thoroughly rinsed and treated in weak acid following washing in alkaline detergent (see Technique No. 1).

3. METHOD FOR TESTING STERILITY OF MEDIA AND GLASSWARE

General

1. *Objects*

There are no degrees of sterility. The object of the tests detailed is to measure the extent of adventitious contamination and/or faults in the methods of preparation of laboratory apparatus.

2. (*a*) *Routine Tests*

A high degree of technical skill is required to ensure that the results adequately reflect the condition of the equipment.

Records should be kept of all such tests showing:

(i) nature of apparatus tested and number of colonies counted;
(ii) batch number and date of sterilization; and
(iii) name of person carrying out the tests.

(*b*) *Reporting*

The total number of colonies per test should be recorded irrespective of the volume of rinse, the number of rinses plated or the number of pieces of

apparatus involved in one test. For example, para. 6 (ii) says 'rinse two pipettes in each blank', but the report should give the total number of colonies, not colonies per pipette.

3. *Standard Check Tests*
In addition to routine tests on apparatus sterilized in their own and other laboratories, control laboratories should carry out 'Standard Check Tests'. These are tests carried out by known skilled workers using apparatus and media sterilized by proven methods and exercising the greatest possible precautions. Such tests serve to check the magnitude of unavoidable error in the techniques given below and should be used to gauge the efficiency of workers carrying out routine sterility tests. *Inoculation chambers must not be used.*

Notes on Technique

4. Specific directions for flaming, etc., have not been included, but the worker shall adopt the usual aseptic precautions and take such steps as he considers necessary throughout the sampling and testing to prevent adventitious contamination. *Care must be taken to avoid excessive flaming which may result in false negative results.*
(*a*) Plates shall be incubated for 72±2 hr at 30±1°, using Yeastrel milk agar.
(*b*) The medium used for these tests shall be tubed in 10 ml quantities and, after filling, the tubes shall be proved sterile by incubating for 72 ±2 hr at 30±1°, followed by 48 hr at room temperature, 15–22°
(*c*) One pipette shall be used for each sample.
(*d*) Ringer's solution denotes one-quarter strength Ringer's solution.
(*e*) A 9 ml blank means 9 ml of sterile one-quarter strength Ringer's solution in a sterile test-tube or 1 oz McCartney bottle.

Technique

5. *Test-Tubes and Sample Bottles with Rubber Closures*
(*a*) *Test-Tubes*
(i) Take at least two tubes at random.
(ii) Remove the closure of the first tube and pour in 9 ml of Ringer's solution from a 9 ml blank contained in a rubber-closured test-tube, or 1 oz McCartney bottle.
(iii) Replace the original closure in the test-tube, shake 12 times with an up and down movement of about 1 ft and return the rinse to the original container. Replace the rubber closure.
(iv) Repeat (ii) and (iii) in turn for each tube selected for testing, using a separate 9 ml blank for each tube. Plate one quantity of 5 ml from each 9 ml rinse.
(*b*) *Sampling Bottles* 3 oz and similar containers
Take at least two sample bottles and proceed as in para. 5 (*a*).
(*c*) *Other Containers.* For larger bottles and measuring flasks use 20 ml Ringer's solution and proceed as in para. 5(*a*), plating two 5 ml quantities.

6. 1 *ml Pipettes*

(i) Insert the tip of the pipette into a sterile 9 ml blank and rinse by sucking up to the 1 ml mark followed by expelling; repeat six times. Air must not be sucked through the pipette. Blowing air through the pipette into the solution must be reduced to a minimum.

(ii) Rinse two pipettes in each blank.

(iii) Plate one quantity of 5 ml.

Pipettes of a larger capacity shall be treated in a similar manner.

7. *Dippers*

(i) If the dippers have been sterilized in cases or wrapped in greaseproof paper, take a dipper from the case or unwrap, touching the handle only, and immediately pour in 9 ml of Ringer's solution from a 9 ml blank. Swirl 12 times and return the Ringer's solution immediately to the original container.

(ii) Plate one quantity of 5 ml.

8. *Water for Resazurin Bench Solution*

(i) Transfer at least 10 ml to a sterile rubber-stoppered test-tube or 1 oz McCartney bottle.

(ii) Plate one quantity of 5 ml.

9. *Resazurin Tablets and Solution*

Tablets, when dissolved according to the instructions given in the Routine Resazurin Test, shall give a 0·005 per cent solution of resazurin. Tablets must not contain any substance other than resazurin, lactose and/or sodium chloride, and the 0·005 per cent solution prepared from the tablet must not give a colony count of more than 10 ml when plated on Yeastrel milk agar, incubated at 30±1° for 72±2 hr.

Additional Techniques

The following additional techniques are designed for the testing of other types of apparatus, etc.

10. (*a*) *Test-Tubes*, with metal caps, or cotton-wool plugs or unstoppered.
(*b*) *Sample Bottles and Measuring Flasks*, with cotton-wool plugs or unstoppered.

(i) These shall be treated as in para. 5.

(ii) For this purpose sterilized rubber closures of suitable size will be required. Such closures must have been wrapped individually in greaseproof or Kraft paper, and autoclaved for 15 min at 121°. Check tests shall be made on closures so treated (see paras. 11 and 12).

11. *Rubber Closures*

(i) With sterile forceps transfer three closures to a sterile 8 × 1¾ in. test-tube containing 50 ml of sterile Ringer's solution and fitted with a sterile rubber closure.

(ii) Invert 12 times by quick turns of the wrist and plate one quantity of 5 ml.

12. *Metal Caps*
Press caps for sample bottles, screw caps for McCartney bottles and loose fitting caps for test-tubes should be tested as for rubber closures (see para. 11).

13. *Petri Dishes*
Add 10 ml of sterile agar medium.

14. 9 *ml Blanks*
(i) If the container has a cotton-wool plug this must be replaced by a sterile rubber closure as in para. 10 (*b*)(ii).
(ii) Plate one quantity of 5 ml.

15. *Ringer's Solution*
(*a*) 500 ml quantities as used for can rinsing.
(i) Plate one quantity of 5 ml.
(*b*) 20 ml quantities as used for bottle rinsing.
(i) Proceed as in para. 15(*a*)(i).

16. *Culture Media*
Take three tubes or bottles at random and incubate for 72 ± 2 hr at $30 \pm 1 \cdot 0°$ followed by 48 hr at room temperature 15–22°.
The report shall show the number of tubes positive in each batch of three.

Basic Bacteriological Media

4. GENERAL INTRODUCTION

1. *Materials*

Distilled water prepared with a glass still or block tin condenser, or water of similar quality, shall be used.

The particular brand of peptone used may influence the results of some bacteriological tests. The following formulae refer to Evans' peptone or its equivalent.

All reagents shall be of analytical quality where obtainable.

Dehydrated media may be used where they have been found to give satisfactory results.

2. *Sterilization of Media*

When an autoclave is used the treatment should be standardized as far as possible so as to ensure sterility without overheating. It may be necessary to modify the autoclave to produce the conditions necessary for uniform heat treatment. These conditions are:

> complete exhaustion of air from the chamber; the control of the process by temperature; rapid heating of the chamber; and the employment of standard loads. Details given by Thiel, Burton & McClemont. (1952). *Proc. Soc. appl. Bact.*, **15**. 53, from which the following recommendations are drawn. See also B.S.2646: 1955.

A mercury-in-glass thermometer should be fitted to the autoclave in a suitable thermometer pocket, sited so that the bulb lies in the path of the steam leading to the vent. This serves to indicate when the chamber is virtually free from air; it also affords a means of checking the holding temperature, provided that a trickle of steam is allowed to bleed past the thermometer bulb during holding. For this purpose a second air-vent with adjustable valve would be necessary (see B.S. 2646: 1955).

(*a*) *Removal of Air*. To ensure complete removal of air from the chamber, the vent should not be closed until the temperature of the exhaust steam shows that it is air-free. Due to variable barometric pressure this temperature may vary a degree or two around 100°, but this difficulty is overcome by closing the vent only when the increase in thermometer reading does not exceed 0·5° during 2–3 min.

Uniform rapid heating and removal of air are impeded by the use of the heavy iron baskets normally supplied with the autoclave. It is recommended that these are replaced by wire spacer trays to support loads in the bottom of the autoclave and to separate layers of media in baskets.

(*b*) *Holding Times Temperature*. A standard treatment of 121° for 15 min is recommended for a load consisting of eight baskets (6 × 6 × 6 in.) each holding 80 tubes containing 10 ml of media, the baskets resting in two layers on a false wire mesh bottom and the layers being separated by 1–1½ in. wire spacers. When larger volumes of liquid are sterilized the holding times may need to be increased: recommended holding times for different types of load are:

Load	*Recommended holding time in minutes to give a treatment equivalent to 15 min at 121°.*
Test tubes (standard load)	
6·4 litres water in 640 tubes	15
8-oz medical flats	
8·4 litres water in 56 flats	15
Waisted Blood bottles	
7·0 litres in 14 bottles	23
3-litre conical flasks	
7·5 litres water in 3 flasks	27

To avoid overheating it is recommended that loads should not exceed the above amounts, except for materials (such as Ringer's solution) on which overheating has no adverse effect.

For media containing sugars or other substances whose nutritive value may be altered at high temperatures, the above recommendations do not apply and instructions for sterilization are given in the appropriate section.

5. RINGER'S SOLUTION

Prepare full strength Ringer's solution of the following composition:

Sodium chloride	9·00 g
Potassium chloride	0·42 g
Anhydrous calcium chloride	0·24 g
Sodium bicarbonate	0·20 g
Distilled water	1,000 ml

Add one part of the above solution to three parts of glass distilled water. Fill into 6 × $\frac{5}{8}$ in. test-tubes fitted with suitable closures and autoclave at 121° for 15 min. With this method it will be necessary to add more than 9 ml to overfill the tubes to allow for evaporational losses during sterilization. The amount of excess used in filling will be determined by experience. Alternatively, sterile tubes may be filled under aseptic conditions with sterile quarter-strength Ringer's solution. The latter method should be attempted only by skilled workers.

Alternatively, Ringer's solution tablets may be used. To prepare quarter-strength Ringer's solution, dissolve one tablet in 500 ml distilled water. Fill into 6 × $\frac{5}{8}$ in. test-tubes and sterilize as above.

6. YEASTREL MILK AGAR

Yeastrel	3 g
Peptone	5 g
Agar*	15 g
Fresh whole milk	10 ml
Distilled water	1,000 ml

(*If New Zealand agar is used 12 g per litre is normally sufficient)

Dissolve the Yeastrel and peptone in the distilled water in the steamer and adjust the reaction at room temperature to pH 7·4, using phenol red as the indicator (see Note 1) or using a pH meter.

Add the agar (see Note 2) and the milk to the broth and autoclave at 121° for 25 min. In order to ensure thorough mixing and that heat treatment of the bulk at this stage is equivalent to the final sterilization of the tubed media, it is recommended that quantities of not more than 1½ or 2 litres are autoclaved in 3-litre conical flasks. The hot medium is then filtered through paper pulp in a Buchner funnel.

The pulp is prepared by mashing up small pieces of filter paper in water and boiling. The funnel should be inserted into an Erlenmayer flask fitted with a side piece. A single layer of filter paper should be laid on the top of the Buchner funnel to prevent the pulp being sucked through. The hot pulp is poured on to the filter paper and a filter pump is applied to suck through the excess water, which is then poured away. The pulp should be firmly packed down just before the last of the water is sucked through. At this stage a layer of filter paper is laid on the filter bed, so that the hot agar can subsequently be poured on to it without disturbing pulp. The filter when ready for use should have a total depth of about 1·5 mm. (A pulp layer of suitable and approximately the same depth for any size of funnel may be obtained by pulping an area of filter paper equal to four times the square of the diameter of the funnel. With ordinary grade filter paper 1 g of the dry paper is required for every 20 sq. cm of filtering area).

The flask and funnel should be thoroughly hot before filtering commences, and these and the agar should be kept hot during filtering. The agar is taken direct from the autoclave, the hot agar poured on to the pulp where the filter paper was laid and the vacuum pump connected.

The reaction of the filtrate is tested at 50° and adjusted if necessary to pH 7·0. Adjustment at this stage should not normally be necessary, but if it is needed at all frequently, the method of preparation should be checked.

The medium is distributed in 10 ml quantities in 6 × ⅝ in. test-tubes (B.S.625: 1959) and autoclaved at 121° for 15 min. Alternatively, for storage, 100–250 ml quantities may be filled into suitable bottles.

The final reaction of the medium *at room temperature* should be pH 7·2.

Notes

1. A brightness screen must be used with the Lovibond phenol red disc 2/IJ.
2. If shredded agar is used, it should be wrapped in muslin and washed in running water for 15 min, the excess water being squeezed out before the agar is added to the broth.

7. MEDIA FOR COLI-AEROGENES ORGANISMS

1. *MacConkey's Broth*

Commercial sodium taurocholate, sodium tauroglycholate or other satisfactory bile salt	5 g
Peptone	20 g
Sodium chloride	5 g
Lactose	10 g
Distilled water	1,000 ml

Heat the bile salt, peptone and sodium chloride in the distilled water until dissolved. Add and dissolve the lactose. Cool to room temperature and check the pH by means of a pH meter or phenol red indicator, adjusting it to 7·4. Filter through paper (Whatman No. 5) or paper pulp (macerated Whatman No. 1) in a Buchner funnel. Add 2 ml of a 1·6 per cent alcoholic solution of bromo-cresol purple. Distribute 5 ml quantities in 6 × $\frac{5}{8}$ in. test-tubes (B.S.625: 1959) each containing a $1\frac{3}{8} \times \frac{5}{16}$ in. Durham tube (B.S.625: 1959). Double strength medium is prepared similarly, using twice the quantity of ingredients in the same quantity of water.

Where required, distribute 10 ml and 50 ml quantities in suitable tubes or bottles (capacity greater than 100 ml) each containing a Durham tube (B.S.625: 1959) a $1\frac{3}{8} \times \frac{5}{16}$ in. for a 10 ml quantity; 3 × $\frac{1}{2}$ in. for a 50 ml quantity.

Autoclave tubes or bottles of media at 115° for 15 min.

2. *MacConkey's Agar*

Prepare MacConkey's broth as above, omitting the bromo-cresol purple. Add 20 g agar per 1,000 ml of broth and heat until dissolved. Filter as detailed for Yeastrel milk agar. Add 10 ml of a 1·6 per cent alcoholic solution of bromo-cresol purple to the medium. Distribute approximately 15 ml quantities in McCartney bottles, or 100–250 ml quantities in suitable screw-cap bottles if numbers of Petri dishes are likely to be poured at one time.

Autoclave at 115° for 15 min.

3. *Eosin-Methylene Blue Agar*

Peptone	10 g
Dipotassium hydrogen phosphate (anhydrous)	2 g
Agar	15 g
Lactose	10 g
Distilled water	1,000 ml

Dissolve the peptone, dipotassium hydrogen phosphate and agar in the distilled water by heating in a steamer. Add the lactose, 20 ml of 2 per cent aqueous solution of yellow eosin, 20 ml of 0·5 per cent aqueous solution of methylene blue and in that order. Neither adjustment of reaction nor filtration is necessary.

Distribute and sterilize as detailed for MacConkey's agar.

Note

Preparation of this medium requires some experience and it may be more satisfactory to employ commercial dehydrated brands when only small quantities are required.

4. *Peptone Water*

Peptone	10 g
Sodium chloride	5 g
Distilled water	1,000 ml

Dissolve the ingredients in the distilled water by heating in a steamer. Cool

to room temperature. Adjust the reaction to pH 7·5 and filter if necessary. Distribute in 5 ml quantities in test-tubes and autoclave at 121° for 15 min.

Note
If the medium is required for the indole test, the peptone used must be known to yield a positive reaction with a known indole-producing strain.

5. *Lactose Broth*
Prepare a 10 per cent aqueous solution of lactose, and sterilize in the steamer for 30 min or by means of a Seitz EK filter. Using aseptic precautions, add the lactose solution to the peptone water, prepared and sterilized as above, in proportions of 5 ml to 100 ml respectively. Add 3 ml aqueous solution of bromo-cresol purple per 1,000 ml of medium. (Prepared by grinding 0·54 g bromo-cresol purple in 100 ml 0·01 N sodium hydroxide until dissolved).

Dispense 5 ml quantities in sterilized test-tubes each containing a Durham tube as for MacConkey's broth (see para. 1) and sterilize at 100° for 30 min.

8. MEDIA FOR LIPOLYTIC ORGANISMS

1. *Basal Medium*

Peptone	10 g
Yeast extract	3 g
Sodium chloride	5 g
Agar	20 g
Distilled water	1,000 ml

Dissolve the peptone, yeast extract and sodium chloride in the distilled water by heating in a steamer. Cool to room temperature. Adjust to pH 7·8 using phenol red indicator or a pH meter. Add the agar, heat to dissolve, and filter as for Yeastrel milk agar. Add 100 ml of a neutral aqueous solution (1:1500) of Victoria Blue* to give a concentration of approximately 1:15,000 Victoria Blue in the medium. Dispense in 6 ml quantities in test-tubes or screw cap McCartney bottles, add 0·3 ml of the melted fat substrate (see para. 2), and sterilize at 115° for 20 min. If preferred, Nile Blue sulphate (Night Blue) may be used instead of Victoria Blue when 100 ml of a neutral aqueous solution (1:1,000) of Nile Blue sulphate should be added to give a concentration of approximately 1:10,000 Nile Blue sulphate in the medium.

N.B. It is important to adhere strictly to the directions concerning pH reaction, quantity of medium per tube, and method of sterilization. The medium will deteriorate on storage and it is recommended that it should not be kept longer than 1 month.

2. *Fat Substrate*
For general purposes olive oil (B.P.) is convenient. Where specific information relating to decomposition of butter fat is required, melt some fresh

*Obtainable from British Drug Houses.

unsalted butter and filter through paper to obtain a clear water and curd free filtrate. Tube and sterilize the olive oil or butter oil in an autoclave at 121° for 15 min.

9. MEDIA FOR YEASTS AND MOULDS

1. *Malt Extract Agar*

Mix 100 g of malt of high diastatic value with 1,000 ml of tap water and hold at 50–55° until no colour reaction is obtained with iodine. Then raise the temperature to 100°, filter the mixture and sterilize by steaming at 100° for 30 min on each of 3 successive days. Alternatively, a 3 per cent solution of commercial malt extract (approximately 80 per cent of malt solids) free from preservative, shall be used.

Add 20 g of agar per 1,000 ml of medium, dissolve by raising to 100° and sterilize by autoclaving at 121° for 15 min.

When the temperature of the medium has fallen to 90–100° adjust the pH to $3{\cdot}5 \pm 0{\cdot}1$ using bromophenol blue as an external indicator by adding aseptically a 10 per cent solution of citric acid monohydrate previously autoclaved at 121° for 15 min.

The medium shall be filled out in 10 ml quantities into sterile test-tubes at this temperature (100°) and no further sterilization is required.

When tubes of medium are required for pouring plates, care shall be taken to melt and cool the medium to 45° as rapidly as possible.

2. *Synthetic Yeast Salt Agar*

Ammonium nitrate	1·0 g
Ammonium sulphate	1·0 g
Dipotassium hydrogen phosphate	4·0 g
Potassium dihydrogen phosphate	2·0 g
Sodium chloride	1·0 g
Dextrose	10·0 g
Yeast extract	1·0 g
Distilled water	1,000 ml

Dissolve the ingredients in the distilled water by heating in a steamer and filter if necessary.

Add 20 g of agar for 1,000 ml of medium, dissolve by raising to 100° and sterilize by autoclaving at 121° for 15 min.

When the temperature of the medium has fallen to 90–100°, adjust the pH to $3{\cdot}5 \pm 0{\cdot}1$ using bromophenol blue as an external indicator by adding aseptically about 57 ml of a 10 per cent solution of citric acid monohydrate previously autoclaved at 121° for 15 min.

The medium shall be filled out in 10 ml quantities into sterile test-tubes at 100° and no further sterilization is necessary.

The tubes should be stored at room temperature but they should not be used after 6 days.

REFERENCE

1. DAVIS, J. G. (1958). *Lab. Practice*, 7, 30.

10. MEDIUM FOR TOTAL SPORES

1. *Starch Milk Agar*

Soluble starch	1 g
Peptone	10 g
Lab. lemco	10 g
Sodium chloride	5 g
Separated milk	10 ml
Agar	20 g
Distilled water	1,000 ml

Dissolve the starch, peptone, lemco, sodium chloride and agar in the distilled water, filter and adjust pH to 7·0. Add the separated milk. Distribute 10 ml quantities in 6 × $\frac{5}{8}$ in. test-tubes and autoclave at 121° for 15 min. See Ref. 1.

Note
If New Zealand agar is used, 15 g will suffice.

REFERENCE

1. GRINSTED, E. and CLEGG, L. F. L. (1955). *J. Dairy Res.*, **22**, 178.

11. MEDIA FOR ANAEROBIC SPORES

1. *Reinforced Clostridial Medium* (*R.C.M.*)

Yeast extract (Yeastrel)	3 g
Peptone (Evans)	10 g
Sodium acetate A.R. (anhydrous)	5 g
Cysteine hydrochloride	0·5 g
Soluble starch	1 g
Agar	0·5 g
Distilled water	1,000 ml

Steam to dissolve all ingredients. Add 10 g of dextrose and dissolve by shaking. Adjust the pH to 7·2. Add aqueous neutral red solution to give a final concentration of 1/10,000 and autoclave at 121° for 15 min.

2. *Clostridial Iron Sulphite Medium*

(*a*) *Basal Medium:*

Tryptone	15 g
Yeast extract powder— moisture less than 5 per cent	10 g
Agar	15 g
Distilled water	1,000 ml

Adjust pH to 7·0±0·1 with 10 per cent caustic soda. Sterilize for 20 min. at 121°.

(*b*) *Sodium Sulphite Solution:*

Sodium sulphite (SO_2 content about 23 per cent)	10 g
Distilled water	100 ml

Sterilize by filtration through a G5 or H5 glass filter.

(*c*) *Iron Citrate Solution:*

Iron citrate	5 g
Distilled water	100 ml

Sterilize by filtration through a G5 or H5 glass filter.

Mix extempore, 1 ml of freshly prepared sodium sulphite solution and 1 ml of iron citrate solution with 100 ml of basal medium.

12. MEDIUM FOR LECITHINASE PRODUCING ORGANISMS

1. *Egg Yolk Media*

Separate the yolk from the white.

To one part of yolk add four parts of distilled water (v/v) and mix thoroughly.

Heat in a water bath at 45° until precipitate is well separated. Two hours is normally sufficient, but sometimes a longer period is necessary.

Centrifuge to deposit precipitate, or alternatively stand overnight in a refrigerator.

Decant supernatant liquid and sterilize by filtration through a Ford Sterimat Grade SB and store in the refrigerator.

For use, add 1–2 ml of the solution to 10 ml of nutrient broth or nutrient agar.

Note

If the salt content of the medium is low, it will become opaque on addition of the egg yolk solution. In the case of 0·3 per cent Yeastrel, 0·5 per cent peptone agar, the inclusion of 0·5–1·0 per cent sodium chloride will remedy this. Higher concentration of salt will retard lecithinase activity.

REFERENCES

1. Billing, E., and Luckhurst, E. R. (1957). *J. appl. Bact.*, **20**, 90.
2. Billing, E., and Cuthbert, W. A. (1958). *J. appl. Bact.*, **21**, 65.

13. LITMUS MILK

1. *Five per cent Litmus Solution*

Use a suitable water soluble grade of litmus and prepare a 5 per cent solution in distilled water. Steam for 1 hr, agitating two or three times during this period. Filter through a pulp filter and distribute into screw-capped McCartney bottles. Steam this concentrated solution at 100° on 3 successive days and store in a cool dark place. Any bottle of solution which has been opened should be resterilized after use.

2. *Fresh Separated Milk*

Separate raw morning's milk of good bacteriological quality known to be free from antibiotics. If sterilization does not commence within 4–6 hr of milking, the milk, which should always be cooled immediately after separation, must be stored at a low temperature, i.e., below 5°. All containers and glassware which will come in contact with the separated milk should be sterilized before use and the separator should be steamed.

If a suitable source of raw milk is not available, litmus milk may be prepared from a reliable source of skim milk powder. Such a medium is not as suitable in many respects as that made from raw milk.

3. *Method*

Add 10 ml of the 5 per cent litmus solution to 1,000 ml of separated milk, mix, and without delay fill out, in approximately 10 ml amounts, into sterilized tubes, preferably plugged with cotton wool. Care should be taken not to wet the inside of the top of the tube when filling as this will cause the cotton wool to stick to the tube. Immediately after tubing, steam for ½ hr at 100° and repeat this steaming on a further 2 successive days. After each steam treatment the medium should be kept at laboratory temperature.

If the milk is to be used for investigations requiring incubation above 45° the medium should be kept at 63° for part of the time between steam treatments to encourage germination of any surviving thermophilic spore-formers. After the medium is removed from the steamer in the morning, place at 63° for about 6 hr until the late afternoon when the medium should be placed at laboratory temperature until it is steamed on the following day.

After sterilization incubate the tubes of litmus milk for 3 to 5 days at 30° and discard any tubes showing contamination. If large numbers of tubes are not sterile, the supply of separated milk is unsatisfactory and a better source must be found. The incubation periods indicated above will not necessarily reveal the presence of all spores, as germination may be delayed for many days and late germination of spores may interfere with subsequent tests. In any case litmus milk which has been stored in the refrigerator should be held at room temperature for a few days prior to use, to encourage the germination of any surviving spores.

Explanatory Notes on the use of Litmus Milk

Growth of organisms in litmus milk, especially if pure cultures are used, provides information on:

(*a*) reduction (decolourization) of litmus;
(*b*) lactose fermentation (production of acid with or without gas);
(*c*) coagulation of protein (with or without acidity) and;
(*d*) digestion of protein.

These reactions are useful diagnostic characteristics of organisms in pure culture and also indicate types likely to cause spoilage in raw or pasteurized milk, and milk products.

Occurence of the reactions should always be related to the temperature and duration of incubation. In general, 22° is appropriate for determination of milk spoilage capacity and reactions should be recorded after 24, 48 and 72 hr. For diagnostic work with certain types of bacteria, temperatures of 30° or 37° may be more suitable.

The reaction should be read in comparison with an uninoculated incubated control tube and recorded using the following code:

NC = no change;
R = reduction; litmus is decolourized, often first evident at the bottom of the tube;
A = acid; litmus is slightly pink or pink;

AC = acid clot; litmus is pink and milk clots; at a later stage whey may appear as the curd shrinks and hardens;
ALK = alkaline; litmus is slightly blue or blue;
SC = sweet clot; often the clot is quite soft and remains so and may readily be broken by tilting the tube; the reaction is *not* acid but may be alkaline;
D = digestion; the milk, whether liquid or clotted, as the protein is peptonized, becomes clear; the reaction may be alkaline;
G = gas production; small bubbles may be seen escaping from medium; if milk is clotted, gas bubbles may be trapped in curd and disrupt it.

More than one reaction often occurs, particularly with longer incubation times. For example, (i) reduction accompanies acid clot, but the medium is seldom entirely reduced and acidity is shown by a pink band at the surface of the medium (RAC); (ii) digestion may follow alkaline reaction or sweet clot (ALK, D or SC, D).

Indefinite or slight reactions, e.g., slight acid, partial digestion (a few mm at the surface of the tube) should be indicated by bracketing the symbol thus: (A) or (D).

14. YEAST DEXTROSE LEMCO BROTH

This medium has been found suitable for the culture of most bacteria isolated from milk.

Peptone	10 g
Lab. lemco	10 g
Yeastrel	3 g
Sodium chloride	5 g
Distilled water	1,000 ml
Dextrose	5 g

Dissolve all ingedients except dextrose by steaming for ½ hr. Cool to room temperature and adjust pH to 7·0. Add dextrose, shake to dissolve and then filter. Tube in 5 ml quantities. Autoclave at 115° for 15 min. Incubate sterilized media at 30° for 3 days and check sterility.

Bacteriological Examination of Milk

15. TOTAL MICROSCOPIC COUNT

1. *Reagents*

All reagents shall be of recognised analytical quality.

(*a*) *Methylene Blue Stain:*

Methylene blue	0·3 g
Ethanol (ethyl alcohol) (95 per cent v/v)*	30 ml
Distilled water . . .	100 ml

Dissolve the methylene blue in the alcohol and add the distilled water.

(*b*) *Newman's Stain:*

Methylene blue	1·0 g
Ethanol (ethyl alcohol) (95 per cent v/v)*	54 ml
Tetrachlorethane	40 ml
Glacial acetic acid	6 ml

Add the alcohol to the tetrachlorethane and heat on a water-bath to a temperature not exceeding 70°. Add the methylene blue and shake until the dye dissolves. Cool, add the acetic acid slowly, mix and filter.

(*c*) *Xylene*, or other suitable fat solvent.

(*d*) *Ethanol* (ethyl alcohol), (95 per cent v/v)

2. *Apparatus*

Pipettes calibrated to deliver 10^{-2} ml milk.

Microscope with 1/12 in. oil-immersion objective.

Microscope slides having an area of 1 sq. cm outlined by etching or otherwise on the surface.

3. *Calculation of Microscope Factor*

Measure the diameter of the microscope field with a stage micrometer using the $\frac{1}{12}$ in. oil-immersion objective, ×10 eyepiece and a standard tube length.

If 0·01 ml of milk is spread over an area of 1 cm^2, the microscope factor is calculated from the formula $\frac{10{,}000}{3 \cdot 142 \times r^2}$

where r is the radius of the microscope field in mm.

The following table shows the microscope factors corresponding to different diameters of field:

Diameter in mm	*Factor for the average number of bacteria/ml*
0·206	300,000
0·178	400,000
0·160	500,000
0·146	600,000

*Industrial methylated spirit of equivalent strength is suitable. It should be noted that the use of industrial methylated spirit is governed by the Methylated Spirits Regulations 1952 (S.I. 1952. No. 2230).

4. *Technique of Test*

(*a*) *Preparation of Film.* Using the pipette, remove 10^{-2} ml of the thoroughly mixed sample of milk, wipe the exterior of the pipette with a clean cloth or piece of filter paper and spread the milk evenly over the area of 1 sq. cm etched on a slide. A template having a cut-out square of 1 sq. cm may be used if preferred. Dry the preparation on a level surface free from dust at a temperature of 40–45°. The drying time shall not exceed 5 min, but should not be too rapid or the film will not be fixed properly.

(*b*) *Defatting and Fixing.* Immerse the slide in xylene for at least 1 min. Remove, drain and allow to dry. Immerse in the ethanol (see para. 1(d) for 1 min, remove, drain and allow to dry.

(*c*) *Staining*

(i) *Methylene Blue.* Dip the slide in methylene blue staining solution for 10–15 sec. Do not exceed this time or the slide will be over-stained. Rinse carefully in water, drain and allow to dry slowly. When properly prepared, the background of the slide will be light blue.

(ii) *Newman's Stain.* The use of Newman's stain (see para. 1(*b*)) permits of defatting and staining at the same time, but the preparations may not be so clear as when separate treatments are used. After preparing the film as in para. 4(*a*), immerse the slide in Newman's stain for 15 sec. Remove and allow to drain until thoroughly dry. Wash gently in water to remove surplus stain, drain and allow to dry slowly and completely.

5. *Examination of Prepared Film*

Examine under the $\frac{1}{12}$ in. oil-immersion objective using a ×10 eyepiece and the standard draw tube used in measuring the diameter of the field. Count the number of organisms in 30 fields taken at random, recording clumps and chains as single organisms. From the average per field, calculate the number of organisms per ml as follows:

Direct microscopic count per ml = average count per field × microscope factor.

REFERENCE

1. Eleventh edition of *Standard Methods for the Examination of Dairy Products*, American Public Health Association, New York.

16. COLONY COUNT

I Petri Dish Method

1. *Preparation of Dilutions*

Thoroughly mix the sample according to the instructions for each particular product. Dilutions should be prepared in one-quarter strength Ringer's solution. Prepare serial one-tenth dilutions by adding 1 ml of inoculum to 9 ml of diluent. Introduce the tip of a 1 ml blow-out pipette into the sample to a depth of $\frac{1}{2}$ to 1 in. below the surface, suck up and down 10 times to the 1 ml mark, in each case withdrawing the pipette until the tip is above the level of the liquid before expelling. Finally, measure 1 ml holding the pipette vertically. Before withdrawing the charged pipette

touch the tip against the neck of the sample container. Transfer the charged pipette to the first dilution tube and, with the tip touching the side of the tube at a point ½ to 1 in. above the level of the diluent, blow out the contents: allow 3 sec to elapse and blow out again. With a fresh pipette, proceed as above and mix the first dilution by sucking up and down 10 times: measure 1 ml and transfer to the second dilution tube: discard the pipette. Prepare further tenfold dilutions in the same way.

2. *Inoculation of Petri Dishes*
Take a fresh 1 ml pipette, and using the mixing technique described in para. 1, inoculate 1 ml from the last dilution to a Petri dish (B.S.611: 1956). Hold the tip of the charged pipette about ½ in. above the bottom of the dish, and blow out the contents of the pipette. Allow 3 sec to elapse: touch the tip of the pipette against the dish at a point away from the liquid already delivered, and blow out the last drop. Inoculate Petri dishes from the next dilutions in the same way, using the same pipette or a fresh one. For general work, one Petri dish may be prepared from each dilution examined, but for special work it is preferable to prepare three plates from each dilution.

Alternatively, the Petri dish may be inoculated when the dilutions are made, i.e., before transfer of the 1 ml quantity to the next tube of diluent; the same pipette may then be used to inoculate the appropriate Petri dish.

3. *Pouring Petri Dishes*
To each dish add 10 ml of melted agar medium (see Technique No. 6) at a temperature of 45–47°. The medium should be poured to one side of the inoculum. If tubes of media are used, dry and flame before pouring. Immediately mix the medium and inoculum by five to-an-fro movements, followed by five circular clockwise movements, followed by five to-and-fro movements at right angles to the first set, followed by five circular anti-clockwise movements. Allow the dishes to stand until the medium has set, invert and then transfer to the incubator. The time elapsing between the preparation of the dilutions and the pouring of Petri dishes should not exceed 15 min. The operations detailed in this para. and in paras. 1 and 2 should not be carried out in direct sunlight. If the poured plates are not placed in the incubator immediately after setting, they should be covered with a clean, dry cloth.

4. *Incubation*
Incubate dishes bottom upwards and do not stack more than six deep. For times and temperatures of incubation the techniques for individual products should be consulted, but for normal colony counts on milk and milk products, swabs and rinses of dairy equipment, cans and bottles, incubation at 30± 1·0°, for 72±2 hr is recommended. Water-jacketed incubators are to be preferred. Other time and temperature combinations for special purposes may be employed at the discretion of the bacteriologist.

5. *Counting of Colonies*
Colonies should be counted within 4 hr of the expiry of the incubation period. A suitable counting chamber is desirable. To facilitate counting, a

lens not exceeding a magnification of $\times 2\frac{1}{2}$ diameter, and a tally counter should be used. Assessment of the colony counts should be made from plates showing 30 to 500 colonies, unless the plate of the lowest dilution or the undiluted sample contains less than 30 colonies. If more than one dilution gives a count within this range, select the plate giving the higher count. To record the count per ml, multiply the count per plate by the reciprocal of the dilution from which the plate was prepared.

II Roll Tube (Astell) Method

1. *General*

In this method, the medium is filled into the bottles at the time of preparation and then sterilized. Before use, the medium is melted and cooled to 45° and, after adding the inoculum, the bottles are spun mechanically until the medium sets in an even film on the inner surface of the bottles.

Introduce 0·5 ml of inoculum to each bottle when using serial tenfold dilutions. Where low counts are expected, as with swabs or rinses, 1 ml quantities may be inoculated. Blow-out pipettes, graduated at 0·5 and 1·0 ml will be found convenient, 1·0 ml diluent being used for preparation of tenfold dilutions and 0·5 ml for inoculation of the bottles.

2. *Preparation of Bottles*

At the time of preparation fill 4·0–4·5 ml of medium (containing 2·0 or 2·5 per cent agar) into each bottle, close the bottle with the seal and sterilize by autoclaving.

Note

The filled bottles may be stored at room temperature for long periods before use without risk of contamination or drying-out of the medium.

When required for use, loosen seals, melt the agar, and then cool to 45–47° in a constant temperature bath.

3. *Preparation of Dilutions*

Proceed as in the Petri dish method (see para. 11) substituting blow-out pipettes graduated at 1·0 and 0·5 ml for the 1 ml pipettes.

4. *Inoculation of Bottles*

Take a fresh pipette and, using the technique described in para. 11, inoculate 0·5 ml of the last dilution into a bottles cooled to 45–47° as follows:

Remove the seal aseptically, insert the tip of the pipette, containing the inoculum into the bottle and deliver 0·5 ml into the melted medium, touching the lower part of the neck to discharge any drops adhering to the tip when withdrawing the pipette.

Proceed as in para. 12.

5. *Spinning the Bottles*

As each bottle is inoculated, place it on one or other of the spinning positions on the spinner, which is so arranged that the bottles may be placed in position or removed while the spinner is in motion. With the cooling arrangement fitted to the spinner, medium solidifies into an even film on the inner surface of the bottle in about 1 min.

6. *Incubation of Bottles*
Place the bottles in divided baskets with the seals upwards. Proceed as detailed previously in para. 14.

7. *Counting of Colonies*
Proceed as detailed previously in para. 15 with the following modifications: a colony illuminator designed for this method is desirable; only bottles with counts between 15 and 250 should be used for assessing the results; and to record the count per ml, multiply the count per bottle by two and multiply the answer by the reciprocal of the dilution from which the bottle was inoculated.

III Drop Plate Method

1. *Introduction*
This method consists of dropping from a capillary pipette a known volume of bacterial suspension on to the surface of an agar plate, allowing the liquid to be absorbed, incubating the plate and counting the resulting colonies. It is based upon a technique originally described by Miles and Misra (see Ref. 7).

When compared with the conventional pour-plate technique it enables a saving of media and glassware to be made, it is quicker and more flexible in its application, it enables the colony form of the bacteria to be studied and it does not expose bacteria to the temperature of molten agar. The same degree of accuracy can be achieved as with the pour-plate method.

2. *The Preparation of Capillary Pipettes*
A suitable method is described by the Medical Research Council (see Ref. 6). Pipettes are made from an 8 in. length of clean, soda glass tubing of 8 mm external diameter. By heating in the centre, the glass can be drawn out to make two pipettes with relatively parallel sides. The size of the drop expelled from such a pipette is governed by the external diameter of the tip, the speed at which the drop is formed and the nature of the medium in which the bacteria are suspended. For performing colony counts a drop size of 0·2 ml is convenient and this may be achieved by the use of a pipette with a capillary tip diameter of 0·043 in. and by dropping at the rate of 20–30 drops per min. This will be correct for suspensions in water, physiological saline or quarter-strength Ringer's solution, but if other suspending media are used, e.g., nutrient broth, correction factors should be determined by comparing the weight of a known number of drops of the particular medium with the weight of the same number of drops of water.

In order to ensure that the capillary tip is of the correct size it is introduced into the No. 57 hole (0·043 in. diam) of a Morse Twist Drill or Starrett Gauge and pressed down until it engages. The pipette is then cut off squarely with a sharp glass cutter at the point of impaction. The pipette should be rotated with the tip at eye level and discarded if seen to be cracked or not cut at right angles to the axis. To ensure that the tip of the pipette is free from grease it is advisable to dip it in acetone. The pipette is plugged at the wide end with cotton wool, placed in a suitable container, and sterilized in the hot-air oven.

Alternatively, pipettes may be made from ground-down hypodermic needles (see Ref. 5) and from platinum tubing (see Ref. 6).

3. *Inoculation of the Plates*

In use the pipette is fitted with a teat and the bacterial suspension drawn up and down five times. The first drop of the final filling should be discarded and the drops delivered by holding the pipette vertically above the plate with the tip 2·0–2·5 cm above the agar surface. The drops should be delivered at the rate of 20–30 drops per min and a little practice using the stop watch will enable this to be done. The tips of the pipettes must be clean so that the drop during formation creeps up the sides of the capillary. If it is necessary for a number of dilutions to be plated, these should be prepared in the usual way and a separate sterile dropping pipette used for plating each dilution.

4. *Medium*

The type of medium used will depend upon the nature of the investigation. The use of 2 per cent agar is recommended (see Ref. 7) but 1·5 per cent has been found satisfactory. The plates should be dried beforehand so that the 0·02 ml drop is absorbed in 15–20 min. In order to achieve this the length of drying required will vary with the type of medium used. Drying for 24 hr at 37° with the lids closed and for a further 2 hr with the lids raised, was recommended by Miles and Misra. It has been found that drying for 2–3 hr at 37° with the lids raised is usually sufficient. Dried plates of the ordinary nutrient media may be stored in the refrigerator for 1 week. Recently Jacobs and Harris (see Ref. 4) have shown that bacteriological agar contains substances which reduce the colony size of *E. coli* and *Staph. aureus* counted by a drop-plate method, but that the inhibition could be removed by treating the agar with activated charcoal or ferric chloride. It would appear that in some types of investigation it would be desirable to treat the agar used as described by these workers, who also found that inhibitory substances in the agar reduced the count of viable cells after they had been treated with phenols.

5. *Incubation*

After the inoculum has been absorbed the plates should be incubated in the inverted position. The temperature and duration of the incubation period will vary with the nature of the investigation.

6. *Replication and Counting*

Unless the viable population is approximately known a number of tenfold dilutions will have to be prepared and plated. The most suitable dilution for counting is that containing the largest number of colonies without signs of confluence or gross diminution of colony size due to over-crowding. Counting should be carried out under a magnification of × 4. The number of colonies counted in one drop may vary from 20–100 depending on the colony size of the species and duration of the incubation period. The degree of accuracy attained depends upon the number of colonies counted. For many purposes it is sufficient to count the colonies in three replicate drops, but if an accuracy of ±20 per cent is required a minimum of 100 colonies

must be counted (see Ref. 2). Assuming that only 20 colonies are present in the *countable* drop it is apparent that replicate drops will be required and these can be accommodated on one plate.

The viable colony count is calculated from the arithmetic mean colony count per drop of suspension × 50 × dilution factor.

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17. EXAMINATION FOR COLI-AEROGENES ORGANISMS

1. *General*

For many dairying purposes the presumptive coli-aerogenes test is adequate and little information of value is likely to be obtained from further confirmatory tests. Under some circumstances (e.g., examinations of dried milk, condensed milk, cheese, butter, plant swabs and rinses), it may be desirable to employ further tests which are sufficient to eliminate errors arising from the possible occurrence of false positive presumptive tests but without proceeding to detailed differentiation of types.

Interpretation of the results is dependent upon the nature, age and previous history of the sample and this information should be available to the bacteriologist.

2. *Presumptive Test*

(*a*) *Preparation of Dilutions*. Prepare serial one-tenth dilutions by the method prescribed in Technique No. 16, para. 11. The choice of particular dilutions shall be determined by the bacteriologist. It is often convenient to use dilutions which have been prepared for inoculation of Petri dishes.

(*b*) *Inoculation of Culture Tubes*. Using the technique prescribed in Technique No. 16, para. 11, inoculate 1 ml quantities from the dilution to be examined, or where appropriate from the original sample, into each of three culture tubes containing MacConkey's broth (see Technique No. 7). For approximate purposes where more than one dilution is being examined, one tube may be inoculated from each dilution at the discretion of the bacteriologist. Inoculation shall commence from the highest dilution chosen for examination. Mix the contents of the tubes by gentle rotation.

Note

If information on the most probable number is required, then five tubes instead of three at each dilution are required in accordance with McCrady's tables*. When detection of very small numbers of coli-aerogenes organisms is required, sufficient 10 ml quantities of the original sample shall be inoculated into 10 ml quantities of double-strength MacConkey's broth or 50 ml of the sample into 50 ml quantities of double-strength broth.

**The Bacteriological Examination of Water Supplies*. Ministry of Health Report No. 71 (1964).

(*c*) *Incubation and Recording of Culture Tubes.* Culture tubes or bottles shall be incubated at 30±1° for 72±2 hr and examined every 24 hr for acid and gas production. Tubes showing the presence of acid and gas shall be recorded as presumptive positive. For a presumptive positive tube the top curved portion of the Durham's tube shall be filled with gas, save that with lesser quantities the tube shall be positive if copious gas bubbles arise on shaking. When three tubes of each dilution have been inoculated, the dilution shall be recorded as positive when not less than two of the three culture tubes are positive. If confirmatory tests are to be carried out the appropriate transfer from presumptive positive tubes shall be performed immediately the reaction becomes evident.

3. *Confirmatory Tests*

(*a*) *Preparation of Petri Dishes of Differential Medium.* Pour about 15 ml of melted MacConkey's agar or eosin-methylene blue agar into each of the number of Petri dishes required, and allow to set. Dry overnight in a 37±1° incubator, or for 1 hr in a 55±5° incubator with the inverted inner dish containing the medium supported on the edge of the cover; overdrying should be avoided.

(*b*) *Inoculation of Petri Dishes.* Transfer one loopful (1–2 mm diameter) of liquid from the appropriate presumptive positive tube to the surface of the plates of the selected medium and distribute over the whole area by parallel and cross striations with the wire, or by spreading with a sterile bent glass rod. Isolated colonies may be obtained readily by diluting one loopful of the liquid with about 1 ml of sterile peptone water before spreading one loopful on the medium.

(*c*) *Incubation of Petri Dishes.* Incubate dishes bottom upwards for 24–48 hr at 30±1°.

(*d*) *Examination of Petri Dishes.* On MacConkey's agar, coli-aerogenes colonies are yellow.

On eosin-methylene blue agar, colonies of *Escherichia coli* are black or dark blue with a greenish sheen, less commonly bluish-red; colonies of *Klebsiella aerogenes* are red with or without a darker centre. On either medium, *K. aerogenes* colonies may be viscous and confluent. Inoculate a portion from suspected coli-aerogenes colonies into tubes of lactose broth and incubate at 30±1° for 72 hr and examine every 24 hr. Acid and gas production is indicative of true coli-aerogenes organisms. No colonies may develop if the presumptive test is a false positive produced by spore-forming bacilli, or alternatively if inoculation from tubes originally containing true coli-aerogenes bacteria has been delayed.

4. *Differential Tests*

For dairy purposes, confirmation of the presence of coli-aerogenes organisms by the above method is normally sufficient, but the procedure does not permit distinction between types. If such information is required for special purposes, additional differential tests are essential. The indole, methyl red, Voges-Proskauer, citrate and gelatin liquefaction tests are recommended; details of these are given in the report of the Coliform Committee, *Proc. Soc. appl. Bact.* (1949), **2**, 3. Careful purification of

selected colonies is an essential preliminary, and the inoculum for all tests should be a 24 hr culture in peptone water.

18. COLI-AEROGENES COLONY COUNT ON VIOLET RED BILE AGAR

The determination of the coli-aerogenes content of milk, dairy products and rinses and swabs of dairy equipment by means of the colony count on violet red bile (V.R.B.) agar has been shown to be a convenient method, which may have certain advantages over the presumptive coli-aerogenes test using MacConkey's broth. It has also been found suitable for investigating the incidence of different types of coli-aerogenes organisms in milk products and on dairy equipment.

British and American brands of dehydrated V.R.B. agar are available and give comparable colony counts. It is recommended that either should be used in preference to V.R.B. agar made up in the laboratory from ingredients.

1. *Preparation of the Medium*

Rehydrate the medium by suspending the recommended quantity in one litre of cold distilled water and steaming till *all granules have completely dissolved*. Dispense the hot medium into sterile containers.

The medium should not on any account be overheated. If the heated medium is stored at 3–5° it may be used up to 3 weeks after preparation.

2. *Preparation of Dilutions*

It is recommended that the following amounts of different materials should be plated.

(*a*) Raw milk: 1 ml–10^{-2} ml.
(*b*) Pasteurized milk: duplicate quantities of 5 ml with an additional plating of 1 ml in duplicate.
(*c*) Rinses and swabs: 1 ml and 10^{-1} ml of rinse or swab solution.
(*d*) Butter and cheese: duplicate 5 ml quantities of a 10^{-1} dilution with an additional plating of 1 ml of a 10^{-1} dilution.

3. *Inoculation of Petri Dishes*

The method prescribed in Technique No. 16 should be used.

4. *Pouring Petri Dishes*

To each dish add 15 ml of melted V.R.B. agar at a temperature of 45–47°. The tubes or bottles of medium should be dried and the mouths flamed before pouring. Immediately mix the inoculum. As soon as the medium has set, an additional 4–5 ml of V.R.B. agar should be poured over the surface of the inoculated agar and allowed to set.

5. *Incubation*

Incubate the Petri plates bottom upwards in stacks of *not* more than six deep. For coli-aerogenes colony counts on milk and milk products, rinses and swabs of dairy equipment, and milk cans and bottles, incubation at

30±1°, for 20–24 hr is recommended. Incubation for a period exceeding 24 hr will allow colonies other than coli-aerogenes colonies to develop.

6. *Counting of Colonies*

Typical coli-aerogenes colonies on V.R.B. agar are 0·5 mm to rather more than 1 mm in diameter, reddish purple in colour and surrounded by a reddish zone of precipitated bile. Colonies should be counted as soon as possible after incubation. Refrigeration of the plates alters the colour of the colonies and makes accurate counting difficult. Counting should be done in an illuminated counting chamber using a low power hand lens and a tally counter, and for ease of counting it is desirable to have a white background to show up the dark colonies, i.e., insert a sheet of white frosted glass in the counting chamber instead of the blue glass. Alternatively, a sheet of filter paper may be fitted on top of the blue glass in a Mattick and Hiscox counting chamber. Assessment of the colony count should preferably be made from plates showing not more than 150 typical coli-aerogenes colonies.

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19. COLONY COUNT OF THERMODURIC ORGANISMS

1. *Collection and Storage of Samples*

(*a*) *Farm Milk.* Representative 3 fl. oz samples shall be taken according to B.S. 809 from milk cans at the farm or on arrival at the collection depot. These shall be tested as soon as practicable and in any case within 24–28 hr of production

(*b*) *Bulk Milk.* Representative samples of bulk tanker milk shall be taken according to B.S. 809: 1963 either before despatch from country collecting depots or on arrival at town depots. Samples shall be tested within 6 hr of sampling; where this is not possible the samples shall be iced and subsequently stored at 3–5° for a period not exceeding 18 hr.

2. *Laboratory Pasteurization*

(*a*) *Milk.* Mix the sample thoroughly and pour 10 ml into a sterile rubber-stoppered test-tube which shall conform to B.S.625:1959, 150 × 16 mm, nominal 6 × $\frac{5}{8}$ in., having an internal diameter of 13·5 ± 0·7 mm and being accurately marked at 10 ml. Remove the stoppers of the sample bottle and test-tube, flame the pouring lip of the bottle and the mouth of the test-tube, pour the milk up to the 10 ml mark and firmly replace the stopper. Aseptic precautions shall be strictly observed during these operations. *Completely* immerse the test-tube in water held at 63·5±0·5° in a thermostatically controlled water bath. Total immersion of test-tubes can be effected by placing them in a test-tube rack laid obliquely in the water bath or by wedging tubes in a sloped position in a 6 × 6 in. wire basket in which a smaller basket or some other weight is placed on top of the tubes. After 35 min remove the tube from the water bath and immediately cool to 10° in iced water. Samples showing any signs of curdling when laboratory pasteu-

rized shall not be plated. A control tube of milk containing a thermometer shall be used to check the rate of heating. Alternatively McCartney bottles may be used.

The temperature recovery rate of water baths varies considerably and it is essential to ensure that the temperature of the milk reaches 63° within 5 min of immersion. To achieve this it may be necessary to limit to 10 the number of tubes in the batch to be pasteurized. The temperature recovery rate of baths used for laboratory pasteurization shall be checked before carrying out the test, and a control tube of milk containing a thermometer shall always be used. The thermometers used in the water bath and in the milk shall be checked at regular intervals with an N.P.L. certified thermometer.

Invert the cooled, pasteurized sample slowly three times and plate 10^{-1}, 10^{-2} and 10^{-3} ml amounts on Yeastrel milk agar. One 10^{-1} and 10^{-2} ml amounts shall be plated when a very low count is anticipated. Incubate the plates immediately at $30 \pm 1°$ for 72 ± 2 hr.

(*b*) *Rinses and Swabs.* Rinses of milk cans, dairy utensils, milking machine clusters and pipe lines, using 500 ml of sterile quarter-strength Ringer's solution, and swabs of dairy utensils and milk plant placed in 25 ml of quarter-strength Ringer's solution shall be laboratory pasteurized within 6 hr of sampling; where this is not possible the sample shall be iced and subsequently stored at 3–5° for a period not exceeding 18 hr. Mix the sample by slowly inverting the bottle holding the rinse solution three times or rotating the tube containing the swab solution between the palms of the hands for 12 sec. Then transfer 5 ml of the well-mixed sample aseptically with a sterile straight-sided pipette to a $6 \times \frac{5}{8}$ in. test-tube or a McCartney bottle containing 5 ml of sterile skim milk and firmly stopper. Invert the tube or bottle slowly three times and then laboratory pasteurize and cool as previously described for milk. Invert the pasteurized sample three times and plate 1, 10^{-1} and 10^{-2} ml amounts in Yeastrel milk agar and incubate at $30 \pm 1°$ for 72 ± 2 hr.

3. *Counting of Colonies*

Calculate the colony count per ml of the rinse or swab sample by multiplying the colony count obtained on the countable plate by two times the dilution factor. The thermoduric colony count of rinse samples shall be recorded as the count per utensil (colonies per ml of rinse × 500) or per sq. ft if the surface area of the equipment rinsed is known. The result for swab samples shall be calculated and recorded as the thermoduric colony count per sq. ft of plant surface (colony count per ml of swab samples × 25 × area factor if not 1 sq. ft).

4. *Interpretation of Results*

For general guidance in the interpretation of results for liquid milk, rinses and swabs, see EGDELL, J. W., THOMAS, S. B., CLEGG, L. F. L. and CUTHBERT, W. A. (1950). *Proc. Soc. appl. Bact.*, **13**, 132 and THOMAS, S. B., DRUCE, R. G., PETERS, G. J. and GRIFFITHS, D. G. (1967). *J. appl. Bact.*, **30**, 265.

20. PSYCHROTROPHIC BACTERIA IN MILK AND RINSES

1. *Definition*

There are several definitions of psychrotrophs (see Ref. 5), but the most practicable for dairy purposes is that they are those bacteria which are active at or below 7°, i.e., refrigeration and dairy cold-store temperatures. The activity of these organisms is normally increased at higher temperatures, e.g., 15–20°. They are usually defined as psychrotrophic in bacteriological literature.

2. *Importance in Milk and Dairy Products*

Psychrotrophs may cause defects in milk and milk products depending on the time and temperature of refrigeration. Examples are fishiness, rancidity, fruitiness and ropiness in milk. Ice-cream may be similiarly affected, while butter held at low temperatures may develop a surface rancidity.

The growth of these organisms in milk may affect its keeping quality as determined either by dye reduction or C.O.B. tests, but many are relatively inert.

Psychrotrophic bacteria are seldom thermoduric and their occurrence in commercially pasteurized milk indicates post-pasteurization contamination.

3. *Source*

Psychrotrophs are widely distributed in soil, water, dairy equipment, etc., but they have not been reported in aseptically drawn milk.

4. *Nature of Psychrotrophs*

Psychrotrophic, milk spoilage bacteria most commonly belong to the following genera: *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Alcaligenes*, *Enterobacter* and *Arthrobacter*. *Pseudomonas* is the genus most frequently encountered. Some are inert in litmus milk, but they may be strongly lipolytic and/or proteolytic in other media.

5. *Enumeration*

For the enumeration of psychrotrophs a number of combinations of times and incubation temperatures have been used depending largely on the definition of psychrotrophs employed by the worker concerned (see Ref. 1).

Most workers with dairy products have used conventional plate count media, e.g., Yeastrel milk agar. Low temperatures and short incubation times restrict the numbers of colonies obtained and result in small colonies which are difficult to count. Long incubation times are impractical for routine purposes, and too high a temperature will permit the growth of bacteria not usually considered to be psychrotrophs

Psychrotrophs are mostly Gram-negative rods, and several workers have employed selective media at higher temperatures in an attempt to obtain an estimate of their numbers in a shorter time. These attempts have not met with general success, and it is suggested that the following method (similar to that recommended in *Standard Methods for the Examination of Dairy Products*, 11th Edition, American Public Health Association, and used by Thomas, (1960) and Thomas *et al*, (1961) see Refs. 2 and 3) be employed as a reasonable compromise.

6. *Method*

Serial tenfold dilutions are prepared and suitable plates poured, Yeastrel milk agar being employed. The plates are incubated at 5–7° for 10 days and the colonies counted.

Alternatively, the roll-tube method may be employed with the same temperature and time of incubation.

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21. THE ENUMERATION AND ISOLATION OF COAGULASE-POSITIVE STAPHYLOCOCCI FROM MILK, CHEESE AND OTHER MILK PRODUCTS

The medium recommended by Baird-Parker (see Ref. 1) can be used for the isolation of coagulase-positive staphylococci from milk, cheese and other products. Its selective action depends upon a combination of potassium tellurite, lithium chloride and glycine, egg yolk emulsion being used as a diagnostic agent.

1. *General Method of Enumeration and Isolation*

Prepare serial tenfold dilutions in quarter-strength Ringer's solution and, using a dropping pipette, inoculate duplicate 0·02 ml amounts of each dilution on to the dried surface of a plate of Baird-Parker medium. Using a wire loop, spread each drop over a quarter of the surface of the plate and after the inoculum has been absorbed (15–20 min) invert the plate and incubate it at 37° for 40–44 hr.

After incubation, select a plate, containing 20–30 colonies from the range of dilutions plated and count and record the number of each type of colony present on both duplicate plates. From each plate pick off 2–4 colonies of each type into nutrient broth, incubate at 37° for 18–24 hr and carry out a coagulase test on each culture. Determine the types of colony which are coagulase positive and calculate the number of these staphylococci in the sample by multiplying the mean colony count of this type by the dilution factor.

Coagulase-positive staphylococci on the B.P. medium form black, shiny convex colonies 1–2 mm in diameter with narrow white entire margins and are surrounded by clear zones extending 2–5 mm into the opaque medium. (Some commercial preparations of egg yolk may not give satisfactory clearing.) Occasionally strains may be encountered in which the colony is surrounded by an opaque zone with a zone of clearing outside it. At first it is necessary to submit all colony types to a coagulase test but, when experience has been gained in recognising the coagulase-positive type of colony, the number of colonies picked for confirmation may be reduced.

2. *Milk*

Serial dilutions of the milk sample are plated on B.P. medium as described. It has been shown (see Ref. 2) that the presence of milk itself at a dilution of less than 1:32 may have an effect on the growth of staphylococci on certain selective media and that coagulase-negative staphylococci may not be inhibited. Consequently when examining the plates inoculated with undiluted milk or of a one-tenth dilution, colonies should always be picked for confirmation by the coagulase test.

3. *Cheese*

The cheese should be sampled and dilutions prepared as in Technique No. 23, and serial dilutions plated as described in the previous para.

4. *Other Milk Products*

Dried milk powder should be reconstituted and examined in the same way as milk.

Cream should be examined in the same way as milk except that the first dilution (one-tenth) should be prepared on a weight/volume, basis, i.e., 1 g cream added to 9 ml quarter-strength Ringer's solution.

Media

5. *Baird Parker Medium* (*see Ref.* 1)

Tryptone (Bacto)	10 g
Lab lemco	5 g
Yeast extract (Bacto)	1 g
Lithium chloride	5 g
Agar	20 g
Distilled water	1,000 ml

Dissolve the ingredients in the water by autoclaving at 121° for 15 min. Adjust the pH to 7·6 at 60°. Steam for ½–1 hr and dispense in 90 ml amounts in screw-capped bottles. Autoclave at 121° for 15 min. The final pH at room temperature should be 7·6–7·8.

Before use, melt a bottle of the medium, cool to 45° and add successively the following solutions sterilized by filtration and warmed to 45°.

(*a*) 5·0 ml of 20 per cent w/v sodium pyruvate (L. Light & Co.)
(*b*) 1·0 ml of 1·07 per cent w/v potassium tellurite (B.D.H.)
(*c*) 6·4 ml of 20 per cent w/v glycine (A.R.)
(*d*) 5·0 ml of concentrated egg yolk emulsion (*Oxoid* or prepared according to the technique given in para. 6).

Mix the contents of the bottle and pour about 15 ml into Petri dishes and allow to set. Before use, dry the plates at 37° for ½–2 hr so that the 0·02 ml inoculum is absorbed in 15–20 min. Excessive drying of the plates must be avoided.

Bottles of basal medium and stock solutions of glycine and tellurite may be stored at room temperature for up to 2 months. The pyruvate solution is best stored at 3–5° and replaced every month. Poured plates of complete medium must be used within 24 hr of preparation.

6. *Preparation of Egg Yolk Emulsion*

To a litre of nutrient broth at pH 7·2 containing peptone (Evans) 1·0 per cent, Lab. lemco (Oxoid) 1·0 per cent, sodium chloride 0·5 per cent, add 200 g of egg yolk. Mix and add 20 g acid-washed Kieselghur (B.D.H.). Shake vigorously and stand overnight at 0–4°. Decant the supernatant liquid and filter this under vacuum through a firm bed of paper pulp using the minimum amount of paper which will produce a clear filtrate. If the filtrate is not clear then filter again through the same filter bed. Filter again through a Seitz K.5 clarifying pad and sterilize by filtration through a Seitz E.K. filter pad.

This emulsion is stable for several months if stored at 0–4°.

Some absorption takes place during the filtering processes and the minimum amount of filtering material should be used, but unless a clear filtrate is obtained prior to sterilization, difficulty will be experienced in obtaining worth while yields.

The Coagulase Test

Mix 1 ml of human or rabbit plasma with 9 ml of isotonic saline (0·85 per cent NaCl) to make a one-tenth dilution. Place 1 ml of this diluted plasma into each of three coagulase tubes and add 0·1 ml of an 18 hr nutrient broth culture. Incubate for 3 hr at 37° and examine. A positive result is shown by coagulation; negative tubes will be clear or only faintly cloudy with no coagulation. If one of the three tubes gives a different result from the other two, the test must be repeated.

It is essential in each batch of tests to include two control tubes, one containing a known coagulase-positive strain, and the other a coagulase-negative one.

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2. Joshi, N., Blackwood, A. C. and Gale, D. G. (1963). *J. Bact.*, **86**, 1119.

22. CLASSIFICATION OF STAPHYLOCOCCI AND MICROCOCCI ISOLATED FROM MILK AND FROM FARM DAIRY EQUIPMENT

Plate samples of milk, and rinse and swab solutions of farm dairy equipment on Yeastrel milk agar (Y.M.A.) and incubate at 30° for 3–5 days.

Pick at random a representative number of colonies from a countable plate and classify according to the method outlined in Technique No. 28.

Gram-positive, catalase-positive cocci are deemed to be staphylococci or micrococci and they can be further examined and classified according to the following scheme:

(*a*) apply the Baird-Parker (1963) modification of the Hugh and Leifson (1953) test, (Appendix I) using a heavy inoculum of the culture from Y.M.A. plates.

Strains showing acid production from glucose under anaerobic conditions, i.e., in the depth of the tube sealed with liquid paraffin, are considered to be anaerobic glucose utilizers (fermentative), and to be members of the genus *Staphylococcus*. They may be further

classified into coagulase-positive (*Staph. aureus*) or coagulase-negative staphylococci on the coagulase test (see Technique No. 21).

Strains failing to show glucose fermentation under anaerobic conditions, but forming acid or failing to do so in the unsealed tube under aerobic conditions (oxidative) are considered to be members of the genus *Micrococcus*.

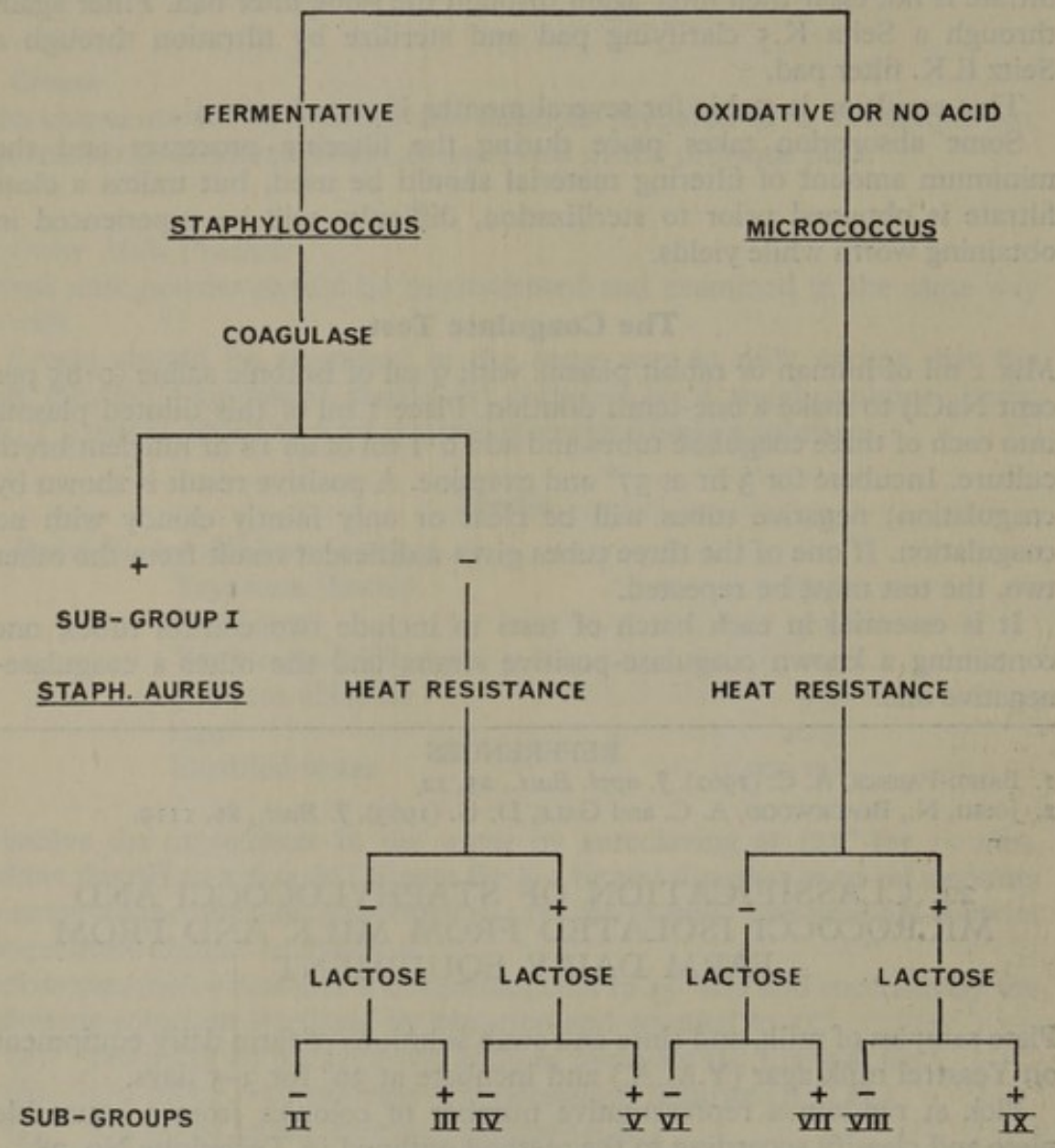

Fig. 1. Classification of staphylococci and micrococci

(*b*) all cultures of staphylococci and micrococci may be further examined by the following tests:

(i) heat resistance in skim milk, laboratory pasteurized at 63·5° for 30 min. Inoculate 0·2 ml of a moderately turbid (18–24 hr at 30°) lactose broth culture into each of duplicate tubes (6 × $\frac{5}{8}$ in.) containing 5 ml of sterile skim milk. Mix and laboratory pasteurize the inoculated skim milk strictly according to the method described in Technique No. 19.

Take one tube of the cooled pasteurized milk and plate out 1·0 ml on Y.M.A. and incubate at 30° for 3–4 days. The development of 50 or more colonies will indicate that the culture is heat-resistant; slight survival is shown by the production of fewer colonies.

Incubate the second, unopened tube of pasteurized skim milk at 30° for 3–4 days. The presence of coagulation or digestion is indicative of survival even if no colonies develop on the plated culture from the first tube. If no coagulation or digestion is observed in this tube, and if less than 50 colonies have grown on the plate inoculated from the first tube, streak a loopful (10^{-2} ml) of the pasteurized culture from the second tube on to Y.M.A. and incubate as before. The presence of growth indicates that the culture is heat resistant;

(ii) acid formation in lactose broth within 14 days at 30°.

The results of these test will enable cultures to be grouped (see fig. 1 on opposite page).

REFERENCES

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2. Hugh, R. and Leifson, E. (1953). *J. Bact.*, **66**, 24.
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23. ENUMERATION AND ISOLATION OF LACTOBACILLI FROM MILK, CHEESE AND STARTER

Lactobacilli can be isolated from these sources, using a selective acetate agar (Ac. A.) medium (see Refs. 1 and 2); or selectively enriched in a broth of the same composition (Ac. B.). The selective action is due to a low pH of 5·4 and a high concentration of acetate ions, which suppress the growth of other organisms.

1. *General Method of Enumeration and Isolation*

Serial dilution of the samples are made in quarter-strength Ringer's solution and duplicate 1·0 ml amounts are plated in Ac. A., using the pour plate technique. These plates are incubated anaerobically in an atmosphere of CO_2 or 90 per cent H_2 + 10 per cent CO_2 without sparking the jars. Alternatively, 90 per cent N may be used instead of hydrogen. If anaerobic jars are not available, double layer plates should be used, a thin layer of 1·5 per cent plain agar being poured on the surface of the solidified Ac. A. Otherwise evaporation during incubation increases the concentrations of acetate in the medium sufficiently to inhibit lactobacilli in addition to other organisms. The presence of CO_2 stimulates the growth of some lactobacilli. Plates are incubated for 4–5 days at 30±1° without being inverted.

Colonies growing on Ac. A. are counted, and those selected for further examination are picked into Ac. B., incubated at 30° for 3 days and purified by streaking on M.R.S. agar (see Ref. 3). Single colonies are then picked from this into M.R.S. broth. Strains to be preserved are cultured into yeast dextrose litmus milk (Y.D.L.M.)+chalk, stored at 4° and subcultured every 3 months.

2. *Milk*
Serial dilutions of the milk samples are plated on Ac. A. as described. Counts of lactobacilli are unlikely to be higher than 10^3/ml, and in clean single herd milks they may be less than 10/ml. If it is desired to detect the presence of very small numbers of less than 1/ml, an enrichment method is used. Ten 1 ml portions of the milk are added to 5 ml amounts of Ac. B. in $\frac{1}{4}$ oz screw-capped bottles. After 5 days incubation, each sample is plated on Ac. A. and the resulting colonies, if any, picked into M.R.S. broth, and then examined further.

3. *Streptococcus Starter Cultures*
Serial dilutions of mother culture or bulk starter are plated on Ac. A. as described.

4. *Cheese*
Two sterilized cheese borers—sizes 4F and 2F (see Note) are required. A core of about 1 in. deep is taken from the top of the cheese with the larger borer and put aside. A core 3 in. deep is then taken with the narrower borer from the inner surface of the cheese thus exposed and transferred with the help of a sterile scalpel to a sterile wide-necked screw-capped bottle (1 oz Universal). The cheese is then plugged up again with the outer core of cheese which was first removed.

The cheese is minced in a previously sterilized Mouli grater into a sterile beaker. Nine grammes of the minced cheese and 80 ml of 2 per cent sodium citrate previously warmed to 50° are homogenised for 3 min. The homogenate is left to stand for 15 min, then serial dilution are made and plated in the usual way. Colony counts may range from 10^2—10^6/g of cheese, depending on such factors as source and heat treatment of the cheese milk, and age and variety of the cheese.

5. *Organisms Isolated on the Selective Medium*
In addition to lactobacilli, pedicocci and some leuconostocs are able to grow in this medium. These organisms cannot be distinguished from lactobacilli by colony appearance, so they may also be included in total counts on Ac. A. For further differentiation, colonies must be picked and examined microscopically and biochemically.

Note
Cheese borers obtainable from Sutherland and Thomson, Wells, Somerset.

Media

6. *Selective Acetate Medium*
Rogosa, Mitchell and Wiseman's (see Ref. 1) medium is the easiest to prepare, and can also be obtained as a proprietary dehydrated product.
(*a*) *Acetate Agar* (*Ac. A.*)

Trypticase (BBL)	10 g
Yeast extract (Difco or Oxoid) . .	5 g
Potassium di-hydrogen phosphate, KH_2PO_4	6 g

Di-ammonium hydrogen citrate, $(NH_4)_2HC_6H_5O_7$	2 g
Salts 'A' solution*	5 ml
Dextrose	20 g
Tween 80	1 g
Sodium acetate, $CH_3COONa.3H_2O$	25 g
Glacial acetic acid, CH_3COOH	1·32 ml
Agar	15 g

*Salts 'A' Solution

Magnesium sulphate $MgSO_4.7H_2O$	11·5 g
Manganese sulphate $MnSO_4.2H_2O$	2·4 g
or	
Manganese sulphate $MnSO_4.4H_2O$	2·8 g
Ferrous sulphate $FeSO_4,7H_2O$	0·68 g
Distilled water	to 100 ml

Dissolve the agar separately in 400 ml hot distilled water.

Dissolve the remaining ingredients together in a further 400 ml distilled water. Bring to the boil and add to the hot agar solution. Adjust the volume to 1 litre with hot distilled water, and dispense immediately whilst hot into sterile tubes or bottles. If the ingredients have been measured accurately, the pH will be 5·4 without further adjustment. It is most important that the final pH should be 5·4, and this should be checked with a glass electrode. Because of its inhibitory properties, this medium need not be sterilized. It should, however, always be stored at 3–5°. Prolonged or repeated heating darkens it and may cause inhibition of lactobacilli. It should, therefore, never be reheated or heated longer than necessary to melt the agar.

(*b*) *Acetate Broth* (*Ac. B.*)

The same medium as above with agar omitted.

(*c*) *de Man, Rogosa and Sharpe's Broth* (*M.R.S. broth*)

Lemco (Oxoid)	10 g
Peptone (Oxoid)	10 g
Yeast extract (Oxoid)	5 g
Sodium acetate, $CH_3COONa.3H_2O$	5 g
Di-potassium hydrogen phosphate, K_2HPO_4	2 g
Di-ammonium hydrogen citrate, $(NH_4)_2HC_6H_5O_7$	2 g
Salts 'A' solution (as for Ac media)	5 ml
Dextrose (add the Dextrose after filtration)	20 g
Tween 80	1 g

Make up in distilled water to 1 litre. Steam to dissolve, adjust to pH 6·5. Filter. Dispense and autoclave at 121° for 15 min.

(*d*) *M.R.S. Agar*

As above with 1·5 per cent agar added.

(*e*) *Yeast Dextrose Litmus Milk + Chalk*

Separated milk + 0·01 per cent litmus	
Yeast extract	0·3 per cent
Dextrose	1·0 per cent
Calcium carbonate, $CaCO_3$(AR)	5·0 per cent

Growth of lactobacilli is improved by the addition of 0·25 per cent liver extract, to this medium, but this is not essential. Dispense in 10 ml quantities and autoclave at 121° for 15 min.

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24. COUNT OF TOTAL AND RESISTANT SPORES

Note
This method is primarily intended for liquid milk.

1. *Collection of Samples*
Samples shall be taken according to B.S. 809: 1963 and they shall be cooled immediately to 10° to prevent spore germination which would have the effect of lowering the count.

(*a*) *Total Spores*
Pour 10 ml of the well-mixed sample into a sterile rubber-stoppered test-tube, which shall conform to B.S. 625: 1959, 150 × 16 mm, nominal 6 × $\frac{5}{8}$ in., having an internal diameter of 13·5±0·7 mm and being accurately marked at 10 ml. Completely immerse the tubes in a suitably controlled water bath and heat at 80° for 10 min. It is necessary to wedge the stoppered tubes sideways in a 6 × 6 in. basket or a suitable rack in order to prevent the bungs coming out. The temperature should be read from a thermometer immersed in a control tube. After this treatment cool the samples immediately to 10° and plate in starch milk agar (see Ref. 1 and Technique No. 10). The concentration of the agar may be 2·5 or 3 per cent to prevent the result being masked by spreading colonies.

(*b*) *Resistant Spores*
Since these are frequently present in a lower concentration than 1 per ml, plating methods are not applicable and the dilution technique must be used.

Five tubes containing 10 ml, five of 1 ml and five of 0·1 ml of the sample will cover the range 2–1800+ per 100 ml in McCrady's tables (Ministry of Health Report No. 71, 1956. *The Bacteriological Examination of Water Supplies*). As the level of the spores to be measured may be below the level of unavoidable aerial contamination, the heating should be done after distribution of the sample in tubes. The addition of litmus assists in reading results. For the 10 ml tubes, autoclaved litmus can be added directly to bulk before distribution. Add the 1·0 and 0·1 ml of the milk sample amounts to 5 ml tubes of autoclaved litmus milk which has previously been in-

cubated to check sterility. Examine larger amounts of the sample where it is suspected that the number of organisms is below 2 per 100 ml.

Immediately after the distribution in 6 × $\frac{5}{8}$ in. tubes cover with grease-proof paper to protect the plugs. Place the tubes in a warm steamer or autoclave, and bring to steaming within 15 min; maintain in flowing steam for a further 30 min. Cool to incubation temperature.

(*c*) *Incubation*

(i) *Total Spores*. mesophiles, 3 days at 30±1° or 2 days at 37±1°; thermophiles, 2–3 days at 55±3°.

(ii) *Resistant Spores*. mesophiles, 21 days at 30±1° or 14 days at 37±1°; thermophiles, 3 days at 55 ± 3°. It is advisable to place plates and tubes incubated at 55° in a cannister containing moist cotton-wool, or in plastic bags. Alternatively, a large dish of water should be placed in the incubator. This prevents drying out.

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25. COUNT OF ANAEROBIC SPORES (DILUTION COUNT TECHNIQUE)

1. *Medium*

The medium recommended is a semi-solid broth suitable for the enumeration of anaerobic spores by a dilution technique (see Ref. 1) *Reinforced Clostridial Medium* (R.C.M,) to which is added aqueous neutral red solution to give a final indicator concentration of 1/10,000 (see Technique No. 11).

2. *Dilution Counts*

Dilution counts on solutions containing a small number of organisms usually necessitate working with turbid solutions, and consequently there may be difficulty in reading positive growth tubes. The addition of a suitable indicator, which gives a colour change when anaerobic growth has taken place, facilitates the identification of positive tubes. Neutral red gives a yellow green fluorescence at an Eh of—0·34 V. at pH 7·0, which is in the range reached by anaerobic growth but lower than the level normally produced by aerobic growth. Neutral red is, therefore, used in detecting the presence of anaerobes. (Streptococci and Lactobacilli may also give fluorescence but as these are not sporing organisms, they should not confuse the result).

The medium is dispensed in 9 ml quantities into 6 × $\frac{5}{8}$ in. test-tubes and sterilized by autoclaving at 121° for 15 min. If they are not used at once the tubes of medium should be boiled for 30 min immediately before use.

The number of anaerobic spores may be determined by making dilutions in R.C.M. broth and subsequent inoculation of these dilutions in 1 ml quantities into replicate tubes containing 9 ml of R.C.M. broth. When the expected count per ml is low, it is advisable to use twofold dilutions with at least four tubes at each level and at least eight levels; where tenfold dilutions are made, five tubes should be put up at each level.

After inoculation, seal the tubes by the addition of one of the following:
sterile liquid paraffin; or
sterile paraffin wax and ceresin, melting point approx. 50°; or
sterile vaseline and agar mixture (see Ref. 1).

Heat the tubes in a water bath at 80° for 10 min to destroy all organisms except spores, cool and incubate at 37±1° for 14 days. Make examinations for gas production and fluorescence daily. The fluorescence, although an Eh effect, is bound up with pH and may fade with prolonged incubation.

Determinations of the most probable number of organisms may be deducted from the number of positive tubes, using McCrady's Tables for tenfold dilutions, or W.L. Stephens' Tables (see Ref. 2) for two-, four- or tenfold dilutions.

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2. FISHER, R. A. and YATES, F. (1963). Densities of organisms estimated by the dilution method.
Statistical Tables for Biological, Agricultural and Medical Research, 6th Edition, p. 62.

26. DETECTION OF LECITHINASE PRODUCING ORGANISMS

(*a*) The fault 'broken' or 'bitty' cream in milk is associated with the production of lecithinase by certain milk organisms and in particular by strains of *B. cereus* and *B. cereus* var. *mycoides*. Some *Pseudomonas* and *Achromobacter* species also produce lecithinase but do not necessarily cause bittiness. A characteristic observed with bittiness produced by *Pseudomonas* species is that in hot tea the particles disperse and oily globules are formed.
(*b*) Lecithinase producing organisms may be identified by inoculating into egg yolk broth or by streaking on the surface of egg yolk agar plates.
(*c*) *B. cereus* renders the egg yolk broth thickly turbid after incubation at 37±1° for 8–10 hr, and after 16–24 hr a thick curd usually appears on top of the medium. With certain strains of *B. cereus*, 22° may be a better temperature of incubation. Lecithinase producing Gram-negative rods produce less marked changes. Lecithinase negative organisms produce no visual change in the egg yolk.
(*d*) On egg yolk agar, colonies of lecithinase producing organisms are surrounded by a *thick opaque* zone usually extending several mm from a colony. With experience, the type of zone produced by *B. cereus* can be distinguished from that produced by other species since it tends to be more opaque and has a less well defined margin.
(*e*) Quantitative estimation of lecithinase producing organisms in milk or other material may be made using the pour plate method. A surface plate method, however, will give more clear cut results. Estimation of *B. cereus* spores may be made by first heating to 80° for 10 min before plating out on egg yolk agar. The numbers present in milk are often too small to be detected by this method, and the following is a more suitable routine method:

(i) distribute the milk as follows:—10 ml into each of five sterile boiling tubes or McCartney bottles, 1 ml into each of five test-tubes

containing 5 ml of sterile litmus milk, 1 ml of 10^{-1} dilution into each of five tubes containing 5 ml of sterile litmus milk. Laboratory pasteurize the 15 tubes of milk by holding at $63 \cdot 5 \pm 0 \cdot 5°$ for 35 min. Cool to 22°;

(ii) incubate at $22 \pm 1°$ for 72 ± 4 hr. At the end of incubation, spot a loopful from the surface of each bottle or tube on to the surface of an egg yolk agar plate and incubate at $22 \pm 1°$ (one plate will accommodate the 15 spots which should be so grouped that dilutions can be identified).

(iii) examine the plates at 24 hr for typical zones of opacity round the spot, and the most probable number per 100 ml of milk can be calculated using McCrady's tables. This figure will indicate the presumptive *B. cereus* spore index.

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27. THE FLOC COUNT TECHNIQUE FOR THE DETECTION OF 'BITTY' CREAM

This technique is intended for assessing the incidence of 'bitty' or 'broken' cream, caused by strains of *Bacillus cereus*, in fresh samples of raw and pasteurized milk. It involves making a direct count of the flocs or flakes of cream which have developed in the milk when incubated under standard conditions. The techinique, therefore, measures the effect produced by *B. cereus* in milk and not necessarily the number of spores of this organism. The spore count can be assessed by using the method given in Technique No. 26.

Although the floc count technique is subject to inherent errors, it can nevertheless be of value for advisory purposes to indicate the probable degree of bittiness likely to develop in bottled milk exposed to warm atmospheric conditions.

1. *Apparatus and Materials*

Heating-bath
22° water bath (or incubator)
4 oz sterile medical flat screw-capped bottles with mark etched at 100 ml
Dropping pipette (or bottle)
Petri dishes, large, 6 in. diameter
Thermometer, 0–100°
10 per cent w/v water-soluble nigrosin in distilled water

2. *Procedure*

Mix the sample well and fill a sterile medical flat bottle up to the 100 ml mark with the milk. Slacken the screw-cap half a turn, place the bottle in warm water (approx. 50°) in the heating-bath so that the level of the water is above that of the milk. Raise the temperature rapidly (until a thermometer

in a control bottle registers 73°) and hold at this temperature for 10 sec. Remove the sample immediately and allow to cool on the bench for 5 min, then complete the cooling to 22° in running water and tighten the screw-cap.

Transfer the sample to the water bath or incubator and incubate at 22° for 24 hr. At the end of the incubation period warm the sample to 40–50° in water in the heating-bath to melt any particles of fat caused by fortuitous mechanical churning or produced by micro-organisms other than *B. cereus*.

Add 5–10 drops of nigrosin solution to the warmed milk and mix gently by inverting the bottle *very slowly* three times. Then pour the contents gently into the Petri dish and count the flocs which are clearly visible against the dark background of the nigrosin. Express the result as the number of flocs per 100 ml milk.

Notes

1. Care must be taken to avoid any undue agitation of the milk at all stages after incubation has commenced, otherwise flocs may be broken up and the count will be too high.
2. Whilst heating the sample some skin may form on the milk and may be confused with flocs during counting. Fragments of skin can be distinguished by touching them with a small wire spade or the tip of a pipette to which they will adhere, whereas true flocs slide off.
3. Occasionally the milk coagulates during incubation. When this occurs it is impossible to obtain a floc count.

For fresh farm milk a floc count in excess of 50/100 ml may be considered unsatisfactory, but if the milk is that of a producer/retailer a smaller number of flocs could be a cause of complaint.

In freshly pasteurized milk a count of more than 10 flocs in 100 ml milk may also be a cause of complaint.

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28. TYPES OF MILK BACTERIA FORMING COLONIES ON YEASTREL MILK AGAR INCUBATED AT 30°

This technique is intended to be used as a general guide for preliminary investigations of the main groups of bacteria in milk and on dairy equipment. It is designed for the examination of colonies developing on Yeastrel milk agar (Y.M.A.) plates inoculated with milk and rinses or swabs of dairy equipment and incubated at 30°. It may be adapted for use with other media and incubation temperatures.

1. Pick 20 to 30 well isolated colonies from a countable Y.M.A. plate (30 to 300 colonies) which has been incubated at 30° for 3 to 5 days.

Draw two radii on the plate to form a wedge-shaped area where there is a typical distribution of well-spaced colonies free from large spreaders. The radii should be drawn so as to enclose about 20 to 30 colonies. Com-

mencing from the angle in the centre of the plate and working towards the periphery, pick every colony from this area with a wire needle or spade.

2. Inoculate into 5 ml Yeastrel dextrose lemco broth (Y.D.L.B.) in 6 × $\frac{5}{8}$ in. tubes, macerating the growth on the side of the tube at the broth surface, and ensuring that it is well mixed. Incubate the Y.D.L.B. culture at 30° for 1 to 3 days until turbidity appears.

3. Pour Y.M.A. plates at 40–45°. Dry by incubating the closed inverted plates at 37° or 30° until the surface is free of condensate, or by holding in a closed container at room temperature for up to 3 days.

Streak the Y.D.L.B. culture with a small loop onto the surface of a dried Y.M.A. plate, in such a way as to produce several well isolated colonies. Incubate the streaked plate at 30° for 1 to 2 days.

4. When more than one type of colony appears on the streaked plate, a colony of each type should be re-streaked on a separate dried Y.M.A. plate.

5. If the culture appears to be pure, well-isolated colonies should be streaked on Y.M.A. slopes and incubated at 30°. The Gram-reaction and morphology should be determined at 24 hr or as soon as growth has occurred. The culture should be returned to the incubator.

6. A catalase test should be applied to the agar slope culture after incubation for 3 or 4 days. To a drop of hydrogen peroxide (10 vol) on a slide, add a substantial quantity of culture from the slope using a wire loop, and mix. If gas bubbles are produced, the result is recorded as positive.

7. When spores have not been observed at the time of Gram-staining in a culture of Gram-positive rods, it should be examined again for spore formation after a further incubation for 3 or 4 days. A simple single stain (e.g., carbol fuchsin or methyl violet) may be employed for this purpose.

8. Examine the agar slope culture for chromogenesis after incubation at 30° for 7 days.

9. Inoculate unpigmented cultures composed of Gram-negative rods into MacConkey's broth and record the formation of acid and gas after incubation at 30° for 72 hr.

10. Inoculate each culture into litmus milk ensuring that the inoculum is well incorporated and observe the kind of fermentation after incubation at 22° for 24, 48 and 72 hr. An uninoculated control should also be incubated.

Record:

No change	=	NC	Reduction	=	R
Acid	=	A	Alkali	=	Alk
Gas production	=	G	Digestion	=	D
Acid clot	=	AC	Sweet clot	=	SC
Acid clot with reduction	=	RAC			

'Slight' should be signified by placing the characters in brackets e.g., (A) = Slight acid

The reactions of litmus milk and their interpretation are more fully described in Technique No. 13.

11. The following scheme shows the criteria which can be used for distinguishing the main groups of predominating bacteria:

				RODS	*COCCI*
CULTURE	CATALASE NEGATIVE	GRAM POSITIVE		LACTOBACILLI	STREPTOCOCCI
		GRAM POSITIVE	AEROBIC SPORE-FORMERS		MICROCOCCI
			NON-SPORING GRAM-POSITIVE RODS		
	CATALASE POSITIVE	GRAM NEGATIVE	COLI-AEROGENES		
			OTHER GRAM-NEGATIVE RODS		

A further distinction of the cultures composed of Gram-negative rods other than coli-aerogenes bacteria can be made if required according to the type of chromogenesis developed on the agar slope culture. These will include *Serratia* (blood red), *Chromobacterium* (violet), *Flavobacterium* (orange or yellow) and fluorescent pseudomonads (yellow, green or blue fluorescent water-soluble pigment). Cultures of Gram-negative rods other than coli-aerogenes bacteria should be streaked onto a slope of Proteose peptone agar (see para. 13b), incubated at 30° for 3 days and examined for fluorescence.

Catalase-positive Gram-positive rods producing small yellowish colonies may be *Corynebacterium* (*Microbacterium*) distinguishable in stained smear preparations by the characteristic arrangement of their cells in angular 'chinese letter' forms.

Streptomycetes do not fit into the above scheme but may be distinguished by their earthy odour. Their much branched hyphae with chains of terminal conidia give the concentrically-ringed colonies a 'chalk powder' appearance. Microscopical preparations from the aerial mycelium may consist solely of conidia.

12. The characteristics of each culture should be recorded on a laboratory day sheet, and a summary of the general classification of the 20 to 30 colonies picked from a single Petri plate recorded on another sheet.

13. The following media will be required:

(*a*) *Yeastrel Dextrose Lemco Broth* (Y.D.L.B.) (see Technique No. 14).

(*b*) *Proteose Peptone Agar*

Proteose peptone (Oxoid L46), 20 g: glycerol A.R., 10 ml: magnesium sulphate crystals A.R., 1·5 g: di-potassium hydrogen ortho-phosphate anhydrous, 1·5 g: agar 15 g: distilled water, 1 litre.

Dissolve the ingredients in the distilled water, do *not* filter, and adjust if necessary to pH 7·2. Distribute into sterile tubes and sterilize at 115° for 15 min.

This medium is based on that by KING, WARD and RANEY (1954). *J. Lab. clin. Med.*, **44**, 301.

14. The following method of Gram-staining is recommended:

BURKE AND KOPELOFF–BEERMAN MODIFICATIONS

SOLUTION 'A'

Methyl Violet 6 B . . 10 g
Distilled water . . .1000 ml
Dissolve; stand overnight; filter into stock bottle.

SOLUTION 'B'

$NaHCO_3$ . . . 25 g
Distilled water . . 500 ml

SOLUTION 'C'

Iodine . . . . 10 g
Potassium Iodide . . . 20 g
Distilled water . . .1000 ml

SOLUTION 'D'

Safranin . . . . 20 g
Distilled water . .1000 ml
Dissolve: stand overnight; filter into stock bottle.

SOLUTION 'E'

Ether . . . .1 vol
Acetone . . . .1 vol

Procedure

1. Dry thin films in air without heat.
2. Mix 15 ml 'A' and 4 ml 'B'. Flood the slide with the mixture and stand 5 min or more.
3. Rinse with 'C'. Flood slide with 'C' and stand for 2 min or more.
4. Rinse with tap water.
5. Place wet slide on Whatman No. 1 filter paper. Take a clean dry No.1 filter paper and *quickly* blot the slide twice, using a clean dry area of the filter paper each time. Free water must be got rid of but the cells must *not* be allowed to dry.
6. *Immediately* flood the slide with 'E', dropping the mixture on the slide until no more blue colour comes away (10–15 sec.)
7. Dry in the air.
8. Rinse in 'D'. Flood with 'D' and stand for 30 sec.
9. Wash in tap water.
10. Blot dry and examine.

REFERENCES

1. Society of American Bacteriologists, (1957). *Manual of Microbiological Methods*, McGraw-Hill Book Co., Inc., London. 15–18.
2. THOMAS, S. B. *et al* (1962). *J. appl. Bact.*, **25**, 107.

Dye Reduction Tests

29. METHYLENE BLUE REDUCTION TEST

1. *Reagent*

Methylene blue tablets manufactured under arrangements made by the Ministry of Agriculture, Fisheries and Food and the Ministry of Health shall be used for the test. A solution shall be prepared by adding one tablet, with aseptic precautions, to 200 ml of cold, sterile, glass-distilled water in a sterile flask graduated at 800 ml, and by shaking until the tablet is completely dissolved. Then make up the solution to 800 ml with cold, sterile glass-distilled water. The resultant solution shall be stored in a sterile stoppered flask in a cool, dark place, and shall not be used if:

(*a*) it has been exposed to sunlight;

(*b*) a period of 2 months has elapsed since the date of preparation; or

(*c*) it is suspected to have become contaminated.

The amount of methylene blue required for a day's work shall be poured off with aseptic precautions from the stock bottle into a sterile glass container. The pipette used for transferring the methylene blue solution to the tubes of milk shall not be introduced into the stock bottle.

2. *Apparatus*

Test-tubes shall conform to B.S.625: 1959, 150 × 16 mm, nominal 6 × $\frac{5}{8}$ in., having an internal diameter of 13·5±0·7 mm and being accurately marked at 10 ml. They shall be plugged with non-absorbent cotton wool, or covered with closely fitting aluminium caps, or stored in such other way as may prevent contamination.

Pipettes shall be 1·0 ml straight-sided blow-out delivery pipettes, and shall be plugged with cotton wool at the upper end.

Glassware and rubber stoppers shall be sterile before use.

3. *Method of Carrying out the Test*

The sample of milk shall be thoroughly mixed in the following manner:

(*a*) If the sample bottle is filled to the level of the stopper, it shall be inverted 25 times by a rapid rotary movement of the wrist in order to mix the cream uniformly with the milk. About one-quarter of the contents of the bottle should be poured away and the bottle shaken 25 times, each shake being an up-and-down movement with an excursion of about 1 ft, the whole shaking lasting about 12 sec. If the bottle is not completely filled it can be shaken directly in the way just described.

(*b*) A sample in a sealed bottle or carton should first of all be inverted 25 times by a rapid rotary movement of the wrist, the cap removed with sterile forceps and the whole contents poured rapidly into a sterile capped or stoppered bottle of larger capacity. This should then be shaken in the way described above.

The milk shall then be poured into a test-tube up to the 10 ml mark, leaving one side of the interior unwetted with milk. One ml of methylene blue solution shall be added without allowing the pipette to come into contact with the milk in the tube or with the wetted side of the interior of the tube. After a lapse of 3 sec, the solution remaining in the tip of the pipette shall be blown out. The tube shall be closed with a rubber stopper with aseptic precautions. The tube shall then be slowly inverted twice so that the whole column of contained air rises above the level of the milk, and placed within 5 min in a water bath. The test shall not be carried out in bright sunlight.

The water in the bath shall be kept above the level of the milk in the test-tubes, and its temperature, which shall be between 37° and 38°, shall be maintained as nearly uniform as possible by means of a reliable automatic thermo-regulator. The interior of the bath shall be kept completely dark.

To indicate when decolorization is commencing, and when it is complete, two control tubes shall be used for comparison with each batch of tubes containing the milk under test. One control tube shall be prepared by immersing in boiling water for not less than 3 min a properly stoppered test-tube containing 1 ml of tap-water and 10 ml of a mixture of milk having a fat content and colour similar to that of the milk being tested, and a second control tube shall be prepared by immersing in boiling water for not less than 3 min a properly stoppered test-tube containing 1 ml of methylene blue solution and 10 ml of a mixture of milk having a fat content and colour similar to that of the milk being tested.

The tubes containing the milk under test and the control tubes shall be inspected at half-hourly intervals. At these inspections:

(i) any tube in which the milk has become decolorized shall be removed from the water bath;

(ii) any tube in which decolorization has begun shall remain without inversion in the water bath until decolorization is complete; and

(iii) all other tubes in the water shall be inverted once and replaced.

The milk shall be regarded as decolorized when the whole column of milk is completely decolorized or is decolorized up to within 5 mm of the surface. A trace of colour at the bottom of the tube may be ignored provided that it does not extend upwards for more than 5 mm.

A record shall be kept of the time taken for complete decolorization of the sample. Incubation need not be extended beyond 6 hr.

The methylene blue test described above has many applications, and when interpreting the result due regard should be given to the object of the test and the age and treatment of the sample.

30. THE HALF-HOUR METHYLENE BLUE TEST

This test is used for the examination of untreated and heat-treated consumer milk and is also useful as a sorting test for raw milk supplies.

1. *Treatment of Sample*

(*a*) On arrival at the testing laboratory the sample of milk shall at once be removed from the insulated container. Thereafter it shall be stored as

follows:

(i) a sample taken at any time during the period from 1st May to 31st October, inclusive, in any year shall be kept at atmospheric shade temperature until 9.30 a.m. on the following day;

(ii) a sample taken at any time during the period from 1st November to 30th April, inclusive, in any year shall be kept in its original container or in a sterile 3 oz sample bottle at atmospheric shade temperature until 5.0 p.m. on the day of sampling and thereafter at a constant temperature at 65±2°F until 9.30 a.m. on the following day;

(iii) if the sample is to be broken down to a 3 oz sample bottle, the breaking down must be carried out on arrival at the laboratory and before the sample is placed in the atmospheric shade temperature cabinet.

(*b*) If during the period of storage at atmospheric shade temperature to which a sample is subjected, this temperatue at any time exceeds 70°F the test shall not be applied.

(*c*) The test shall be begun between 9.30 and 10.0 a.m. on the day after the sample is taken.

2. *Reagent—Methylene Blue*

(*a*) Tablets manufactured under arrangements made by the Minister shall be used for the test. A solution shall be prepared aseptically by adding one tablet to 200 ml of cold, sterile, glass-distilled water in a sterile flask, shaking until the tablet is completely dissolved, and making up the solution to 800 ml with cold, sterile, glass-distilled water. The resultant solution shall be stored in a stoppered sterile flask in a cool, dark place, and shall not be used if:

(i) it has been exposed to sunlight, or

(ii) a period of 2 months has elapsed since the date of preparation.

(*b*) The amount of methylene blue required for a day's work shall be poured off from the stock bottle into a suitable glass container. The pipette used for transferring the methylene blue solution to the tubes of milk shall not be introduced into the stock bottle.

3. *Apparatus*

Test-tubes shall conform to B.S.625: 1959, 150 × 16 mm, nominal 6 × $\frac{5}{8}$ in., having an internal diameter of 13·5±0·7 mm and being accurately marked at 10 ml. They shall be plugged with cotton wool or covered with closely fitting aluminium caps or stored in such a way as to prevent contamination.

Pipettes shall be 1·0 ml straight-sided blow-out delivery pipettes, and shall be plugged with cotton wool at the upper end.

Glassware and rubber stoppers shall be sterile immediately before use.

The water bath shall be fitted with a reliable automatic thermo-regulator capable of maintaining the water at a temperature of 37·5±0·5°.

4. *Method of Carrying Out the Test*

(*a*) The sample shall be mixed thoroughly by inverting and shaking, and the milk shall be transferred to a test-tube up to the 10 ml mark in such a manner

that one side of the interior of the test-tube is not wetted with milk. One ml of methylene blue solution shall be added without letting the pipette come into contact with the milk in the tube or with the wetted side of the interior of the tube. After a lapse of 3 sec, the solution remaining in the tip of the pipette shall be blown out. The test-tube shall be closed with a rubber stopper, aseptic precautions being taken, and shall then be inverted twice, slowly, so that the whole column of contained air rises above the level of the milk. Within a period of 5 min the test-tube shall be placed in a water bath. The water in the bath shall be kept above the level of the milk in the test-tube, and its temperature, which shall be between 37° and 38°, shall be maintained as nearly uniform as possible by means of a reliable automatic thermo-regulator. The interior of the bath shall be kept completely dark.

(*b*) A control tube shall be used for comparison with each batch of tubes under test to indicate when decolourization is complete. The control tube shall be prepared by immersing in boiling water for 3 min a stoppered test-tube containing 1 ml of tap water and 10 ml of mixed milk having a fat content and colour similar to that of the milk being tested.

(*c*) The milk shall be regarded as decolourized when the whole column of milk is completely decolourized or is decolourized up to within 5 mm of the surface. A trace of colour at the bottom of the tube may be ignored provided that it does not extend upwards for more than 5 mm.

5. *Interpretation*

The test shall be deemed to be satisfied by milk which fails to decolourize methylene blue in 30 min.

REFERENCE

S.I. (1963) No. 1571. *The Milk (Special Designation) Regulations.* London. H.M.S.O.

31. RESAZURIN TESTS FOR THE BACTERIOLOGICAL GRADING OF FARM MILK SUPPLIES

Several modifications of the resazurin reduction test at 37° have been used at creamery and advisory laboratories in England and Wales, for the examination of farm milk supplies since the ten-minute platform test and the routine temperature-compensated test were introduced by the Ministry of Agriculture and Fisheries in 1942.

Outlines of the methods of application of the various tests, and their attributes in relation to bacteriological control work and farm advisory work are given in this memorandum.

It has been claimed that the various resazurin tests can be used as indicators of one or more of the following:

1. suitability of farm milk supplies for bulking prior to processing or manufacture at creameries;
2. marketability of raw milk;
3. unhygienic methods of milk production and inefficient cooling on the farm;
4. keeping quality; and

5. incidence of high udder cell content due to sub-clinical mastitis or end of lactation.

The different applications of the resazurin test which have been used in England and Wales are described below.

It is recommended that whenever disc readings are taken a suitable North Daylight Cabinet should be used.

Ten-Minute Platform Rejection Test

1. *Application*

Since 1942 this test has been applied to samples taken from cans of milk which are set aside by the platform examiner at creameries because of appearance, smell, or which from previous records are suspected to be of doubtful bacteriological quality. The object is to detect the relatively small quantities of milk of really poor keeping quality, which, if added to a much larger bulk of average milk at a country creamery, would be likely to cause the loss of the bulked consignment through souring or tainting. This test continues to be used at all creameries.

2. *Technique*

Cans of producer's milk set aside by the platform examiner are sampled by the creamery laboratory staff and the test commenced within half an hour of arrival of the milk on the creamery platform. One ml of standard resazurin solution is added to 10 ml of the milk sample in a 6 × $\frac{5}{8}$ in. test-tube, closed with a rubber stopper. After inversion, the tube of milk is placed in a water bath at 37° and after 10 min incubation the stage of resazurin reduction (disc reading) is determined, using a comparator. Full details are given in Technique No. 32.

3. *Guide Standards*

Cans of milk giving disc readings of $3\frac{1}{2}$ or less are rejected and returned to the producer, whereas milk giving a disc reading of 4–6 is accepted, though milk with pronounced taint or defects (blood, etc.) likely to affect the bulk is rejected even if the results of this test are satisfactory.

4. *Interpretation*

It can be safely assumed, from the farm advisory point of view, that milk rejected on the ten-minute platform resazurin test has either been produced under unhygienic conditions, handled in heavily contaminated equipment, insufficiently cooled, or held overnight at high atmospheric temperatures.

High cell counts (more than 750,000/ml) in bulk milk rarely affect the reduction of resazurin to such an extent as to give a disc reading of $3\frac{1}{2}$ or less within 10 min.

Most milk samples giving disc readings of $\frac{1}{2}$ or 0 on this test have colony counts of more than 10^8/ml and are usually tainted or sour.

One-Hour Test

5. *Application*

This test has sometimes been employed for routine sorting at receiving depots to detect raw milk supplies of doubtful bacteriological quality, and

for this purpose a composite sample may be taken from each farm supply in the weigh tank or from cans; but with the introduction of the 2 hr 'hygiene' test it is probable that the test will not longer be used for this purpose.

It is used for the examination of bulk tanker milk before despatch from receiving depots or on arrival of road or rail tankers at processing or manufacturing creameries. (see Appendix IVD).

It may also be applied to samples taken at intervals from the milk in the balance tank below the weigh tank as a check to ensure that the platform inspection and the ten-minute rejection test are being properly applied.

6. *Technique*

Full details of the test are given in Technique No. 33, where it is pointed out that the selection of 1 hr incubation at 37° is purely arbitrary, and that the duration of incubation may be varied to suit particular circumstances, although disc readings are usually recorded in 1 hr in all cases.

7. *Guide Standards*

Milk samples giving disc readings of 4 and over are usually regarded as satisfactory.

8. *Interpretation*

The action taken by the creamery will depend on the purpose for which the test has been used.

Routine Temperature-Compensated Test

9. *Application*

This test was introduced by the Ministry of Agriculture and Fisheries in 1942, and was used at creamery and advisory laboratories for the fortnightly examination of all farm milk supplies in England and Wales during 1942–49.

Some creameries used the results of this test for bonus quality payments for milk giving disc readings of 4 and over.

10. *Technique*

Milk samples were pre-incubated at atmospheric shade temperature for different times depending on whether or not they were taken from cans of a.m., p.m., or mixed meals of milk. The test was set up at 9 a.m. on the day following production, and the tubes of milk and resazurin were incubated at 37° for different times ranging from 15 min during warm summer weather to 2 hr during winter according to the mean of the maximum and minimum shade temperature. Full details of sampling, pre-incubation and times of incubation at 37° are given in Technique No. 34.

11. *Guide Standards*

Farm milk supplies were graded into three categories on this test. Those giving disc readings of 4 and over were placed in Category A and were considered to have a satisfactory keeping quality; those giving disc readings of 3½ to 1 (both inclusive) were placed in category B and considered to be of

doubtful keeping quality; and those giving disc readings of ½ or 0 were placed in Category C and were considered to be of unsatisfactory keeping quality.

12. *Interpretation*
Persistent Category C results were taken to be an indication of poor hygienic milking methods, inefficient cleansing of equipment or inadequate cooling of milk on the farm, and advisory work was concentrated on such producers.

Hygiene Test

13. *Application*
Since 1st October, 1962, farm milk supplies have been examined at creamery laboratories in Emgland and Wales by means of a 2 hr resazurin test, and a hygenic quality scheme, based on this test operated by the Milk Marketing Board, came into full operation on 1st October, 1964.

14. *Technique*
A composite sample of each farm milk supply (i.e., of mixed evening's and morning's milk) is taken, *each month*, from the weigh bowl at the creamery and examined within 30 min of sampling, no tests being applied to milk arriving at the creamery after 3.00 p.m. Samples of farm bulk tank milks are tested *weekly*.

The tubes of milk are incubated at 37° and inverted every 30 min. They are examined for decolourization after 2 hr incubation.

15. *Guide Standards*
In the present Milk Quality payment scheme (October 1964) tubes of milk which decolourize to white or pink and white mottling are deemed to have failed the test.

16. *Interpretation*
From the farm advisory point of view, a high proportion of the milk supplies failing on this test during summer months will have been produced under unhygienic conditions and/or have been inadequately cooled. In winter, when atmospheric temperatures are at their lowest and bacterial proliferation in milk much less active, high numbers of udder cells may be partially responsible for failures of this test. This may be due to a high incidence of sub-clinical mastitis in a high proportion of the cows in the herd or to end of lactation milk. Nevertheless, failures in winter will in many cases be due to a combination of high cell counts, unhygienic milking methods, poorly cleaned dairy equipment and ineffective cooling.

The Advisory Test

17. *Application*
An extended resazurin test at 37°, in which disc readings are recorded every hour up to 3 or 4 hr has been introduced at advisory laboratories in England and Wales for the examination of milk samples taken during visits to dairy farms following failure on the 'hygiene' 2 hr resazurin test at creameries. This is not unlike the 'triple-hour' test which has been extensively used at creameries as well as in public health laboratories in North America.

18. *Technique*
A sample of a.m. or p.m. milk, taken at the farm from the first can to be filled, is brought to the laboratory, preferably within 4 hr of milking. The milk sample is divided on arrival at the laboratory into two portions, and one portion is either examined immediately (a.m. milk) or held overnight for 18 hr at 3–5° (p.m. milk), and the other portion is held for 18 hr at 18·5±1° before examination by the resazurin test at 37°. The tubes are inverted at intervals of 30 min and disc readings are recorded at 1, 2, 3 and 4 hr. See Appendix VA for further details.

19. *Guide Standards and Interpretation*
Bacterial reducing activity is indicated if, in the resazurin test applied before pre-incubation, disc readings of 2 or 3 are obtained within 1 hr followed by rapid reduction to ½ or 0. On the other hand, if disc readings of 2 or 3 are obtained in 2 hr and reduction subsequently proceeds at a slower rate, abnormal milk is indicated, and this may be confirmed by the results of the udder cell count or Whiteside test. The sub-sample pre-incubated at 18·5±1° overnight before examination may sometimes give higher disc readings than the initial test. This indicates the loss of leucocyte induced reduction due to ageing of the udder cells.

REFERENCES
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3. Thomas, S. B., Jones, T. I. and Griffiths, D. G. (1963). *Dairy Inds.*, **28**, 812.
4. Thomas, S. B. and Makinson, P. E. (1964). *Dairy Inds.*, **29**, 432.

32. THE TEN-MINUTE (REJECTION) RESAZURIN TEST

Buyers often wish to use a standard test for the appraisal of producers' milk at the time of arrival at the point of first delivery (dairy, creamery or milk depot) in order to determine whether the milk is acceptable. The most suitable test for this purpose at the present time is the ten minute Resazurin test.

Sampling

1. *Sampling Equipment*
Long-handled dippers should be used for sampling. The cup of the dipper should have a capacity of at least 3 oz and the handle be at least 15 in. long. At least three dippers should be provided for each sampler. Dippers must be sterilized before use, and this may be done on the platform as follows:

immediately before use, rinse each dipper in cold water; wherever possible running cold water should be used, otherwise the cold rinse water must be changed frequently. Then immerse the dipper to within 3 in. of the hook end of the handle in water at a temperature of at least 90° (194°F) for not less than 30 sec. Repeat this procedure after taking each sample. By always taking the dipper from the left and replacing on the right, sterilization between samples is ensured.

2. *Taking the Sample*

The sample should be taken into sterile bottles or direct into the sterile 6 × $\frac{5}{8}$ in. test-tubes used for the test. The bottle or test-tube should be provided with a sterilized closure. Using the long-handled dipper, the milk in the can should be vigorously stirred and plunged for at least 5 sec and the sample then taken from well below the surface of the milk. The sample should be poured into the sterile bottle or test-tube (up to the 10 ml mark) and stoppered immediately; the part of the stopper or cap which may come into contact with the milk must not be allowed to come into contact with any unsterile object or surface.

Notes

1. It is advisable that two persons should be present when a number of samples is to be taken rapidly, one to take the sample and the other to handle the bottle and check the identity of the sample.
2. Caps may be used for sample bottles if stoppers are not available.

3. *Identification of Samples*

The bottle or tube should be numbered or labelled with the particulars necessary to enable the sample to be identified in the laboratory.

Testing

4. *Apparatus*

Sampling dippers with a capacity of at least 3 oz and handles at least 15 in. long:
Sample bottles B.S.809: 1963
Test-tubes shall conform to B.S.625: 1959, 150 × 16 mm, nominal 6 × $\frac{5}{8}$ in., having an internal diameter of 13·5±0·7 mm and being accurately marked at 10 ml.
1·0 ml straight sided blow out pipettes
A water bath capable of being maintained at a temperature of 37·5±0·5°
Standard resazurin tablets
Resazurin disc and comparator stand

5. *Preparation of the Resazurin Solution*

Glass distilled water or water of similar purity should be used. The water must be sterilized by one of the following methods and must be protected from subsequent contamination:

(*a*) autoclave at 121° for 15 min (preferable); or
(*b*) steam sterilize for one hour at 100°; or
(*c*) boil for 30 min.

If either methods (*b*) or (*c*) are adopted, the water must be used within 24 hr of sterilizing.

Add one standard resazurin tablet to 50 ml cold sterile water in a clean sterile bottle by allowing the tablet to fall from the container or by transferring with sterile forceps. The resazurin solutions must not be used if more than 4 hr old, and when not actually in use must be kept in a cool dark place.

6. *Mixing and Transferring the Samples*
Where samples have been taken into sample bottles, they must be shaken 25 times, each shake being an up and down movement with an excursion of about 1 ft and the milk then poured into sterile test-tubes (see para. 4, third item). In doing this, the stopper or cap of the bottle should be removed with aseptic precautions and the pouring lip of the bottle flamed. The tube stopper is then removed, the mouth of the test-tube flamed, the milk poured into the sterile test-tubes up to the 10 ml mark and the stopper replaced.

7. *Method of Testing*
The test must be started within ½ hr of arrival of the milk on the creamery platform. When ready to test, measure 1 ml of resazurin solution with a sterile pipette; remove the stopper from the first tube in the rack, taking care not to touch the mouth of the tube. Insert the pipette about ½ in. into the mouth of the tube and expel the solution by blowing, taking care that the tip of the pipette does not touch the test-tube. Replace the stopper; mix by inverting the tube twice in four sec and return to the rack. When resazurin has been added to a batch of not more than five tubes, place immediately in the water bath, and note the time.

Notes
Aseptic precautions must be observed in these operations.
Any pipette becoming contaminated must be discarded.
If the bench resazurin solution becomes contaminated with milk, it must be discarded.
Use a fresh sterile pipette for every batch of samples.
See also para. 10 (General Precautions).

8. *Incubation of Samples*
The temperature of the water bath must be maintained at $37{\cdot}5 \pm 0{\cdot}5°$. At the end of 10 min ± 30 sec, remove the batch of tubes from the water bath and examine.

9. *Examination of Tubes*
Any tube showing complete reduction, i.e., white, is recorded as **0**. Any tube showing extremely pale pink, pink and white mottling or a deeper pink band at the top above a paler pink below, is recorded as ½. Other tubes are inverted twice and immediately matched in the comparator as follows:

place a 'blank' tube of mixed milk without dye in the left section of the comparator and the incubated tube in the right section. The comparator must face a good source of daylight, if possible a north window or an artificial daylight source of illumination. Direct sunlight must not be allowed to fall on the comparator or tubes during matching. The comparator and stand are placed on a bench at such a height that the operator is able to look down on the two apertures. The disc is then revolved and the operator determines between which two discs the colour lies, and records the appropriate ½ disc value, e.g., if between 2 and 3 record **2½** and if between 3 and 4, record **3½** etc. A whole disc must only be recorded when the colour is considered an exact match. If, in the opinion of the operator, the colour is above or below the disc by no matter how little, the appropriate ½ disc reading must be recorded. Readings must be recorded immediately, tube by tube.

10. *General Precautions*

(i) All testers must be examined for ability to match the colours used in the comparator.

(ii) An initial reading immediately after adding resazurin to good quality low count milk should give a disc number of not less than 6, otherwise the resazurin solution is faulty and must be discarded.

(iii) Test-tubes used in the comparator should be of the same colour and thickness of glass.

(iv) Appropriate 'blank' samples must be used for highly pigmented milk, e.g., Guernsey milk.

(v) During the sampling and testing, the milk, the resazurin solution or milk to which resazurin has been added must not be exposed to direct sunlight, particularly where the test is carried out on the creamery platform.

(vi) The level of water in the bath must be maintained above the level of the milk in the tubes.

(vii) The water bath must be kept closed during the test.

(viii) The temperature of the water bath must be checked before commencing each batch of tests.

(ix) Baths must be cleaned out, the racks scrubbed and fresh water added at least once a week.

Use and Interpretation of the Test

11. The test can be applied to all milk supplies immediately on arrival at the point of first delivery and should be applied to any milk which has an abnormal smell or taste or is suspected from previous records of the supplier to be unsatisfactory. The results should be interpreted as follows:

Ten-Minute Resazurin Disc Reading:

4–6	Accepted
$3\frac{1}{2}$ or less	Rejected

Even though the results of the test are satisfactory, milk with pronounced taints likely to affect the bulk should be rejected.

33. THE ONE-HOUR RESAZURIN TEST

The one-hour resazurin test may be useful when employed as a routine sorting test at receiving depots to detect raw milk supplies of doubtful bacteriological quality. The technique set out below is recommended for this purpose. The selection of 1 hr is purely arbitrary, and the duration of incubation may be varied to suit particular circumstances. If incubation is carried out for 2 or 3 hr the tubes should be inverted at hourly intervals.

Sampling

1. *Sampling Equipment*

Long-handled dippers should be used for sampling. The cup of the dipper should have a capacity of at least 3 oz and the handle should be at least 15 in. long. At least three should be provided for each sampler. The dippers should be sterilized before use, and this may be done on the platform as

follows:

immediately before use rinse each dipper in cold water; wherever possible running cold water should be used, otherwise the cold rinse water must be changed frequently. Then immerse the dipper to within 3 in. of the hook end of the handle in water at a temperature of at least 90° (194°F) for not less than 30 sec. Repeat this procedure after taking each sample. By always taking a dipper from the left and replacing on the right, sterilization between sampling is ensured.

Sample bottles should comply with B.S.809: 1963 and be of 3 oz capacity. A large size, e.g., 8 oz will be necessary if the same sample is to be used for chemical tests. Sample bottles and bungs should be sterile before use.

2. *Taking a Sample*

Normally a representative sample of the whole consignment from a producer is required and this is conveniently obtained from the weigh-bowl. When a composite sample is taken from all the cans in the consignment, the dipper need not be sterilized between the taking of aliquot portions. The aliquots should be approximately in proportion to the volume of milk in the cans sampled. The milk should be vigorously stirred and plunged for at least 5 sec with the long-handled dipper and a sample taken from well below the surface of the milk. The sample should be poured into the sterile bottle which is stoppered immediately; that part of the stopper which may come into contact with the milk must not be allowed to come into contact with any unsterile object or surface.

When a number of samples is being taken, two persons should be present; one to take the sample and the other to handle the bottle and check the identity of the sample.

3. *Identification of Sample*

The bottle containing the sample should be numbered or labelled with the particulars necessary to enable it to be identified in the laboratory.

4. *Storage of Samples*

The samples should be kept at atmospheric shade temperature until a convenient time of testing, which might be, say, 2 p.m.

Testing

5. *Apparatus*

Test-tubes conforming to B.S. 625: 1959, 150 × 16 mm, nominal 6 × $\frac{5}{8}$ in., having an internal diameter of 13·5±0·7 mm and being accurately marked at 10 ml

1·0 ml straight sided blow out pipettes

A water bath capable of being maintained at a temperature of 37·5±0·5°

Standard resazurin tablets, as used for milk testing in Britain

Resazurin disc and comparator stand

Note

All glassware should be clean and sterile before use.

6. *Preparation of Resazurin Solution*

Glass distilled water or water of similar purity should be used. The water should be sterilized by one of the following methods and must be protected from subsequent contamination:

(i) autoclave at 121° for 15 min (preferable);
(ii) steam sterilize for 1 hr at 100°; or
(iii) boil for 30 min.

If either methods (ii) and (iii) are adopted, the water must be used within 24 hr of sterilizing.

Add one standard resazurin tablet to 50 ml cold-sterile-glass-distilled water in a clean-sterile-stoppered bottle by allowing the tablet to fall from the container or by withdrawal with sterile forceps. The resazurin solution must not be used if more than 4 hr old and, when not is use, must be kept in a cool dark place.

7. *Mixing the Sample*

The bottle should be shaken 25 times, each shake being an up and down movement with an excursion of about 1 ft, the whole shaking lasting about 12 sec.

8. *Transferring the Samples to Test-tubes*

After mixing, the milk is poured into a test-tube conforming to B.S. 625:1959, 150 × 16 mm, nominal 6 × $\frac{5}{8}$ in., having an internal diameter of $13{\cdot}5 \pm 0{\cdot}7$ mm and being accurately marked at 10 ml. In doing this the stopper or cap of the bottle must be removed with aseptic precautions, the pouring lip of the bottle flamed, the test-tube stopper removed, the mouth of the test-tube flamed and the milk poured into the sterile test-tube up to the 10 ml mark. If the transfer to test-tube has been made more than 3 hr before testing, the test-tubes containing the milk must be shaken immediately before testing as described above.

9. *Method of Testing*

Place the tubes in numerical order, from left to right in a rack. Measure 1 ml of the resazurin solution with a sterile pipette. Remove the stopper from the first test-tube in the rack, taking care not to touch the mouth of the tube. Insert the pipette about $\frac{1}{2}$ in. into the mouth of the tube and expel the solution by blowing, taking care that the tip of the pipette does not touch the test-tube. Replace the stopper, mix by inverting the tube twice in 4 sec and return to the rack. When resazurin has been added to a batch of not more than 10 tubes, place immediately in the water bath and note the time.

Notes

1. Full aseptic precautions must be observed in these operations.
2. Any pipette becoming contaminated with milk or otherwise must be discarded immediately.
3. If the bench resazurin solution becomes contaminated with milk it must be discarded immediately.
4. Use a fresh sterile pipette for every batch of 10 samples.
5. See also para. 12 (General Precautions).

10. *Incubation of Samples*
The temperature of the water bath must be maintained at $37 \cdot 5 \pm 0 \cdot 5°$. Tubes must be held in the bath for a period of 1 hr.

11. *Examination of Tubes*
At the end of the 1 hr incubation time remove and examine each tube. Any tube showing complete reduction, i.e., white, is recorded as **0**. Any tube showing an extremely pale pink, pink and white mottling or a deeper pink band at the top, above a paler pink below, is recorded as $\frac{1}{2}$. Other tubes are inverted and immediately matched in the comparator as follows:

place a 'blank' tube of mixed milk without dye in the left section of the comparator and the incubated tube in the right section. The comparator must face a good source of daylight, if possible a north window or an artificial daylight source of illumination. Direct sunlight must not be allowed to fall on the comparator or tubes during matching. The comparator and stand are placed on a bench at such a height that the operator is able to look down on the two apertures. The disc is then revolved until the sample is matched and the disc reading noted. When the colour falls between two disc numbers, it must be recorded as the $\frac{1}{2}$ value, e.g., a reading between 3 and 4 is recorded as $3\frac{1}{2}$. Readings must be recorded immediately, tube by tube.

12. *General Precautions*
The general precautions to be observed during testing and the method of preparation of the resazurin solution shall be as described in Technique No. 32.

13. *Interpretation of Results*
Samples giving a disc reading of 4 and over may be regarded as satisfactory.

14. This test is useful for checking the quality of bulked raw milk at creameries.

34. THE TEMPERATURE-COMPENSATED RESAZURIN TEST

In the routine resazurin test (see Technique No. 33) incubation of the samples is carried out for 1 hr at 37° irrespective of the time of the year and varying atmospheric temperatures. Since this may lead to misleading deductions being drawn from a producer's record over the year, because dye reduction results of this type are mainly a reflection of atmospheric temperature, it is recommended that the time of incubation should vary accordingly to the mean atmospheric temperature during the period of storage prior to incubation. Results based on such a procedure would give a better indication of the level of milk quality throughout the year by eliminating as far as practicable the effect of atmospheric temperature. This technique was used in the National Milk Testing and Advisory Scheme in England and Wales during 1942–46.

Treatment of samples

1. *Sampling*

Sampling should be carried out as described in Technique No. 33.

The following table gives the possible times of testing and the two temperature readings to be used for various classes of samples:

Meal	Time of testing	Temperature readings
Evening milk (E)	(a) 4 p.m. day following date of production. or (b) refrigerated at 4 p.m. on day following production until 9 a.m. next day.	9 a.m. Min. and 4 p.m. Max. on day *following* date of of production. Ditto
Morning milk (M)	9 a.m. day following date of production.	4 p.m. maximum on day of production and 9 a.m. minimum on day following date of production.
Mixed milk in which the evening milk is the older (E.M.X.)	(a) 4 p.m. day following date when *evening* milk was produced. or (b) refrigerated at 4 p.m. until 9 a.m. following day.	9 a.m. minimum and 4 p.m. maximum on day following date when evening milk was produced. Ditto
Mixed milk in which morning milk is the older (M.E.X.)	9 a.m. day following day of production.	4 p.m. maximum on day of production and 9 a.m. minimum on day following production.

Mixing the sample, transferring to test-tubes and the method of testing are all as described in Technique No. 32.

2. *Storage*

The milk may be transferred to sterile 6 × $\frac{5}{8}$ in. test-tubes at any time prior to testing, provided that the prescribed temperature conditions given below are observed.

(*a*) P.M. and Mixed (E.M.X.) Milk

Samples will be kept at atmospheric shade temperature from the time of sampling until 4 p.m. on the day following production of the p.m. milk. Any samples which are not tested at 4 p.m. must be cooled by placing in iced water in a cold store or refrigerator at a temperature of 32–40°F. until 9 a.m. the following morning and then tested immediately. Testing of p.m. and mixed (E.M.X.) milk should be carried out at 4 p.m. whenever possible.

(*b*) A.M. and Mixed (M.E.X.) Milk

Samples will be held at atmospheric shade temperature until 9 a.m. on the day following production and then tested.

(*c*) Atmospheric Shade Temperature

This is defined as the temperature in an approved atmospheric shade cabinet (see Technique No. 75). An approved maximum thermometer and an approved minimum thermometer (see Technique No. 76) must be kept in this cabinet. The minimum temperature read at 9 a.m. and the maximum temperature read at 4 p.m. are recorded daily in a log book. The styles of both thermometers must be reset at 9 a.m. and 4 p.m. For a.m. samples tested at 9 a.m. on the day following production take the arithmetic mean of the maximum temperature read at 4 p.m. on the previous day (i.e., the day of production) and the minimum temperature read at 9 a.m. on the day of testing. For p.m. samples tested at 4 p.m. on the day after production or at 9 a.m. on the following day take the arithmetic mean of the minimum temperature recorded at 9 a.m. and the maximum temperature recorded at 4 p.m., both on the day after production.

3. *Incubation of Samples*

The temperature of the water bath must be maintained at 37·5±0·5°. Tubes must be held in the bath for the time prescribed below plus or minus 1 min. Tubes incubated for 90 min and 120 min must be inverted at 1 hr.

Mean of maximum and minimum shade temperatures	Period of incubation at 37·5±0·5°
40°F and under	120 min
Over 40°F and up to and including 50°F	90 min
Over 50°F and up to and including 55°F	60 min
Over 55°F and up to and including 60°F	30 min
Over 60°F	15 min

4. *Examination of Tubes*

At the end of the incubation time remove and examine each tube as described in Technique No. 33.

5. *Interpretation of Results*

The results may be used to grade milk supplies as follows:

Disc reading after prescribed period of incubation	Grade
4 and over	Satisfactory
3½ to 1 (both inclusive)	Doubtful
½ or 0	Unsatisfactory

6. *General Precautions*

The general precautions to be observed during testing and the method of preparation of the resazurin solution shall be as described in Technique No. 32.

Keeping Quality Tests

35. KEEPING QUALITY OF MILK AT 22° (C.O.B. TEST) FOR INVESTIGATIONAL PURPOSES

KEEPING quality results are more reliable when the clot-on-boiling (C.O.B.) test is made on the same sample every 3 hr. Where that is not possible, this Technique, No. 35, is the one to use.

1. *Collection of Samples*

Milk samples of not less than 6 oz should be taken within 1 hr of production. They should be iced and transported as quickly as possible to the laboratory. If necessary, samples may be kept for a maximum of 3 hr in the ice box (see Note 1).

(*a*) *Evening Milk*

Keep the sample on ice until 5.30 p.m. Shake and divide into two 3 oz bottles marked A and B.

Transfer A immediately to a water bath at 22°. Place B in the refrigerator (in water+ice) overnight and transfer to the 22° water bath at 8.30 a.m. next day.

(*b*) *Morning Milk*

Keep the sample on ice until 8.30 a.m. Shake and divide into two bottles A and B. Transfer A immediately to the water bath at 22°. Place B in refrigerator (in ice+water) until 5.30 p.m.

2. *Determination of End Point*

Examine samples at the times indicated in the table in the following manner:

(i) mix the sample thoroughly by inverting and shaking the sample bottle;

(ii) pour 5 ml into a clean test-tube and place in boiling water for 5 min; then record the results.

Look for precipitation or curdling. Note granular appearance of milk or flecks on the side of the tube or complete clot. The following are suggested for the laboratory record sheet:

No change	=	−
Slight granulation	=	+ end point
Clot	=	++ end point

Slight granulation (+) should be regarded as the end point, but only if it is succeeded by a clot (++);

(iii) at 5.30 p.m. apply the alcohol test if the C.O.B. test is negative.

3. *Alcohol Test* (*A.P.T.*)

To 1 ml of milk add 1 ml of bromo-cresol purple alcohol. Mix and examine for precipitation of protein and any colour change.

Precipitate:	None	=	−
	Slight	=	+
	Definite	=	++

B.C.P. reaction: The change in colour is not recorded but is a useful guide to denote slight change. A++alcohol test on milk which does not clot on boiling may be taken as C.O.B. three hours later where no other readings are available. (For preparation of B.C.P. alcohol see Note 2).

The keeping quality can be determined from the appropriate table. Samples can be discarded at 48 hr.

It should be appreciated that, where milk is of poor keeping quality, the result from the B sample may not compare with that from the A sample. In such cases, while the B results should be noted, the A results should be used wherever possible.

EVENING MILK

C.O.B. at	'A' Sample K.Q. in hr	'B' Sample K.Q. in hr
8.30 a.m.	15	0*
11.30 a.m.	18	3*
2.30 p.m.	21	6*
5.30 p.m.	24 (A.P.T.27)	9*
8.30 a.m.	39	24
11.30 a.m.	42	27
2.30 p.m.	45	30
5.30 p.m.	48	33 (A.P.T.36)
8.30 a.m.		48

*Tests at these times are seldom necessary

MORNING MILK

C.O.B. at	'A' Sample K.Q. in hr	'B' Sample K.Q. in hr
8.30 a.m.	0	
11.30 a.m.	3*	
2.30 p.m.	6*	
5.30 p.m.	9*	0
8.30 a.m.	24	15
11.30 a.m.	27	18*
2.30 p.m.	30	21*
5.30 p.m.	33 (A.P.T.36)	24*
8.30 a.m.	48	39
11.30 a.m.		42
2.30 p.m.		45
5.30 p.m.		48

*Tests at these times are seldom necessary

4. *Suggested Groups of Keeping Quality at 22°*

27 hr and under	=	Unsatisfactory
30–33 hr	=	Fair
36–42 hr	=	Good
45 hr and over	=	V. good

Notes

1. It is desirable that incubation of samples at 22° should start at the times given. If, however, evening samples arrive at the laboratory after 5.30 p.m. or morning samples after 8.30 a.m., appropriate adjustments must be made to the incubation times shown in the tables, e.g., where an evening milk sample is put into the 22° water bath at 6.30 p.m. instead of 5.30 p.m., 1 hr should be deducted from the K.Q. times given for the A sample: Adjustment will not be necessary for the B sample.
2. *Preparation of B.C.P. alcohol.* Dissolve 0.05 g B.C.P. powder in 500 ml 95 per cent alcohol (industrial methylated spirits). Add N/10 NaOH drop by drop until colour changes to greyish purple. Adjust the S.G. to 0.895 (68 per cent alcohol) with distilled water.

36. KEEPING QUALITY TEST FOR ADVISORY MILK SAMPLES

This techinque for determining the keeping quality of raw milk provides temperature compensation for the sample during the period 9 a.m. to 4 p.m., and assesses whether or not the milk would have clotted on boiling had it been kept at 22° for 24, 33 and 48 hr from the assumed time (9 a.m.) of collection (see the Interpretation Table).

1. *Sampling*

(*a*) When the milk is in containers not exceeding 1 qt in capacity the sample shall consist of one such container which shall be delivered intact to the testing laboratory.

(*b*) When the milk is in containers exceeding 1 qt in capacity the sample shall consist of approximately 6 fl. oz of milk. This quantity should not be greatly exceeded as this is the most convenient quantity for the C.O.B. test. The metal dipper of at least 3 oz capacity with a handle approximately 15 in. long will normally be used for sampling. After the milk in the can has been vigorously stirred and plunged for at least 5 sec with the dipper, the dipper should be emptied and the sample taken from well below the surface of the milk.

(*c*) The dipper used for stirring and sampling shall be sterile and the sample shall be poured into a sterile bottle which shall be immediately stoppered. The part of the stopper which may come into contact with the milk shall be sterile. A choice of stoppers is available but the longer type makes aseptic handling easier. Additional protection to the lip of the sterile bottle may be given by covering the stopper and lip with sterilized foil or grease-proof paper, or by the use of overlapping stoppers of the Subaseal type.

2. *Treatment of Samples*

A.M. and mixed (E.M.X.) milk: store the sample in S.A. box until 4 p.m.

P.M. milk:
- (*a*) *sampled on day of production*: store the sample at 3–5° overnight and at 9 a.m. transfer to A.S. box until 4 p.m.
- (*b*) *sampled following morning*: store the sample in A.S. box until 4 p.m.

3. *Treatment of Sub-Samples*
Immediately prior to 4 p.m. divide the sample into A and B sub-samples.
A sub-sample: At 4 p.m. place at 22° for the next 2 days.
B sub-sample: At 4 p.m. place in ice-water and store at 3–5° until 9.30 a.m. next day when it should be transferred to 22° until tested.

4. *Times of Applying Tests*
At the following times indicated, approximately 5 ml portions of the A and B sub-samples should be boiled:

Mean A.S.T. (°F)	67.6 and over	64.6–67.5	60.6–64.5	55.6–60.5	50.6–55.5	43.6–50.5	under 43.6
24 *hr K.Q.* Boil A at	9.30	10.30	11.30	12.30	1.30	2.30	3.30
Following Day 33 hr K.Q. Boil B at	9.30	10.30	11.30	12.30	1.30	2.30	3.30
48 *hr K.Q.* Boil A at	9.30	10.30	11.30	12.30	1.30	2.30	3.30

If additional information is required, an A.P.T. test can be applied. A positive test will indicate that the sample will clot on boiling within 3 hr (for example, above—36 hr).

5. Interpretation

	Sample clots on boiling at			
	24 hr	33 hr	48 hr	over 48 hr
Estimated K.Q. at 22° from 9 a.m.	Under 24 hr	Over 24 and under 33 hr	Over 33 and under 48 hr	Over 48 hr
Sample examined: A.M.	Unsatisfactory. Major improvements in production methods necessary.	Could be improved. Margin small. Considerable improvement needed in production methods.	Satisfactory.	Very good. Margin ample.
E.M.X. (can sampled at farm in morning)	Unsatisfactory. Considerable improvement needed in production methods.	Could be improved. Margin is insufficient. Milk liable to fail future tests.	Satisfactory. Margin ample.	Very good.
P.M. (can sampled at farm next morning)	Unsatisfactory. Improvement needed in production methods.	Reasonable margin but could be improved.	Satisfactory. Margin ample.	Very good.
P.M. (can sampled on day of production) and P.M. or A.M. or E.M.X. from refrigerated bulk tank.	Interpretation as for A.M. milk above.			

Taints and Defects

37. LABORATORY INVESTIGATION OF TAINTS IN MILK

IT is essential at the outset to determine whether the taint is bacterial in origin. Bacterial taints are normally absent from milk when first produced, but develop on storage. They may be reproduced on subculture of the tainted milk into fresh milk. On the other hand, food and weed taints may be detected immediately after milking. Some taints, including lipase and oxidation taints, often increase in intensity on storage at low temperatures and may be less marked in milk that has been kept warm.

The results of the laboratory should be correlated with conditions at the farm, and a comprehensive Field Report should accompany samples. In addition to tests for taint, milk samples, and rinses and swabs of dairy equipment should be examined, as in ordinary advisory cases, by the colony count on Yeastrel milk agar (Y.M.A.) incubated at 30°, and the coli-aerogenes test at 30°. Lower incubation temperatures may be required depending upon the circumstances under which the taint has developed.

1. *Examination of Milk*

About 1 ml of the tainted milk should be transferred to 50 ml amounts of fresh, cooled, laboratory-pasteurized milk in 3 oz bottles with suitable covers, such as beakers or kali tops (rubber or composition caps should not be used). Bottles should be held at room temperature unless circumstances suggest otherwise. An uninoculated control should be held at each temperature. The milk should be examined for the presence of the taint at intervals of about half a day.

Milk samples which are not tainted on receipt should be tested for taint development by holding portions at suitable temperatures, e.g., 3–5° and 22°, and examined for taint at half-day intervals (e.g., 9.0 a.m. and 5.0 p.m.) If a taint develops, subculture and proceed as above.

2. *Detection of Taints*

Some taints can be detected by smell alone. As they are often due to volatile substances, they are sometimes more readily detected when the milk is heated. A quantity (10–20 ml) should be poured into a small clean beaker, heated to about 60° and any odour noted. If the taint cannot be detected by smell it may be detected by tasting, but this should be resorted to only when absolutely necessary and the milk should not be swallowed.

3. *Detecting Sources of Infection*

Quantities of 5–10 ml of *rinse*, *swab solution* or *water* may be added to 50 ml of laboratory pasteurized milk and stored and tested as above. Uninoculated controls should be set up.

4. *Isolation of Causative Organisms*

Poured or streak plates should be prepared from a sample of milk in which the taint is well developed, and incubated at 22° and 30°. When plates are examined, it should be remembered that the taint-producing organism is not always dominant. It sometimes happens that two organisms act together to produce a taint. Pure cultures should be obtained and tested for their taint-producing ability by inoculation into fresh laboratory pasteurized milk.

Further test may be made to establish the identity of the organism.

38. LABORATORY INVESTIGATION OF ROPY MILK

Ropiness is a fault occurring in raw milk and is occasionally found in pasteurized milk. It usually develops in 12 to 24 hr, and milk which appears normal at the time of delivery to the consumer may become ropy before it is used.

The results of the laboratory examination should always be correlated with conditions at the farm or creamery, and a comprehensive field report should therefore accompany samples submitted for examination. In addition to tests for ropiness, samples of milk and rinses or swabs of dairy equipment should be examined for colony count and coli-aerogenes organisms as a general check on production methods.

1. *Testing Milk for Ropiness*

(*a*) *Sampling*

A 3 oz sample of the milk, taken from the milk can by means of a sterile long-handled dipper, is adequate for most cases in which laboratory investigation is called for.

(*b*) *Incubation*

Ropiness normally develops most rapidly in milk at temperatures of 16–22°, and it can be best detected in covered Petri dishes containing 10 to 15 ml of milk which is examined at 12, 24 and 48 hr from the time of milking or pasteurization. The Petri dishes should either be incubated at 22° or held at room temperature and protected from light. A lower temperature of incubation may be necessary if indicated by the circumstances of the outbreak.

(*c*) *Testing*

A sterile wire loop dipped under the surface of the milk and lifted will reveal viscous threads of $\frac{1}{2}$ in. long or more if ropiness is present. The approximate length of the thread should be recorded. In some milks the natural viscosity of the cream may cause it to adhere on the loop as thickish columns of $\frac{1}{8}$ in. which will break before it can be drawn into thin threads.

2. *Detecting Sources of Infection*

If farm or creamery *water supplies* are to be tested, 5 ml of the water may be added to 15–20 ml of sterile skim or laboratory pasteurized milk and incubated in a Petri dish at 22° or at room temperature, and tested for ropiness at 24, 48 and 72 hr.

The detection of ropy organisms on farm dairy equipment, milk plant, milk cans and milk bottles should be done by inoculating 5 ml of *rinse or swab* solutions into 20 ml of sterile skim milk held in a Petri dish or of 10 ml of swab or rinse solution into 50 ml sterile milk, and incubating and testing as previously described. The time between sampling and inoculation should not exceed 6 hr.

If *hay, straw or cattle feed* is suspected, samples should be brought to the laboratory and either chopped or broken up into small portions. About 5 g are then placed in 50 ml sterile milk, shaken well and allowed to stand for some time (15 min) and shaken again. Ten-ml portions of the 'infusion' are then poured aseptically into a number of Petri dishes and tested for the production of ropiness, as previously described.

3. *Isolation of Ropy Organisms*

Where the caustive organisms are to be isolated from infected material and identified, plating on Yeastrel milk agar (Y.M.A.) or Yeast dextrose agar (Y.D.A.) and incubation at 22° for 2 to 4 days has been found to be suitable Viscous colonies should be picked with a sterile loop and transferred to litmus milk, incubated at 22°, and tested for ropiness in 12, 24 and 48 hr.

As soon as the litmus milk shows ropiness, the cultures should be purified by inoculating a loopful into peptone water incubated for 6 to 18 hr at 22°, and high dilutions (10^{-4} to 10^{-8}) plated on Y.M.A. or Y.D.A. incubated for 2 to 4 days at 22°. A well isolated slimy colony should again be picked from the agar plate and inoculated into peptone water. Capsulated, ropy organisms are generally difficult to purify and this purification process should be repeated *at least three times*.

4. *Identification of Caustive Organisms*

The purified culture should be examined by the following tests:

(*a*) Gram staining reaction and morphology;
(*b*) formation of acid and gas in MacConkey's broth within 5 days at 30°;
(*c*) reaction in litmus milk at 22°;
(*d*) ability to produce ropiness in sterile or laboratory pasteurized skim milk incubated at 22°;
(*e*) heat resistance; ability to survive laboratory pasteurization in milk.

The Gram-negative organisms most commonly found to cause ropy milk are coli-aerogenes bacteria (usually *Klebsiella aerogenes I*) and *Alcaligenes viscolactis*. The former produce acid and gas in MacConkey's broth, and develop acidity in litmus milk. The latter is distinguished by its failure to form acid and gas in MacConkey's broth and by the characteristic development of an alkaline reaction in litmus milk.

Less frequently, micrococci are found to be the caustive organisms. They occasionally survive laboratory pasteurization.

On a few occasions ropy strains of aerobic spore-forming rods (e.g., *Bacillus cereus* and *B. subtilis*) have been found to cause ropiness in commercially pasteurized milk. Their spores survive heating in milk for 10 min at 80°.

REFERENCE

THOMAS, S. B. (1960). *Dairy Inds.*, **25**, 202.

39. THE WHITESIDE TEST

The test is an indirect measure of the cell (leucocyte) content of milk, and it is useful for detecting samples that are abnormal in this respect. It should not be carried out on milk which is more than 24 hr old.

1. *Apparatus and Materials*
   N/1 NaOH solution
   Glass plate or watch glasses
   Glass stirring rods
   1 ml pipettes

2. *Procedure*
With a 1 ml pipette deliver five drops of the well-mixed sample on to a glass plate or a clean watch glass resting on a black background. Using a similar pipette place two drops of N/1 NaOH on to the glass plate or watch glass in close proximity to the milk. Using a glass rod, mix the milk and NaOH solution by *stirring briskly* for 15 sec and read the result immediately.

If the milk is normal, no change occurs in the consistency or appearance of the mixture and the result is recorded as negative (−). When the mixture remains unchanged in consistency but a few white flecks are seen, the result is recorded as doubtful (±).

Positive results are recorded according to the intensity of the change in the mixture, on the following basis:

(+) thickening of the milk and the appearance of numerous, but discrete, small, white particles;

(++) a slightly more gelatinous appearance with more numerous and larger, but mainly discrete, white particles;

(+++) a further increase in the viscosity with large white particles tending to form clumps;

(++++) the mixture becomes very thick with the formation of a large white viscous mass surrounded by a clear fluid.

40. TOTAL CELL COUNT TEST OF MILK

In normal milk the cell content consists largely of epithelial cells with only small numbers of leucocytes and lymphocytes, but in mastitis milk the leucocyte content is increased and becomes markedly predominant. Although the number of cells in normal milk varies to some extent, the total count in fore-milk rarely exceeds 300,000–400,000/ml, and in udder samples it is normally less than 100,000–150,000/ml. Milk from young cows and from cows in mid-lactation shows a lower count than milk from old cows and at the beginning and end of lactation. With the establishment of inflammatory processes in the udder the cell content of milk rises sharply, the counts often exceeding 500,000/ml in fore-milk and 250,000/ml in udder samples.

The technique outlined below is designed to measure the approximate total cell count. This is sufficient for advisory purposes where it is only

intended to differentiate between normal, doubtful and abnormal milk. If, for special purposes, separate estimates of the different types of cells are required a differential staining technique must be applied.

1. *Apparatus*

(*a*) *Microscope*

An instrument with a $\frac{1}{12}$ in. oil-immersion objective, and a 6 × or 10 × ocular fitted with a stop from which a central square has been cut is required. A suitable stop can be cut from sheet photographic film. Cut the aperture in the stop so that the square field of vision when measured with a stage micrometer has a side of 0·10–0·11 mm. The microscopic factor to convert count/field or count/strip to count/ml milk can then be calculated. For example, with a field of vision of 0·012 sq. mm (side = 0·11 mm) the respective factors in round numbers to give the approximate total cell count /ml milk are 810,000 and 9,000. Where large numbers of tests are carried out, a binocular microscope is an advantage.

(*b*) *Microscope Slides*

Good quality, 3 × 1 in. slides with lightly etched areas of 1 cm^2 should be used. Four squares can be conveniently etched on a slide with the aid of a template and glass writing diamond.

(*c*) *Nickel-Chromium Loop*

Suitable loops can be made from 75–80 mm of 21 SWG nickel-chromium wire. Square off one end of the wire with a file. Lay a sharp knife firmly across the wire 15–16 mm from this end and bend the wire upwards at right-angles. To ensure a sharp angle in the bend, tap the wire gently over the right-angle corner of a piece of metal. With round-nosed pliers, the 15–16 mm length of wire is bent to form a circular loop with the squared end of the wire close against the right-angle bend. Fix the wire stem to a suitable holder.

The capacity of the loop must be checked by delivering quickly 10 loopfuls of milk of known specific gravity (the loop being operated as described in para. 5) at 20° to weighed, dry slide, and determining without delay the weight of milk delivered. The volume of milk delivered per loopful is then calculated; this should be as near as possible to 0·010 ml, and in any event must be within the range of 0·009–0·011 ml. The arithmetic mean of three separate determinations should be used.

(*d*) *Spreading Needle*

A dissecting needle bent to an angle of 135 degrees at 0·6 cm from the tip is suitable for spreading the milk evenly on the slide.

2. *Reagents*

(*a*) *Formalin* (40 per cent solution of formaldehyde)

(*b*) *Newman's Stain*

Methylene blue	1·0 g
Industrial methylated spirit (95 per cent v/v)	54 ml
Tetrachloroethane	40 ml
Glacial acetic acid	6 ml

Add the spirit to the tetrachloroethane and heat on a water bath to a temperature not exceeding 70°. Add the methylene blue and shake until the dye dissolves. Cool, add the acetic acid slowly, mix and filter.

3. *Sampling*

As the total cell count of herd milk is of little value for advisory purposes, sampling is normally confined to milk from individual cows or single quarters. Samples may consist of fore-milk only or of the complete milking of the udder or quarter. When fore-milk is sampled, the teats should be washed thoroughly with water containing disinfectant and wiped. If from a single teat, the sample should consist of 1 oz milk approx.; when a composite sample of all four quarters is taken it should consist of 1 oz approx. from each teat.

For an udder sample of the complete milking, mix the milk directly after milking and take a sample from the bucket. Separate quarter samples of a complete milking can be taken in like manner if a machine-milking bucket designed with four compartments is used. Three-ounce sample bottles are suitable, except for the composite samples from the four quarters when a bottle of larger capacity is necessary.

4. *Treatment of Samples*

Samples should reach the laboratory within 2 hr of milking. If this is not possible, cool the bottles in water immediately after sampling and keep the samples cool during transit.

On arrival at the laboratory shake the sample 25 times through an excursion of 1 ft. and pour 10 ml milk into a sterile 6 × $\frac{5}{8}$ in. test-tube with a mark at 10 ml. Add 1 drop of formalin solution, stopper with a sterile rubber closure and invert once to mix. Label the tubes for identification.

If films cannot be prepared on the day of sampling, store the tubed samples in the refrigerator overnight and make the films the following morning. The cell count is carried out on the formalised sample, but the remainder of the original sample should be stored in the refrigerator for reference purposes or for the application of other tests, e.g., Whiteside test.

5. *Preparation and Staining of Film*

Write a number on the left-hand end of each slide and a letter below each square. Thus each square can be readily identified, e.g., Ia, Ib, Ic and 1d, 2a, 2b, 2c, 2d, etc., with the sample numbers on the laboratory record sheet. Where large numbers of samples are to be tested, it is preferable to write the numbers and letters with a diamond when the squares are etched.

Unless the film is prepared immediately after dispensing and formalising the milk in the test-tube, bring the temperature to 15–20° and shake the tube thoroughly as described in para. 4.

After the foam has completely dispersed, invert the test-tube slowly once, introduce the standard loop vertically into the milk to about 1 in. below the surface and withdraw vertically with a crisp movement so that the loop is completely and evenly filled. It is important that no thin area or bubble of milk is visible within the loop.

Transfer the loopful of milk to the centre of the square on the slide by touching the glass with the edge of the loop and then allowing the loop to turn on to its flat side to break the surface tension. Return the loop to its edge and lift it slowly from the slide. Without delay distribute the milk

evenly over the square with the spreading needle; this operation must be done with care to obtain a uniform film.

Between transfers, thoroughly rinse the soiled loop and needle in clean tepid water in a beaker, and remove as much water as possible by tapping gently against the side of the beaker. Milk residues must not be allowed to dry on the loop or needle, which should not be flamed. After each series of film has been made, the loop and needle should be cleaned with detergent solution and rinsed in distilled water.

Dry the film immediately on a level surface at 40–45°. Rapid and even drying is essential to minimize the drift of cells towards the centre of the film.

When completely dry, stain with Newman's stain for 15 sec, then drain off the surplus stain by holding the slide in a near-vertical position. Allow to dry thoroughly, then rinse the slide gently with cold water to remove surplus stain, drain and allow to dry.

Notes

1. The loop must be handled with care at all times and its accuracy checked periodically.
2. With formalised milk there is less likelihood of the film slipping off the slide during staining and rinsing. If, however, any part of the film is so lost, another film should be prepared.

6. *Counting the Cells*

Using an oil-immersion objective, and commencing at the left-hand side, count the cells on a strip passing through the centre of the film. If the first 10 fields contain more than a total of 30 cells, stop counting at that point and record the results as 'over 2½ millions/ml milk'. When the total count for the first 10 fields is 30 or less, continue to count the remainder of the strip. Multiply the strip count by the microscope factor (for a field of vision of 0·012 sq. mm this is 90 × 100 = 9,000) to obtain the total cell count/ml milk.

Notes

1. The count on a single strip is sufficiently accurate for routine advisory purposes; for more critical work, duplicate films can be made and the mean count of one strip on each film determined.
2. Since it is necessary to keep a note of the first 10 fields as well as counting the cells therein, it is advisable to use a tally counter for recording the cells.
3. Staining jars may be used when a large number of slides is being examined, but stain should be discarded after each day's use.

41. EXAMINATION FOR BLOOD IN MILK

The presence of blood in milk indicates that the milk is abnormal. If the cow's udder is diseased or injured, or if she has recently calved, the cream may show a slight pink colouration in the can, but this may not be noticed with highly-coloured Channel Island milk. The milk may be rejected by the creamery if blood is detected.

For advisory purposes blood may be detected visually in the bulk (approx. 250 p/m) or in the deposit after centrifugation in the laboratory, or chemically by the 'Occultest' which may be used both on the farm and in the laboratory.

1. *Centrifugation*

Warm 10 ml of a well-mixed sample to 37° in a reductase or centrifuge tube. Place the tube in a centrifuge, and spin at not less than 1,100 r.p.m. for at least 5 min. A Gerber centrifuge is suitable for this purpose. Examine the deposit for the presence of red blood cells.

2. '*Occultest*'

Using a pipette place one drop of the well-mixed sample on to the centre of the square of filter paper provided. With forceps, place one reagent tablet directly on the moist area. Add two drops of distilled water to the top of the tablet so that they overflow on to the filter paper. After exactly 2 min. note any colour change in the paper surrounding the tablet. A blue colour denotes the presence of blood. Any colour developing after 2 min. should be disregarded.

It is essential to make the milk and water drops of equal size and the water drops should overflow on to the paper. Formalised milk should not be tested.

Notes

1. For both tests a control should be put up with milk known to be free of blood.
2. Neither of these tests can be used quantitatively though both give positive results with milk containing 20 p/m blood (1 ml blood in 10 gal milk).

Milk Products

42. BACTERIOLOGICAL EXAMINATION OF CREAM

1. *Sampling*

SAMPLES of cream to be tested bacteriologically should be taken to the laboratory as soon as is practicable. If for any reason a sample cannot be delivered to the laboratory within 2 hr of collection, it should be packed in ice in an insulated container and delivered to the laboratory before 5 p.m. on the day of collection.

When a sample is taken from a bulk, mix well, using a sterile 3 oz dipper, fill a sterile 3 oz bottle and securely close, using a sterile rubber bung. All samples, including cartons or other small packs, should be labelled and transported to the laboratory where they should be retained in the refrigerator until testing commences.

2. *Testing*

Because of the difficulty experienced in using a pipette with some cream samples, the '*Gravimetric*' method should be used to obtain the first dilution.

Weigh 5 g of the well-mixed sample into a sterilized weighing bottle and dilute with 20 ml quarter-strength Ringers solution warmed to 37° and mix well. This gives the 1 in 5 dilution necessary for carrying out the methylene blue test. It is best to make this original dilution between 3 and 5 p.m. to allow time for the tests to be properly carried out.

(*a*) *Methylene Blue Test.* To 10 ml of the 1/5 dilution in a test-tube, add 1 ml methylene blue solution, stopper and invert to mix as for milk (see Technique No. 29). This tube is then incubated for 18 hr at 18·3±1° (65±2°F) after which it is transferred to a water bath maintained at 37·5±0·5°. The tube is then examined strictly according to the method given in Technique No. 29 and the time taken for complete reduction recorded. A control tube should be set up for each sample using 1 ml distilled water in place of the methylene blue solution.

Tentative standards of quality are suggested as follows:

Decolourized in 1 hr . .	Unsatisfactory
Decolourized in 1½–4 hr .	Fairly satisfactory
Not decolourized in 4 hr . .	Satisfactory

(*b*) *Colony Count.* Using the 1 in 5 dilution, prepared by the gravimetric method, immediately make four serial tenfold dilutions and plate on Yeastrel milk agar by the method described in Technique No. 16. Incubate at 30±1° for 72 hr and express the result as colony count/g cream. On cold-stored pasteurized cream a psychrotrophic colony count (see Technique No. 20) should be carried out.

There is insufficient evidence to consider numerical standards for the colony count of cream at the present juncture.

(*c*) *Coli-Aerogenes Organisms*. Using the four serial tenfold dilutions already prepared, inoculate tubes of MacConkey's broth as recommended in Technique No. 17; alternatively the technique recommended in Technique No. 18 may be employed.

REFERENCES

1. Specification for Cream, fresh and canned. (1964). Canadian Government Specifications Board. 32-GP, 170c.
2. *Monthly Bulletin* (April 1958). Ministry of Health and Public Health Laboratory Service. **18**.

43. COLONY COUNT OF LIPOLYTIC ORGANISMS

Petri Dish Method

1. *Collection of Samples*

Samples shall be taken in accordance with B.S.809 and transferred to suitable sterile containers. They should be examined within 6 hr of sampling or stored at 3–5° and the examination carried out within 24 hr.

2. *Dilutions*

Serial dilutions should be prepared at the discretion of the bacteriologist, but it is generally sufficient to plate 1 ml quantities of the 10^{-1}, 10^{-2} and 10^{-3} dilutions. In the case of butter, add 1 g to 9 ml Ringer's solution containing 0·15 per cent agar. Warm in a water bath at 40–45° until the butter is melted and then shake well.

3. *Pouring of Plates*

Take 1 tube of basal medium (see Technique No. 8) for each Petri dish to be inoculated and add 0·3 ml of melted fat substrate per tube. Melt the medium, cool to 48° and fit the tubes with sterile rubber stoppers. Alternatively, $\frac{1}{2}$ oz screw-capped McCartney bottles may be used instead of test-tubes with rubber stoppers. Disperse the fat in the basal medium by vigorous shaking through about 1 ft at the rate of 20 to 30 times for 10 sec. Return the tubes to a 45° water bath for a few moments and allow froth to subside. Pour the plates as described in Technique No. 16.

4. *Incubation of Plates*

Plates shall be incubated at $22 \pm 1°$ for 5 days. It is important to avoid exposure of the plates to light which may cause fading of the colour of the medium. N.B. In some cases, the colour of the medium develops after only a few hours in the incubator.

5. *Examination of Plates*

Colonies which show a distinct colour change in or around colonies should be considered to be lipolytic.

REFERENCE

JONES, A. and RICHARDS, T. (1952). *Proc. Soc. appl. Bact.*, **15**, 82.

44. COLONY COUNT OF YEASTS AND MOULDS

Petri Dish Method

1. *General*
The yeast and mould count may be regarded as an index of the care taken in preparation of the product.

2. *Collection of Samples*
Samples shall be taken in accordance with B.S.809 and transferred to suitable sterile containers. As the micro-organisms on the surface of the product are often of importance, the surface layer may be examined separarately. For this purpose the surface shall be removed to a depth of $\frac{1}{4}$ in. by means of a sterile scraper and tested in the same way as the bulk sample; all samples should be examined within 6 hr of sampling or stored at 3–5° and the examination carried out within 48 hr.

3. *Media*
The media used shall be acidified to pH $3{\cdot}5 \pm 0{\cdot}1$ to suppress the growth of bacteria. Citric acid monohydrate is a suitable acidifying agent. For use with dairy products, the most suitable media are malt extract agar and yeast salt agar (see Technique No. 9).

4. *Dilutions*
The choice of dilutions for plating shall be at the discretion of the bacteriologist, but it is generally sufficient to plate 1 ml quantities of the 10^{-1}, 10^{-2} and 10^{-3} dilutions. In the case of butter, add 1 g to 9 ml Ringer's solution containing 0·15 per cent agar. Warm in a water bath at 40–45° until the butter is melted and then shake well.

5. *Pouring of Plates*
Melt the medium and cool to between 45° and 48° as soon as possible, as prolonged heating at 100° will destroy the gelling properties of the medium.

6. *Incubation of Plates*
Invert the plates and transfer to an incubator at 22° and incubate for 3–5 days.

7. *Counting of Colonies*
After incubation for the specified time, count the colonies using a counting chamber. Record the result, which shall be reported as number of yeasts and moulds per gramme of sample.

45. BACTERIOLOGICAL EXAMINATION OF STARTER

In the control of starters used for cheesemaking, daily examination for activity as described in Technique No. 46 is essential. In addition, a few other bacteriological tests can, from time to time, advantageously be made, particularly when contamination is suspected.

1. *Microscopical Examination*
As described in Technique No. 15 this may be useful in detecting contaminating organisms when present in very large numbers. These will differ in morphology from the starter streptococci and can easily be recognized.

2. *Plating on Yeastrel Milk Agar*
Incubating at 30° for 72 hr or at 22° for 72 hr, and using dilutions of 10^{-5} to 10^{-7} is a more useful method of detecting large numbers of contaminating bacteria, which will usually form much larger colonies than the starter streptococci. A simple method, suitable for creamery laboratories, is to streak a loopful (10^{-2} ml) of starter over a dried Yeastrel milk agar slope in a 6 × $\frac{3}{4}$ in. test-tube and to incubate at 30° and/or 22°.

3. *Coli-Aerogenes Organisms*
These may be found in starters subjected to contamination. The inoculation of duplicate 5 ml amounts of starter into separate tubes of double-strength MacConkey's broth or inoculation of triplicate 1 ml and of 1 ml of 10^{-1} and 10^{-2} amounts into separate tubes of MacConkey's broth, incubated at 30°, will detect the presence of these organisms in 18–24 hr.

4. *Yeasts and Moulds*
These can be detected by spreading 1 ml of the starter over the surface of a dried plate of malt extract or synthetic yeast salt agar (see Technique No. 9) incubated at 22° for 3–5 days.

46. TESTS FOR THE ACTIVITY OF CHEESE STARTERS AND THE PRESENCE OF EITHER BACTERIOPHAGE OR INHIBITORY SUBSTANCES

The tests outlined below are intended to supply information regarding the activity of a starter and also give warning of the presence of bacteriophage (phage) or inhibitory substances, including antibiotics, in the milk used for cheese-making.

When advice has been sought regarding the cause of starter failure or slowness in cheese-making, samples should be obtained of an 18–24 hr culture of the starter, of the normal vat milk in use, and of the whey from the vat where the trouble was experienced. If several vats are involved, and if cutting takes place at different times, the sample of whey should be taken from the vat in which the curd was cut first. Where several starters are under investigation, it is essential to obtain samples of whey from curd produced by each starter. If a suspected starter is no longer in use it should still be tested. A sample of the whey from cheese made with suspected starter can sometimes be obtained from the previous day's cheese in press.

Samples should be collected in sterile 4 oz bottles, transported to the laboratory, and tested as soon as possible—preferably within 1 hr of collection. If the samples arrive too late to be tested on the day of sampling, 1·0 per cent of the starter must be inoculated into sterile milk, incubated at 22° overnight and the test made the following morning.

1. *Apparatus and Materials*

Sterile 4 oz bottles
10 ml and 1 ml sterile pipettes
6 × ¾ in. sterile boiling tubes
Rubber bungs to fit tubes
Burette
Porcelain basins with stirring rods
Water bath at 30°
N/9 sodium hydroxide (NaOH) carbonate free solution
0·5 per cent neutral phenolphthalein solution (see Technique No. 53)
Rosaniline acetate solution (see Technique No. 53)
Sterile quarter-strength Ringer's solution (see Technique No. 5)
A supply of sterilized skimmed milk *known not to contain* antibiotics or other inhibitory substances. This should be sterilized by intermittent steaming and not by autoclaving.

2. *Procedure*

In carrying out the following tests, all dilutions should be made according to the technique given in Technique No. 16.

Filter about 10 ml of the whey through a sterile G.S. grade Seitz filter. About half of this and 25 ml of the vat milk should be placed in boiling tubes and held in boiling water for 5 min. and then cooled immediately. A further 25 ml of the vat milk should be autoclaved at 121° for 15 min.

Prepare a 1 in 4 dilution of the starter in quarter-strength Ringer's solution, using 6 × ¾ in. boiling tubes, and add 1 ml to each of the following tubes which have been pre-heated to 29–30°:

Tube 1 containing 25 ml sterilized skimmed milk
2 „ 25 ml „ „ „ + 5 drops filtered whey
3 „ 25 ml „ „ „ + 5 drops heated filtered whey
4 „ 25 ml vat milk
5 „ 25 ml „ „ + 5 drops filtered whey
6 „ 25 ml „ „ + 5 drops heated filtered whey
7 „ 25 ml boiled vat milk
8 „ 25 ml autoclaved vat milk

The initial acidity of both the sterilized skimmed milk and of the vat milk should be determined as they may differ by as much as 0·1 per cent lactic acid.

The tubes should be closed with sterile rubber bungs and incubated in a water bath at 30°±1° for 6 hr. In order to prevent the starter organism concentrating in the cream layer of the vat milk and so giving a low final activity reading, all the tubes should be inverted once every hour during incubation.

This hourly inversion of the tubes can be dispensed with if, where it is available, 2·5 ml of 3·0 per cent sterile melted agar, cooled to 50°, is added to each tube and immediately mixed with the milk by inverting the tube before incubation. If agar is used, however, it should be borne in mind that, in the presence of agar, the starter organisms may produce slightly more acidity than in milk alone.

At the end of the incubation period, mix well by inverting gently to avoid trapping air bubbles, pipette out 10 ml from each tube into a porcelain

basin and titrate for acidity, using 10 ml normal milk for the colour control. The quantity of N/9 NaOH solution required, until the pink colour matches that of the control, divided by 10, is the percentage of lactic acid in the milk. The acidity developed by the starter should be calculated by subtracting from this figure the initial acidity of the milks used in the test. It is essential that the titrations should be completed as quickly as possible because of the rapid changes in acidity which take place at the end of this period.

3. *Interpretation of Results*

The activity of the starter is indicated by the amount of acidity produced in tube 1. A developed acidity reading of 0·45 per cent or above indicates that the starter is satisfactory. If the starter is slow, producing significantly less than 0·45 per cent lactic acid, then advice regarding the introduction of a new starter should be given.

If the starter has been found to be satisfactory the presence of inhibitors in the vat milk and whey may be indicated by comparing the acidity produced in tube 1 with that produced in tubes 2 to 8. If, the acidity in any of the tubes 2 to 8 is 10 per cent lower than that produced in tube 1, inhibition can be said to have taken place. Such a 10 per cent reduction of acidity in the vat milk—tube 4—shows that the milk contains one or more inhibitory agents.

If an antibiotic is present, tubes 5, 6 and probably 7 will show a similar loss of activity, although boiling the vat milk (tube 7) may reduce the amount of antibiotic present and consequently the degree of inhibition. When present in the vat milk, some antibiotic will be found in the whey derived from it and, if present in large enough amounts, will affect the amount of acid produced in tubes 2 and 3. The presence of an antibiotic may be confirmed by making enquiries at the farms involved, and by carrying out one of the tests recommended for the purpose, e.g., Technique No. 54.

The presence of bacteriophage in the vat is indicated by the complete elimination of inhibition in tube 7 in which the phage has been destroyed by boiling. If the difficulty in cheese-making is due to phage, however, it is more likely to be found in the whey, and inhibition will be observed in tube 2, and, if the acidity developed in tube 4 is normal, also in tube 5. The presence of phage in the whey is confirmed by normal acidity developing in tube 3 and in tubes 4 and 6 since the phage in the whey added to tubes 3 and 6 has been destroyed by boiling. When the presence of phage has been confirmed, advice should be given to change the starter immediately and to keep the present one out of use for at least 1 week. The introduction of a rotation of starters, each having a different phage sensitivity pattern, should be considered. The conditions under which the starter is propagated should be investigated and if necessary a stricter aseptic technique recommended.

During the winter months an inhibitory effect may be present in milk which is not due to either antibiotics or phage. It is not destroyed by boiling, and consequently tubes 4, 5, 6 and 7 will all show inhibition. This effect, known as 'winter slowness', is destroyed by autoclaving the milk at 121° for 5 min., thus eliminating any inhibition in tube 8. The nature of this inhibition is not fully understood and no advice can therefore be given regarding its elimination from milk.

Bacteriological Examination of Farm Dairy Equipment

47. USE OF RINSES, SWABS AND MILK SAMPLES IN FARM ADVISORY WORK

BACTERIOLOGICAL examination of rinses or swabs of dairy equipment and of milk samples can be of considerable assistance in milk advisory work. In order that the results may be comparable and correctly interpreted, it is necessary that sampling techniques are standardised and uniformly adopted. It is not intended to prescribe a sterotyped approach, but it is recommended that the procedures outlined in this Technique be adopted unless circumstances clearly justify a departure from them.

Investigational Procedure

(*a*) The premises, plant and facilities should be examined in the presence of the producer or his representative. The routine methods employed should be discussed and defects in them pointed out.
(*b*) If the investigation is carried out with thoroughness, it will often be possible to trace the cause of the contamination and to offer sound practical advice for its prevention *without resort to sampling*. It is a waste of time and materials to take samples when the methods are obviously at fault.
(*c*) A clean white coat and cap should be worn and the hands should be thoroughly washed immediately before the milk utensils are examined. When examining utensils, it is essential to handle them with care to avoid extraneous contamination. Any piece of equipment which has been dismantled for visual inspection or has been in contact with brushes or rods during inspection should not be rinsed or swabbed.
(*d*) Sterile sampling equipment is supplied by the testing laboratory. The equipment and samples for examination should always be carried in suitable containers. If the equipment has to be stored pending use, a clean, cool place should be chosen.

1. *Choice of Samples*
(*a*) When samples are considered necessary, their type and number will depend on the nature of the problem. When the investigation concerns failure to produce milk of marketable quality or designated standard, the number of samples should be restricted to that considered necessary for locating the fault or faults. If information on the efficacy of cleansing the milking utensils and plant is desired, rinses or swabs of judiciously selected pieces of equipment are appropriate. If the milking methods, cooling and handling of the milk are under investigation, milk samples, taken at selected points can provide valuable information. For involved problems, e.g., taints and other abnormalities in milk, it may be necessary to take a larger number of samples.

(*b*) If the problem appears to be of a complex nature, it may be necessary to extend the investigation or adopt special methods of examination.
(*c*) The laboratory should always be notified in advance when it is proposed to bring in a large number of samples.

2. *Rinse Samples*
(*a*) For rinsing purposes, sterile quarter-strength Ringer's solution is used. When disinfectants are used for cleansing dairy equipment, an inhibitor must be used to neutralize any residual disinfectant carried over into the sample. In the case of chlorine and iodine disinfectants, the inhibitor is added to the Ringer's solution during preparation, but for quaternary ammonium compounds it is added to the rinse sample immediately after it is taken.
(*b*) The Ringer's solution is normally dispensed in 500 ml quantities and a quantity smaller than 500 ml should not be used. If the utensil to be rinsed will not contain 500 ml, the surplus Ringer's solution should be left in the bottle and the rinse subsequently returned to it. In transferring the solution to the sample bottle after rinsing the utensil, as much as possible of the 500 ml should be collected. For rinsing the entire system of a pipeline milking machine, a larger quantity of solution will be necessary, depending on the size of the plant. The sample collected for testing must be representative of the total quantity used for rinsing.
(*c*) As far as possible, the whole surface of the equipment being rinsed should be thoroughly wetted with rinse solution. Agitation of the solution assists in dislodging the organisms. A single rinsing will only remove a part of the contamination, and, except for certain equipment which is difficult to rinse, *the rinse should be repeated* in order to recover a larger proportion of the organisms.
(*d*) Items for rinsing should be selected so that they are representative of the equipment. The conditions of the rubber parts should be noted and the least satisfactory chosen for rinsing. If any equipment to be tested by the rinse or swab method has not received the final rinsing of the cleansing operation, this should be done in order to remove detergent-disinfectant residues before samples are taken. Equipment which is visibly dirty, greasy, or in a bad state of repair is obviously unsatisfactory and should not be rinsed.
(*e*) Milk cans which show the presence of milk solids or serious physical defects should not be rinsed but would justify a complaint by the producer to the buyer. When cans in apparently good condition give high rinse counts the producer should be advised to apply a suitable cleansing procedure.
(*f*) Items of equipment may be rinsed separately, or, alternatively, two or more associated pieces of equipment, e.g., strainer, receiver and cooler, may be tested by a single quantity of rinse.
(*g*) Certain sections of milking machines, such as cluster and long milk tube, bucket, recorder jar and releaser, can be rinsed separately. An overall picture of the condition of the machine may be obtained by passing a rinse through the entire system. Parts that are difficult to rinse can be tested by the swab method.
(*h*) The number of rinses to be taken will depend on the nature of the problem and the type of equipment, but the parts to be rinsed should be

selected judiciously with the object of keeping the samples to a minimum. Where the aim is to check the efficiency of the routine cleansing procedure, rinses of the following pieces of equipment should provide the necessary information.

For Hand Milking: (i) a milking pail or carrying bucket, (ii) strainer, receiver and surface cooler together, unless there is a specific reason for rinsing them separately, and (iii) a milk can if considered necessary.

For Bucket Milking Machines: rinse a cluster in addition to (i), (ii) and (iii) above. For direct-to-can milking, only two rinses are necessary, a cluster assembly and a milk can.

For Pipeline to Releaser Milking Machines: rinse (i) a cluster, (ii) the entire plant from clusters to discharge point, (iii) any ancillary equipment, i.e., strainer, receiver and cooler, and (iv) a milk can if necessary. In addition, separate rinses of a recorder jar and releaser vessel can also be taken if required.

For Pipeline to Can Milking Machines: a cluster assembly and a milk can are rinsed separately prior to rinsing the assembled plant.

Where machines of four or more units are in use, it may be desirable to rinse more than one cluster. Where milk is bottled on the farm, washed bottles should be taken to the laboratory for testing.

(*i*) A bulk milk tank should first be thoroughly inspected. Advice on cleaning can be given without resort to sampling if there is any sign of film or scale on the sides, bridge, lids, on the bottom of the tank, under the arms of the thermometer and thermostat, on the rubber bungs holding these in position, or on the rubber bung at the tank outlet. The ancillary equipment inside the tank (e.g., dipstick, agitator) should be specially inspected. Where all the parts of the tank and its accessories appear to be clean, one rinse may have greater value than several swabs in assessing the general cleanliness.

(*j*) If any rinse contains milky residues it should be discarded, and an effective cleansing routine recommended.

3. *Swab Samples*

(*a*) Although the bacteriological condition of most items of farm milk plant can be checked adequately by the use of rinses, some equipment, e.g., parts of farm bulk milk tanks, milking machine bucket lids, in-can coolers, bottling plant, cocks, gaskets, etc., has to be examined by the swab method. Sections of pipelines may also be swabbed if they cannot conveniently be rinsed. Standard swabs are used, and the swab solution is dispensed in 25 ml quantities. The use of inhibitors in the swab sample applies as for rinse samples (see para. 2 (*a*) above). A swab can also be useful as a visual aid for demonstrating the presence of residues in parts of the plant which are difficult to clean.

(*b*) If it is necessary to swab a bulk tank, the important parts to examine are the outlet and rubberware, as these points are the most frequently contaminated. If it is decided to rinse the tank, a swab of the outlet should be taken first. Other parts of the equipment which may be swabbed are the side of the tank, the bridge, the paddle blade and the stems of the thermometer, thermostat, paddle, dipstick and outlet plug stems.

4. *Milk Samples*

(*a*) Milk samples may be taken when an assessment of the general methods of production is required. For routine purposes it will suffice to take one sample, preferably from the first can to be filled. In the case of special problems, e.g., taints, it may be desirable to trace the stage of production at which contamination is occurring, and for this purpose serial milk samples can be taken. For some abnormalities, samples of milk from individual cows may be required, but they should not be taken until all other possible sources of contamination have been investigated and unless there is good reason to suspect that the milk from some cows in the herd is the direct cause of the abnormal condition of the milk.

(*b*) It is important to record the temperature of the milk from which a sample has been taken; and the bulb and lower part of the thermometer stem must be cleaned and disinfected before use. For serial samples, all the samples in the batch should be thoroughly cooled in the bottle in running water to the same temperature to ensure that temperature variation is not the cause of any differences in results between samples.

(*c*) *Field Report*

In order that the appropriate tests can be applied and the results interpreted correctly, it is essential that a concise and complete description of the conditions on the farm be given on the Field Report. The methods of production should be carfully noted, and the degree of thoroughness with which the work is done should be assessed and recorded, since often it is not the cleansing system which is at fault, but rather the manner in which it is applied. The section of the Report dealing with samples taken should be completed with care; the quantity of rinse used, the type and capacity of the equipment rinsed, the area rinsed or swabbed and the temperature of the milk sampled must always be given so that the results of the tests can be accurately evaluated.

(*d*) *Transport*

Milk samples should be transported to the laboratory in insulated containers with as little delay as possible. Routine advisory samples of morning milk should reach the laboratory not later than 4 p.m. on the day of production, and samples of afternoon milk should be conveyed to the laboratory on the same evening. Where unusual problems are being investigated, it is desirable that the samples be examined within 2 hr of production; this may necessitate making a special journey to the laboratory with the samples. Rinse and swab samples should also be conveyed to the laboratory in suitable containers with a minimum of delay so that tests can be applied as soon as possible, and in any event within 6 hr of sampling.

(*e*) *Testing and Reporting*

There must be co-ordination between laboratory findings and advice in the field. From the information supplied on the Field Report the Bacteriologist will decide the type and number of tests to give the best possible information required for the solution of the problem.

The bacteriological reports are intended primarily for the guidance of the adviser, but they should be worded so that they are suitable for passing on to the milk producer. It must be realised, however, that the person taking the samples, in view of his knowledge of the farm conditions, is in the best

position to transmit the reports to the producer. It may be desirable in some cases that the reports should be taken to the farmer and explained to him. The sense of the bacteriological reports should never be altered, but the adviser should make additional remarks to indicate means of correcting faults

Sampling Techniques

5. *Rinses*

(*a*) *Milk Can*. Pour the rinse solution into the lid, swirl and pour into the can. Replace the lid. Lay the can on its side and roll to and fro so that the can makes 12 complete revolutions. Allow the can to stand for 5 min and repeat the rolling. Pour the rinse directly from the can into the original bottle. If more convenient, pour the rinse back into the lid and then into the bottle.

(*b*) *Bucket* or *Receiver*. Pour the rinse solution into the vessel. Swirl the vessel 12 times in such a manner that as much as possible of the internal surface is thoroughly wetted. After a pause of 2 min repeat the swirling and transfer the rinse to the original bottle.

(*c*) *Strainer*. Rinse in conjunction with the receiver, but if rinsed separately place it over a bucket or receiver which has been previously rinsed. Pour the rinse solution carefully and slowly over the internal surface and return from the bucket to the original bottle. After 2 min repeat the process using the same rinse.

(*d*) *Surface Cooler*. Place the plug in the bottom tray, then pour the rinse slowly into the top tray and spread it with a sterile swab over the corrugations to assist thorough wetting of the whole of the cooler surface. Collect the rinse in the bottom tray, remove the plug and collect the sample in the original bottle. Repeat the whole process after 2 min using the same rinse.

(*e*) *Strainer, Receiver and Cooler Combined*. Pour the rinse slowly over the internal surface of the strainer placed over the receiver, and allow the rinse to collect in the receiver with the tap turned off. Swirl the receiver as indicated in para. 5 (*b*), open the tap and allow the rinse to pass slowly over the cooler using a sterile swab, as described in para. 5 (*d*), to assist thorough wetting of the whole of the surface. Collect the rinse from the bottom tray into the original bottle and repeat the whole procedure after a lapse of 2 min using the same rinse.

(*f*) *Milk Bottles*. Select at least four bottles at random, stopper with sterile rubber bungs and take to the laboratory for rinsing.

(*g*) *Teat-Cup Cluster Assembly*. It makes for easier and more efficient rinsing if a simple rack is used for holding the teat-cups. Insert a short length of sterile glass or suitable metal tubing bent at an angle of about 130 degrees into the free end of the long milk tube to facilitate pouring. With the teat-cups and free end of the long milk tube held in a vertical position, pour the rinse into the cup orifices until the system is about two-thirds full (approx. 300 ml rinse). Leave the remainder of the rinse in the bottle. Manipulate the long tube carefully up and down so that the solution is agitated and the whole system bathed with rinse. Take care not to allow the rinse to overflow. Collect the rinse in the original bottle by pouring from the free end of the long milk tube. Repeat the rinsing after a lapse of 2 min using the same rinse.

(*h*) *Recorder Jar*. When the jar is fitted with an ordinary spreader (not of the swivel type) below the central vacuum port on the lid, detach the tube from the inlet milk port, then detach the tube from the vacuum port and attach it to the inlet milk port. Close the discharge milk tube cock or the pinch clip on the discharge tube. Fix to the vacuum port a 2½ ft length of sterile rubber hose fitted with 1 ft of sterile glass or suitable metal tubing which has been sterilized in greaseproof paper. Remove the greaseproof paper and insert the end of the tube into a bottle of the Ringer's solution. Then turn on the vacuum and draw two 500 ml quantities into the jar, breaking the flow from time to time. Turn off the vacuum, disconnect the milk discharge tube at the connection with the overhead, horizontal pipeline, insert a short length of sterile glass tubing in the end and return 500 ml rinse solution to the original bottle. Rinse the jar once only. When the jar is fitted with a swivel spreader it is only necessary to turn the spreader into the spray position in preparing the jar for rinsing. Alternatively, the jar can be tested without the discharge tube by inserting a sterile rubber bung fitted with a short piece of glass tubing and Mohr clip connection. Thus, the rinse solution can be collected directly from the jar. Jars not fitted with a spreader cannot be properly rinsed, and it is better to swab the glass surface, lid and gaskets separately.

(i) *Pipeline Releaser Milking Machine*. Rinse one or more cluster assemblies as described in para. 5 (*g*). Ensure that the plant has been completely drained and proceed as follows:
assemble the plant for milking, except that the inlet milk tube to the recorder jar is attached to the vacuum port, and the vacuum tube attached to the milk port on the jar lid. It is not necessary to reverse these tubes when a swivel spreader is used, as this should be in the washing position. Close the pinch clip or cock of the milk discharge tube of the jar. Using 500 ml of rinse solution, fill one set of teat cups and draw it into the jars by turning on the vacuum, flushing through with the remainder of the rinse. Using a further 500 ml of rinse, repeat the process with each set of teat cups in use. Then open the pinch clips or cocks to allow the solution to flush the pipeline and pass into the releaser or interceptor jar, and also through the pump if installed. Collect the solution at the discharge point into a 3 litre flask with the aid of a funnel, or into a wide-mouthed bottle, or a can, which has been sterilized. Transfer a representative 500 ml quantity of the rinse into one of the original bottles for testing. Rinse the plant once only. Depending on the purpose of the investigation it may not be necessary to pass rinse solution through all the cluster assemblies. An alternative method of checking the pipeline and after-part of the plant without the clusters is to attach a short length of sterile rubber hose with a glass or suitable metal connection (see para. 5 (*b*)) to the pipeline connection furthest from the releaser, and draw 1,000–2,000 ml solution, depending on the size of the plant, through the system from that point. When the recorder jar is not fitted with a spreader, it may be desirable to swab it before passing the rinse solution through the plant. Likewise, the releaser jar, pump, and sections of the milk pipeline can be swabbed separately if considered necessary.

(*j*) *Pipeline to Can Milking Machine*. Rinse one or more clusters as described

in para. 5 (*g*) and a milk can as described in para. 5 (*a*), and swab the vacuum lid. Ensure that the plant has been completely drained and assemble as for milking. Pour 500 ml Ringer's solution into each cluster and draw through the plant into the can, and take a representative sample of the rinse from the can. Do not repeat the rinsing. It may not always be necessary to pass rinse solution through all the cluster assemblies. Alternatively, the pipeline system can be rinsed by attaching a short length of sterile rubber hose fitted with a sterile glass or suitable metal connection to the pipeline connection furthest from the can and drawing the rinse solution from the bottle direct as shown in para. 5 (*i*). If recorder jars are used, these can be examined separately if necessary.

(*k*) *Bulk Milk Tank.* Ensure that the tank is drained completely by unscrewing the outlet cap and removing the plug or opening the tap. Replace the plug or shut the tap and rinse the whole tank, wetting as much of the surface as possible. For tanks with a capacity up to and including 200 gal, use two bottles of rinse, and for tanks of 250 gal capacity or over use three bottles. Allow the rinse to drain down and then collect at the outlet in a suitable sterile container. Shake and pour off 500 ml into one of the original sterile rinse bottles.

6. *Swabs*

(*a*) *General.* Wherever possible an area of 1 sq. ft should be swabbed. In every case the surface area swabbed should be estimated and recorded on the Field Report so that the correct conversion factor may be used in the evaluation of the results. For pipes and cocks, the surface area can be calculated by multiplying the diameter by the length and by 3·14. Where the item swabbed is small, such as a bung closing the outlet of a farm bulk milk tank or the rubber bung holding the thermometer or thermostat on such a tank, the result shall be expressed as colony count per bung. The general procedure for swabbing a surface can be applied to all equipment that is difficult to rinse, e.g., milking machine bucket lid, in-can cooler, bottling plant, cocks, gaskets, sections of pipeline, blades of pumps and those parts difficult of access on a bulk milk tank.

(*b*) *Bulk Milk Tank.* When it is considered necessary to swab the surface of a bulk milk tank, two sections, each 1 sq. ft in area either below or overlapping the milk line, should be swabbed using one swab only. If the tank outlet is to be swabbed, remove the bung and allow the tank to drain, then swab in the usual way. Items such as the underside of the bridge, the paddle blades and stem, the dipstick and stems of thermometer and thermostat can all be swabbed with the usual technique.

7. *Bulk Milk Samples*

(*a*) *From Milk Can.* Mix the milk thoroughly in the can with a sterile long-handled dipper for at least 5 sec, then take a sample from well below the surface and transfer to the sample bottle. Fill the bottle as full as practicable and place it in an insulated box for transport to the laboratory. When sampling, care must be taken to ensure that the part of the bottle stopper or cap which comes in contact with the milk does not touch any unsterile surface. Record the temperature of the milk in the can after sampling.

(*b*) *From Farm Bulk Tank.* Operate the paddle for 2 min to mix the milk

in the tank, then take a sample with a sterile dipper in the manner described in para. 7 (*a*). Without delay, place the sample into an insulated box. Record the temperature of the milk in the tank.

(*c*) *Bottled Milk.* A filled bottle, properly capped, should be taken at random and brought intact to the laboratory.

8. *Serial Milk Samples*

(*a*) Milk should be sampled direct from the cow, the milking pail or machine unit bucket, the releaser, cooler inlet to farm bulk tank, milk can or at other points as appropriate. It is important that the same batch of milk is sampled at the various points, and all samples cooled immediately to the same temperature.

(*b*) *From the Cow.* One or more clean, quiet, healthy cows in mid-lactation and giving at least 1 gal per milking, should be selected. The udder should be thoroughly washed, first in clean water, and then in water containing approved hypochlorite solution (1 oz to 2 gal). Special attention should be paid to the washing of the teats. The milker's hands should be washed in dilute hypochlorite solution. Two or three squirts of milk should be drawn from each quarter into a strip cup and examined. If free from abnormalities, the sample should then be taken by drawing a few squirts of milk from each quarter into the sample bottle, taking all possible precautions to avoid contamination. The cow should then be milked out, and her milk sampled from the bucket, cooler, can or other selected points.

(*c*) *From Bucket, Receiver or Bottle Filler.* Stir the milk with the sterile dipper and transfer the sample to the bottle as for can samples.

(*d*) *From the Base of the Cooler.* Place the plug in the cooler tray outlet and allow the milk to flow over the cooler, spreading it with a sterile swab. When the tray is almost full, ease the plug and allow the milk to flow into the sample bottle until it is one quarter to one third full. The remainder of the milk in the tray is allowed to flow into the milk can and the process repeated until the bottle is almost full.

Note

Further details of the application and bacteriological examination of rinses and swabs are given in Techniques Nos. 48—52.

48. RINSE TECHNIQUE FOR EXAMINATION OF FARM DAIRY EQUIPMENT

1. *General*

For rinsing purposes, sterile quarter-strength Ringer's solution is dispensed in 500 ml quantities. This quantity will suffice for rinsing most pieces of equipment, but when it is desired to rinse the entire system of a releaser type milking machine, a larger volume may be necessary. Quantities of rinse smaller than 500 ml should not be used. If the utensil to be tested will not contain this quantity, the surplus Ringer's solution should be left in the bottle and the rinse subsequently returned to, and mixed with it. In transferring the solution to the original bottle after rinsing, as much as possible of the 500 ml should be collected.

As far as possible the whole surface of the piece of equipment to be rinsed

should be thoroughly wetted with the rinse solution. Agitation of the solution by rotary or other movement assists in dislodging the organisms. A single rinsing is not likely to remove more than half of the contamination, and to recover a reasonable proportion the rinsing should normally be repeated.

Items of equipment for rinsing should be carefully selected; for example, where there is a choice of buckets, the vessel in the poorest condition should be rinsed. Again, with teat-cup clusters the condition of the rubber should be noted and one or more of the poorest chosen for rinsing. Equipment which is visually dirty, greasy or in a bad state of repair should not normally be rinsed. For the rinsing of milk cans see Technique No. 50.

A separate rinse can be taken from the bucket, but since the strainer, receiver and cooler are normally cleansed together, a single rinse of the three should suffice. An exception can be made if it is considered that one piece of the assembly is likely to be particularly prone to contamination.

With milking machines it is desirable that the cluster and the bucket should be rinsed separately. The cleanliness of the lid can be tested by the swab method.

Where the aim is to check the efficiency of the general cleansing procedure on the farm, rinses of the following pieces of equipment should provide the necessary information:

for hand milking—(*a*) a milking pail or carrying bucket, (*b*) strainer, receiver and cooler, collectively, unless it is decided to rinse them separately, and (*c*) a milk can if considered necessary; for a bucket milking machine plant, a rinse of the teat-cup cluster should also be taken; for releaser plants, rinses should be taken from a cluster and from the entire milking plant in addition to (*b*) and (*c*) above; for bail plants, separate rinses should be taken of the cluster and the milk can, then another rinse should be taken of the assembled plant, including the can; for direct-to-can milking machines, the cluster and the can should be rinsed separately. A swab can be taken of the vacuum lid of direct-to-can milking machines of bail plants if considered necessary. Where milk is bottled on the farm, washed bottles should be taken for testing. (see Technique No. 51).

2. *Rinse Technique*

The detailed techniques are as follows:

(*a*) *Bucket or Receiver*. Pour the rinse solution into the vessel. Swirl the vessel 12 times in such a manner that as much as possible of the internal surface is thoroughly wetted. After a pause of 2 min, repeat the swirling and transfer the rinse to the original bottle.

(*b*) *Strainer*. Rinse in conjunction with the receiver but, if rinsed separately, place it over a bucket or receiver which has been previously rinsed. Pour the rinse carefully and slowly over the internal surface and return from the bucket or receiver to the original bottle. After 2 min repeat the process using the same rinse.

(*c*) *Surface Cooler*. Place the plug in the bottom tray, then pour the rinse slowly into the top tray and spread it over the top corrugation to obtain a more even flow. Use a sterile swab to spread the rinse. Collect the rinse in the bottom tray, remove the plug and collect the sample in the original bottle. Repeat the whole process after 2 min using the same rinse.

(*d*) *Strainer, Receiver and Cooler Combined.* Pour the rinse slowly over the internal surface of the strainer placed over the receiver, and allow the rinse to collect in the receiver with the tap turned off. Swirl the receiver as indicated in para. 2 (*a*), open the tap and allow the rinse to pass slowly over the cooler. Collect the rinse from the bottom tray into the original bottle and repeat the whole procedure after a lapse of 2 min using the same rinse.

(*e*) *Milk Bottles.* Select at least four bottles at random, stopper with sterile rubber bungs and take to the laboratory for rinsing (see Technique No. 51).

(*f*) *Teat-Cup Cluster.* With the cups and the free end of the long milk tube held together in a vertical position, pour the rinse into the cup orifices until the system is about two-thirds full (approx. 300 ml rinse). Leave the remainder of the rinse in the bottle. Manipulate the long tube carefully up and down so that the solution is agitated and the whole system is bathed with rinse. Take care not to allow the rinse to overflow. Collect the rinse in the original bottle by pouring from the end of the long milk tube. Repeat the whole process after a lapse of 2 min using the same rinse. The procedure is easier to carry out if a sterile glass tube about 6 in. long is inserted into the end of the long milk tube.

(*g*) *Releaser Milking Plant.* Detach the furthest cluster from the releaser and rinse as in para. 2 (*f*). Replace and with the vacuum turned on pour 1,000 ml at least into the same cluster and draw it through the plant, including the recorder jars, to the releaser. If it is not practicable to sample the rinse direct from the releaser, collect the solution in a previously rinsed bucket or a sterile wide-mouthed jar, and transfer 500 ml to one of the original bottles. Because of the volume of solution and difficulties involved *do not repeat the process.*

(*h*) *Bail.* Pour 500 ml rinse over the vacuum lid into the can, swirl 12 times to wet as much of the inside of the can as possible, and return direct to the bottle. Repeat the process after 2 min, using the same rinse. Detach the cluster furthest from the can and rinse as in para 2(*f*). Replace and with the vacuum turned on pour another 500 ml quantity of rinse into the cluster. Draw it through the assembled plant and pour the rinse from the can direct into the sample bottle. *Do not repeat the process.*

(*i*) *Direct-to-Can Milking Machine.* Detach the cluster and rinse as in para 2(*f*). Rinse the vacuum lid as in para. 2 (*h*) with a second quantity of 500 ml. When the machine is provided with an arm necessitating the use of an additional length of milk line, test the latter by drawing a third 500 ml quantity of rinse through the assembled plant and collect the rinse from the can direct into the bottle *Do not repeat the process.*

3. *Testing*

Rinse samples should be tested as soon as possible and in any event within 6 hr of sampling. The tests normally applied are the colony count on Yeastrel milk agar at $30 \pm 1°$ for 72 ± 2 hr and, if desired, the coli-aerogenes test at the same incubation temperature and time.

Mix the sample by inverting the bottle slowly three times. Normally prepare 1 ml and 0·1 ml Petri dishes using Yeastrel milk agar. The preparation of the dilutions, inoculation, pouring and incubation of the Petri dishes and the counting of the colonies is carried out according to Technique No. 16. Where the area of equipment rinsed is 1 sq. ft or less, it may be

desirable to plate a larger quantity. Record the results as colony count per sq. ft of surface rinsed or per utensil.

For the coli-aerogenes test it will generally suffice to inoculate three tubes of single strength MacConkey's broth each with 1 ml of rinse or to plate 1 ml on violet red bile agar.

If desired, the thermoduric colony count may be carried out as described in Technique No. 19.

Testing for milk spoilage organisms may provide useful additional information; quantities of 1 ml, and if desired 0·1 ml are inoculated into separate tubes of 5 ml sterile skim milk containing litmus or bromo-cresol purple dye and incubated at 22±1° for 72±2hr.

Rinses of milk bottles should be examined according to the technique described in Technique No. 51.

Notes

1. When sodium hypochlorite or other approved chlorine compound has been applied to any surface, crystalline sodium thiosulphate must be added to the Ringer's solution for rinsing before autoclaving so as to give a concentration of 0·05 per cent, i.e., 0·25 g crystalline sodium thiosulphate to each 500 ml of Ringer's solution, or 1 ml of a 25 per cent solution of sodium thiosulphate.
2. If a detergent-steriliser containing a quaternary ammonium compound has been used for cleansing, 5 ml of a 4 per cent lecithin and 6 per cent Lubrol W. solution in distilled water shall be sterilized, and added to the rinse solution after rinsing.
3. Rinse solutions containing sodium thiosulphate may be used for rinsing equipment which has been cleansed with a detergent-steriliser containing a quaternary ammonium compound, but the lecithin-Lubrol W. solution must be added as indicated in Note 2.
4. The addition of the lecithin-Lubrol inactivator immediately after rinsing should always be recorded on the Field Report.

49. SWAB TECHNIQUE FOR EXAMINATION OF MILK PLANT AND EQUIPMENT

1. *General*

This technique is intended for assessing the bacteriological condition of plant and equipment where the rinsing technique is not applicable. Such plant includes tanks, large coolers, pipe lines, cocks, agitators, air vents, milking machine bucket lids, in-can farm coolers, bottling plants, etc. A swab can also be useful as a visual aid for demonstrating dirty conditions in inaccessible parts such as claw pieces and vacuum check valves of milking machines, fixed covers of in-can coolers, etc.

2. *Apparatus*

10 × 1 in. test-tubes of heavy resistance glass;
14 in. of 12 G. (0·104 in.) stainless steel wire, formed into a loop at one end, leaving a straight length 12 in. long, and notched at the other end to hold the ribbon gauze; and
2 in. wide unmedicated ribbon gauze.

3. *Preparation of Swab*

The swab shall be 2 in. in length and shall consist of 6 in. of the gauze wound round the notched end of the wire and secured with thread.

4. *Sterilization of Swab*

Place the swab in 25 ml quarter-strength Ringer's solution in the test-tube, plug with cotton wool or suitable rubber closure, cover the plug with greaseproof paper and sterilize by autoclaving at 121° for at least 15 min. To obtain a final quantity of 25 ml quarter-strength Ringer's solution, it is necessary to start initially with a larger quantity to allow for evaporation during autoclaving. The actual quantity must be found by trial and error with each individual autoclave.

5. *Swabbing Technique*

Where possible, an area of 1 sq. ft shall be examined. Press the swab with a rolling motion against the side of the test-tube to remove excess liquid. Remove the swab, and with heavy pressure rub back and forth over the area to be examined so that all parts of the surface are treated twice. Rotate the swab so that all parts of it make contact with the surface under test. Return the swab to the test-tube and insert cotton-wool plug or rubber closure.

6. *Testing Swab Samples*

Swab samples should be tested as soon as possible and in any event within 6 hr of sampling. The tests normally applied are the same as those for rinses. After not less than 5 min contact of swab and liquid, mix the sample by twirling the swab vigorously in the Ringer's solution six times. Remove the swab, taking care to express the liquid by pressing against the side of the tube. After thorough mixing by rotation between the palms of the hands, prepare 1 ml and 0·1 ml Petri dishes and incubate at 30±1° for 72±2 hr. If desired, the coli-aerogenes, thermoduric organisms, and milk spoilage organisms tests can be applied in the same manner as for rinse samples.

Record the colony count as the count per sq. ft of surface tested (Colony count per ml × 25 × area factor if not 1 sq. ft).

Notes

1. Swabs may be sterilised and used again, but swabs which have been used for very greasy surfaces shall not be used more than once.
2. When sodium hypochlorite or other approved chlorine compounds have been used for sterilization, crystalline sodium thiosulphate shall be added to the Ringer's solution for swabbing before autoclaving so as to give a concentration of 0·05 per cent, i.e., 0·5 g crystalline sodium thiosulphate to each litre of Ringer's solution, or 1 ml of a 25 per cent solution of sodium thiosulphate.
3. When a detergent-sterilizer containing a quaternary ammonium compound has been used for cleansing, 0·5 ml of a 4 per cent lecithin and 6 per cent Lubrol W. solution in distilled water should be sterilized, and added to the swab solution after rinsing.
4. Swab solutions containing sodium thiosulphate may be used for swabbing surfaces cleansed with detergent sterilizers containing a quaternary ammonium compound, but the lecithin-Lubrol W solution must be added as indicated above.
5. Stainless steel wires may show a tendency to rust when kept in quarter-strength Ringer's solution containing 0·05 per cent sodium thiosulphate. If FMB stainless steel wires are used no rusting should occur.

50. EXAMINATION OF WASHED MILK CANS

1. *General*

The examination is designed to give information on the condition of cleansed cans before leaving the buyer's premises, or in connection with advisory

work on the farm. When examined at the buyer's premises to assess the efficiency of the can-cleansing, the cans should be examined within an interval of not less than ½ hr and not more than 1 hr after treatment.

The cans shall be examined by a rinsing technique. Any cans with open seams or containing milky water or easily removable milk solids as distinct from hard scale shall be regarded as unsatisfactory without testing.

2. *Rinsing Technique*

Pour 500 ml of sterile quarter-strength Ringer's solution into the lid and then into the can. Replace the lid. Lay the can on its side on a clean floor or on a can-roller and roll to and fro so that it makes 12 complete revolutions. Allow the can to stand upright for 5 min and then repeat the rolling. Pour the rinse solution from the can into the lid and then into the original bottle. In transferring the solution to the original bottle as much as possible of the 500 ml should be collected.

3. *Testing*

Rinse samples shall be tested for colony count as soon as possible, and in any event within 6 hr of sampling. Mix the sample by inverting the bottle slowly three times. Inoculate 1 ml and 0·1 ml of the rinse into Petri dishes, and to each dish add 10 ml of Yeastrel milk agar. Incubate at 30±1° for 72±2 hr. Record the results as the colony count per can (colonies per ml of rinse × 500).

51. EXAMINATION OF WASHED MILK BOTTLES

The method outlined is designed to give information on the condition of bottles immediately after washing. The number of bottles examined at each test is left to the discretion of the bacteriologist concerned, but it is suggested that at least four selected at random should be tested. When a machine is giving occasional unsatisfactory results it may be necessary to test a complete row of bottles.

1. *Sampling*

Bottles for examination should be selected immediately after washing and closed with a sterile rubber bung. Rubber bungs should be wrapped in greaseproof paper and sterilized in the autoclave for 15 min at 121°. After closing the bottle with the bung, the greaseproof paper should be used as a cover over the bung and neck of the bottle, being held in position by a rubber band. Bottles shall be taken to the laboratory and examined within 6 hr.

2. *Rinsing*

Twenty millilitres of sterile quarter-strength Ringer's solution shall be added to the bottle and the bung replaced. This amount shall be used irrespective of the size of the bottle. Where bottles are taken from a hot section of a machine for special purposes, they shall be fitted with a bung and allowed to cool before rinsing.

Sodium thiosulphate shall be added to the Ringer's solution before autoclaving to give a concentration of 0·05 per cent, i.e., 0·25 g crystalline sodium thiosulphate to each 500 ml of Ringer's solution.

When a detergent-sterilizer containing a quarternary ammonium compound has been used for cleansing, 0·5 ml of a 4 per cent Lecithin and 6 per cent Lubrol W solution in distilled water should be sterilized, and added to the solution after rinsing.

The bottle shall be held horizontally in the hands and rotated gently 12 times in one direction so that the whole of the internal surface is thoroughly wetted. Allow the bottle to stand for not less than 15 and not more than 30 min and again gently rotate 12 times so that the whole of the internal surface is thoroughly wetted.

3. *Testing*

Inoculate 5 ml amounts of rinse into each of two Petri dishes. Pour 10 ml Yeastrel milk agar into each Petri dish and incubate at 30±1° for 72±2 hr. before counting.

Additional information may be obtained by testing for the presence of coli-aerogenes bacteria and for milk spoilage organisms.

For the coli-aerogenes test it will generally suffice to inoculate 5 ml of rinse into 5 ml of double-strength MacConkey's broth incubated at 30±1° for 72±2 hr.

Testing for milk spoilage organisms can be done by inoculating quantities of 1 ml (and if desired, 0·1 ml) rinse into tubes of 5 ml sterile skim milk containing litmus or bromo-cresol purple and incubating at 22±1° for 72±2 hr.

4. *Recording Results*

The results shall be recorded as the colony count per bottle, i.e., the sum of the counts on the two plates multiplied by two.

5. *Interpretation of Results*

Colony Count per bottle	*Classification*
Not more than 200	Satisfactory (S)
Over 200 to 1,000	Could be improved
Over 1,000	Unsatisfactory (US)

52. THE DETECTION OF MILK SPOILAGE ORGANISMS (M.S.O.) IN RINSE AND SWAB SOLUTIONS

1. *Introduction*

The M.S.O. test was introduced by Mattick and Hoy (1937) who used it during their investigations on bottle washing. Hoy and Rowlands (1948) also used this test for the examination of rinses of milking machine clusters and Thomas and Druce (1961) have described the various modifications of the test which have been used for the examination of rinses of farm dairy equipment.

Brief descriptions of the M.S.O. test (72 hr. at 22°) are given in previous Techniques. A more detailed account of the test and interpretation of results is given in this Technique.

2. *Method*

(*a*) Inoculate 1 ml and higher dilutions of rinse or swab solution if required into separate tubes of 5 ml sterile skim milk containing litmus (see Technique No. 13) or bromo-cresol purple indicator to give a final concentration of 0·004 per cent. Mix by rolling the tube between the hands. Set up an uninoculated control tube with each batch of tests.

(*b*) Incubate at 22° and record the reactions in 24, 48 and 72 hr. A detailed scheme for recording reactions of pure cultures of bacteria in litmus milk is given in Technique No. 13, but the following will suffice for the M.S.O. test:

Acid clot	AC
Acid clot with entrapped gas bubbles	ACG
Acid only	A
Digestion (proteolysis) . . .	D
Reduction only	R
No change	NC

3. *Interpretation of Results*

This test enables the presence of milk souring or casein digesting bacteria to be detected in rinses or swabs of dairy equipment, i.e., it gives useful additional information to that provided by the colony count and coli-aerogenes test. It thus detects not only streptococci, coli-aerogenes organisms, staphylococci and other types capable of producing acid from lactose, but also actively proteolytic types of Gram-negative rods and aerobic spore-formers. There are three main categories of results which may be used for advisory purposes.

Reactions	*Indicating presence of*:
Acid clot within 24 or 48 hr.	Rapid milk souring organisms
Either acid clot or acid only within 72 hr.	Milk souring organisms
Either acid clot, acid only or digestion within 72 hr.	Milk spoilage organisms

REFERENCES

MATTICK, A. T. R. and HOY, W. A. (1937). *Bottle Washing and Bottle Washing Machines* Reading: National Institute for Research in Dairying.

HOY, W. A. and ROWLANDS, A. (1948). *Proc. Soc. appl. Bact.* 40.

THOMAS, S. B. and DRUCE, R. G. (1961). *Dairy Inds.* **26**, 565.

Chemical Tests

53. THE DETERMINATION OF TITRATABLE ACIDITY OF MILK

1. *Apparatus*

1 Burette fitted with a soda-lime guard tube
1 Pipette to deliver 10 ml milk
2 Pipettes to deliver 1 ml
1 Glass stirring rod
2 White porcelain basins (60 ml capacity)
1 Fluorescent lamp as in B.S.950 (If test cannot be made in north daylight).

2. *Reagents*

(*a*) N/9 sodium hydroxide solution (carbonate-free).

(*b*) 0·5 per cent neutral phenolphthalein. Dissolve 1 g of phenolphthalein in 110 ml of ethanol (95 per cent v/v in water), and add 80 ml distilled water. Add sodium hydroxide solution (app. 0.1N) drop by drop until one drop gives a faint pink colouration. Make up to 200 ml with distilled water.

(*c*) Rosaniline acetate solution. Dissolve 0·12 g of rosaniline acetate in 50 ml ethanol (95 per cent v/v in water) to which 0·5 ml glacial acetic acid has been added. Dilute to 100 ml with 95 per cent ethanol. Dilute 1·0 ml of this solution to 500 ml with a 1:1 mixture of 95 per cent ethanol and water. Both solutions should be stored in the dark, in brown bottles securely closed with rubber stoppers.

3. *Procedure*

Pipette 10 ml of the milk into each of the two basins. To one, add 1 ml of the dilute rosaniline acetate to act as a colour control. To the other add 1 ml of the phenolphthalein solution, and add rapidly 1 ml of the N/9 sodium hydroxide from the burette. Continue the addition drop by drop until the colour matches the pink tint of the control. Stir the milk vigorously throughout the titration, which should be completed in 20 sec. The titration should be carried out in north daylight or under illumination from the fluorescent lamp.

Express the acidity as 'lactic acid' g/100 ml milk by dividing by 10 the number of millilitres of N/9 sodium hydroxide solution required.

If the rosaniline colour control is not used, the end-point is determined by adding the sodium hydroxide drop by drop with constant stirring until the milk assumes a faint pink colour which persists for 5 sec.

54. THE ESTIMATION OF PENICILLIN IN MILK (T.T.C. METHOD)

The presence of residual penicillin in milk can give rise to problems in the dairy industry, particularly in the manufacture of cheese and in the produc-

tion of yoghourt. The present test provides a simple yet sensitive routine method for the estimation of penicillin.

Other antibiotics and inhibitory substances may also be detected by the test, but the presence of penicillin can be established if the inhibitory effect is removed by adding penicillinase.

The test, known as the T.T.C. (2–3–5 triphenyl-tetrazolium chloride) test, depends on the fact that the growth of a sensitive organism—*Streptococcus thermophilus* 'BC'—is inhibited by traces of penicillin, the inhibition being indicated by the failure of the T.T.C. to change from colourless to the red colour of formazone. The concentration of antibiotic in the milk is thus inversely proportional to the intensity of the red colour produced on incubation and an estimate of the amount of penicillin present can be obtained by matching the colour against prepared standards using Lovibond disc 4/22.

1. *Apparatus*

10 ml sterile reductase tubes and bungs
5 ml sterile graduated pipettes
1 ml sterile graduated pipettes
1 ml sterile blow-out pipettes
Water bath at 44–45°
All-purpose Lovibond comparator with stand and disc 4/22

(The disc contains four standards marked 0, 1, 2 and 3 respectively, corresponding to the colours of standards prepared according to Liska and Calbert, 1958).

2. *Materials*

(*a*) T.T.C. solution. 1 per cent in distilled water in brown glass bottles as it is sensitive to light. Store in refrigerator (3–5°) and renew monthly.

(*b*) Penicillinase solution. Dissolve contents of one vial of A.V.M. Penase (100,000 units) in 100 ml of distilled water to give a working solution of 1,000 units/ml. Store in refrigerator (3–5°) and renew fortnightly.

(*c*) Penicillin solution. Benzylpenicillin tablets (B.D.H.) each containing 15,000 I.U. Dissolve one tablet in 150 ml distilled water. Store this stock solution in refrigerator (3–5°) and renew monthly. To prepare positive controls dilute 1 ml of stock solution to 100 ml with distilled water and use 1 ml of this diluted solution (1 ml=1 International Unit).

(*d*) Penicillin-free sterilized milk. This is prepared by sterilizing in the autoclave at 121° for 15 min pre-tested penicillin-free raw milk. Alternatively, use sterilized milk pre-tested for freedom from penicillin.

(*e*) Test culture of *Str. thermophilus* 'B.C.' Supplied as a freeze-dried culture. Open the vial under aseptic conditions and use the culture after two subcultures in the penicillin-free sterilized milk (see Note 1).

3. *Procedure*

(*a*) If possible milk should be tested within 2 hr of sampling. Failing this, the sample should be cooled immediately to 3–5° and maintained at this temperature until tested, which should not be later than 24 hr after sampling. Not more than nine samples should be tested in any one batch.

(*b*) Pipette 5 ml of the sample into each of two test-tubes and make up to the 10 ml mark with penicillin-free sterilized milk. Return the remainder of the sample to the refrigerator until the results are known. To one tube add 0·2 ml of the penicillinase working solution (control tube). Inoculate each tube with 1 ml of *Str. thermophilus* culture (see Note 2), stopper with sterile rubber bungs, mix by inverting twice and place in the water bath at 44–45°.

(*c*) After incubating for 1½ hr add 1 ml of T.T.C. solution to each tube, mix by inverting and replace in the water bath for a further 1 hr.

(*d*) Remove tubes from water bath, invert once and place in succession in right-hand compartment of comparator stand. Place in the left-hand compartment as a blank, a similar test-tube containing 10 ml of milk diluted to the same degree with the same sterilized milk as the sample but not incubated.

(*e*) Match the colour of the incubated sample against the permanent glass standards in the disc, using north daylight and record to the nearest half disc. Tubes should be read as quickly as possible, avoiding exposure to strong light.

Table giving colour intensities developed in the presence of various concentrations of pencillin

Penicillin I.U./ml of milk	T.T.C. disc reading after 2½ hr incubation at 44-45° Samples diluted with penicillin free milk				
	1:1 (Penase control)	1:1	1:4	1:19	1:99
Nil	3	3	3	3	3
0·01	3	2½	3	3	3
0·02	3	1½	3	3	3
0·03	3	<1	2½	3	3
0·05	3	0	1½	3	3
0·075	3	0	<1	2½	3
0·10	3	0	0	2	3
0·20	3	0	0	1	3
0·30	3	0	0	<1	2½
0·50	3	0	0	0	2
1·0	3	0	0	0	1
1·5	3	0	0	0	<1

4. *Interpretation of Results*

(*a*) If both the sample and the penicillinase treated control tube match disc reading 3 after incubation, then no penicillin is present in the sample.

(*b*) If the penicillinase treated control matches disc 3 and the sample matches or lies between one of the disc readings 1–3, then the penicillin content can be obtained from the table following para. 3(*e*).

(*c*) If the sample has a colour disc 1 then the test should be repeated using a dilution of 1:4, 1:19 or 1:99 as necessary, so that the colour falls within the disc range of 1–3. Prepare similar dilutions as blanks for use in the comparator.

(*d*) If the colour of the control tube falls below that of disc 3 after incubation, then either the activity of the penicillinase has decreased or an antibiotic other than penicillin is present. The penicillinase activity should be checked by the method given in para. 5, and if found to be normal, then another inhibitory substance is present.

5. *Checking Reagents*

With each batch of samples tested, set up an extra tube containing sterilized milk only and inoculate with the test organism. Carry out the test as described above under para. 3 (*b*) and (*c*). If the colour of this tube after incubation is below disc 3, then the activity of the organism is below standard and a new freeze-dried culture should be used.

Weekly checks to test the activity of the organism and the efficiency of the penicillinase solution should be carried out as follows:

(*a*) add 1 ml of penicillin solution containing 1 I.U. to 25 ml of the sterilized penicillin-free milk and mix well. This milk now contains approximately 0·04 I.U./ml. Label three sterile test-tubes 1, 2 and 3 respectively. Add 0·2 ml of penase solution to tube 2. Pipette 5 ml of the sterilized milk containing 0·04 I.U./ml of penicillin into tubes 1 and 2 and 2 ml into tube 3. Make each tube up to the 10 ml mark with penicillin-free sterilized milk, inoculate with 1 ml of the culture and incubate as in para. 3 (*b*) and (*c*);

(*b*) after incubation, tube 1 should show no activity (disc 0), tube 3 should show some activity (disc 2 approx.) while tube 2 which contains added penicillinase should show good activity (disc 3);

(*c*) low or no activity in tube 2 indicates that the penicillinase is unsatisfactory, always provided the activity of the culture is normal. High activity in all three tubes shows that either the penicillin and/or the culture are unsatisfactory in which case they should be renewed. Low activity in all three tubes shows the culture to be unsatisfactory, provided that the bath temperature has been correctly maintained and the sterilized milk is satisfactory.

Notes

1. To transfer the freeze-dried culture to milk, immerse the tube containing the culture in 1 per cent hypochlorite and lay it on a square of muslin soaked in the same solution. With a glass cutting file nick the tube across about 1 in. below the sealed tip and, holding it over a gas flame, snap the tube open, keeping the open end of the tube close to the flame. With a sterile 1 ml pipette fill the tube almost to the brim with sterile quarter-strength Ringer's solution by placing the tip of the pipette on the inner edge of the tube and allowing the solution to flow in slowly. Then, holding the open end of the tube close to the flame, give the tube several sharp twists to dislodge the particles of culture and assist their solution. Using an aseptic technique, transfer the contents of the tube to a bottle containing about 10 ml penicillin-free sterilized milk, by placing the lip of the tube on the inner surface of the bottle. Mix in the in-

oculum and incubate for 24 hr at 37°. At the end of this period subculture into penicillin-free sterilized milk at the rate of 1 drop of culture to 10 ml milk and again incubate for 24 hr at 37°. This culture should then be subcultured as described in Note 2 following.

2. Propagation of *Str. thermophilus* 'B.C.' should be carried out daily by inoculating penicillin-free sterilized milk at the rate of one drop per 10 ml and incubating overnight at 37° or over the weekend for 48 hr at 30°. If propagation cannot be carried out for several days, inoculate penicillin-free sterilized milk with 10 per cent of the clotted culture and store at 3-5°. On the day before the culture is required, the inoculated milk is transferred from the refrigerator into the water bath until clotted. From this culture, 50 ml is inoculated at the usual rate, followed by overnight incubation at 37°. This will provide sufficient inoculation for 10 samples. The test organism must always be cultured in penicillin-free milk. In the case of loss of activity open a new freeze-dried culture.

REFERENCES

1. WRIGHT, R. C. and TRAMER, J. (1961), *J. Soc. Dairy Technol.*, **14**, 85.
2. LISKA, B. J. and CALBERT, H. E. (1958), *J. Dairy Sci.* **41**, 776.

55. PHOSPHATASE TEST FOR PASTEURIZED MILK

1. *Sample*

Milk samples should be examined as soon as possible after arrival at the laboratory. If they are not examined immediately on arrival at the laboratory, they shall be kept at a temperature of between 3° and 5° until examined. All samples shall be raised to room temperature immediately before being tested.

2. *Precautions*

The following precautions shall be taken:

(*a*) samples which are tainted or clot on boiling shall not be tested;
(*b*) all glassware shall be chemically clean before use;
(*c*) a fresh pipette shall be used for each sample of milk. Pipettes shall not be contaminated with saliva;
(*d*) tests shall not be carried out in direct sunlight;
(*e*) distilled water shall be used throughout.

3. *Reagents*

Whenever possible reagents of recognized A.R. quality should be used.

(*a*) Buffer solution: dissolve 3·5 g of anhydrous sodium carbonate and 1·5 g of sodium bircarbonate in distilled water, and make up to one litre;
(*b*) Substrate: disodium p—nitrophenyl phosphate. The solid subtrate shall be kept in a refrigerator;
(*c*) Buffer-subtrate solution: place 0·15 g of the substrate into a 100 ml measuring cylinder, and make up to 100 ml with the buffer solution. The solution shall be stored in a refrigerator and protected from light. It shall give a reading of less than the standard marked 10 on the comparator disc A.P.T.W. or A.P.T.W.7 when viewed in transmitted light through a 25 mm cell in the 'all purposes' comparator, using distilled water for comparison. The solution shall not be more than 1 week old.

4. *Apparatus*

A Lovibond 'all purposes' comparator complete with stand for work in reflected light

A Lovibond comparator disc A.P.T.W. or A.P.T.W.7

Two fused glass cells, 25 mm depth

A water bath or incubator that can be maintained at $37{\cdot}5 \pm 0{\cdot}5°$

A pipette to deliver 5·0 ml

A supply of 1·0 ml straight sided pipettes of an accuracy equal to that of N.P.L. grade B

A 1,000 ml graduated flask

A 100 ml measuring cylinder

A supply of test-tubes conforming to B.S.625: 1959, 150 × 16 mm, nominal 6 × $\frac{5}{8}$ in., having an internal diameter of $13{\cdot}5 \pm 0{\cdot}7$ mm and being accurately marked at 10 ml. The test-tubes should have rubber stoppers to fit.

5. *Care of Apparatus*

(*a*) After use each test-tube shall be well washed in hot water containing soda, rinsed in hot clean water and then washed with 50 per cent commercial hydrochloric acid. The acid wash shall be carried out by filling one tube with the acid and passing it from tube to tube, the acid being replenished when necessary. The tubes shall then be well rinsed again in hot clean water, then in distilled water and finally dried.

(*b*) New glassware shall be cleaned in chromic acid prepared in the following manner:

dissolve 90 g of powdered potassium dichromate in 200 ml of hot water contained in a four litre conical flask. Cool, add two litres of commercial sulphuric acid (not less than 90 per cent). Stir until the precipitate has dissolved. Keep the solution covered and discard when it becomes green. After cleaning in chromic acid, new glassware shall be well rinsed in hot water, then rinsed in distilled water and finally dried.

(*c*) Pipettes shall be well rinsed in cold clean water and shall be cleaned by soaking for 24 hr in chromic acid solution in a 250 ml glass cylinder or other suitable container. They should be then well rinsed in warm water, rinsed in distilled water and dried.

(*d*) Glassware used for the test shall not be used for any other purpose, and shall be kept apart from all other apparatus in the laboratory.

6. *Method of Carrying Out the Test*

The test shall be carried out in the following manner:

pipette 5 ml of the buffer-substrate solution into a test-tube, stopper, and bring to 37°. Add 1 ml of the milk to be tested, stopper and mix well. Incubate for exactly 2 hr at 37°. Incubate with each series of test samples one blank prepared from boiled milk of the same type as those undergoing the test. (With highly coloured milk, such as homogenised milk or milk from Channel Island cows, a separate blank shall be prepared). After incubation for 2 hr, remove the test-tubes from the water bath, and mix well. Place the blank on the left hand ramp of the comparator stand and the test sample on the right. Take readings in reflected light by looking down on to the two apertures with the comparator facing a good

source of daylight (preferably north light). If artificial light is needed for matching, a 'daylight' type of illumination must be used. Revolve the disc until the test sample is matched; record readings falling between two standards by affixing a plus or minus sign to the figure for the nearest standard.

7. *Interpretation*
The test shall be deemed to be satisfied by milk which gives a reading of 10 u.g. or less of p-nitrophenol/ml of milk.

REFERENCE

The Milk (Special Designation) Regulations (1963). *S.I.* (1963) *No.* 1571. London, H.M.S.O.

56. TURBIDITY TEST FOR STERILIZED MILK

1. *Examination*
Samples may be examined at any time after delivery to the laboratory, but shall be at room temperature when the test is begun.

2. *Reagent*
Ammonium sulphate A.R. shall be used.

3. *Apparatus*
A supply of conical flasks, 50 ml capacity
A supply of graduated cylinders, 25 ml capacity
A supply of test-tubes conforming to B.S.625: 1959, 150 × 16 mm, nominal 6 × $\frac{5}{8}$ in., having an internal diameter of 13·5±0·7 mm and being accurately marked at 10 ml
A supply of filter funnels, 6 cm diameter
Two beakers, 400 ml capacity
A supply of Whatman folded filter papers, 12·5 cm No. 12

4. *Method of Carrying Out the Test*
The test shall be carried out in the following manner:
weigh 4±0·1 g of ammonium sulphate into a 50 ml conical flask. Measure out 20±0·5 ml of the milk sample and pour into the conical flask. Ensure that the ammonium sulphate dissolves by shaking for 1 min. Leave for not less than 5 min and then filter through a folded paper into a test-tube. When not less than 5 ml of a clear filtrate have collected, place the tube in a beaker of water, which is kept boiling, and keep it therein for 5 min. Transfer the tube to a beaker of cold water and, when the tube is cool, examine the contents for turbidity by moving the tube in front of an electric light shaded from the eyes of the observer.

5. *Interpretation*
The test shall be deemed to be satisfied when a sample of milk treated as in para. 4 above gives a filtrate showing no sign of turbidity.

REFERENCE

The Milk (Special Designation) Regulations (1963). *S.I.* (1963) *No.* 1571. London. H.M.S.O.

57. DETECTION OF CHLORATE IN MILK

1. *Introduction*

All chlorine compounds approved for the cleansing of dairy utensils are required to contain chlorate as a tracer.

The test cannot be made quantitative on the basis of a constant content of chlorate because, although a minimum of 0·7 per cent sodium chlorate must be present in hypochlorite on despatch from the manufacturer, the amount slowly increases with storage.

2. *Reagents and Apparatus*

The reagents used shall be of recognised A.R. quality.

73·5 per cent sulphuric acid containing 0·025 per cent stannous chloride
To prepare—Dissolve 0·025 g of stannous chloride in 25 ml water and add carefully 75 ml of concentrated sulphuric acid.

6 × ¾ in. test-tubes. B.S.625: 1959

12·5 ml centrifuge tubes

All glassware shall be chemically clean.

3. *Method of Testing*

To 3 ml of milk in a 6 × ¾ in. test-tube cooled to 0–5° add 3 ml of reagent, (sulphuric acid containing stannous chloride) also cooled to 0—5°. Agitate the tube thoroughly in a freezing mixture of ice and salt, and allow to stand in the mixture for 3 min. Transfer the contents to a 12·5 ml centrifuge tube and spin for 3 min at approximately 2,500 r.p.m. Examine the tubes immediately in ultra-violet light from a mercury vapour lamp fitted with a Wood's filter, for the presence of any yellow fluorescence. (Examination should be carried out in a dark room or cupboard.)

Notes

1. The temperature of the freezing mixture should not be allowed to fall so low that the contents of the tubes begin to freeze. (It is important to prevent the temperature rising above 5° to avoid charring.)
2. Two control tubes should be set up for each batch of samples: one from a milk supply known to be free from sodium hypochlorite; the other from milk containing sufficient fresh approved hypochlorite to give an approximate concentration of 20 p/m available chlorine.
3. It is possible to detect 1 part sodium chlorate in $2·5 \times 10^{-6}$ parts of milk or 0·0004 mg per ml of milk.
4. Milk which gives a positive test when fresh may give a negative one after storing, but the apparent decrease in chlorate content is not appreciable until the milk gives a positive alcohol precipatation test.

REFERENCE

WRIGHT, R. C. and ANDERSON, E. B. (1938). *The Analyst*, **63**, 252.

58. DETECTION OF SMALL AMOUNTS OF QUATERNARY AMMONIUM COMPOUNDS (Q.A.Cs) IN MILK AND RINSE WATER

This method is both qualitative and quantitative; the presence of about 1 p/m (1 mg/litre) Q.A.C. is detectable. For a more accurate quantitative estimation of Q.A.C. in milk, the procedure given in Official Methods of Analysis, A.O.A.C. (see Ref. 2) should be followed.

1. *Reagents*

(*a*) Tetrachloroethane, technical grade. Store in a brown glass bottle in the dark.

(*b*) Lactic acid solution, 50 per cent w/w. Dilute 57 g A.R. lactic acid (about 88 per cent) to 100 g using distilled water.

(*c*) Eosin solution, 0·05 per cent. Dissolve 25 mg eosin (tetrabromofluorescein) in 50 ml distilled water.

(*d*) Sodium hydroxide solution, 4N. Dissolve 32 g NaOH in distilled water and dilute to 200 ml.

(*e*) Citric acid buffer solution pH 4·5. Dissolve 25 g citric acid in 75 ml distilled water and adjust to pH 4·5 by adding 50 per cent w/w NaOH solution (about 13 ml).

(*f*) Anionic reagent, 0·0001 N. Prepare a 1 in 10 dilution of the anionic reagent, 0·001 N sodium dioctyl sulphosuccinate, described under 'Estimation of Q.A.C.s in Detergent-Sterilizer Solutions' (see Technique No. 63).

2. *Procedure*

(*a*) *Milk*

Place 5 ml tetrachloroethane, 2 ml lactic acid solution and 5 ml milk sample in a test-tube (B.S.625: 1959) fitted with a stopper. (Rubber stoppers should not be used.) Shake vigorously for 1 min. Add 2 ml 4N NaOH solution, invert six times, shake for 30 sec. Transfer to a suitable centrifuge tube fitted with a stopper. (Rubber stoppers should not be used.) Centrifuge at 3,200 rev/min in a 10 in. machine (or other type machine giving equivalent force) for 5 min. Three distinctive layers should be formed. Decant or aspirate off the top aqueous layer which should be clear. Loosen the middle layer, consisting chiefly of white precipitated protein, with a thin glass rod and pour or pipette off as much as possible of the bottom layer, which consists of clear tetrachloroethane containing any Q.A.C. originally in the milk sample, to another test-tube. Transfer 2 ml of this tetrachloroethane to a clean test-tube containing 0·5 ml citric buffer solution and 0·2 ml eosin solution. Close the tube and shake vigorously for a few seconds. A pink-to-red colour in the tetrachloroethane indicates the presence of Q.A.C. Samples of milk containing about 1 p/m Q.A.C. develop a faint pink colour, which can be distinguished from a control prepared by using milk known to be free from Q.A.C.

If deep pink or red is observed, an approximate estimation of the amount of Q.A.C. present can be made by titrating with anionic reagent (see para.1 (*f*)) until the pink colour disappears (see procedure for Rinse Water). The titration found represents the quantity of Q.A.C. in 2 ml sample.

(*b*) *Rinse Water*

Place 5 ml rinse water sample in a glass stoppered test-tube containing 2 ml tetrachloroethane, 2 ml citric buffer and 0·5 ml eosin solution. Shake vigorously. The development of a pink or red colour in the tetrachloroethane layer indicates about 0·5 p/m or more Q.A.C.

Estimate the amount of Q.A.C. present by adding anionic reagent (see para. 1 (*f*)) drop by drop, shaking vigorously after each addition until a colourless end point is reached.

Calculate the concentration of Q.A.C. present as below:

1 ml anionic reagent 0·0001 N $= \frac{M}{20}$ p/m Q.A.C. in 2 ml sample

$= \frac{M}{50}$ p/m Q.A.C. in 5 ml sample

where M is equivalent wt (normally = molecular wt of Q.A.C.)

An alternative method for the determination of small amounts of Q.A.C. in water is given under 'Estimation of Q.A.C.s in Detergent-Sterilizer Solutions' (see Technique No. 63).

REFERENCES

1. FURLONG, T. E. and ELLIKER, P. R. (1953). *J. Dairy Sci.* **36**, 225.
2. Association of Official Agricultural Chemists (1955). *Official Methods of Analysis*, 8th Ed., 502. Washington D.C.

59. THE DETECTION OF IODOPHOR IN MILK AND RINSE WATER

This method is qualitative; it cannot be used for the quantitative estimation of free iodine. It is possible to detect 5–10 p/m (mg/litre) iodine in milk and about 1 p/m (mg/litre) iodine in water.

1. *Reagents*
   - (*a*) Concentrated hydrochloric acid, sp. gr. 1.16.
   - (*b*) 0·5 per cent w/v 7:8–benzoflavone (a-napthoflavone) in a mixture of equal volumes of ethanol (ethyl alcohol) 95 per cent v/v and ether. The solution should be stored in the refrigerator and be discarded when it has become distinctly yellow.
   - (*c*) 20 vol. (6 per cent) hydrogen peroxide.

2. *Method*
   - (*a*) *Milk*

     Cool 1 ml of milk in a 6 × $\frac{5}{8}$ in. test-tube in iced water. Add 1 ml hydrochloric acid, shake to mix and replace in iced water. Add five drops benzoflavone solution followed by four or five drops of the hydrogen peroxide and shake to mix. A lilac or blue colour developing within a few seconds indicates the presence of more than 5 p/m iodine. A control using milk known to be free from iodophor should be prepared at the same time for comparison.

   - (*b*) *Rinse Solution and Water*

     To 5 ml of the sample add 0·25 ml hydrochloric acid, and five drops of the hydrogen peroxide. Shake to mix and add five drops of the benzoflavone solution. A lilac or blue colour developing within a few seconds indicates the presence of about 1 p/m iodine or more. A control using tap or distilled water should be prepared at the same time for comparison.

REFERENCE

1. EISSES, J. and VRIES, H. de (1959). *Off. Org.K. ned. Zuivelb.* **51**, (47) 1009.

60. ESTIMATION OF AVAILABLE CHLORINE IN SOLUTIONS OF SODIUM HYPOCHLORITE

This method can be used for undiluted stock solutions, e.g., approved sodium hypochlorite. A titration with sodium arsenite is the official method used for the analysis of approved hypochlorites, but titration with standard sodium thiosulphate solution is sufficiently accurate for most purposes and is more convenient.

1. *Apparatus and Reagents*

50 ml burette
10 ml volumetric pipette
10 ml graduated pipettes
25 ml volumetric pipette
500 ml volumetric flask
100–200 ml conical flasks
N/10 sodium thiosulphate solution
10 per cent w/v potassium iodide solution (Discard as soon as a yellow tinge is observed.)
50 per cent w/v acetic acid solution
1 per cent starch indicator solution (To 1 g soluble starch in a beaker add sufficient water to make a paste. Add 100 ml cold water, stir, and bring to the boil. Cool before use. The addition of 0·2 g thymol to the cold water will help to prevent mould growth.)

2. *Procedure*

Using the volumetric pipette, transfer 10 ml of the stock hypochlorite solution to the volumetric flask, make up to 500 ml and mix thoroughly.

Measure 10 ml acetic acid and 10 ml potassium iodide solutions into the conical flask and add 25 ml diluted hypochlorite solution measured with the volumetric pipette.

Titrate immediately with N/10 sodium thiosulphate solution to a pale yellow colour. Add two to three drops of starch solution and titrate until the blue colour disappears.

Note the volume of N/10 sodium thiosulphate solution used. (A ml). Now 1·0 ml N/10 sodium thiosulphate solution = 0·00355 g available chlorine. Therefore A ml = 0·00355 × A g available chlorine and available chlorine content of stock solution will be $0{\cdot}00355 \times A \times \frac{500}{25} \times \frac{100}{10}$ per cent w/v = 0·7 × A per cent w/v

If the per cent w/w is required, divide this figure by the specific gravity of the stock hypochlorite solution

$$\text{i.e., per cent w/w} = \frac{0{\cdot}7 \times A}{\text{S.G. of sample}}$$

A similar method can be used to determine the available chlorine content of more dilute hypochlorite solutions. For solutions expected to contain 200–300 p/m available chlorine, titrate 10 ml of the solution in the presence of 5 ml acetic acid and 5 ml potassium iodide with N/100 sodium thiosulphate solution as before. Let the titration figure be B ml.

Now 1 ml of N/100 sodium thiosulphate solution = 0·000355 g available chlorine. Therefore B ml = 0·000355 × B g available chlorine. Thus the available chlorine content of the solution will be

$$0{\cdot}000355 \times B \times \frac{10^6}{10} \text{ p/m}$$

or $35{\cdot}5 \times B$ p/m

61. A FIELD TEST FOR THE ESTIMATION OF RESIDUAL CHLORINE IN DAIRY EQUIPMENT STERILIZING SOLUTIONS

This method depends on the power of available chlorine to liberate iodine from potassium iodide with the production of a yellow or brown colour.

It is not suitable for the estimation of traces of free chlorine of the order of less than five p/m, but is very convenient for the checking of available chlorine in detergent-sterilizer solutions and hypochlorite rinsing solutions where the concentration of available chlorine is of the order of 50 to 250 p/m. It is thus a valuable test *for use on the farm* as the equipment required is simple and easy to carry.

1. *Apparatus*

Lovibond comparator *or* all-purposes Lovibond comparator
B.D.H. Lovibond disc 3/2 A.R.P. range 5–50 p/m chlorine
B.D.H. Lovibond disc 3/2 I range 5–250 p/m chlorine
Lovibond comparator test-tubes graduated at 10 ml
0·5 ml pipette.

2. *Reagents*

B.D.H. potassium iodide tablets (5 grains)
50 per cent w/v acetic acid solution

3. *Method of Testing*

Fill two graduated test-tubes to the 10 ml mark with the solution to be tested and place one in the left-hand compartment of the comparator. To the other add one potassium iodide tablet (5 grains) and 0·5 ml of 50 per cent w/v acetic acid solution and mix thoroughly. Place this tube in the right-hand compartment and compare the colour produced with the standards in the disc by rotating the disc until a colour match is obtained. The amount of available chlorine present is indicated in the aperture in the right-hand bottom corner of the comparator. Readings can be carried out immediately as there is no time lag in the reaction.

62. A FIELD TEST (PALIN'S) FOR THE PRESENCE OF RESIDUAL CHLORINE IN BACTERIOLOGICAL RINSES OF DAIRY EQUIPMENT

1. *Introduction*

Where milking plant has received a final hypochlorite rinse which cannot be completely drained off, the thiosulphate present in the bacteriological rinse solution may be insufficient to inactivate completely the residual

hypochlorite. Even a trace of residual chlorine will reduce markedly the numbers of viable bacteria collected by the rinse from the plant and will give a lower colony count than would have been obtained with excess thiosulphate.

2. *Reagents and Apparatus*

D.P.D. chlorine tablets No. 4 (or No. 1 and No. 3) (Palin test)
Two clean test-tubes marked at 10 ml and fitted with rubber bungs

3. *Method*

After rinsing the plant, and avoiding further contamination of the rinse solution, pour 10 ml into one of the test-tubes. This is the control tube. Rinse the second tube with a small quantity of the rinse solution, leaving a drop or two in the bottom of the tube. Drop into this tube one No. 4 tablet; alternatively one No. 1 and one No. 3 tablet may be used. Leave for about 1 min until the tablet(s) start(s) to disintegrate. Make up to the 10 ml mark with rinse solution, stopper, shake vigorously for about 2 sec, remove the bung and compare the tubes.

A pink colour developing within 2 min in the tube containing the tablet(s) indicates the presence of residual chlorine. If a pink colour is not immediately obvious the tubes should be viewed from above against a white background when a trace of colour, if present, can readily be detected in comparison with the control. As little as 0·1 p/m residual chlorine can be detected in this way and often 0·05 p/m if the colour is examined by looking vertically down into the sample rather than viewing it horizontally through the test-tube.

Notes

1. It is important to keep the tablets dry and to avoid handling them. Dispensing the tablets individually into small, clean, dry, well-stoppered containers in the laboratory is a great help for a test which has to be carried out on the farm. A ¼ oz screw-capped bottle is suitable to hold one No. 4 tablet or one No. 1 and one No. 3 tablet.
2. Residual chlorine present immediately after rinsing is likely to decrease slowly in concentration.
3. A chlorine content in excess of 10 p/m may give a slight pink colour while the tablets are disintergrating but within 2 min considerable fading may occur with the development of a pale yellow to brown colouration. Repetition of testing, using two No. 4 or two No. 1 and one No. 3 tablets, should show a marked increase in colour indicating that the solution should be diluted if an estimate of the chlorine content is to be made.

The following table gives a guide to the colour changes which may be observed in the Palin test and relates these to the residual chlorine content of the rinse solution:

Colour developing in 2 min	Residual chlorine in p/m
Very faint pink (just detectable)	0·05—0·2 } less than 1
Slight pink	0·25—0·5 }
Pink	1 } 1 to 10
Bright pink	2 }
Deep pink	5—10 }
Faint pink—pale yellow	10—25 } more than 10
Yellow	25—50 }
Deep yellow	100 }

63. ESTIMATION OF QUATERNARY AMMONIUM COMPOUNDS (Q.A.Cs) IN DETERGENT-STERILIZER SOLUTIONS

This method is applicable to solutions of detergent-sterilizers and sterilizers containing Q.A.C., the error is within ± 10 p/m Q.A.C.

The same reagents can be used for qualitative and quantitative estimation of small amounts of Q.A.C. in water (see para 2 (*b*))

1. *Reagents*

(*a*) Dichloroethane. Store in a brown glass bottle in the dark.

(*b*) Indicator solution:

Bromophenol blue . . .	0·04 g
Sodium chloride . . .	100·00 g
Sodium carbonate . . .	10·00 g

made up to 1,000 ml with distilled water.

(*c*) Anionic reagent, 0·001 N sodium dioctyl sulphosuccinate. Dissolve 0·4444 g of solid in distilled water and dilute to 1,000 ml. A fresh solution should be prepared every 2 months.

Note

Sodium dioctyl sulphosuccinate (100 per cent) may be obtained as Manoxol OT. This should be purchased in small quantities and kept dry.

2. *Procedure*

(*a*) *Detergent-Sterilizer and Sterilizer Solutions*

Add to a 25 ml glass stoppered measuring cylinder 3 ml dichloroethane and 5 ml indicator. Add by means of a calibrated pipette 2 ml anionic reagent. Add the Q.A.C. solution under test, shaking vigorously after each addition, until the dichloroethane layer changes from colourless to a clear blue. Read off the volume of Q.A.C. solution added. This is the total cylinder contents, less 10 ml.

For a closer estimation, the Q.A.C. solution should be added to the cylinder by means of a burette or calibrated pipette.

Calculate the concentration of Q.A.C. in the solution as below:

$$\frac{2M}{V} = \text{p/m (mg/litre) Q.A.C.}$$

where M is the equivalent weight (normally equal to the molecular weight of the Q.A.C.) and V is the volume of Q.A.C. solution added.

Where the molecular weight of the Q.A.C. is unknown, prepare a solution containing a known quantity of the original detergent-sterilizer, and titrate as above. From the titration a comparative figure for the concentration of the unknown solution can be obtained. If the known strength solution is made according to manufacturers' instructions then the result for the unknown sample can be quoted as the percentage of the strength when manufacturers' instructions are followed.

(*b*) *Water Containing Traces of Q.A.C.*

Add 50 ml water sample to a glass stoppered 100–150 ml conical flask containing 3 ml dichloroethane and 5 ml indicator. Shake vigorously. Allow

to stand until the dichloroethane separates out. A blue colour indicates the presence of about 0·1 p/m or more Q.A.C.

Dilute the anionic reagent (see para. 1 (*c*)) 1 in 10 to make 0·0001 N solution. Shake vigorously. Add 1·5 ml of this reagent and shake. Disappearance of the blue colour indicates that less than 1 p/m Q.A.C. is present.

If the blue colour persists, continue the addition of anionic reagent until the blue colour disappears.

Calculate the concentration of Q.A.C. as below:

$$1 \text{ ml } 0{\cdot}0001 \text{ N anionic reagent} = \frac{M}{500} \text{ p/m (mg/litre) Q.A.C.}$$

where M is the molecular weight of the Q.A.C.

REFERENCE

CHINNICK, C. C. T., and LINCOLN, P. A. (1954), *Lab. Prac.*, **3**, 364.

64. ESTIMATION OF IODINE IN DAIRY EQUIPMENT CLEANSING SOLUTIONS

This method is applicable to use-solutions of iodophors which are detergent-disinfectant solutions containing free iodine. At least 250 ml of the solution should be taken and protected from light.

1. *Apparatus and Reagents*

100 ml pipette
150–200 ml conical flask
Burette
N/100 sodium thiosulphate solution.

2. *Method*

Pipette 100 ml into a conical flask and titrate with N/100 thiosulphate solution to disappearance of the yellow colour.

Then 1 ml N/100 thiosulphate solution ≡ 12·7 p/m iodine in the sample.

Notes

1. Starch solution cannot be used as an indicator of the presence of iodine because the wetting agents in the iodophor solution may interfere with development of the characteristic blue colour.
2. The strength should be compared with that of a freshly prepared solution made up as recommended by the manufacturers.

65. DETERMINATION OF ALKALINITY AND ACIDITY OF DAIRY EQUIPMENT CLEANSING SOLUTIONS

Many solutions used in cleansing dairy equipment, i.e., most detergents, are alkaline in nature, while others, such as descaling solutions, are acidic in reaction. To determine whether the correct concentration of material is being used, a determination of the alkalinity or acidity of the used solution can be carried out.

1. *Apparatus and Reagents*

25 ml burettes and stand
10 ml volumetric pipettes
250 ml conical flasks
N/10 hydrochloric or nitric acid
N hydrochloric or nitric acid
N/10 caustic soda
Phenolphthalein solution
(1 per cent w/v in 50 per cent v/v alcohol)

2. *Procedure*

(*a*) *Alkaline Solutions*

Pipette 10 ml of the well-mixed sample into a 250 ml conical flask. Add five drops of the phenolphthalein solution and run in N/10 acid from the burette until the pink colour disappears. The solution in the flask should be well shaken while the titration is in progress.

Note the number of ml of acid required to neutralize the solution and multiply by 0·04 to obtain the percentage of available alkalinity expressed as caustic soda.

Example

10 ml of detergent solution required 6 ml of N/10 nitric acid for neutralization.

Then caustic alkalinity $= 0{\cdot}04 \times 6$
$= 0{\cdot}24$ per cent

It may be useful to compare the result with that obtained from a freshly made solution of the same product prepared according to the manufacturer's instructions.

When checking the strength of caustic soda immersion solution use normal (N) acid and multiply by 0·4.

Example

10 ml of immersion solution required 6·0 ml of N acid for neutralization to phenolphthalein end point.

Then caustic alkalinity $= 6 \times 0{\cdot}4$
$= 2{\cdot}4$ per cent

(*b*) *Acid Solutions*

Descaling solutions usually consist of solutions of inorganic acids such as nitric or phosphoric, or an organic acid such as gluconic. For most purposes it is convenient to express the acidity in terms of hydrochloric acid.

Pipette 10 ml of the well mixed descaling solution into a 250 ml flask, add five drops of phenolphthalein indicator and titrate with N/10 caustic soda until the appearance of a pink colour. Note the number of ml of N/10 caustic soda required and multiply by 0·0365 to obtain the percentage acidity in terms of hydrochloric acid.

Example

10 ml of descaling solution required 4 ml of N/10 caustic soda for neutralization to phenolphthalein end point.

Acidity expressed as hydrochloric acid $= 4 \times 0{\cdot}0365$
$= 0{\cdot}146$ per cent

It may be useful to compare the result with that obtained from a freshly prepared solution of the same product made up according to the manufacturer's instructions.

Note

Where the alkalinity of detergent-hypochlorite solutions is to be determined, the chlorine must first be destroyed by the addition of sodium thiosulphate. The addition of 1 ml N/10 sodium thiosulphate solution or 0·025 g of $Na_2S_2O_3.5H_2O$ (a few small crystals) is sufficient to inactivate 350 p/m available chlorine in 10 ml of the detergent solution. An excess of thiosulphate crystals (up to 0·25 g) will not affect the subsequent determination of alkalinity.

Examination of Dairy Farm Water Supplies

66. DIRECTIONS FOR SAMPLING WATER SUPPLIES FOR BACTERIOLOGICAL EXAMINATION

1. Sampling equipment consists of suitable sterile glass bottles of 20 oz capacity (containing sodium thiosulphate) in insulated containers, sterile dippers and well-sampling outfits. Sterile equipment is supplied by the laboratory on request. Prior to use the equipment should be stored in a clean, cool, dry situation.

2. Samples of water for bacteriological examination must be taken with great care to avoid extraneous contamination. Select sampling points carefully; never take unnecessary samples or samples of obviously polluted water.

3. When the source works and distribution system appears satisfactory, one sample taken from a distribution point, normally the dairy tap, may suffice. If it is suspected that the water is being polluted after it leaves the source, a sample from the source and another from a distribution point should be taken for comparison. Where there is definite evidence of pollution after the water leaves the source, take only one sample at the source on that particular visit. Check samples should always be taken from the point of use for the dairy before the supply is approved.

4. Ensure that the hands are clean before taking samples. Do not unwrap or open sampling equipment until needed and do not disturb the water to be sampled. The sample bottle should not be rinsed. When ready to collect the sample, unplug the bottle or remove the cap without touching the neck. Do not allow the cap to touch anything. Fill the sample bottle to the shoulder, replace the cap tightly, label the sample clearly and transfer to the insulated container as soon as possible.

5. When sampling from a tap or pump allow the water to run to waste for 2–3 min in order to flush the interior of the tap and discharge any stagnant water in the pipes. Collect the sample directly into the bottle without splashing. Do not allow waste water to run back into the well. Avoid sampling from hosepipes and also from cowshed taps if possible. If a hose pipe is connected to the only available tap, remove the hose, clean the tap orifice with sterile cotton-wool (obtainable from the laboratory), and flush thoroughly before sampling. The same cleaning treatment can be given to any tap or pump orifice which appears to be soiled. Normally there is no advantage in flaming the orifice although in some instances it may be advisable to do so.

6. When sampling from a collecting chamber, storage tank or reservoir, use a dipper or well-sampling outfit. Care must be taken not to contaminate the water when removing the cover. In some cases it may be more convenient to collect the sample from an outlet or overflow directly into the bottle.

7. When it is necessary to sample directly from a well, use a well-sampling

outfit. Lift the weighted, unplugged bottle from its container by means of the attached cord or chain without touching the bottle, and lower it carefully down the well so that it does not touch the sides. When the bottle is at water level, lower it quickly to about 2–3 ft below the surface and allow it to fill completely. Before capping the bottle pour off a little water so that the bottle is filled to the shoulder only.

8. When sampling from a stream, plunge the bottle completely below the surface mouth downwards and towards the current, and depress the base until the mouth is pointing slightly upwards. If no current exists the flow should be artificially created by moving the bottle slowly in a forward direction. Alternatively, a sterile dipper or well-sampling outfit may be used.

9. When assessing the efficiency of chlorination, it is advisable to take a sample of the untreated water in addition to the chlorinated sample. A sample of water can also be taken in a bottle not containing thiosulphate for the determination of residual chlorine.

10. The bacteriological condition of supplies can be influenced by weather conditions and, whenever practicable, at least one sample should be taken within a few days after a period of heavy rainfall. Contamination is normally at a minimum during the first 4 months of the year and, if possible, at least one sample should be taken outside this period.

11. As it is imperative that samples be examined before changes in the bacteriological condition of the water occur, they should arrive at the laboratory together with the Field Report with as little delay as possible. In any event they should arrive so that the examination can be commenced within 6 hr of sampling.

67. BACTERIOLOGICAL EXAMINATION OF SAMPLES OF WATER SUPPLIES

This technique is designed primarily for the examination of samples from private supplies used on farms, and is based on *The Bacteriological Examination of Water Supplies*, Ministry of Health Report No. 71 (1956), London: H.M.S.O.

Samples should be examined within 6 hr of sampling, and testing should commence immediately on arrival at the laboratory. If this is not practicable the samples may be stored at 3–5° provided they are tested within the 6 hr time limit. Testing should not be carried out in direct sunlight, and aseptic precautions must be observed.

1. *Preparation of Sample*

Shake the bottle 25 times through an excursion of about 1 ft, the whole shaking to last for about 12 sec. If the bottle has been filled to the brim invert 25 times by a rapid rotary movement of the wrist and then pour off about 2 fl. oz before proceeding to shake as described above.

2. *Preparation of Dilutions*

Dilutions should be prepared in quarter-strength Ringer's solution. Prepare serial one-tenth dilutions by adding 1 ml of inoculum to 9 ml of diluent.

Introduce the tip of a sterile 1 ml blow-out pipette into the sample to a depth of ½ to 1 in. below the surface, suck up and down 10 times to the 1 ml mark, in each case withdrawing the pipette above the level of the liquid before expelling. Finally, with the pipette held vertically, measure 1 ml and touch the tip of the pipette against the inside of the neck of the sample bottle before withdrawing. Transfer the charged pipette to the first dilution tube and, with the tip touching the side of the tube at ½ to 1 in. above the level of the diluent, blow out the contents. Allow 3 sec to elapse and blow out again. This constitutes the 10^{-1} dilution. With a fresh pipette mix the 10^{-1} dilution by sucking up and down 10 times, then measure 1 ml and transfer to the second dilution tube as described above. This is the 10^{-2} dilution. Discard the pipette.

3. *Presumptive Coli-Aerogenes Test*

After mixing the 10^{-1} dilution by sucking up and down 10 times, inoculate each of five tubes of single-strength MacConkey's broth with 1 ml, transferring the charged pipette in the same manner as described in para. 2. With the same pipette similarly inoculate 1 ml of the sample to each of five tubes of the same medium.

Using a sterile 10 ml pipette, transfer 10 ml of the sample to each of five 6 × ¾ in. test-tubes containing 10 ml double-strength MacConkey's broth after sucking up and down several times to ensure that the inner surface of the pipette is thoroughly wetted.

Incubate the 15 inoculated tubes at $37 \pm 0{\cdot}5°$ for 48 ± 2 hr. Inspect the tubes after 24 hr, and record and remove any tube showing the presence of acid and gas for the subsequent application of the differential test (see para. 4). Return any remaining tubes to the incubator and, at the end of a further 24 hr record those tubes showing acid and gas and again apply the differential test. A tube is positive for gas production when the curved part of the Durham tube is filled with gas or with a lesser volume of gas if copious gas bubbles arise on tapping the tube.

4. *Differential Test for* Escherichia coli (E. coli I)

The tubes which are positive at the end of 24 hr or 48 hr in the presumptive coli-aerogenes test should be subcultured immediately into single-strength MacConkey's broth, using a triple loop or a 5 mm diameter loop, and incubated in a water bath at $44 \pm 0{\cdot}5°$ for 24 ± 2 hr. Inspect the tubes and record the results. Record any tube showing acid and gas as *E. coli I* positive.

The most probable number of presumptive coli-aerogenes and *E. coli I* organisms in 100 ml of the sample can be determined from the number of positive tubes at 37° and 44° respectively, by reference to McCrady's tables. (Ministry of Health Report No. 71 (1956)).

5. *Colony Count Tests at* 37° *and* 22°

In practice these test are set up at the same time as the presumptive coli-aerogenes test after the dilutions have been prepared. Thus one pipette only need be used for inoculating the 10^{-2} dish, the 10^{-1} dishes and tubes and the 1 ml dishes and tubes in that order.

(*a*) *Inoculation of Petri Dishes.* When the dilutions have been made as in para. 2, take a sterile 1 ml pipette and mix the 10^{-2} ml dilution by sucking

up and down 10 times and inoculate 1 ml into a Petri dish (B.S.611: 1956). Hold the tip of the charged pipette about ½ in above the bottom of the dish, and blow out the contents of the pipette. Allow 3 sec to elapse, touch the tip of the pipette against the dish at a point away from the liquid already delivered and blow out the last drop. Using the same pipette, mix the 10^{-1} ml dilution by sucking up and down two or three times and inoculate two Petri dishes each with 1 ml. Following the same procedure inoculate two dishes each with 1 ml of sample.

(*b*) *Pouring Petri Dishes.* To each dish add 10 ml of melted Yeastrel milk agar medium at 45–47°. As each dish is poured, immediately mix the medium and inoculum by five to-and-fro movements, followed by five circular clockwise movements, followed by five to-and-fro movements at right angles to the first set, followed by five circular anti-clockwise movements. When the medium has set, invert the dishes and transfer to the incubator. The time elapsing between the preparation of the dilutions and the pouring of Petri dishes should not exceed 15 min. If the poured dishes are not placed in the incubator immediately after setting, cover them with a clean cloth.

(*c*) *Incubation.* Incubate one dish inoculated with 1 ml of water and one with 10^{-1} ml at $37 \pm 0{\cdot}5°$ for 48 ± 2 hr. Incubate the other three dishes inoculated with 1 ml, 10^{-1} ml and 10^{-2} ml of water at $22 \pm 1°$ for 72 ± 2 hr.

(*d*) *Counting of Colonies.* At the end of the incubation period count the colonies within 4 hr using a suitable counting chamber, a lens not exceeding a magnification of × 2½ diameter, and a tally counter.

The colony count/ml is assessed by multiplying the count of the dish containing 30–500 colonies by the reciprocal of that dilution. However, if the dish inoculated with 1 ml of the sample contains less than 30 colonies the colony count must be calculated from this. If more than one dilution gives a count within the 30–500 range use the count of the lower dilution for the calculation.

REFERENCE

1. Ministry of Health Report. No. 71 (1956).

68. DETERMINATION OF pH VALUES OF WATER

For all general purposes the pH value can be regarded as a number used to express the concentration of ionised hydrogen in an aqueous solution. It is thus indicative of the reaction of that solution, i.e., its neutrality or its degree of acidity or alkalinity.

For an accurate determination of hydrogen ion concentration, a potentiometric method is recommended, but pH values obtained by colorimetric methods are sufficiently accurate for routine purposes.

1. *Reagents*

The indicators most generally useful for the colorimetric determination of pH values of water are:

Chlorphenol red,	pH *range*	4·8–6·4
Bromothymol blue,	pH *range*	6·0–7·6
Cresol red,	pH *range*	7·2–8·8

All indicators should be stored in resistant glass containers with glass stoppers. Corks should not be used. They should be kept in the dark or away from strong light when not in use.

Indicator solutions are most accurate in the middle of their range, so that in carrying out a pH determination an indicator giving a reading near the middle of its range should be chosen.

Reliable indicator solutions can be purchased, and colour discs for these indicators are available for use with either the Lovibond comparator or the all purpose Lovibond comparator. The special round test-tubes or all glass fused cells of the same internal dimensions, i.e., 13·5 mm, can be used, with the latest models of either comparator. The discs required are:

2/1F Chlorphenol red,	*range*	4·8– 6·4
2/1H Bromothymol blue,	*range*	6·0– 7·6
2/1K Cresol red,	*range*	7·2– 8·8
2/1P Universal indicator,	*range*	4·0–11·0

2. *Method*

Obtain an approximation of the pH value of the sample by means of the Universal indicator and select the appropriate indicator, avoiding the extremes of the range. Pipette 0·2 ml of the indicator solution into a clean test-tube or cell. Add 10 ml of the water, cover with a clean glass slide and mix by inversion. Place the tube or cell in the right-hand compartment of the comparator. In the left-hand compartment place a test-tube or cell containing 10 ml of the sample. Hold the comparator before a north window or other suitable source of white light, and by rotating the disc match the colour of the test liquid. The pH is given in the aperture at the bottom right-hand corner when the match is obtained.

The sensitivity of this colorimetric method is usually about $\pm$ 0·1 pH unit, but many waters are only very slightly buffered and there is always some danger that the indicator may merely set up its own pH in the solution.

With waters low in bicarbonate it is therefore advisable to check the pH, using a second indicator of somewhat different range. Such errors may also be minimized by using 50 ml of the sample in a Nessler cylinder instead of 10 ml in a comparator, thus reducing the concentration of indicator. In this case the Lovibond Nessleriser will be required and the *appropriate discs* must be used. These are:

NLO 4460 indicator,	*range*	4·4– 6·0
NLB Bromo-cresol purple,	*range*	5·2– 6·8
NLC Bromo thymol blue,	*range*	6·0– 7·6
NLE Cresol red,	*range*	7·2– 8·8
NLH Universal indicator,	*range*	4·0–11·0

Note

It is essential to use the special test-tubes graduated at 10 ml with the comparator as these are of colourless glass. It is also necessary to see that the correct disc is used with the right instrument, e.g., the 2/ series of discs with the comparator and the N/ series with the Nessleriser.

69. DETERMINATION OF THE HARDNESS OF WATER

Originally the power of water to destroy the lather of soap was called its hardness. Although the most frequent cause of destruction of lather is the presence of dissolved salts of calcium and magnesium in waters, any metal forming an insoluble soap, for example, iron, aluminium, manganese, barium, etc., will impart hardness to water. Furthermore, the presence of acid, e.g., carbonic acid, in a water may cause decomposition of sodium or potassium soaps by precipitation of free fatty acids. As, however, in most waters the hardness is due primarily to calcium and magnesium salts the term 'hardness' has by custom become synonymous with the calcium and magnesium content.

In British Standard 2690: 1956, temporary hardness is now termed *alkaline hardness* and permanent hardness *non-alkaline* hardness.

1. *Apparatus*

50 ml burette
1 ml pipette
2 ml pipette
5 ml pipette
50 ml graduated flask
100 ml graduated flask
250 ml graduated flask
250 ml conical flask
500 ml conical flask

2. *Reagents*

N/10 nitric acid
N/50 disodium ethylenediaminetetra-acetate (EDTA)
Ammonium buffer
Total hardness indicator
Standard hard water
Screened indicator

All the above (with the possible exception of screened indicator) can be purchased from recognised suppliers or alternatively can be prepared as detailed in Appendix V.

Where interfering substances are present in the water to be tested for total hardness, additional reagents are needed. For details of interfering substances see Appendix VI.

Procedure

3. *Alkaline Hardness*

Measure 100 ml of the sample in the 100 ml graduated flask and transfer to a 250 ml conical flask. Add one or two drops of screened indicator and against a white background titrate with N/10 nitric acid to the end point (first appearance of permanent pink). The colour change is from green through grey to pink.

The alkaline hardness in terms of p/m $CaCO_3$ is obtained by multiplying the titration figure (ml N/10 nitric acid used) by 50.

4. *Total Hardness*

(*a*) *Hardness greater than about* 10 *p/m up to* 250 *p/m in terms of* $CaCO_3$
Measure 100 ml of the sample in the 100 ml graduated flask and transfer to a 250 ml conical flask. Add 2 ml of ammonium buffer and six drops of total hardness indicator and titrate immediately with N/50 EDTA against a white background until the red colour changes to blue. The colour at the end point is usually pure blue but with some waters a neutral grey is obtained.

The total hardness in terms of p/m $CaCO_3$ is obtained by multiplying the titration figure (ml N/50 EDTA) by 10.

(*b*) *Hardness less then about* 10 *p/m* $CaCO_3$
Measure 50 ml of the sample in the 50 ml graduated flask and transfer to a 500 ml conical flask. Add 5 ml of standard hard water, 10 ml of ammonium buffer, six drops of total hardness indicator and titrate immediately with N/50 EDTA until the solution has lost all traces of red colour. Note the burette reading. The colour at the end point is usually pure blue but with some water a neutral grey is obtained. Then add 250 ml of the sample and a further 0·5 ml of indicator and immediately, without refilling the burette, continue the titration until the end point is again reached.

The total hardness in terms of p/m $CaCO_3$ is obtained by multiplying the titration difference in ml by four.

5. *Non-Alkaline Hardness*

The non-alkaline hardness is the total hardness less the alkaline hardness.

6. *Excess Alkalinity*

Sometimes, particularly in some areas, it will be found that the screened indicator titration expressed in terms of alkaline hardness exceeds the total hardness. This is due to the presence of sodium bicarbonate or free calcium hydroxide neither of which however can be classed as alkaline (or bicarbonate) hardness. This excess is termed *excess alkalinity* and is found by subtracting the total hardness from the alkaline hardness.

Note

Interfering substances. Some substances occasionally present in water samples cause interference with the end point at certain concentrations, whilst others cause no interference but slightly affect the accuracy of the determinations. The concentrations at which serious interference occurs are higher than are normally found, but the information given in Appendix VI is for guidance in unusual cases.

70. CLASSIFICATION OF WATERS ON THE BASIS OF HARDNESS

All water is originally derived from rain and in passing through the atmosphere it takes into solution various gases, including carbon dioxide, which are also absorbed from the soil and rock on which it falls.

The purest natural water contains varying amounts of organic matter in solution, but if pollution is present some of this organic matter is usually in the colloidal form.

Water derives its characteristics from the land on which it falls and through which it passes and, on occasions, descriptive names are given to waters with definite mineral characteristics. For example waters containing an excess of calcium are termed 'calcarious' and, if the calcium is chiefly in the carbonate form, the waters are also termed 'carbonated'. If a water contains more than the normal levels of sodium chloride associated with other salts of sodium and/or potassium, it is called 'saline'. Waters with magnesium salts in excess are named 'magnesia' waters. If these salts are in the form of sulphates they are called 'sulphated'. These have medicinal properties and are usually malodorous. A water containing an undesirable excess of nitrates is 'nitrated' and, if iron is present and discernable by taste, the water is described as 'ferruginous'.

As a general rule, however, water is categorised by its 'hardness' according to its effect on the amount of soap required to produce a lather. The harder the water the more soap is required.

There are, roughly speaking, three main types of water, based on the pH, the mineral content and electrical conductivity (E.C.). These types are:

1. *Soft or Acid Water*
   This water has a pH of 6·0 or less, contains usually 50 p/m total solids or less and has an E.C. of less than 200. This type of water is generally derived from moorlands, shallow grassland, woodlands or from mountain slopes composed of impervious rock, such as granite. The acidity is mainly due to such readily soluble acids as humic, formic, acetic and oxalic, which are met with during the water's passage to the spring or reservoir, with little or no dissolved mineral matter. These waters may be discoloured or turbid, they may dissolve lead and other injurious metals and are known to attack some types of concrete.
2. *Neutral Water*
   This is a very wide group embracing most of the public water supplies in this country. The pH is between 6 and 8. The hardness of this group varies from fairly soft to moderately hard, depending on the amount of mineral salts in solution with an E.C. between 200 and 1,000. These waters are derived from all types of land where the mineral content of the soil is partially soluble.
3. *Hard or Alkaline Water*
   Water with a pH of over 8 and containing an excess of mineral salts usually calcium and magnesium, and an E.C. of over 1,000. This water is characteristic of all limestone country.

Hardness is usually expressed as parts per million, parts per hundred thousand or grains per gal (degrees Clark) of total hardness expressed as calcium carbonate ($CaCO_3$). *Total Hardness* is the sum of alkaline or temporary hardness and non-alkaline or permanent hardness. *Alkaline hardness* is the amount of hardness or solids lost upon boiling when the bicarbonates of calcium and magnesium, being the least soluble of the carbonates, are thrown out of solution. The hardness left after boiling is composed of the

soluble sulphates, (and on occasion the chlorides and nitrates,) of calcium and magnesium and is termed the *non-alkaline* or permanent hardness of the water. Some waters contain sodium bicarbonate or free calcium hydroxide and the alkaline hardness will then exceed the total hardness. The difference is termed *excess alkalinity*.

Because of the wide range of mineral content in natural waters it is not possible to have a hard and fast line of demarcation between hard and soft waters, and it only possible to categorize them in a series of relative hardness figures, as given below.

Less than 50	p/m	total	hardness	—	Soft
50–100	,,	,,	,,	—	Moderately soft
100–150	,,	,	,,	—	Slightly hard
150–250	,,	,	,,	—	Moderately hard
250–350	,,	,,	,,	—	Very hard
More then 350	,,	,,	,,	—	Excessively hard

When it is necessary to convert parts per million to grains per gal (° Clark) the following conversion factors should be used.

(*a*) Parts per million (p/m) to grains per gal (g/g)

multiply p/m by $\frac{7}{100}$

e.g., 50 p/m $= \frac{50 \times 7}{100}$ or 3·5 g/g or ° Clark.

(*b*) Grains per gal or ° Clark to parts per million (p/m)

multiply g/g by $\frac{100}{7}$

e.g., 3·5 g/g or ° Clark $= 3{\cdot}5 \times \frac{100}{7}$ or 50 p/m.

71. DETERMINATION OF CHLORIDE IN WATER

Sodium chloride in water supplies is unobjectionable in small quantities. Up to 200 p/m are barely detectable by taste, but at 200–500 p/m it becomes noticeable and values in excess of 500 p/m indicate a brackish water. As an indicator of possible pollution the amount present must be related to the local normal chloride content. It is not uncommon to find a water with a natural chloride content of up to 50 p/m. Human urine contains about 1 per cent of sodium chloride and town sewage will contain about 50 p/m more chloride than the original water supply. Sodium chloride is, however, a common mineral and may be present in appreciable quantities in bricks, concrete rings and mortar etc., used in developing a new source of water.

1. *Apparatus*

25 ml burette
250 ml conical flasks
100 ml graduated flask
Glass rod.

2. *Reagents*

(*a*) *Standard Silver Nitrate* (*N*/35·5)

Dry approximately 5 g silver nitrate (A.R.) at 105° to constant weight. Weigh accurately 4·791 g, dissolve in distilled water or water of equivalent purity, and make up to 1,000 ml.

One ml of this solution is equivalent to 1 ml chlorine expressed as chloride.

(*b*) *Potassium Chromate Solution*

Dissolve 5 g potassium chromate (A.R.) in distilled water and make up to 100 ml. Add silver nitrate solution drop by drop to produce a slight red precipitate and filter.

(*c*) *N*/50 *Nitric Acid*

(*d*) *N*/50 *Sodium Carbonate*

(*e*) *Precipitated Calcium Carbonate* (*A.R.*)

(*f*) *Methyl Orange Indicator*

Dissolve 0·04 g methyl orange in 20 ml industrial methylated spirits (85 per cent ethanol). Add 80 ml water. Mix well.

These reagents can be made up or purchased from recognised suppliers.

3. *Procedure*

Measure 100 ml of the sample in the 100 ml graduated flask, pour it into a 250 ml conical flask and add three drops of methyl orange. If alkaline (orange) add N/50 nitric acid drop by drop until a red colour is obtained. If acid (red) add N/50 sodium carbonate drop by drop until just orange.

Add approximately 0·01g precipitated calcium carbonate, stirring thoroughly. Then add 0·5 ml potassium chromate indicator and titrate with N/35·5 silver nitrate mixing well during the titration until the contents are tinged reddish brown. The detection of the end point can be facilitated by, having alongside the sample being titrated, a flask containing a second sample which has been treated in the same way as the test sample before titration.

The p/m chloride content is obtained by multiplying the titration figure in ml by ten.

72. DETERMINATION OF RESIDUAL CHLORINE IN WATER: COLORIMETRIC METHODS USING ORTHO-TOLIDINE

The following methods can be used.

(*a*) *The Standard O-Tolidine Method*

This method measures total residual chlorine (free and combined), but various substances may interfere by producing with the reagent a yellow colour similar to that given by chlorine. Nitrite, ferric compounds and manganic compounds interfere if present in a sufficient quantity, in which case this standard O-tolidine method does not give an accurate measure of the available chlorine.

It may be applied, however, without modification or correction when the ferric iron content and the nitrous nitrogen content are both under 0·2 p/m and when not more than 0·1 p/m of manganese is present in oxidised form. The method given will only measure chlorine residual to 1·0 p/m. For higher concentrations the determination should be made on a dilution of the

sample in distilled water; in such a case it is best to add the O-tolidine solution first, followed immediately by the water under test.

(*b*) *The O-Tolidine Arsenite Method*

This method is a more complicated variation of the standard O-tolidine method. Its use is necessary when either or both of the following conditions apply:

(i) when nitrites, ferric compounds or manganic compounds are present in interfering amounts; and/or

(ii) when it is desired to distinguish between 'free residual chlorine' and 'combined residual chlorine'.

1. *Reagents and Apparatus*

O-tolidine reagent. 0·1 per cent in 10 per cent v/v hydrochloric acid

Sodium arsenite solution. Dissolve 0·5 g of sodium meta-arsenite ($NaAsO_2$) in distilled water and make up to 100 ml

B.D.H. Lovibond Nessleriser with standard disc and matched glasses

Disc NCA, range 0·01–0·09 p/m

Disc NCB, range 0·1–0·5 p/m

2. *Methods*

(*a*) *Standard O-Tolidine Method*

Fill one of the Nessler glasses to the 50 ml mark with the sample of water under examination and place in the left-hand compartment of the Nessleriser. Fill the other Nessler glass to the 50 ml mark, add 0·5 of the O-tolidine reagent, mix rapidly by swirling the liquid in the cylinder and place in the right-hand compartment of the Nessleriser. Stand the instrument facing a uniform source of white light—a north window is the best—and compare the colour produced in the test solution with the colours in the standard disc, rotating the latter until a colour match is obtained. The markings on the disc represent the concentrations in parts per million of residual chlorine when 50 ml of water is taken for the test.

(*b*) *O-Tolidine Arsenite Method*

Use three 50 ml Nessler cylinders marked A, B and C respectively.

A.

Add 0·5 ml of O-tolidine solution.

Add 50 ml of the sample, mix and add immediately 1 ml of sodium arsenite. Mix. Match the colour after 2 min by placing in the right-hand compartment of the Nessleriser and having 50 ml of the water under examination in a Nessler cylinder in the left-hand compartment. This reading represents the 'free residual chlorine' plus interfering substances—*A*.

B.

Add 1 ml of sodium arsenite solution.

Add 50 ml of the sample, mix, and add immediately 0·5 ml of O-tolidine solution. Mix and match the colour after 2 min as before. This reading represents the interfering substances only—the *B*1 value. Retain the solution and match the colour again after 15 min to obtain the *B*2 value which is the blank for interfering substances after 15 min standing.

C.

Add 0·5 ml of O-tolidine solution.

Add 50 ml of sample, mix and match the colour after 15 min. This reading gives the total residual chlorine plus interfering substances—*C*.

Then:

Free residual chlorine	=	*A* minus *B*1
Combined residual chlorine	=	(*C* minus *B*2)—(*A* minus *B*1)
Total residual chlorine	=	*C* minus *B*2

All tests should be carried out at 15 to 20°, as the formation of colour is accelerated by rise in temperature. The various tubes must be kept in the dark or subdued light between readings. With certain saline waters containing traces of bromide a fictitiously high value for free residual chlorine may be obtained.

3. *Comparator Methods*

Where the B.D.H. comparator or all-purpose comparator is used with either the special test tubes or 13·5 mm cell, 0·5 ml of O-tolidine is added, as recommended in the standard procedure issued by B.D.H.

When using the arsenite method with the comparator, add 0·5 ml of sodium arsenite solution.

Discs required for use with either comparators are:

(*a*) when using special 10 ml test-tubes or 13·5 mm cell:
KDA range 0·1–1·0 p/m
KDB range 1·2–2·0 p/m

(*b*) when using 0·5 cm cell in the all-purpose comparator:
APDA 3 range 1·0–5·0 p/m

The above methods are based on those recommended by a Joint Committee on 'Approved Methods for the Physical and Chemical Examination of Water'. *Journal of the Institution of Water Engineers* (1949), **3**, (7), 555.

Laboratory Equipment

73. CARE OF EQUIPMENT IN BACTERIOLOGICAL LABORATORIES

1. *General*

THE importance of keeping laboratory equipment in good working order cannot be over-emphasized. The manufacturers' instructions for operating equipment should be available in all laboratories and should be closely followed. Faults in equipment should be reported promptly and defective parts replaced without delay. Where possible, spares, e.g., capsules, pilot bulbs, etc., should be kept of those parts of apparatus which are liable to damage or breakdown.

2. *Incubators*

There are two types of incubator, anhydric and water-jacketed. Water-jacketed incubators are capable of more accurate temperature control and have a more even temperature distribution than anhydric models. They are therefore to be preferred for most bacteriological work.

(*a*) *Heating*. May be by electricity or gas. With gas heated incubators care is necessary to keep the jet clear. Any deposits which form on the under-part of the incubator over the flame should be regularly removed, otherwise a particle may drop on to the flame and extinguish it. There should be a shield to protect the flame. Incubators should be sited away from draughts and vibration.

(*b*) *Temperature Control*. Thermostats are normally of two types: capsule or bi-metallic rod.

For adjusting the temperature in all types of incubators the manufacturers' instructions should be carefully followed. If a pilot light indicator is provided, see that the bulb is functioning correctly.

It takes time to set the temperature of an incubator correctly and the process should not be hurried. After each adjustment of the thermostat, adequate time should be allowed for the temperature in the incubator to reach equilibrium.

(*c*) *Capsules*. It should be noted that some capsules are marked in degrees Farenheit with a temperature which is approximately 10°F lower than that at which the incubator is to be maintained. In other words, these capsules are marked with the boiling point of the capsule fluid. Other capsules, on the other hand, are marked in degrees Centigrade with the temperature at which the incubator is to operate.

A capsule which has been damaged or overheated should be replaced. To test a capsule to see if it is working properly, it should be placed in water, the temperature of which is 1 or 2 degrees above its boiling point. The capsule should then be seen to expand and there should be no emission of bubbles.

(*d*) *Adjustments*. Major adjustments should only be made by a qualified electrician or other experienced worker. The current should be switched off

and the plug pulled out before any alteration is made other than the adjustment of temperature mentioned above.

Electric points will stick or 'chatter' when dirty or worn. They may require cleaning very lightly with a very fine grade of emery paper, or even replacing. Wear on points can be minimized by fitting condensers to the apparatus.

The thermostatic control mechanism of an incubator should be protected from dust and disturbance by providing a suitable cover.

(*e*) *Maintenance*. Using a funnel, you should top up every week or as required the water in the jacket. On some incubators there is a float to indicate when topping up is necessary. A very hard water or water of an acid or corrosive nature should not be used for filling or circulating through incubators.

The interior of the incubator should be kept free of dust by wiping when necessary with a clean damp cloth (switching off current during the operation). If a culture is spilled in the incubator, it may be wiped off with cotton wool soaked in a very weak hypochlorite solution, but a persistent disinfectant such as formaldehyde should *not* be used.

(*f*) *Temperature Readings*. Temperature readings should be made and recorded regularly, at least at the beginning and end of each day. It should be made a practice always to read the thermometer before opening the inner incubator door on each occasion. Alternatively it should be placed in a tube of water so that it can be quickly read on opening the incubator. Occasionally thermometers should be placed on each shelf so that temperature differences within the incubator can be noted. Thermometers should always be checked against a standard thermometer.

3. *Water Baths*

(*a*) There are several types of water-bath which may be electrically or gas heated. Temperature control can be of the capsule or bi-metallic strip.

Siting of water baths is important. They should be placed in the part of the laboratory where there is the least possible vibration or temperature variation, away from draughts and direct sunlight. There should be adequate air space round the bath (4–6 in. gap).

(*b*) *Adjustment of Temperature*. The manufacturers' instructions should be carefully followed. It should be ensured that the indicator light (if provided) is working and that the temperature required is in the range of the thermostatic control. The water level should be at least $\frac{1}{2}$ in. above the level of the capsule or thermostat arm. As with incubators, plenty of time should be allowed between thermostat adjustments to allow the bath to settle down. Depending on circumstances this may take several hours.

(*c*) *Maintenance*. Water baths should be emptied and cleaned out as often as is necessary, at least once weekly, and whenever anything is broken or spilled in them. If the water supply is excessively hard, or corrosive, it is advisable to use distilled water.

The water should be adjusted to the correct level, i.e., about $\frac{1}{2}$ in. above the level of fluid in culture tubes and this may need adjustment for varying loads in the bath. Care should be taken that racks or baskets used in the bath are not made of a metal antagonistic to that of the bath. The points of capsule operated baths need cleaning occasionally with very fine emery

paper; care should be taken not to overfile the points. This should normally be done by an electrician or other experienced worker.

(*d*) *Low Temperature Water Baths.* When it is required to run a water bath at a temperature lower than that of the surrounding atmosphere, it may be possible to do so by circulating water at a lower temperature through the bath. It is essential for the water flow to be controlled. The water temperature should be at least 4° lower than the temperature at which the bath is to operate.

(*e*) *Temperature Readings.* For information on methods of checking the temperature control of water baths, reference should be made to Technique No. 74. Routine temperature checks will depend on the purpose for which the bath is required. It is usually advisable to place a second thermometer in the bath away from the position of the thermometer provided in the bath. Maximum and minimum temperature checks are also desirable.

4. *Autoclaves*

Reference should be made to Technique No. 4, and to the paper by Thiel, Burton, and McClemont (1952), *Proc. Soc. appl. Bact.*, **15**, 53.

The autoclave should be sited so that the lid is easily and safely lifted and the pressure guage and thermometer easily read. The steam outlet should be directed away from operators, but it is not normally desirable to extend the pipe outside the laboratory unless a thermometer has been fitted to register steam temperature.

Autoclaves should be insured and will be regularly inspected and tested by the agent appointed by the insurance company.

An adequate gas supply is necessary for gas autoclaves and suitable burners to allow rapid heating are essential.

Autoclaves should be cleaned out regularly and a check made that the steam outlet valve and safety valve are working properly. Care should be taken that the correct water level is maintained; usually this is about $\frac{1}{2}$ in. above the junction of the curved bottom and the side of the autoclave.

It is a great advantage if the autoclave can be fitted with a thermometer. This should be held in a copper or brass thermometer pocket situated in the steam outlet pipe, fitted with a petcock so that steam can be allowed to bleed past the bulb of the theremometer during operation.

It is important that the sealing ring or gasket is in good condition and a spare should be kept in the laboratory.

When closing down the lid of the autoclave, the opposite bolts should be tightened in turn so that even pressure is exerted all round. Tools should not normally be used for tightening the bolts—if the gasket is sound, hand pressure is adequate.

5. *Care of Balances*

(*a*) A balance should be chosen with a capacity and sensitivity suited to the work for which it is intended.

(*b*) *Siting and Setting Up.* Balances should be kept in a dry atmosphere and should be placed on a firm level bench where a minimum of vibration occurs. A self-indicating silica gel placed in the balance helps to maintain dry conditions and may be revived by heating. The balance should not be

moved unless absolutely necessary; if it has to be moved, the pans and balance arm should first be removed.

The balance should be set level as indicated by the plumb line or spirit level. When the pans are in balance the pointer should swing freely. If the pointer does not swing equidistant either side of the centre, adjustments should be made to the screws on the extremities of the arm.

Pan arrestments when fitted on the floor of the balance case should be adjusted to ensure that the weight of the pans is removed from the knife edges when the balance is at rest.

(*c*) *Use and Maintenance*. The balance must be kept clean at all times. A camel hair brush should be kept in the balance case for dusting purposes. Spilled chemicals should be cleaned up immediately, otherwise corrosion may take place. The knife edges should be cleaned occasionally.

The pans should always be at rest when chemicals or weights are added. Weights should be placed carefully and not dropped on to the pan. When using a sensitive balance the front of the case should be kept closed during weighing.

Chemicals should never be placed directly on to the pans. For small quantities a watch glass, weighing bottle or scoop should be used.

The weights should be kept scrupulously clean. They should be dusted frequently with a soft cloth and occasionally cleaned with warm soapy water, rinsed in distilled water and dried. Abrasive or polish should never be applied to the weights.

Because of the care necessary to avoid damage to knife edges, assistants should not be allowed to use a balance until properly trained.

A sensitive balance should be checked by the manufacturer's representative once a year.

6. *Stills*

The manufacturers' instructions for the maintenance and adjustment of stills should be available in every laboratory.

The majority of stills in use in laboratories are the type which pre-heats the mains water, some of which then overflows and runs to waste and some of which goes into the boiling chamber, replacing water which has been evaporated.

In hard water areas frequent descaling is required. After descaling the still should be allowed to run for some hours, the distilled water going to waste.

In soft water areas, or in areas where the water is corrosive, the jacket or inner tube of the still may become perforated with very fine holes. It is advisable therefore to check periodically that no 'distilled' water appears at the distilled water outlet when no distillation is taking place.

Care should be taken to ensure that the outlet temperatures of the overflow water and distilled water (at bottom of still) are within the manufacturers' limits.

The bottom of the still should be open to allow gases, previously dissolved in the water, to escape.

7. *Microscopes*

Adjustment or repair of microscopes by non-specialists should be discouraged. For a small fee, microscopes can be inspected, overhauled and

cleaned in the department by visiting technicians of microscope manufacturing companies. This service should be engaged whenever possible. Daily maintenance is then limited to keeping them free from aggressive chemicals and to keeping clean the outside surfaces of the lenses. Lubrication is not necessary. Manufacturers' instructions relating to the care and use of microscopes should be carefully followed.

The instrument should be kept under cover when not in use.

Modern microscopes are heavy. Both hands should be used to carry them, one hand on the limb and the other under the foot supporting the weight.

The eyepieces should be kept in position because dust is difficult to remove when it falls on the prisms of a binocular or on the back lens of an objective.

Rack upwards to bring the slide into focus. This is most important with objectives of short-working distance. Some microscopes have a safety stop built in to prevent damage to objectives and valuable specimens. Remove immersion oil and clean the stage after use. Lens tissues should be used to remove immersion oil and clean lens surfaces. Solvents should not be used.

74. WATER BATHS FOR BACTERIOLOGICAL PURPOSES

Water baths and incubators are used to maintain a controlled temperature for the propagation of cultures and for physiological tests. Water baths can be controlled within narrower temperature limits than can incubators and should therefore be used where closer temperature control is required, e.g., an incubator can be used for the initial isolation of coli-aerogenes organisms but the critical 44° confirmatory test for *E. coli I* can only be achieved in a water bath.

It is important to remember that the temperature of a water bath varies within limits and a constant temperature is not maintained. However, some water baths which have continuous heating either by gas or oil will maintain what is virtually a steady state between heat input and heat losses. Electric water baths on the other hand have alternating phases of rising and falling temperature.

Thermostatic control of water baths for bacteriological purposes is usually based on either the expansion of a capsule containing a volatile liquid or on the rotary motion produced by changes of temperature in a bimetallic spiral. The working temperature of water baths fitted with a capsule can be adjusted to a limited extent (2° or 3°) and any major change usually requires a different capsule. The bimetallic element is however capable of adjustment over a very wide range and is often therefore described as 'universal'.

The extent of variation above and below the desired temperature is governed by the sensitivity of the thermostat and its proximity to the heater. In some baths the sensitivity of the thermostat can be varied by adjusting the gap between a small magnet (on one contact) and its keeper (on the other).

In modern water baths the manufacturers allow for a thermometer to be maintained in a fixed position in the bath. This thermometer should be of

such a kind and fixed in such a position that the temperature of the bath can be read without it being necessary to move or lift the thermometer. The thermometer should be graduated in 0·1° and cover a range at least five degrees above and below the working temperature of the bath. The accuracy of the thermometer should be checked against a standard (N.P.L.) thermometer before being used.

All water baths should be stirred and modern types have built-in stirrers which are independent of the on/off cycle of the heater. Stirrers eliminate hot and cold spots and prevent thermal stratification which otherwise develops, because hot water, being lighter, rises and overlies the cooler water.

1. *Testing the Working Temperature*

The incorporation of stirrers has greatly reduced the need for temperature distribution tests, but if no stirrer is fitted these tests should be done by inserting a number of thermometers (checked against a N.P.L. certificated thermometer) at various places and depths in the bath and recording the readings. A suggested arrangement of these thermometers and instruction for calculating the accuracy of the bath is given in Appendix II.

2. *Testing Temperature Recovery*

Temperature recovery is the ability of a water bath to recover its working temperature quickly after a load at a different temperature is introduced into it. It is rare in bacteriological laboratories for hot samples to be placed into a cooler bath, but if this contingency arises, it is best dealt with by pre-cooling the samples immediately before placing them in the water bath. It is more usual for cold materials to be placed into a warm bath, e.g., methylene blue tubes into a 37° bath and 2 oz milk samples into a 22° bath. Quick temperature recovery demands a powerful heater and this may lead to 'over-shoot' due to residual heat in the heater after the current has been cut off. Often a compromise is necessary, and some baths incorporate a separate booster heater which is manually operated and which is used only when starting from cold or where shortening of recovery time is required. A method of checking and recording recovery time is given in Appendix III.

3. *Control of Temperature*

Ideally the temperature of water baths should be recorded using a recording apparatus which gives a continuous traced record. These are expensive and for most purposes maximum and minimum thermometers will suffice. When direct reading thermometers of a suitable temperature range are used, the temperature should be read and recorded at the beginning and end of every working day. Intermediate check readings should be taken occasionally, but need not be recorded. It is important to remember that the ambient temperature of laboratories can reach its highest as late as 7 or 8 p.m.

4. *Low Temperature Water Baths*

Difficulty is sometimes experienced in maintaining a water bath at 22° in hot weather. This temperature has the peculiar distinction that a temperature above it (e.g., 25°) can be fairly easily controlled, while any temperature below it (e.g., 18°) necessitates some form of refrigeration. Most laboratories

depend on the water supply to keep the bath down to 22°. It is difficult to maintain a slow steady flow of cold water from the ordinary cold water tap and the following points may be helpful:

(*a*) a direct connection from the underground mains is an advantage but may not comply with local regulations. The pressure may vary, but a small header tank with a ball valve will help to minimize any fluctuations. A small tank of 1 or 2 gal has the advantage that there is no need to insulate it as the rate of flow is high. Should a larger 5 to 10 gal tank be used, insulation will be necessary. The tank should be connected to the bath with a fixed metal pipe rather than one of rubber;

(*b*) the tap controlling the flow to the bath should be set to deliver 800–1,000 ml/min and this should be further cut down by a needle valve (in preference to a fibre washer type) to allow a flow rate into the bath of about 250 ml/min;

(*c*) the temperature of the water entering the bath should preferably be below 19° and the inlet should be in close proximity to the stirrer. The outlet port should be of sufficient diameter to prevent air bubbles forming and blocking the flow;

(*d*) the outlet pipe should be a fixed metal pipe, as short as possible, leading directly to a sink or drain which is not liable to be stopped up by debris or by freezing in winter.

5. *Maintenance*

The working level of the bath is such that the top of the liquid sample in the test-tube is about 2 cm below the water level in the bath. In static water baths this level should be maintained by topping up at frequent intervals with distilled water at or slightly below the working temperature of the bath. The temperature of the bath should be checked frequently, quite apart from the daily recordings. To keep it sweet the water should be changed frequently.

75. NOTES ON ATMOSPHERIC SHADE BOX

1. The introduction of the temperature compensated clot-on-boiling (C.O.B.) test has emphasised the importance of suitably designed and properly sited atmospheric shade boxes for the pre-test storage of milk samples. For the C.O.B. test the time of testing is determined by the temperature recorded within the box and it is most important that, as far as practicable, the design and siting of the boxes at different laboratories should be standardized. The following notes will be found useful in this connection.

2. The ideal box should be constructed so as to form a uniform temperature enclosure in which the temperature should be the same as that of the external air. It should completely surround the thermometers and be unaffected by unnatural sources of heat. These conditions are very difficult to achieve but are approximated by the use of louvres with ample air circulation and by painting the whole structure white.

3. Size and design will depend on the site and the work load of the particular laboratory but generally speaking a rectangular wooden box approximately 3 ft wide, 2 ft 6 in. high and 2 ft deep will be found to be suitable.

This type (see Fig. 2) with a removable centre shelf will meet the demands of most laboratories and will be found easier to use, keep clean and maintain than a smaller sized model.

4. The box should be provided with louvres on sides, back and front. These should be made of $\frac{3}{8}$ in. timber, 3 in. wide with a $\frac{1}{2}$ in. sloped edge and $\frac{1}{2}$ in. between each louvre. The use of wire mesh, perforated zinc or other similar material instead of louvres is not considered suitable. These materials have been found to restrict air circulation and allow rain and dust to enter the box.

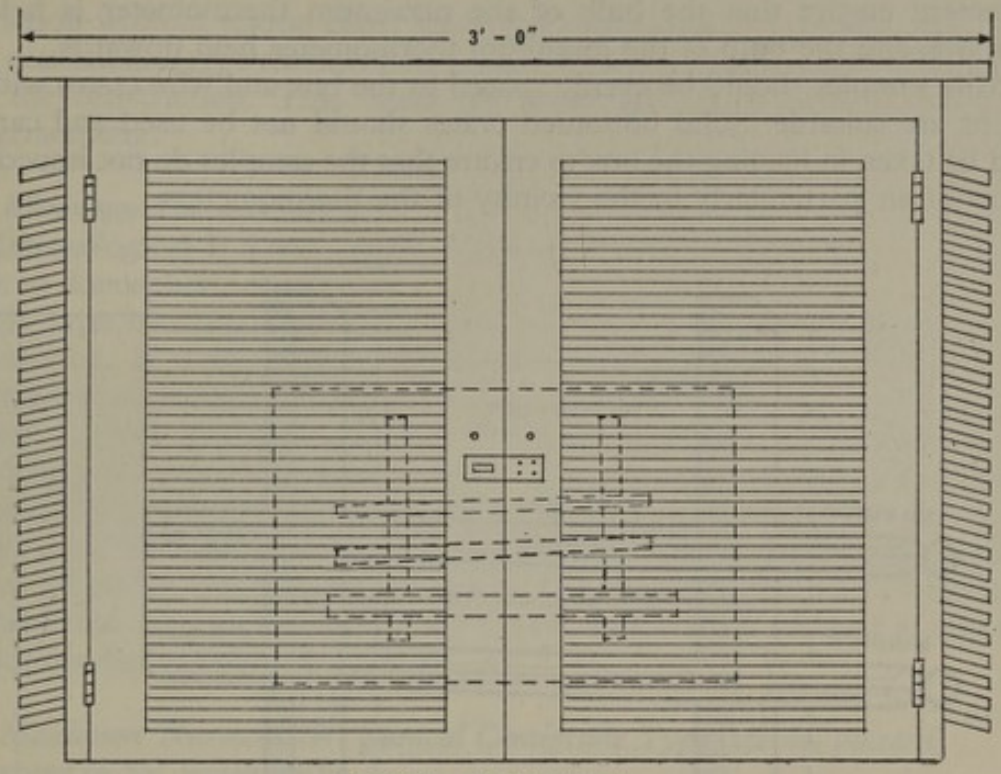
5. The base of the box should be made of 2 in. slats, $\frac{1}{2}$ in. apart, fitted lengthwise. The centre and bottom shelves should be made of expanded metal or other material such as pegboard or well perforated boarding allowing free air circulation. The shelves should be 1 ft 3 in. and 3 in. from the base respectively. The roof should be waterproof and sloped, with 1 in. fall from front to back. Two doors, which open from the centre, should be fitted and a strong catch and lock are essential. The box and stand should be finished with good quality white gloss paint.

6. Correct siting is most important and may be a problem where laboratories are accommodated in single-storey buildings or in houses in built up areas. The technique recommends that the box shall be 'so situated on the outside of a wall on the north side of a building or other comparable position so that it is at all times in the shade'. A suitable location may be difficult to find since it must be readily accessible to the laboratory. The box must be freely exposed to the air and ideally should not be shielded to the front and sides by trees and other obstacles. A site in a confined space is not recommended and one too high from ground level is subject to exceptional atmospheric conditions and should be avoided.

7. The box should be securely fixed on a firm and level stand so that it is not subject to excessive vibration. The base should be at least 3 ft from ground level, whenever practicable 3 ft 6 in., and there should be at least 3 in. of air space between the back and the wall. The immediate surroundings should be clean and tidy. The box should be scrubbed out with soap and water at regular intervals and repainted as often as is necessary to preserve its condition and appearance.

8. The box must be fitted with maximum and minimum thermometers of the approved meteorological pattern and these should be securely mounted on a wooden frame behind four spring brass clips. The bulbs should be at least 2 in. apart and 3 in. from any wall, the bottom thermometer being sited 6 in. from the bottom shelf at the back of the box. The maximum thermometer is clipped in the upper pair of brackets and the minimum thermometer in the bottom pair at an angle of approximately 2 degrees to the horizontal, a fall of $\frac{1}{4}$ in. in 7 in., the bulbs being at the lower end and to the left when facing the box.

9. Thermometers must always be fixed firmly in position. Any jolting either by strong winds or opening of the doors may lead to displacement of the index or mercury column. This may also occur when removing the thermometers and it is important to ensure that temperature readings are made whilst they are still in position. The readings should be recorded immediately before removing and resetting the thermometers. After resetting and until



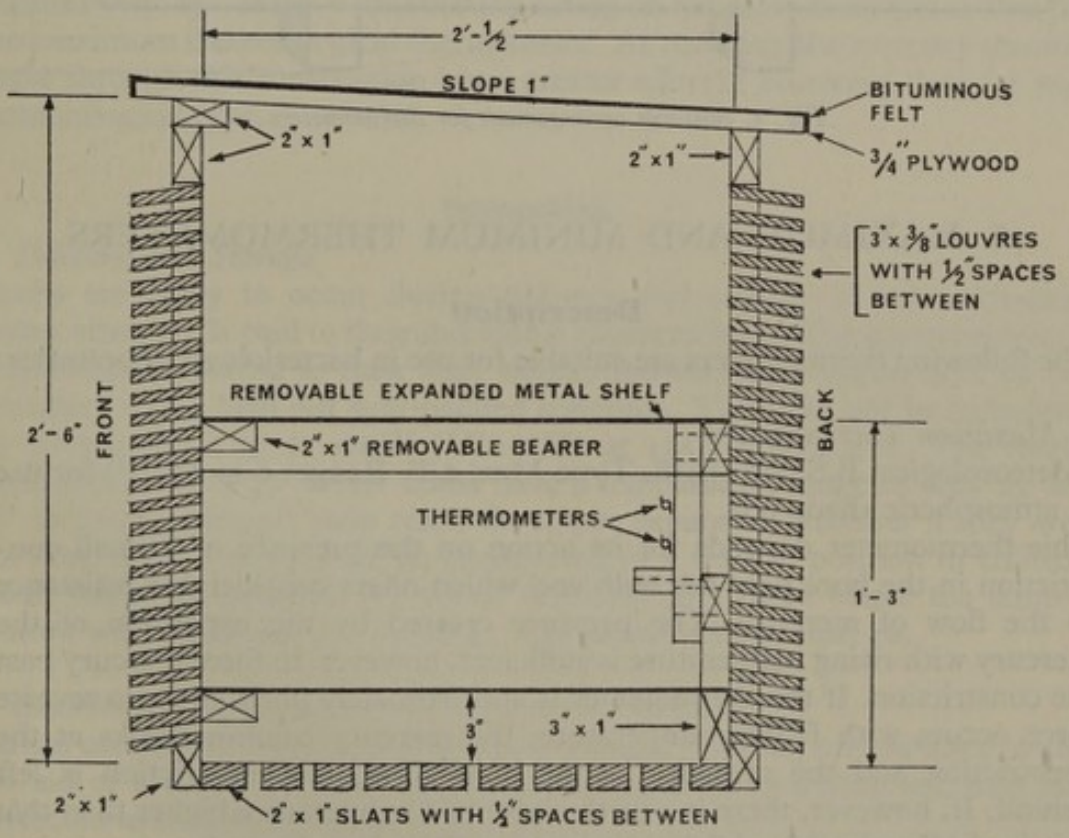


Fig. 2. Atmospheric Shade Box. Detail drawing of the arrangement for thermometers (within the boxed area of the front elevation) is shown in Fig. 3.

replacement ensure that the bulb of the maximum thermometer is held downwards and the bulb of the minimum thermometer held upwards.

10. Milk samples should be evenly spaced in the box and wire crates with divisions are suitable. Solid bottomed crates should not be used and care should be taken in loading the box to ensure that the samples do not impede the flow of air particularly in the vicinity of the thermometers.

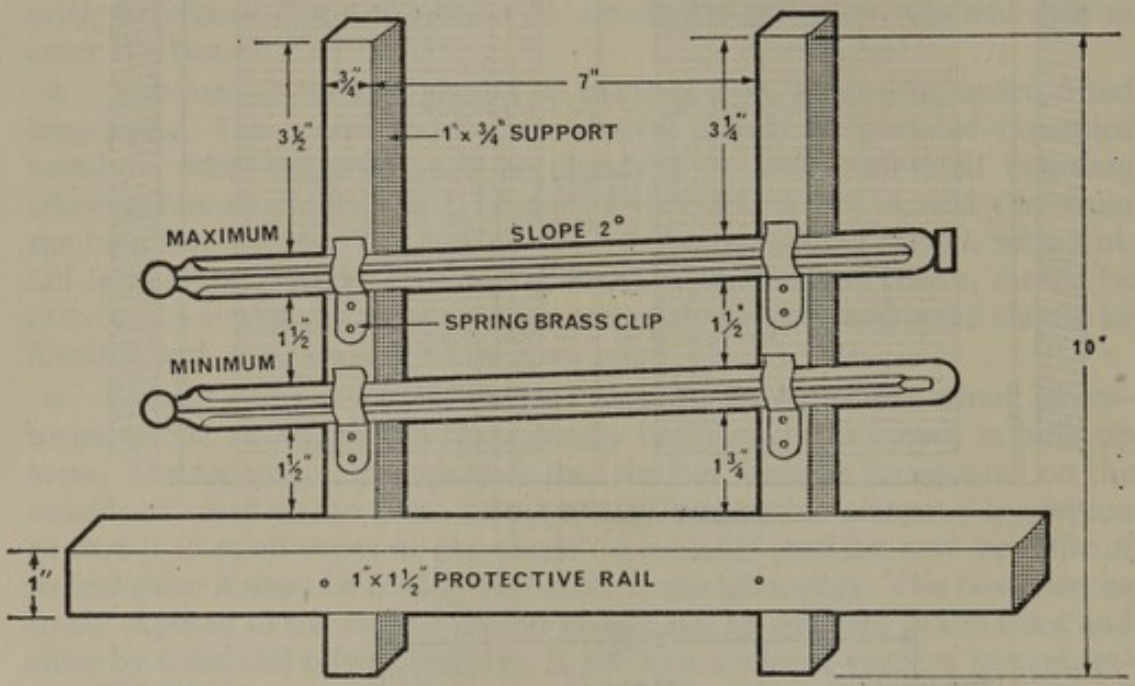


Fig. 3. Suitable arrangement for thermometers

76. MAXIMUM AND MINIMUM THERMOMETERS

Description

The following thermometers are suitable for use in bacteriology laboratories:

1. *Maximum Thermometer*

(Meteorological B.S.692: 1958. Type Max. 2/F. Range: 0 to 130°F) for use in atmospheric shade box.

This thermometer depends for its action on the presence of a small constriction in the bore near the bulb end which offers considerable resistance to the flow of mercury. The pressure created by the expansion of the mercury with rising temperature is sufficient, however, to force mercury past the constriction. If the thermometer is approximately horizontal, no reverse force occurs with falling temperature, the mercury column breaks at the constriction and the mercury in the bore above the constriction is left behind. If, however, there is a further rise to a temperature higher than that existing before the fall, additional mercury will be forced past the constriction. Owing to this 'one-way' action, the reading of the far end of the mercury column in the bore indicates the highest temperature reached since the thermometer was last set. The mercury should shake through the con-

striction without undue effort when the thermometer is being reset, but in all constriction type thermometers there is an apparent break in the mercury at the constriction. This break has been allowed for in calibrating the thermometer.

2. *Minimum Thermometer*
(Meteorological B.S.692: 1958. Type Min. 2/F. Range: 30 to 100°F) for use in atmospheric shade box.
This type contains alcohol in which a light dumb-bell shaped index is immersed. If a minimum thermometer is tilted bulb end upwards, the index will slide along the bore until it reaches the meniscus at the end of the spirit column, where it will stop because of the resistance offered by the surface tension of the meniscus. If the thermometer is placed in an approximately horizontal position, the index will be dragged back by the surface tension of the meniscus when the temperature falls, but if the temperature rises the index will remain stationary. Consequently, the end of the index nearest the meniscus indicates the lowest temperature reached at any time since the thermometer was set.

3. *Maximum Thermometer. Clinical Centigrade Type* (*special pattern*)
For use in 22° water baths.
This thermometer covers a limited range of temperatures from 19–25° and possesses an expansion chamber into which the mercury enters at temperatures over 25°. It has a constriction acting on the same principle as that of the maximum meteorological thermometer. At resetting the mercury should shake through this constriction but a greater effort is necessary than for the meteorological type.

Inspection

4. *Delivery and Storage*
Faults are likely to occur during delivery and storage of thermometers unless attention is paid to their individual characteristics. The meteorological thermometers, owing to their size, weight and very thin bulbs, need to be transported in a firm but well-padded container. They will not be damaged by temperatures up to 120°F (Min 2/F) or 135°F (Max 2/F). The thermometers used in 22° water baths have a temperature range of only up to 25° and some mercury may reach the upper expansion chamber if they are not kept cold. They should be transported in a vertical position in chilled water whenever possible and stored vertically in a place where the temperature will not rise above 25°, e.g., cold cellar, refrigerator, etc.

5. *Examination Before Use and Correction of Faults*
Before use and at regular intervals thermometers should be inspected with the aid of a hand lens. Certain faults noticed then or during use may be corrected as indicated below.
(*a*) *Maximum Meteorological Thermometer*. The mercury column should be unbroken and the upper part of the bore should be devoid of mercury. Any discontinuity of the mercury column is best remedied by thoroughly chilling the thermometer and vigorously shaking down. Should this fail the thermom-

eter should be placed alternately in warm (130°F approx.) and cold (35°F approx.) water and again shaken down. It may even be necessary to chill the thermometer to 0°F or less before successfully shaking down.

Occasionally, with falling temperature, the mercury tends to move back into the bulb through the constriction, and should this occur when the thermometer is at an angle of 10 degrees or less the thermometer should be discarded. This fault can be detected during cooling following gentle warming in the hand.

(*b*) *Minimum Meteorological Thermometer.* The spirit column of a minimum thermometer should be free from 'bubbles', and the upper part of the bore and the expansion chamber (if any) should be devoid of drops of liquid. The column of spirit is very liable to become separated during transit so that detached sections are formed, which may be difficult to observe. Any discontinuity of the column may be remedied by holding the instrument vertically downwards and tapping the top against the other hand, by swinging it bulb downwards or by correcting as for a maximum thermometer, i.e., chilling and shaking down. Patience and perseverance are necessary. If the break is near the meniscus, correction may be easier by holding the thermometer upright in warm weather and tapping or shaking the stem from time to time so as to introduce some of the spirit into the expansion chamber at the top of the tube. The bulb must be removed from the warm water before there is any risk of the expansion chamber becoming full otherwise the bulb may burst. By a combination of heating and shaking, the 'bubbles' can be driven up the bore and into the chamber. When all the 'bubbles' have been eliminated, the thermometer should be clamped bulb downwards for at least an hour so that any alcohol adhering to the side of the bore may drain down as the thermometer cools. Certain individual thermometers may be especially prone to develop 'bubbles', e.g., after exposure to temperatures well below 32°F while undisturbed in the atmospheric shade box, and, after frequent trouble, the only solution is to discard the faulty thermometer.

The volatile spirit may condense on the wall of the bore above the column. This can be corrected by warming (e.g., with a wet warm cloth) the upper end of the thermometer thus causing the spirit to evaporate off the wall and recondense lower down the bore. During this process it is helpful to place the bulb in chilled water, holding the thermometer vertically.

The index must be entirely immersed in the spirit otherwise the thermometer should be shaken as for resetting a maximum thermometer so as to correct the fault, and tested to see that the index is not liable to stick at any point.

(*c*) *Maximum Thermometer, Clinical Type, for Use in a 22° Bath.* If mercury is present in the expansion chamber, the bulb should be warmed in the fingers until the mercury column is continuous with that trapped in the chamber when the thermometer should be chilled in the refrigerator. The cold thermometer should be placed with its bulb on cotton wool in a cardboard thermometer case and the whole firmly packed with cotton wool into a Gerber or other centrifuge of suitable type, which should then be balanced and spun for 2–3 min (or longer if necessary). This procedure should pull all the mercury from the chamber so that the mercury column

is continuous except at the constriction. Repetition of the procedure may be necessary to correct this fault completely. A visual check should show that the only gap in the column is present at the constriction, and it may be possible to remove this by shaking down the mercury below the constriction. On warming in the hand the gap should disappear from the column as the mercury passes through the constriction in short bursts. If on repetition the procedure fails to correct the fault the thermometer should be rejected.

If the gap is very near the top of the mercury column, the bulb should be warmed in the hand to move the mercury to the top of the bore when the mercury should then be shaken down to the normal position, and a check made that no mercury is trapped in the chamber (it may be necessary to re-centrifuge if this has occurred).

If during use the mercury appears to be 'sticking' at some temperature, e.g., 19°, the thermometer should be re-examined with a hand lens to make sure that the expansion chamber has remained empty.

As in the maximum meteorological thermometer, the mercury may tend to move back into the bulb through the constriction and when this fault occurs the thermometer should be rejected.

Testing

6. New thermometers should be tested before use and at about 2 year intervals.

The recommended method needs no special apparatus other than a standard N.P.L. certified thermometer or one standardised against such a thermometer over the range 35–90°F and with a total range approximating to that of the thermometer to be tested, 0–120°F and calibrated for 3 in. immersion.

After visual examination and correction of faults, the thermometer to be tested should be immersed in not less than 2 gal of water and the standard thermometer should be placed vertically with its bulb as close as possible to that of the thermometer under test. The water should be stirred and, following adjustment to the requisite temperature, the readings on the thermometer under test recorded when a steady reading is obtained on the standard thermometer. In practice it is easier to read the specified temperature on the thermometer (when of the meteorological type) under test and then read the difference, if any, on the standard thermometer, since the scale of the latter is marked in fractions of a degree (usually fifths whereas the scale of the former is graduated in whole degrees only.)

The standard thermometer and the thermometer under test will be at different angles, and *care must be taken to avoid parallax. The eye must be directly in front of the thermometer and at 90 degrees to the extreme end of the mercury column or index.*

(*a*) *Maximum Meteorological Thermometer*

The thermometer under test should be first placed in clamps so that the bulb is 3 in. below the surface of the water and at least 2 in. from the side of the container. The engraved face should be uppermost, and the stem at an inclination of 2 degrees to the horizontal. The standard thermometer should be placed vertically with the bulb as near as possible to that of the

maximum thermometer and at least 2 in. from the side of the container. The water in the container must be continuously and vigorously stirred throughout.

When the reading on the standard thermometer is 35°F, and only after the reading of the standard thermometer has been steady for at least 60 sec, the reading taken from the top of the meniscus on the maximum thermometer should be recorded. The water should then be warmed and similar pairs of readings recorded at 5°F intervals from 40°F to 90°F.

While the maximum thermometer is under test the temperature of the water should not be allowed to fall. If the temperature does fall, the maximum thermometer must be withdrawn, cooled to a temperature below that of the water, and the mercury column reset in the usual way by a few downward shakes of the thermometer.

After the calibration has been checked the thermometer should be examined to see that as the temperature is lowered the mercury is not drawn past the constriction when the thermometer is inclined with the stem sloping upwards at an angle of 10 degrees.

The maximum errors permitted are as follows:

At range	*Error*		*Change of Error in 20°F*
	low	*high*	
Below 32°F	−0·5	+0·3	0·4
32°F and above	−0·3	+0·1	0·2

Greater minus errors than plus errors are permitted to allow for the tendency of the zero to rise slowly with age caused by the slow contraction of the bulb. The change of error in 20°F refers to the algebraic difference between the errors at opposite ends of the 20°F interval, e.g., if the errors are −0·3°F at 40°F and +0·1°F at 60°F the change in error is 0·4°F, which exceeds the permitted change in the range.

If, after correction of faults and testing, the errors are greater than those permitted the thermometer should be rejected.

(*b*) *Minimum Meteorological Thermometer*

The thermometer under test and the standard thermometer should be placed in water as described above for the maximum meteorological thermometer.

When the reading on the standard thermometer is 35°F, and only after the reading of the standard thermometer has been steady for at least 60 sec, the reading taken from the bottom of the alcohol meniscus on the minimum thermometer should be recorded. The water should then be warmed and similar pairs of readings recorded at 5°F intervals from 40°F to 90°F.

After the calibration has been checked with rising temperatures, further pairs of readings at 5°F intervals should be recorded as the water is cooled gradually to 35°F, using ice when necessary. For this series the reading must be recorded from the meniscus end of the index, but again only after the reading on the standard thermometer has been steady for at least 60 sec. While the minimum thermometer is under test, the temperature of the water should not be allowed to fall below the desired reading. If this should occur, *either* the reading must be taken at the actual temperature of the water, and a record made of this temperature, *or* the minimum thermometer

must be removed from the water, raised to a temperature above that required and the index reset. At the same time the water should be adjusted to the required temperature before the thermometer is replaced.

Errors in reading spirit-in-glass thermometers under test may occur if the minimum thermometer is not cooled slowly, since the spirit will adhere to the wall of the bore. Consistently low readings may be due to this fault and can be corrected by warming the stem with a warm cloth.

The maximum errors permitted are as follows:

At range	*Error*		*Change or Error in 20°F*
	low	*high*	
Below 32°F	−0·4	+0·4	0·4
32°F–80°F	−0·2	+0·2	0·2
Over 80°F	−0·4	+0·4	0·4

If, after correction of faults and testing, the errors are greater than those permitted the thermometer should be rejected.

(*c*) *Maximum Thermometer, Clinical Type, for Use in a 22° Bath*

The mercury in the thermometer under test should first be shaken down below 20° and placed in a vertical position by clamps so that the bulb is about 2 in. below the surface of the water and at least 2 in. from the side of the container. The standard thermometer should be placed at an angle so that 3 in. is immersed, and with the bulb as near as possible to that of the thermometer under test and at least 2 in. from the side of the container. The water in the container must be continuously and vigorously stirred throughout.

The thermometer under test should be read when the standard thermometer records 20° (68·0°F), 22·5° (72·5°F) and 25° (77·0°F). The whole operation at 22·5° should be repeated. The readings must be taken from the top of the meniscus, and only after the standard thermometer has been steady for at least 60 sec. Special care should be taken to avoid errors of parallax with lens fronted thermometers. The temperature of the water should not be allowed to fall during this examination, otherwise the test thermometer should be withdrawn, cooled to a temperature below that of the water and the mercury column reset in the usual way by a few downward shakes of the thermometer.

The thermometer should be examined to see that as the temperature is lowered the mercury is not drawn past the constriction when the thermometer is mounted vertically. A gradual rather than an abrupt fall in temperature is more likely to reveal this fault, e.g., in the bath for the temperature-compensated Clot on Boiling Test (C.O.B.) after switching off the heat.

The maximum permitted error is ±0·1° at 22·5°.

If, after correction of faults and testing, the error is greater than that permitted the thermometer should be rejected.

Resetting and Reading

7. *Maximum and Minimum (Meteorological) Thermometers*

The details of mounting these thermometers are given in the 'Notes on Atmospheric Shade Boxes'. The thermometers cannot be permanently

fixed in position as they must be reset each day, but when correctly mounted at an angle of about 2 degrees to the horizontal, with the bulb at the lower end, strong winds and jolts (as when closing the door) must not be capable of moving the indices.

Both thermometers should be kept clean and bright.

(*a*) *Resetting*. The maximum thermometer should be reset by holding it near the end opposite to the bulb and swinging vigorously downwards at arms length (standing well clear of all fixed objects).

The minimum thermometer should be reset by tilting it until the index contained in the spirit touches the meniscus.

After setting meteorological thermometers a check should be made that both instruments give the same reading within a fraction (a fifth) of a degree. Any discrepancies should be investigated.

When placing in the atmospheric shade box following resetting, the maximum thermometer should be inclined bulb downwards and the minimum thermometer bulb uppermost so that the lower end and the upper end respectively reach their supports first, thus ensuring that the mercury in the former does not break and flow up the bore and the index in the latter does not flow down. While replacing in the atmospheric shade box the thermometer bulbs should not be touched with the hands.

(*b*) *Reading*. As the scale and the mercury or index are not in the same plane, errors of parallax will occur when observing unless the eye is directly in front of the thermometer and at 90 degrees to the extreme end of the index or mercury. Readings should be taken while the thermometers are still in position and should be estimated to tenths of a degree. The correction for the error of the individual should be applied and the corrected reading recorded to the nearest degree, readings of 0·5 or greater being raised to the next highest degree.

The thermometer should be read as rapidly as is consistent with accuracy in order to avoid changes of temperature due to the presence of the observer. Special care should be taken that the warmth from a lamp or from the hand does not raise the temperature.

After recording the reading the thermometers should be reset and replaced in the atmospheric shade box.

8. *Maximum Thermometer, Clinical Type, for Use in 22° Bath*

(*a*) *Resetting*. Provided mercury has not entered the expansion chamber the thermometer should be reset by holding it near the end opposite the bulb and swinging vigorously downward at arms length (standing well clear of all fixed objects). If the air temperature is over 20° the thermometer should be chilled in cold water prior to shaking. After resetting to a reading below 21° the thermometer should be placed in the control sample which itself should be below 21°.

(*b*) *Reading*. The temperature-compensated Clot on Boiling (C.O.B.) Test technique lays down that the maximum thermometer, clinical type, should be contained in 2 oz of water in a 3 oz bottle placed in the 22° bath. The thermometer should be read immediately after removal from the bath but while still immersed in the 2 oz of water. Any correction factor which may be necessary as a result of testing should be applied and the corrected reading should not be rounded off before recording.

Detergents

77. DETERGENTS

DETERGENTS may be described broadly as materials added to water to improve its power of removing dirt from surfaces. Another property, which may be just as important in special cases, e.g., bottle washing, is their bactericidal power. The water softening power of certain detergents is also important.

The varieties of dirt to be dealt with in the milk industry are:
liquid milk films;
air dried milk films;
heat precipitated films;
heat hardened films;
hard water deposits; and
miscellaneous foreign matter.

In order to deal with these the detergent should have the following properties:
power to wet the dirt and the surface on which it lies;
disintegration, suspension and/or solution of milk solids over a range of conditions; and
free rinsing.

This brief outline should make it apparent that no one chemical compound can fulfil all the essential requirements and a mixture may be necessary to obtain the desired properties. The materials available may be put in seven groups

1. Hydroxides and alkaline salts.
2. Synthetic surface active agents.
3. Water softening salts.
4. Acids.
5. Soaps.
6. Colloids.
7. Abrasives.

Of the above groups, 1, 2 and 3, whether separately or in formulated products are by far the most generally used. Group 4 compounds have special application for scale prevention or removal. The remaining groups are largely out-dated but may find use in special cases.

Hydroxides and Alkaline Salts

In this group the main products in common use are:
sodium hydroxide (caustic soda)
sodium carbonates (usually as soda ash or washing soda)
trisodium phosphate
sodium silicates (ortho, meta and sesqui salts)

The detergent properties of each of these materials are reviewed in succeeding sections.

Where laboratory experimental results are quoted it should be appreciated that these have not in all cases been substantiated in the less simple conditions of commercial practice. Differences of opinion in publications may be due to variations in laboratory technique.

1. *Wetting*

The alkaline salts in themselves are relatively poor wetting agents when compared with the synthetic surface active agents.

It is necessary to wet surfaces to remove films from them and to bring the detergent compounds into intimate contact with the soiling. In compound detergents, synthetic surface active agents are frequently included. In bottle washing, this effect is augmented by the production of soap from the action of caustic soda on fatty residues.

2. *Solution*

Solution as far as milk protein is concerned is a function of the caustic alkalinity (NaOH), also referred to as 'Available alkalinity', or in the notation of alkali manufacturers as 'active Na_2O', which represents the end point of the titration with standard acid using phenolphthalein as the indicator. The figures for the common commercial detergents are given in the table as caustic alkalinity and active Na_2O.

Table I

	Caustic alkalinity as NaOH	Activity alkalinity as Na_2O
Caustic soda (NaOH)	96·8	75·0
Soda ash (Na_2CO_3)	36·3	28·1
Washing soda ($Na_2CO_3 10H_2O$)	14·0	10·8
Sodium sesquicarbonate ($NaHCO_3$. $Na_2CO_3 3H_2O$)	14·3	11·1
Trisodium phosphate ($Na_3PO_4 11H_2O$)*	11·2	8·7
Sodium ortho silicate ($2Na_2O\ SiO_2 3H_2O$)	59·7	46·2
Sodium metasilicate ($Na_2O\ SiO_2 5H_2O$)	32·1	24·9
Sodium sesquisilicate ($3Na_2O\ 2SiO_2 11H_2O$)	40·0	31·0

*Commercial sample (normal composition, $12H_2O$)

Solution occurs because of the formation of soluble sodium salts of the proteins, but there is a secondary action due to the fact that these salts are weakly surface active and therefore assist in the emulsification of fats and oils.

Deposits formed from heat denatured and coagulated protein are more difficult to remove than air dried films, and the factors of concentration of detergent, time of application and temperature all require consideration.

In general the effectiveness of alkaline detergents in removing such deposits is related to their caustic alkalinity, but other factors, such as pH, also play a part. For pasteurizing equipment it is also important to maintain an adequate concentration of sequestering agents.

3. *Emulsification*

Emulsification is necessary to facilitate the complete removal of fats and oils. An essential preliminary is the wetting of the surface of the fat or oil and the addition of a surface active agent, causing the liquid to spread over the surface, is necessary. Scrubbing action will then disperse the fat in the form of droplets which tend to remain in suspension because of the reduction of interfacial energy between them and the solution. The practical result is that the fat is carried away in suspension in the detergent solution.

Hydroxides and alkaline salts play little part in emulsification except so far as they may react with fatty deposits to form surface active compounds.

4. *Buffering Power*

The caustic alkalinity of caustic soda, ortho silicates and phosphates is less prone to inactivation by the acidity of the milk residues than that of the carbonate and sesqui salts. This property is termed buffering power.

5. *Rinsability*

In the interests of avoiding corrosion of equipment and/or actually tainting milk products subsequently handled, it is important that the detergent should be readily rinsed from the surface after use. That there are big differences in this respect is illustrated by the slimy feel of a surface washed with caustic soda solution compared with one on which trisodium phosphate has been used. This property of free rinsing is associated with a good wetting power and the degree to which the detergent is absorbed on the washed surface. There is no standard method of determining this factor, but it is generally recognised that among inorganic alkalis trisodium phosphate is the best in this class, followed by sodium hexametaphosphate and the silicates.

Most of the synthetic surface active agents possess good rinsability and are able to impart this property to alkaline detergents when used in conjunction with them.

6. *Scale Prevention*

A point to be remembered is that alkaline detergents may give insoluble precipitates with hard water, which may lead to troublesome deposits. The detergent consumed in precipitating 'hardness' is lost as regards detergent powers and allowance may have to be made for this.

These deposits can, of course, be avoided by the use of softened water or steam condensate. Under certain conditions water softening salts can be used (see page 152).

Conditions resembling those of very hard water result from traces of refrigerator brine. These can easily occur when cleaning plate coolers and coil vats of modern design.

7. *Corrosion*

With developments in the use of stainless steel, corrosion is likely to fall into the minor class of dairy problems, but as there is still much

aluminium and tinned copper plant in existence, corrosion must still be considered. Although the effect of alkalis on aluminium is generally known, it is not so widely appreciated that 'active Na_2O' also attacks tin. To protect tin, 1 part of sodium sulphite should be added for every 10 parts of washing soda present. If caustic soda or soda ash is used, the proportion should be increased to 1 in 4. Some idea of the limiting concentration can be obtained from the following figures, representing the maximum percentages of detergent which did not etch tin plate at 60°F.(16°).

Table II

	Per cent detergent not etching in	
	30 min	60 min
Caustic soda	0·005	0·005
Sodium carbonate	0·01	0·005
Trisodium phosphate	0·20	0·01
Sodium metasilicate	0·25	0·15

As the alkalinity of the silicates increases the safe concentration falls. For the same concentration of 'active Na_2O' the silicates are less corrosive than other alkalis.

Silicates, therefore, form the basis of alkaline cleaners for aluminium. The general properties of the common alkalis are summarised in Table III. On the whole this reflects published opinion, but striking differences are recorded and the table should be examined in the light of current opinion. It is obvious that much further work remains to be done on the use of common alkalis in the dairy industry, particularly in respect of optimum times and concentrations and variations in properties arising from admixtures.

Synthetic Surface Active Agents

Surface active agents are chemicals which have the characteristic of accumulating and concentrating at surfaces, such as the surface between a liquid and air, the surface between two liquids which will not mix, or the surface between a liquid and a solid. This accumulation of foreign matter at the surface weakens the latter making it easier to break down, and this is useful in a number of the aspects of detergency, particularly wetting, emulsification and rinsing. Foaming is also a factor much affected by the presence of surface active agents.

Ordinary soaps, which have been known and used for many years, are surface active agents, but they suffer from certain disadvantages inherent in their chemical nature. Some of these difficulties are avoidable in synthetic products and since World War II many thousands of synthetic surface active agents have been made in order to develop ones with a more nearly ideal balance of surface active properties. Most of these have proved

Table III

Agent	Wetting power	Solution of heat-deposited milk solids	Emulsifica-of fats	Buffering power	Rinsability	Scale prevention	Freedom from corrosive action
Caustic soda	poor	very good	fair	*poor	poor	poor	poor
Carbonates	poor	good	poor	poor	fair	poor	fair
Trisodium phosphate	fair	good	poor	good	good	good	fair
Ortho silicate	fair	very good	poor	very good	fair	poor	poor
Metasilicate	fair	good	good	good	good	fair	good
Sesquisilicate	fair	good	poor	fair	fair	fair	good

*In the strictly scientific meaning of the word caustic soda is a poor buffer. However, because of its strong alkalinity it is in effect a good buffer in practice except when used in very dilute solutions.

uneconomical or otherwise unacceptable to industry, and the major consumption has now largely settled down to the eight types listed in Table IV. The field is still evolving, however, and an important property now being sought in new products is the capacity to be broken down by bacteria in sewage plants and river waters, so as to avoid persistent foam which is a nuisance and interferes with water purification processes.

Surface active agents are used in dairy detergents in two main ways. In the first they are used to produce materials relying wholly on surface active agents for detergency, i.e., liquid detergents and those powdered ones in which the bulk of the remainder is inert filler. In the second method, surface active agents are used to accentuate the properties of other constituents and to impart new properties such as wetting and rinsability.

In manual operations foam is usually desirable; in most circulation or spray cleaning operations foam is undesirable and this limits the potential choice of surface active agents.

Certain incompatibilties must also be avoided; undiluted hypochlorite will react with many surface-active agents in concentrated form, leading to a loss of both detergency and bactericidal activity.

Another important factor is that in many mixtures containing surface active agents the activity of the mixture is much greater than the activity which might be expected from the total of activities of the separate components.

To obtain maximum efficiency, the choice of a mixture of surface active agents becomes a very complicated procedure, but in Table IV the major properties of different types are summarised. It must be remembered that each type may cover a whole range of chemicals and it has therefore been necessary to describe certain properties as a range rather than a particular level. Also the table considers only the main types in current use in this country. For more detailed information on types of material and trade names, McCutcheon (see Ref. 1) lists several thousand materials.

Water Softening Salts

From the dairy aspect, polyphosphates, e.g., sodium hexametaphosphate, are products of outstanding interest. Also included in the water softening group are organic sequestering agents, such as ethylene diamine tetra-acetic acid and gluconates. They form soluble complexes with calcium and magnesium salts, thereby both preventing the formation of insoluble compounds from hard waters and dissolving any deposits which may have formed. Their applications, therefore, lie mainly in preventing scale.

The polyphosphates, like other phosphates, are free rinsing but are subject to decomposition at high temperatures in the presence of alkalis or acids.

Acids

The hard scale 'milk stone' in addition to containing denatured protein is largely composed of inorganic calcium salts, and these are usually removed from equipment with phosphoric acid, which is effective at a concentration of about 1 part syrupy acid in 160 parts of water, i.e., 1 pt in 20 gal.

In addition to phosphoric acid, nitric acid, sulphamic acid and lactic acid have been used. Corrosion is an important factor in the use of acids on

Table IV

Summary of Properties of Major Types of Surface Active Agents when used in Normal Detergent Formulations

Chemical type	Ionic nature	Wetting ability	Foaming	Dislodgement of soiling from surfaces	Emulsification	Bactericidal activity
1. Alkyl benzene sulphonates	anionic	good	high	good	moderate to good	poor
2. Alkyl sulphates	anionic	good	very high	good	moderate to good	poor
3. Ether sulphates	anionic	good	very high	good	moderate to good	poor
4. Alkyl aryl ethers	nonionic	good	moderate to low	moderate to good	good	nil
5. Fatty alcohol ethers	nonionic	good	moderate to low	moderate to good	good	nil
6. Fatty acid amides	nonionic	moderate	very high	moderate	moderate	nil
7. Propylene oxide/ethylene oxide condensates	nonionic	good	moderate to low	moderate	poor to good	nil
8. Quaternary ammonium compounds	cationic	poor	moderate	poor	poor	good

metal plant and makers' instructions should be rigidly observed. *Undiluted acid should not be allowed to come into contact with the skin, clothing or metal utensils, and should be out of reach of children and animals.*

Soaps

Although excellent emulsifiers, soaps possess no other outstanding property which would justify their use for dairy purposes. With hard waters they have the disadvantage of forming insoluble glutinous deposits.

Colloids

Carboxymethyl cellulose may be used in compound alkaline detergents to assist in keeping in colloidal suspension the insoluble matter removed from the cleaned surface. Its use is in practice generally limited to detergents used in mechanical bottle and churn washers, where the detergent is recirculated.

Abrasives

In plant which is thoroughly cleaned each day there should be no need to use abrasives to remove milk residues unless the conditions are peculiar to a certain piece of plant. Even then their use should be avoided if possible, both because of the danger of removal of surface coatings, e.g., on copper, and the formation of scratches which provide a basis on which scale can build up.

Miscellaneous Information

The following points should be noted:

(i) *Pre-Rinsing*
The efficient rinsing of the plant with warm or cold water, before the use of detergents, is a valuable preliminary to cleaning. Very hot water should not be used to rinse plant which has only contained cold milk, otherwise scale may be deposited.

(ii) *Mechanical Action*
Scrubbing either by brushing or vigorous agitation greatly assists all cleaning processes.

(iii) *Concentration*
In all detergent operations it is essential to use the concentrations recommended by the manufacturers.

(iv) *Storage*
Detergents should be stored under moderately cool and dry conditions.

(v) *Lubrication*
Caustic soda is frequently relied upon to aid lubrication of machinery, e.g., in bottle washers, and drastic changes in the compounding or concentration of detergents must take this aspect into account.

(vi) *Skin Irritation*
This may become a problem and is best overcome by the use of rubber gloves and general care to avoid skin contact with the detergent, particularly by those persons who have an allergic predisposition. In those cases where skin sensitivity has developed to a particular detergent it may be helpful to change to another.

Bactericidal Properties

For all practical purposes, of the alkalis considered only caustic soda and perhaps ortho silicate have any appreciable bactericidal power at moderate temperatures up to 120°F(50°). Above such temperatures one is soon into the range in which some organisms are killed by heat alone and most detergents will help this process. Strongly-acid cleaners are also rather effective bactericides, but are only used at the present time for special purposes.

The practical efficiency of any process of sterilization will be greatly increased by proper detergency, resulting in the removal from the surface to be sterilized of all adherent soiling and as many micro-organisms as possible.

(*a*) *Caustic Soda.* This is an excellent, non-selective bactericide in its own right, its efficiency being dependent on the strength of solution, the temperature of application and the time for which it acts. These factors, in various degrees of relative importance, govern the performance of all disinfectants. The germicidal properties of caustic soda find two very important applications in dairy cleaning.

(i) In bottle washing, where bottles are either jetted by and/or soaked in caustic soda based detergent solutions, often of some 0·75 per cent caustic alkalinity at perhaps 150°F(66°) Such a treatment, lasting for a matter of minutes will kill all vegetative bacteria and a useful proportion of spores. If temperatures can be raised to, say, 180°F(82°), then a reduction in the concentration of caustic soda can be made whilst reduction in temperature would necessitate an increase in concentration.

In bottle-washing machines, frequent control of detergent strength and temperature is essential, as these can fluctuate rapidly with a high throughput of bottles.

(ii) In immersion cleaning of stainless steel and rubber milking utensils, these are left soaking continuously in cold caustic soda solution, only being removed for use and occasional adjustment and replacement. The contact time is long, but the temperature is low: an initial concentration of 2·5 per cent is needed to ensure both cleaning and sterilizing. Usually a small proportion of an anti-scale agent (e.g., ethylene diamine tetra-acetic acid) is included to prevent hard water scale deposition.

As previously stated, the uses of caustic soda are severely limited by its corrosiveness of metal and its acute irritancy of skin.

(*b*) *Other Detergents.* Whilst substantially non-bactericidal themselves, they can be used in conjunction with bactericides, either being compounded together as single commercial products or being added as separate products to the same solutions. Detergents and bactericides cannot be mixed indiscriminately because of certain incompatabilities. The following is a guide to the type of detergent which can be used with different bactericides:

(i) *Sodium Hypochlorite Solution*

Any of the mild alkalis can be used with hypochlorites. Hypochlorite, although usually regarded as purely a bacteri-

cide, can improve the detergency of alkalis against proteinaceous deposits. For light duty detergency, synthetic surface active agents are often used alone; hypochlorite can be added to such solutions at use dilution to render them bactericidal.

Many surface-active agents interact with hypochlorite when mixed in the undiluted form. Such products should therefore not be mixed undiluted, unless specifically recommended by the manufacturer.

(ii) *Organic Chlorine Compounds*

These are normally sold as proprietary detergent-sterilizer products. They are compounded with synthetic anionic surface active agents, but not with nonionic surface active agents, in the presence of which they are unstable. Acid, neutral and alkaline products are available.

(iii) *Quaternary Ammonium Compounds* (*Q.A.C.*)

These are usually obtained as compounded detergent-sterilizer powders, but plain acqueous solutions can be obtained. The Q.A.C.'s are mutually incompatible with anionic surface active agents, e.g., soaps, alkyl sulphates, alkyl aryl sulphonates, and the uninformed should not attempt to mix Q.A.C.'s with detergents, except perhaps sodium carbonate. Q.A.C.'s are themselves surface active, but, at the concentrations normally used for bactericidal purposes, this gives negligible detergency.

(iv) *Iodine*

Iodine is a powerful sterilizing agent. For utensil treatment it will usually be purchased in the form of formulated products commonly referred to as iodophors, containing selected synthetic wetters and mineral acids. These products should be used according to instructions and never mixed with other detergents.

REFERENCE

1. John W. McCutcheon Inc. (1963 and annually). *Detergents and Emulsifiers*, 236, Mount Kemble Avenue, Morristown, New Jersey, U.S.A.

Appendix I

BAIRD-PARKER MODIFICATION OF HUGH AND LEIFSON TEST (1963)

The production of acid from glucose aerobically and/or anaerobically is tested for by the Hugh and Leifson method (1953) using the following medium:

Ammonium di-hydrogen phosphate ($NH_4H_2PO_4$)	1·0 g
Potassium chloride (KCl)	0·2 g
Magnesium sulphate ($Mg\ SO_4\ 7H_2O$)	0·2 g
Yeast extract (Bacto)	1·0 g
Bromo-cresol purple	0·04 g
Agar	2·0 g
Distilled water	1,000 ml
pH	7·0

Sterilize the medium in 90 ml amounts and add 10 ml of a 10 per cent (W/V) Seitz-filtered solution of glucose to the molten base (about 45°) immediately before use. Dispense the medium aseptically into sterile $6 \times \frac{5}{8}$ in. test-tubes so that they are two-thirds full. Steam the tubed medium for 15 min to drive off the dissolved oxygen and hold at 5° until set.

Stab inoculate duplicate tubes throughout their length with a heavy inoculum from Yeastrel milk agar plates.

After inoculation cover one tube of each pair with a $\frac{1}{2}$ in. layer of sterile liquid paraffin. Incubate for 5–10 days at 30° and examine for acid production in both tubes.

Fermentative organisms will produce an acid reaction throughout in both tubes.

Oxidative organisms will produce an acid reaction in the open tube only, leaving the covered tube unchanged with little or no apparent growth. The acid reaction produced by the oxidative organisms is apparent first at the surface and extends gradually downward into the medium. Where the oxidation is weak or slow, it is usual to observe an alkaline reaction at the surface of the open tube. This may persist for a variable length of time, up to several days of incubation before turning acid and must not be mistaken for a negative reaction.

Non-fermenters and *non-oxidisers* produce no change in the covered tube and only an alkaline reaction in the open tube.

Appendix II

WORKING TEMPERATURE AND TEMPERATURE DISTRIBUTION TEST

For this test the bath should be loaded with its maximum working capacity of racks and test-tubes. The test-tubes should each contain about 10 ml of water and should have been warmed previously to approximately the required working temperature of the bath. After the bath has been loaded it should be allowed to attain equilibrium for 1 hr before commencing the test.

1. The cover must be replaced by a sheet of wood, cardboard, or other suitable material drilled as in Fig. 4 to accommodate thermometers A—G.

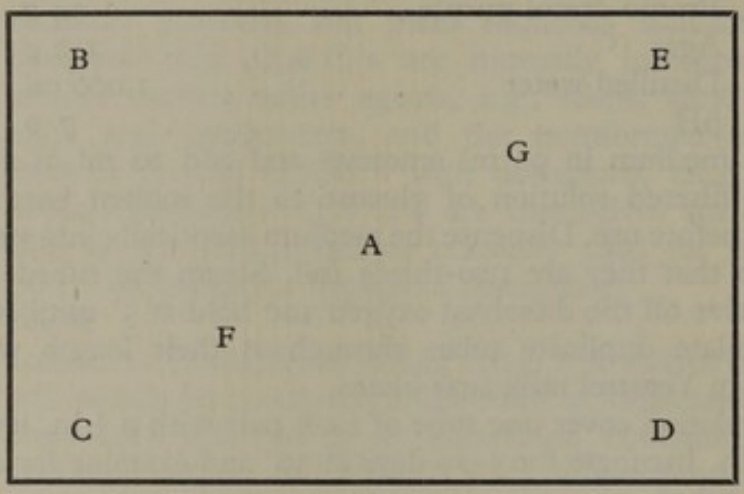


Fig. 4. Plan. Thermometers B, C, D, and E are fitted into the corner test-tubes

2. Eight thermometers are required, one of which is used in the position (if any) indicated by the manufacturer of the bath. All must have been calibrated against a standard (N.P.L.) thermometer.

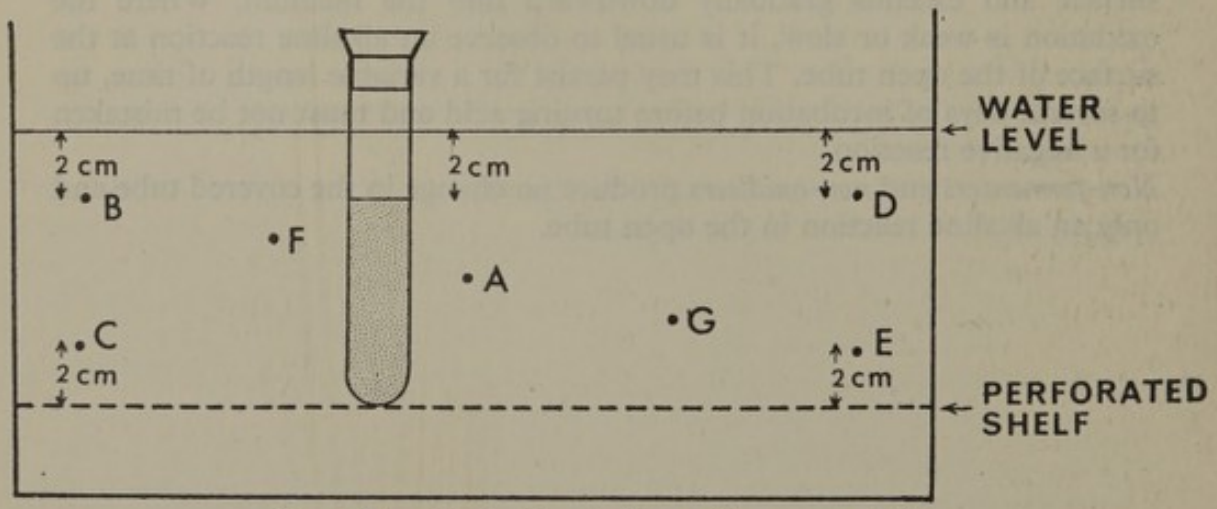


Fig. 5. Elevation to show positions of thermometer bulbs

3. The centre of the thermometer bulbs must be immersed to the depths shown in the elevation diagram Fig. 5.

4. After allowing 1 hr for the bath to reach equilibrium, the temperature shown by all thermometers must be recorded at 5-min intervals over a further period of 1 hr. An example of this type of recording, for a 37° bath, is given in Table I. The letter 'M' in this table indicates the thermometer placed in the fixed position provided by the manufacturer.

Table I

Min.	B.	C.	F.	A.	G.	D.	E.	M.
5	37·7	37·7	37·8	38·0	37·8	37·8	37·4	37·7
10	37·7	37·6	37·8	38·0	37·8	37·8	37·2	37·7
15	37·0	37·6	37·7	37·8	37·7	37·7	37·2	37·6
20	37·3	37·3	37·4	37·6	37·4	37·4	37·0	37·3
25	37·1	37·1	37·2	37·4	37·2	37·2	36·9	37·2
30	37·0	37·0	37·0	37·2	37·0	37·0	36·8	37·0
35	39·5	39·2	40·0	40·0	39·5	39·5	39·0	39·5
40	40·1	40·0	40·0	40·2	40·0	40·0	39·7	40·2
45	39·9	39·9	39·9	40·1	39·9	39·9	39·4	39·9
50	39·7	39·7	39·7	39·9	39·7	39·7	39·1	39·7
55	39·4	39·4	39·4	39·8	39·4	39·4	39·0	39·4
60	39·2	39·2	39·2	39·5	39·2	39·2	38·7	39·2

5. The *Working Temperature* is the mean temperature of thermometer A and in the example given is 38·8°
6. The 'permissible temperature limits' are ±0·5° of the working temperature. This means that in the example given, that all readings falling between 38·3° and 39·3° (both inclusive) are permissible.
7. If, at this point, the mean working temperature is found to be too high or too low, the thermostat should be adjusted and the test repeated until a satisfactory mean is recorded.

Table II

Min.	B.	C.	F.	A.	G.	D.	E.	M.
5	−0·6	−0·6	−0·5	−0·3	−0·5	−0·5	−0·9	−0·6
10	−0·6	−0·7	−0·5	−0·3	−0·5	−0·5	−1·1	−0·6
15	−1·3	−0·7	−0·6	−0·5	−0·6	−0·6	−1·1	−0·7
20	−1·0	−1·0	−0·9	−0·7	−0·9	−0·9	−1·3	−1·0
25	−1·2	−1·2	−1·1	−0·9	−1·1	−1·1	−1·4	−1·1
30	−1·3	−1·3	−1·3	−1·1	−1·3	−1·3	−1·5	−1·3
35	+0·2	0	+0·7	+0·7	+0·2	+0·2	0	+0·2
40	+0·8	+0·7	+0·7	+0·9	+0·7	+0·7	+0·4	+0·9
45	+0·6	+0·6	+0·6	+0·8	+0·6	+0·6	+0·1	+0·6
50	+0·4	+0·4	+0·4	+0·6	+0·4	+0·4	0	+0·4
55	+0·1	+0·1	+0·1	+0·5	+0·1	+0·1	0	+0·1
60	0	0	0	+0·2	0	0	0	0

8. Calculate from the temperatures recorded in Table I the extent to which these differ from the permissible temperature limits (see Table II). In the example given, the temperatures in Table I falling between 38·3° and 39·3° inclusive are within the permissible temperature limits and the difference is recorded as 0. The differences for temperatures below 38·3° are recorded as –ve values and those for temperatures above 39·3° are recorded as +ve values.
   It must be borne in mind that the horizontal readings in each line indicate temperature variation over the whole area of the bath at any one time, while the vertical readings indicate variation in any one place over the given time.
9. Proper adjustment of the thermostat should enable the correct working temperature to be realized and, if the bath is satisfactory, the variation shown by all the other thermometers should not be outside the permissible temperature limits. If this is not the case the manufacturers of the bath should be consulted.

Appendix III

TEMPERATURE RECOVERY TEST

THIS test is necessary to check the speed at which the water bath recovers its working temperature after the insertion of the tubes under test, and is especially important in such short time tests as the resazurin and methylene blue, milk quality tests.

1. During this test all test-tube racks must be in position.

2. A thermometer must be set between the two central holes of the central rack in such a way that the bulb is positioned half way up the column of milk in an imaginary test-tube. A special hole may have to be bored in the rack in order to accommodate this thermometer.

3. Ten tubes, each containing 11 ml of milk at 5°, must then be placed without delay in the same row as the thermometer and equally disposed on either side. If the row has less than 10 holes, the remaining tubes must be placed in the end holes of an adjacent row.

4. Readings must be taken on the thermometer every 30 sec for the 10 min period immediately following the entry of the tubes.

5. The test must then be repeated as in paragraphs 3 and 4, positioning the tubes in the outermost rack placings.

6. Satisfactory recovery is indicated if the lower permissible temperature limit is achieved, for each of the two positions of the rack, within 5 min of taking the first reading.

7. Unsatisfactory recovery times should be taken up with the manufacturers.

Appendix IV

ADVISORY METHODS

A. The Advisory Approach to Modern Methods of Milk Production

WITH the abolition in October, 1964, of the clot-on-boiling test for statutory purposes, the introduction of the 'hygiene' test in creameries for the payment of market milk on a quality basis and changes during recent years in methods of milk production and handling on the farm, a reappraisal of the advisory approach to the bacteriological problems associated with milk production is required.

Since the 'hygiene' test as used at present is a resazurin dye reduction test, involving the incubation of the milk at 37°, the result may be determined by factors different from those affecting the C.O.B. test at 22° and these must be borne in mind in the interpretation of the results.

Changes in methods of production on the farm include the increasing use of fixed and mobile pipeline installations involving cleansing *in situ*, the widespread use of chemical detergent-sterilizers, bulk storage on the farm of refrigerated milk and measures recently introduced for the more effective control of mastitis. All these factors may affect the type of bacteriological problems encountered and consequently the nature of the advice given.

While it must be emphasised that contaminated equipment is still a primary cause of unsatisfactory milk, nevertheless, when a sample fails to satisfy a bacteriological test it can be the result of several factors operating conjointly. Therefore, on the farm, due attention must also be paid to udder health, milking hygiene and the efficiency of cooling and storage of the cooled milk, since test failures or spoilage may be due to weaknesses in these respects in addition to contaminated equipment.

Close attention must, of course, be paid to cleansing of the equipment. Disappointing results are invariably due to the incorrect application of the cleansing procedure, lack of attention to detail and to the fact that the equipment either does not lend itself to effective cleansing or because individual items are not maintained in a satisfactory physical state.

Milking hygiene has perhaps not had as much attention as it warrants, not only from the aspect of the bacteriological quality of the milk, but also in the control of spread of mastitis infection. The efficiency of udder-washing, proper withdrawal of foremilk, correct handling and management of milking machines and general cleanliness of the environment—all have a bearing on the bacteriological condition of the milk. This may be affected either by contamination from external sources or, when mastitis infection is present, directly from the udder.

The increasing use of refrigeration, often in conjunction with bulk storage and collection of the milk, has provided a means whereby bacterial proliferation in the bulk milk can be effectively prevented. Where refrigeration is not available more attention should be paid to making the

best possible use of the cooling water. If the milk can be cooled to 50°F and kept cool, little bacterial proliferation will occur while the milk is stored on the farm; but for much of the year the milk cannot be cooled below 55–60°F and in very warm weather it may be difficult to cool it to 65°F. It is under these latter conditions in particular that advice on making the best possible use of the cooling water, and on the subsequent storage of the milk in a cool situation, may serve to prevent test failures.

In the investigation of any problem a systematic and thorough inspection of all items of the dairy equipment, and of the production methods practiced, is the *first essential*. For most routine problems this will be all that is necessary to allow sound practical advice to be given for the satisfactory correction of the faults. For a complete assessment of the detailed routine followed it is necessary to visit the farm at milking time, so that the equipment can be inspected prior to use, and the milking and subsequent cleansing of the equipment witnessed.

The adviser must use his discretion regarding the taking of advisory samples, but these should be reserved for occasions when a diagnosis of the problem cannot be made by thorough inspection and full discussion with the producer or his herdsman. When it is necessary to take samples this should always be done so that the maximum amount of information on which reliable advice can be based is obtained from the laboratory examinations.

It is most important that the advice given, either verbally or in writing, should be clear, concise and of a strictly practical nature and designed to suit the conditions on the particular farm, otherwise it is unlikely that it will be effectively applied.

The bacteriological problems associated with modern methods of milk production can be many and varied and each has to be investigated in the light of the particular circumstances. Therefore in describing a procedure for the investigation of faults and problems it is not possible to generalise, but recommended procedures for the investigation of some of the more common advisory problems are outlined below.

1. *Failure of Creamery Dye Reduction Tests*

When advice is sought as a result of a failure of the rejection (10-min resazurin) or 'hygiene' tests a visit should be made to the farm just before milking and the problem discussed with the producer. Notification of the test failure may have caused the producer to improve his milking technique, the efficiency of cleansing the equipment and cooling of the milk; any such changes should be noted.

The milking equipment should be examined carefully for physical deterioration and evidence of unsatisfactory cleansing. Physical deterioration of rubber parts will be indicated by distortion, the presence of cracks, roughness of the surface and loss of resilience; of metalware by pitting, corrosion, scratching of the surface, or the presence of open seams. An unsatisfactory cleansing procedure may result in the presence of milk residues or a build-up of scale particularly in the more inaccessible places. The possibility of contamination from the vacuum line should also be investigated.

The temperature of the water used for cooling and, if possible, the temperature of the cooled milk should be recorded. The temperature at which the milk is stored should be noted.

After a thorough inspection of the equipment and observation of the methods, the reasons for the test failure may be apparent and advice can be given without recourse to sampling. If sampling is necessary the relevant information should be entered in detail on the Field Report, since it will have a bearing on the type and extent of the laboratory examination applied to the samples and the interpretation of the results obtained and advice given.

Rinses should only be taken when the equipment is visually clean and in a satisfactory physical condition, and should be subjected to a bacteriological examination only if free from visible residues.

When it is necessary to take rinses of the equipment, the object should be to obtain an overall picture and, therefore, all items should be rinsed using as few rinses as possible. For example, considering a four-unit bucket plant, all four clusters may be rinsed using one 500 ml amount and the buckets similarly treated. The strainer, D-pan and cooler should also be combined in one rinse. Where pipeline systems are involved, one rinse should be taken of the clusters and pipeline, and if the cooler is cleaned *in situ* as part of the system this should also be included, but if cleansed separately it should be rinsed separately. The areas of the surfaces rinsed should be stated on the Field Report.

When a bacteriological examination of the rinse is carried out this should normally be limited to a colony count and sufficient dilutions should be plated to ensure that a *countable* plate is obtained. Additional useful information may be obtained by examinations for coli-aerogenes and milk spoilage bacteria.

In order to obtain information about factors other than contaminated equipment, which may be partly responsible for milk test failures, a milk sample may be taken. This should preferably be taken from a can filled with the first 10 gal milk over the equipment and which has been cooled in the way normal for that farm. If the condition of the can is in doubt it may be cleansed on the farm before use, or a rinse taken beforehand to determine its bacteriological condition, or the sample taken from the cooler. After sampling, the temperature of the milk in the can should be recorded and the sample transported to the laboratory with the least possible delay, preferably within 4 hr of milking. If the presence of udder cells is thought to have affected the 'hygiene' test, a milk sample should be examined for total cell count (see Technique No. 40.).

On arrival at the laboratory a p.m. sample should be divided and both portions held overnight for 18 hr, one at 3–5° and the other at $18{\cdot}5 \pm 1°$ ($65 \pm 2°$F).

The refrigerated sub-sample should be examined for colony count, coli-aerogenes organisms and cells (Whiteside test or cell count). A resazurin test at 37° should also be performed during which the tubes should be inverted at intervals of 30 min and disc readings taken at 1, 2, 3 and 4 hr. When the resazurin has been reduced to white, or pink and white mottling (disc reading 0 or $\frac{1}{2}$), this should be recorded and the tubes discarded.

An a.m. sample should be divided on arrival at the laboratory and one

portion examined immediately for colony count, coli-aerogenes organisms and cells (Whiteside test or cell count) and the resazurin test at 37° should also be performed. The other portion should be held at 3–5° until 5.0 p.m. and then at 18·5±1° for 18 hr overnight.

Sub-samples of a.m. and p.m. milk which have been stored at 18·5° should be examined by the resazurin test and for colony count.

The bacteriological results should be interpreted having regard to the information supplied on the Field Report.

In interpreting the bacteriological results, if the colony count of the equipment is less than 50,000/ft² then it is possible that milking methods, inadequate cooling or high cell counts may be responsible for the test failures *assuming that conditions have not changed on the farm.* The coliform content of the milk, the temperature of the cooling water and milk, and the results of the Whiteside test or cell count may help to determine the influence of these various factors.

When comparing the results obtained on the two milk sub-samples, an increase of more than tenfold in the colony count obtained on the sample stored at 18·5° could indicate bacteriological contamination from the equipment or unsatisfactory milking methods. The effect of these factors can be distinguished as indicated above. If there is no great increase in the colony count then the flora present is probably derived mainly from the udder, and if both colony counts are high the presence of an udder abnormality may be indicated by the Whiteside test or cell count.

A high cell count may also be reflected in the results of the two resazurin tests. In general there will be a higher proportion of test failures due to this cause in winter. If in both of the resazurin tests a disc reading of about 3 is obtained in 2 hr and reduction subsequently proceeds at a slower rate, abnormal milk is indicated and may be confirmed by the Whiteside test or cell count. If reduction proceeds more rapidly in the sub-sample stored at 18·5° and to disc ½ or 0 within 3–4 hr it is evident that there is contamination of the equipment or that milking methods are poor.

2. *Failure of the Half-hour Methylene Blue Test or Customer Complaints regarding Keeping Quality (Producer-Retailer)*

Unlike the producer who sends his milk to a processing creamery, the milk of the producer-retailer will often be subjected to a longer storage period between its production and delivery to the customer and, in addition, an intermediate distributor may be involved or the milk sold from a vending machine. The conditions under which the milk is kept during this period between production and delivery to the customer are very important and often determine the shelf life of the milk after delivery. In addition more equipment such as fillers, bottles, cartons and crates is used and thus there are more possible sources of contamination.

As in the recommended procedures for the investigation of resazurin test failures, the discussion of the problem with the producer, the temperature of the water used for cooling, the temperature to which the milk is cooled, the inspection of the equipment and observation of methods—all are important. Also, the conditions under which the milk is kept between production and delivery to the customer should be determined and recorded on the Field Report.

Rinses should be taken only if inspection shows that the equipment is clean and should be discarded if cloudy. The additional equipment, e.g., the filler should not be overlooked, and two to four washed bottles, depending on the number in use, should be taken for examination.

Bacteriological examination of the rinses should consist of a colony count; tests for coli-aerogenes and milk spoilage organisms will also be found to be of considerable value.

A milk sample should always be taken, (preferably of the first milk over the equipment) and is best collected in a sterile milk bottle inserted into the filler. After filling, the bottle should be closed with a sterile rubber bung and transported to the laboratory without delay. Depending on the circumstances, the bacteriological examination of other milk samples, taken for example from the cold store, may be of additional value.

At the laboratory the sample should be divided into two portions and one portion examined immediately or, if this is not convenient, after overnight refrigeration. The other portion should be examined after a period of storage which simulates as far as possible the conditions under which the milk is stored after bottling and until it reaches the customer. It is not possible to prescribe these storage conditions as they will vary from farm to farm, but they should have been given in detail on the Field Report.

After the required storage periods both sub-samples should each be examined by the half-hour methylene blue test (which should be continued for 3–4 hr), for colony count, and by the investigational C.O.B. test to end-point (see Technique No. 35). At the times of testing for the ability of the milk to clot on boiling the milk should also be examined for taint or other defect. If a taint or defect develops before the milk clots on boiling the reduction in practical keeping quality should be recorded.

The examination for coli-aerogenes organisms should only be carried out on the sub-sample examined immediately.

The results of the test applied to the rinses and milk sub-samples examined immediately will indicate whether or not the methods of production are satisfactory, and will enable the importance of particular factors such as milking methods, contaminated equipment, and the presence of milk spoilage organisms to be determined. In addition the temperature of the cooling water and cooled milk will indicate whether or not the cooling process is satisfactory. If it is evident that the methods of production are not satisfactory then advice may be given without recourse to the results of the incubated sample. If the methods of production are satisfactory then the results of the test applied to the incubated sample will indicate the effect of storage conditions, whether the milk will pass or fail a statutory methylene blue test and the margin of safety present, and whether there is sufficient shelf life (24 hr) remaining after delivery to the customer.

3. *Taints in Milk*

Particular points to be investigated are (*a*) whether the equipment had a different or more thorough cleansing routine applied to it since the taint became apparent and (*b*) whether the cows have had any change of diet or in their feeding routine prior to the appearance of the taint. A sample of the tainted milk (if available) and of the fresh milk should be obtained and taken to the laboratoy, together with rinses of the equipment if

these are considered appropriate, and tested according to the prescribed procedures.

4. *Blood in Milk*

Particular points to be noted are (*a*) the presence of any recently calved cows in the herd and (*b*) the presence of any cows with any udder injury, infection, or teat sores.

Samples of milk should be taken from all suspect cows. It is essential to trace the cow or cows responsible as soon as possible and it may suffice if the samples are stored only for a few hours in a cool situation and then inspected for traces of pink colouration. In small herds the producer can be advised to take individual cow samples in cups, and after storage to examine them for the presence of blood himself. When the amount of blood is insufficient to be observed by direct inspection it may be detected by centrifugation or by using the 'Occultest' procedure.

5. *Mastitis*

If mastitits is suspected as a probable cause of test failures in the herd the farmer should be advised to consult his veterinary surgeon. Nevertheless the opportunity should be taken to give advice on milking hygiene in order to reduce the spread of infection.

6. *Antibiotics in Milk*

Problems concerning the possible presence of antibiotics in milk should be thoroughly investigated at the farm when appropriate samples can be taken for laboratory examination.

B. The Bacteriological Examination of Farm Dairy Advisory Samples

The extent and nature of the bacteriological examination of advisory samples depends on the particular problem under investigation, and the type of samples submitted to the laboratory. Each advisory case is a separate problem to be treated with an open mind, and the type and number of tests to be applied to any or all of the samples should not be determined until the Field Report has been carefully studied.

It is important that, where appropriate, standard testing techniques should be used so that results obtained at different laboratories are comparable. Apart from unusual problems, the application of not more than three tests to each sample will normally provide the information required for advisory purposes.

1. *Rinse and Swab Samples*

(*a*) The tests applied to rinse and swab samples, taken according to the recommendations given in Technique No. 47, should normally be confined to the colony count on Yeastrel milk agar incubated at 30°, the coli-aerogenes test at 30° and the milk spoilage organisms (M.S.O.) test at 22°.

(*b*) The colony count should be determined according to the method recommended in Technique No. 48 for rinses of farm dairy equipment, Technique No. 51 for rinses of washed milk bottles, Technique No. 50 for rinses of washed milk cans and Technique No. 49

for swab solutions. Where large surface areas, e.g., pipelines, are rinsed with relatively small volumes, e.g., 1–2 litres, it may be advisable to plate out higher dilutions of the rinse e.g., 10^{-2} or 10^{-3} ml.

(*c*) For the coli-aerogenes test, it will generally suffice to inoculate three tubes of single-strength MacConkey's broth, each with 1 ml of rinse or swab solution, although useful information can be obtained, if heavy contamination is expected, by inoculating 1 ml–10^{-3}ml into single tubes of MacConkey's broth. As an alternative method, the coli-aerogenes colony count on violet red bile (V.R.B.) agar, incubated for 20–24 hr at 30°, described in Technique No. 18, is recommended.

(*d*) Testing for the presence of milk spoilage organisms (see Technique No. 52) in rinse and swab solutions may provide useful information, particularly in the case of washed milk bottles, if the result is recorded after 24 and 48 hr as well as after 72 hr incubation at 22°.

(*e*) If desired, the thermoduric colony count may be carried out as described in Technique No. 19.

2. *Milk Samples*

(*a*) When the primary purpose is to assess the overall care taken in applying clean milk production methods, the bacteriological exmination of milk samples is recommended. The selection of appropriate samples, sampling technique, transport of samples, storage and testing of advisory milk samples are described later in this Appendix in Section C.

(*b*) Examination by means of the colony count at 30°, the coli-aerogenes test at 30° together with the determination of keeping quality by means of the clot-on-boiling test at 22° should normally suffice.

(*c*) The temperature compensated method of determining keeping quality recommended in Technique No. 36 can normally be applied to advisory milk samples. This indicates whether the milk would have clotted on boiling had it been kept at 22° for 24, 33 and 48 hr from the assumed time of collection (9 a.m.).

Keeping quality results are more reliable when the C.O.B. test is made on the same milk sample every 3 hr. When samples are taken within 1 hr of production, transported to the laboratory in an ice-box and the test set up within 3 hr of production, the C.O.B. test to end-point at 22° as described in Technique No. 35 should be employed. This test is particularly useful for investigational purposes or in the case of difficult advisory problems.

(*d*) The thermoduric colony count at 30° (see Technique No. 19) may be determined with advantage when it is considered that the milk equipment has not been cleansed satisfactorily for a period and that a build-up of thermoduric organisms may have occurred in consequence. This test can be set up on milk samples at any time within 28 hr of production, as long as the samples are kept at a temperature not exceeding 65°F (18°).

(*e*) The psychrotrophic colony count (5–7° for 10 days), as described in Technique No. 20, is another additional test which may be used

for the examination of raw milk, particularly when it has been held at low temperatures (under 45°F) for 48 hr or more.

(*f*) Dye reduction tests have a limited value for advisory purposes, especially during cold weather or for refrigerated milk, and should be applied with discretion.

(*g*) The colony count at 30°, the coli-aerogenes test and one of the keeping quality tests should always be applied, but one or more of the additional tests may be carried out to assist in the interpretation of the primary tests, particularly in the case of difficult problems.

(*h*) The examination of milk samples for taints or other abnormalities requires special tests which can only be determined in the light of the particular circumstances. Brief outlines of the methods recommended for the laboratory investigation of ropy milk and of taints in milk are given in Techniques Nos. 38 and 37 respectively.

3. *Recording Results*

A complete and exact record of all results should be made on the Laboratory Record Sheet so that a correct 'picture' can be reconstructed if queries should arise later. Apart from the usual details, any unusual circumstances or features should be noted.

4. *Reporting Results*

(*a*) The reported results will be derived from the entries on the Laboratory Record Sheet. They should be entered in the simplest possible form with a clear precise entry for each test result. Furthermore, the bacteriological report must be so presented that results of rinses, swabs and milk samples are readily distinguished. Ambiguous or indefinite entries should be avoided except where 'doubtful' is a recognized form of result.

(*b*) It is recommended that the colony counts of rinses and swabs should be expressed as counts per sq. ft of equipment.

(*c*) When a definite result cannot be given for a colony count, it should be given as 'under' or 'over' the lowest or highest definite count which could have been obtained from the quantities plated. For example, where rinse results are given for a milking machine cluster (approx. one sq. ft), indefinite results would be given as 'Under 500/ft²' or 'Over 2,500,000/ft²' when 1 ml and 10^{-1}ml of the 500 ml rinse solution are the largest and smallest quantities plated. Similarly, indefinite swab counts would be reported as 'Under 25/ft²' or 'Over 125,000/ft²' when 1 ml and 10^{-1}ml of a 25 ml swab of one sq. ft of surface are plated.

For indefinite colony counts for milk samples, 'Under 10/ml' or 'Over 500,000/ml' would be correct when 10^{-1}ml and 10^{-3}ml are the lowest and highest dilutions plated.

(*d*) The coli-aerogenes organisms content, when determined by inoculation of single serial decimal dilutions into MacConkey's broth, should be reported as follows:

Not found in 1 ml
Found in 1 ml
Found in 10^{-1}ml

When three tubes of each dilution have been inoculated, the dilution shall be recorded as positive when not less than two of the three culture tubes are positive.

5. *Interpretation of Results*

(*a*) The correct interpretation of the results requires sound bacteriological training and experience as well as good judgement. All information relating to the nature of the problem, the method of sampling, and the conditions to which the samples have been exposed prior to examination should be considered.

(*b*) Since the bacteriological results are influenced by several factors, e.g., time of sampling, age of sample on examination and prevailing atmospheric temperature, it would be *unwise to adopt rigid numerical standards* for general application. Nevertheless, it is desirable that certain guide limits, based on the joint experience of dairy bacteriologists and results given in published work, should be borne in mind so that, after allowance has been made for the variable factors, a general degree of uniformity in the interpretation of results may be achieved. Due consideration should also be given to the suitability of milk for processing or manufacture and a purely academic outlook should be avoided.

(*c*) Experience has shown that the majority of results can be classified into those which are obviously satisfactory or are so poor that it is evident that there are gross weaknesses in production methods which give rise to heavy contamination of both the dairy equipment and the milk, necessitating immediate remedial measures. For the few advisory samples giving results between these limits, discretion has to be excercised in deciding from the information available whether further improvement is required. It cannot be over-emphasized that adherence to rigid bacteriological standards is neither desirable nor justifiable in view of the varying factors which may affect test results.

6. *Observations on Reports*

(*a*) It is not intended that each test result should be individually commented upon in the report. When more than one test is carried out on a rinse, swab or milk sample, the results of the different tests may have varying significances, and to avoid confusion it is desirable that an overall assessment be made on the sample or series of samples. In the case of rinse or swab samples, the colony count will usually be given most weight, but the sample should not be classed as satisfactory if one of the supplementary tests gives an unsatisfactory result. In such a case the observations should be worded to the effect that certain results indicate need for improvement in production methods.

(*b*) Care should be exercised in making observations on the results for rinses and swabs of individual pieces of equipment. Significant variations in the bacteriological results obtained for individual pieces of dairy equipment, or between milking machine clusters, may indicate a lack of thoroughness in the general application of cleansing methods.

(*c*) The observations in the bacteriological report should be so worded that there is no doubt as to their meaning. When utensils are reported as satisfactory it may sometimes be advisable to emphasize that this condition applied at the time of rinsing or swabbing, and that this standard should be maintained if consistently satisfactory results are to continue.

(*d*) Bacteriological tests do not distinguish between faulty washing and faulty sterilization, and the observations should indicate that contamination is not attributed solely to inefficient sterilization. Where combined detergent-sterilizers are used for cleansing, poor results are usually due to faulty washing.

(*e*) The keeping quality test, should be given priority in assessing the results of the examination of milk samples.

(*f*) In most cases, the producer and the adviser are mainly concerned with the overall conditions of production and their effect on the quality of the milk, and an assessment should be made accordingly. This does not prevent attention being focussed on any obvious defects or sources of contamination, but the primary observations should indicate whether milk production methods are regarded as satisfactory or unsatisfactory. This will usually be possible but, where there is doubt, it may be better to indicate that the results are border-line and that further improvement is necessary if a consistently satisfactory standard is to be achieved. Emphasis should be placed on the need for consistently good methods and the maintenance of hygiene in the cowhouse or milking parlour and dairy.

C. Examination of Advisory Milk Samples.

Raw Milk

1. *Selection of Samples*

For advisory purposes milk samples are normally taken at the farm from (*a*) the first can, bottle or carton to be filled, or (*b*) a representative can, bottle or carton of the evening's or morning's milking. On occasions a sample may have to be taken from a representative container of mixed evening's and morning's milk.

Where it is necessary to trace the stage of production at which contamination occurs, serial milk samples may be taken.

In order that the results of serial samples should not be influenced by differences in temperature, it is important that immediately after sampling all samples be cooled in the bottle to the temperature of the cooling water.

The investigation of taints or of abnormal milk may require the sampling of individual udder or udder quarter milk.

2. *Technique of Sampling*

All precautions should be taken to prevent contamination during sampling, and all sampling equipment must be sterile. Equipment should be sterilized according to the recommendations given in Technique No. 1.

Metal dippers, with a capacity of at least 3 oz, and with a handle approximately 15 in. long, will normally be used.

Sample bottles of good quality glass suitable for sterilization and of 3 oz nominal capacity and type as described in B.S.809: 1958 should normally be used, but 6 oz or 8 oz sample bottles of the same type may be necessary on occasions. The sample bottles should be sandblasted over a suitable area for inscription.

The milk in the can should be vigorously stirred and plunged with the dipper for at least 5 sec and the sample then taken from well below the surface of the milk. The sample should be poured into the sampling bottle and stoppered immediately; the part of the stopper or cap which may come in contact with the milk must not be allowed to come into contact with any unsterile object or surface.

The sample should be labelled with the particulars necessary to enable it to be identified in the laboratory, e.g.:

(*a*) name or code number of the producer, and
(*b*) whether A.M., P.M., or mixed milk (E.M.X. or M.E.X.), and
(*c*) time of sampling.

Full particulars of the samples, including a concise description of conditions of milk production, etc., should be given on the accompanying Milk Production—Field Report.

3. *Transport of Samples*

Milk samples may be seriously affected by the temperature at which they are held before testing. Therefore, they should be transported in a suitable container so as to arrive at the laboratory as soon as possible and in any case within 6 hr of sampling.

If the bacteriological condition at the time of sampling is required, the milk should be examined within 3 hr of sampling or transported in an ice box and maintained at a temperature not exceeding 5° until examination, which should be within 24 hr of sampling.

4. *Storage of Advisory Milk Samples*

Representative samples of morning's milk should be examined after storage at atmospheric shade temperature (A.S.T.) until 4.00 p.m. on the day of production, i.e., when 8–10 hr old.

Evening's milk sampled on the day of production should be examined after storage at A.S.T. until 10.00 a.m. the following day, and examined when 16–18 hr old.

Evening's milk sampled on the day following production should be examined at 10.00 a.m. on the day of sampling, i.e., when 16–18 hr old.

Mixed evening's and morning's milk (E.M.X.) should be treated as morning's milk.

Serial milk samples should be held at A.S.T. according to the recommendations for representative meal samples.

Atmospheric shade temperature (A.S.T.) is defined as the temperature in a suitable well-ventilated cabinet, box or cupboard situated on the outside wall on the northern side of a building, so that it is at all times in the shade. The bottom of the A.S.T. cabinet should be not less than 3 ft from the ground. Suitable maximum and minimum thermometers with errors not exceeding 0·5° must be kept in the A.S.T. cabinet.

Maximum and minimum temperatures should be recorded at 9.0 a.m. and 4.0 p.m. daily and entered in the appropriate log book.

The mean atmospheric shade temperature is the arithmetic mean of the maximum and minimum temperature in the atmospheric shade box during the period of storage before testing.

5. *Mixing of Milk Samples prior to Examination*

The sample of milk should be thoroughly mixed according to the recommendations given in Technique No. 29 immediately prior to bacteriological examination.

6. *Testing*

The choice of the tests to apply will normally be governed by the nature of the advisory problem under investigation. When the primary purpose is to assess the overall care taken in applying clean milk production methods, the colony count at 30° and coli-aerogenes test at 30°, together with the determination of keeping quality by means of the clot-on-boiling test at 22° should normally suffice.

When it is suspected that the milk equipment has not been cleansed satisfactorily for a period, a build-up of thermoduric organisms may have occured in consequence, and the thermoduric colony count should be carried out.

Dye reduction tests have a limited value for advisory purposes, especially during cold weather, and should be applied with discretion.

The examination of milk samples for taints and other abnormalities requires special tests, which can only be determined in the light of the particular circumstances.

Pasteurized Milk

1. *Introduction*

The tests to be applied to samples of pasteurized milk submitted for bacteriological examination will depend upon the particular problem under investigation. Samples may be taken as a routine check on keeping quality and in the course of investigations into problems associated with poor keeping quality, bitty cream and non-specific taints.

Recommended procedures for the investigation of particular problems are outlined below.

When recording the results of laboratory examinations, full technical details of all test results including type of sample, dilutions, colony counts, disc readings, etc., should be clearly entered on the laboratory working sheet.

The results from the working sheets should be typed clearly on to the appropriate report sheet.

2. *Quality Control*

Bottles or cartons of the pasteurized milk should be collected from the first crate to be filled from each filler bowl. Samples should also be taken of Channel Island milk which has been bottled separately and of

any milk pasteurized on the previous day and held in the creamery cold store.

Samples should be placed immediately in an insulated container and transported to the laboratory with a minimum of delay. Whenever possible tests should be set up within 6 hr of sampling.

Immediately upon arrival at the laboratory the samples must be thoroughly mixed in accordance with the recommendations given in Technique No. 29. Pour, with aseptic precautions, five sub-samples into sterile 3-oz bottles labelled 'A', 'B', 'C' 'D', and 'E'.

The remainder of the sample should be examined for colony count, coli-aerogenes organisms, and by the phosphatase test (see Technique No. 55).

Sub-samples 'A' and 'B' are used in the determination of keeping quality by the C.O.B. test as follows:

'A' Transfer immediately to a water bath at 22°

'B' Place immediately into iced water in a refrigerator until 5.30 p.m. on the day of sampling and then transfer to the water bath at 22°

Examine both sub-samples at three-hourly intervals for keeping quality by C.O.B. to end point, in accordance with the technique recommended in Technique No. 35, the times of application being as indicated in the table below. The keeping quality in hours can be determined by reference to this table. The sub-samples may be discarded after 72 hr incubation at 22°. In freshly pasteurized milk, differences of more than 3 hr in K.Q. between the 'A' and 'B' sub-samples can occur because of the uneven distribution of organisms in small samples. The shorter K.Q. should be recorded.

Milk pasteurized before noon

C.O.B. test appled at:—	'A' sub-sample	'B' sub-sample
	K.Q. in hr	K.Q. in hr
8.30 a.m. on day after sampling	24	15
11.30 a.m. „ „ „ „	27	18
2.30 p.m. „ „ „ „	30	21
5.30 p.m. „ „ „ „	33 (A.P.T. 36)	24
8.30 a.m. on 2nd day after sampling	48	39
11.30 a.m. „ „ „ „ „	51	42
2.30 p.m. „ „ „ „ „	54	45
5.30 p.m. „ „ „ „ „	57 (A.P.T. 60)	48
8.30 a.m. on 3rd day after sampling	72	63
11.30 a.m. „ „ „ „ „		66
2.30 p.m. „ „ „ „ „		69
5.30 p.m. „ „ „ „ „		72

Sub-sample 'C' is used for the methylene blue test (see Technique No. 30)

Place immediately in a refrigerator (not in ice water) and proceed as detailed in the following table. The methylene blue test should be extended to 3 hr in order to determine the margin of safety.

Season	Treatment of sub-sample 'C' on:		
	Day of sampling	Day after sampling	Second day after sampling
SUMMER (1st May to 31st October)	Place immediately in a refrigerator at 3-5°. (Not in ice water).	Transfer to the A.S.T. cabinet at 9.30 a.m.	Remove from the A.S.T. cabinet at 9.30 a.m. and set up. M.B. test.
WINTER (1st November to 30th April)	Place immediately in a refrigerator at 3-5°. (Not in ice water).	Place in the A.S.T. cabinet at 9.30 a.m. hold until 5.30 p.m. and then transfer to a water bath at 18·3±1° (65±2°F).	Remove from water bath at 9.30 a.m. and set up M.B. test.

Sub-sample 'D' is placed in a water bath at 26±1° for 24 hr and the titratable acidity determined (see Technique No. 53).

Sub-sample 'E' is placed in a water bath at 18·3±1° for 24 hr and the coli-aerogenes organisms content determined (see Techniques Nos. 7 and 17).

When further control is necessary it is useful to take in-line samples at the entry to the pasteurized milk storage tank, from within it and at the bottle filler. These samples should be tested as indicated above.

It is also useful to take samples at intervals during the daily run. These are best taken from the bottle filler and should be held at 26±1° for 24 hr and the titratable acidity determined.

Laboratory control should also include regular checking of the cleanliness of the plant and washed bottles by means of rinses or swabs.

It is essential that raw stock milk which has been held overnight should be examined for taint.

3. *Investigation of Poor Keeping Quality*

Since the quality of the final pasteurized milk may be influenced by that of the incoming raw milk a sample of bulked raw milk ('X') should be taken, and this same bulk milk should also be sampled immediately after pasteurization (sample 'Y') and after it has been filled into bottles or cartons (Sample 'Z').

The bulked raw milk ('X') should be examined for colony count, thermoduric organisms and by the 'half-hour' methylene blue test extended to 3 hr.

The same milk after pasteurization ('Y') should also be examined for colony count and by the 'half-hour' methylene blue test. In addition the acidity should be determined after 24 hr incubation at 26±1° and coli-aerogenes organisms estimated after 24 hr incubation at 18·3±1°. The keeping quality should also be determined by the C.O.B. test. These tests should also be applied to the same bulk of milk sampled after it has been bottled or cartoned ('Z').

If the routine quality control does not include the testing of in-line samples and samples taken during the course of the run, these can usefully be employed to locate sources of trouble.

Poor keeping quality of the pasteurized milk may also be due to the storage of the milk under unsatisfactory conditions. Therefore at the time of the visit the duration of the storage period between bottling and delivery to the customers and the temperature at which the milk has been stored during this period should be determined. Samples of bottled or cartoned milk should be taken from the filler and brought back to the laboratory where each sample should be divided, a portion being examined immediately for colony count, K.Q. by C.O.B. and by the methylene blue test. The other portion should be stored under conditions which simulate as closely as possible the conditions which apply in practice. At the end of this period the stored sample should be tested in the same way, and a comparison of the results will reveal the effect of the storage conditions on the colony count, keeping quality and methylene blue test.

4. *Investigation of Bitty Cream*

Samples of the incoming raw milk and samples of pasteurized milk taken at the filler may be examined for the development of bitty cream by means of the floc count (see Technique No. 27). Further confirmatory evidence may be supplied by microscopic examination or by applying the techniques described in Technique No. 26.

It is necessary to check whether the conditions under which the milk is stored are satisfactory, i.e., less than 5° (41°F). Samples of milk taken from the filler should be stored under conditions which simulate those occuring in practice and then tested to see whether the defect has developed.

The possibility of contamination of the milk with spores of *Bacillus cereus* from the cans and bottles should also be investigated by applying the techniques in Technique No. 26 to rinses of these items of equipment.

5. *Investigation of Non-Specific Defects*

(*a*) *Taints*

The laboratory investigation of taints in milk is described in Technique No. 37 and, using the techniques described, it should be determined whether or not the taint is bacterial in origin.

(i) *Bacterial Origin*

If shown to be bacterial, the causative organisms will most probably be derived from post-pasteurization contamination from the equipment, and the examination of in-line milk samples and appropriate rinses and swabs will enable the source of these organisms to be determined.

(ii) *Non-Bacterial*

Of the non-bacterial taints, that caused by the enzyme lipase can be most serious. The enzyme is secreted by some cows and attacks the milk fat, forming free fatty acids which give the milk an undesirable aroma when heated. The activity of the enzyme is not markedly retarded by low temperatures so that the taint may develop in raw milk cooled by refrigeration and stored overnight. The activity of the enzyme is increased by aeration so that if the enzyme is present, undue agitation, such as that given to the milk during its passage through and discharge from pipelines on the farm and at the dairy, may promote the development of the taint.

Pasteurization will destroy the enzyme so that, provided the milk is free from taint at the time of pasteurization, no trouble will be experienced. If the taint is present in pasteurized milk it must have been developed before pasteurization and it will usually be found to be associated with raw milk which has been cold-stored overnight. The milk chosen for overnight storage is usually that derived from farm bulk tanks since it is colder on arrival at the dairy, but such milk has usually been exposed to excessive agitation since it is conveyed to the bulk tank on the farm by pipeline and, although cooled and stored at a low temperature, the taint may develop if the enzyme is present.

In investigating an outbreak of lipase taint, attention should, in the first instance, be directed to individual producers supplying milk from bulk milk tanks and whose milk is stored overnight at the dairy. A bulk sample from each producer should be agitated by violent shaking or by bubbling air through it, and stored in the refrigerator. After 18–36 hr the milk should be examined for lipase taint by smelling a portion of the warmed milk when the taint may be recognized by its characteristic 'baby-being-sick' smell. If the taint develops in the milk of a particular producer but is not evident on arrival at the creamery, the milk of that producer should be pasteurized as soon as it is received.

If the proportion of this type of producer is not high the investigation should be extended to cover those producers using pipelines on the farm whose milk is stored overnight and, again, if these do not represent a significant number, the milk of all producers whose milk is stored overnight at the creamery may be so examined.

(*b*) *Defects*

(i) *Ropy Milk*

The investigation of a ropy milk outbreak is described in Technique No. 38.

(ii) *Oxidised Flavour*

This taint is sometimes described as a 'cardboard' flavour. Its development is promoted by sunlight, and milk samples taken from the filler should be exposed to strong sunlight for 3–5 hr and then tasted to detect the taint.

D. Testing of Raw Milk in Transport Tankers and Bulked Raw Milk in Cans

1. *Raw Milk in Tankers*

(*a*) *Before Despatch*

(i) *Sampling*. Sampling shall be carried out within 30 min of completion of filling the tanker. The milk shall be thoroughly agitated either by compressed air or by vigorous plunging for 5 min. The plunger should have a handle at least 6 ft in length and a diaphram (disc) of 16 in. in diameter perforated with up to 18 holes, 1 in. in diameter. The plunger must be thoroughly cleaned and then rinsed in clean tap water immediately before use.

Immediately after mixing, a sample shall be taken with a sterile

dipper, attached if necessary to a suitable rod by means of a metal clip. The sample, of at least 8 oz, shall be poured into a sterile bottle immediately on withdrawal of the dipper.

Alternatively a sample rod may be used. It should consist of a metal rod 4 ft in length with a looped handle at one end and a short right-angle bend at the other, to form a support for a sterile sample bottle of at least 8 oz capacity, which is held in place by a strong clip around its neck. In use, the rubber stopper is removed, the bottle dipped quickly into the milk, withdrawn and re-stoppered.

(ii) *Testing*. Whenever possible the sample shall be tested within 30 min of sampling. If the sample cannot be tested within this period it should be immediately immersed in water at a temperature below 40°F (5°) for 15 min and held at 32°–40°F (0–5°) for not more than 18 hr.

The one-hour resazurin test should be applied, but disc numbers should be recorded after 10, 30 and 60 min incubation.

The titratable acidity should also be determined.

In addition, a record should be made of the temperature of the milk in the tanker after mixing, the number of the tanker and the date and time of sampling.

(*b*) *On Arrival*

(i) *Sampling*. Before commencing to empty the tanker, the milk shall be mixed by vigorous plunging for 15 min, or by agitation with compressed air, and then sampled as in (*a*) (i) above.

(ii) *Testing*. Testing shall be carried out as in (*a*) (ii) above.

2. *Bulked Raw Milk in Cans*

(*a*) *Before Despatch*

(i) *Sampling*. When bulked raw milk is to be despatched in cans, at least every twentieth can shall be sampled within 30 min of filling the cans. If less than 20 cans are despatched daily, at least one shall be sampled.

(ii) *Testing*. The samples shall be tested in the same way as the tanker milk in 1 (*a*) (ii) above.

In addition a record should be made of the temperature of the milk in each can after plunging and the date and time of sampling and testing.

(*b*) *On Arrival*

(i) *Sampling*. When bulked milk is received in cans, the proportion to be sampled at random is as follows:

No. of cans in the consignment	*No. of cans to be sampled*
1	1
2 to 4	At least 2
5 to 9	At least 3
10 to 20	At least 4
Over 20	At least 5

Sampling shall be carried out as described in Technique No. 33.

(ii) *Testing*. The samples shall be tested in the same way as the tanker milk in 1 (*a*) (ii) above.

The identity of the milk, the temperature of the milk in the can after plunging and the date and time of sampling shall be recorded for each can sampled.

Appendix V

PREPARATION OF REAGENTS

All solutions must be prepared using distilled water or water of equivalent purity.

Disodium Dihydrogen Ethylenediaminetetra-Acetate, (EDTA), N/50. Dissolve 3·72g crystalline dihydrate in water and dilute to 1 litre. Check against standard hard water. (1 ml N/50 EDTA=1 ml standard hard water).

Ammonium Buffer. Add 67·5g ammonium chloride to 570 ml ammonia, sp. gr. 0·880, and dilute to 950 ml with water. Dissolve 0·616g magnesium sulphate, $MgSO_4.7H_2O$, in 50 ml water and add 0·93g solid EDTA reagent. When this is in solution add it to the ammonia and ammonium chloride mixture.

The addition of 2 ml buffer in 100 ml of water is equivalent to the addition of 5 p/m magnesium in terms of $CaCO_3$ together with its equivalent of EDTA.

Total Hardness Indicator. Add 0·5g Solochrome Black WDFA or Eriochrome Black T to 100 ml industrial methylated spirit, warm to dissolve the dyestuff and add 4·5g hydroxylamine hydrochloride. Allow to stand overnight and filter. This indicator will not keep for more than 1 month.

Standard Hard Water. Dissolve 1g calcium carbonate in 20 ml N hydrochloric acid and dilute to 1 litre. 1 ml=1 mg $CaCO_3$.

Screened Indicator. Weigh out 0·06g brom-cresol green and 0·04g methyl red and dissolve in 100 ml industrial spirit (85 per cent ethanol).

Appendix VI

INTERFERING SUBSTANCES*

Interfering Substances	Maximum permissible concentration p/m	Effect
Copper as Cu^{2+}	0·2	In the presence of more than 0·2 p/m Cu^{2+} no end point can be obtained. A slightly red end point is obtained in the presence of up to 0·2 p/m Cu^{2+}
Iron as Fe^{3+}	20	As the iron content increases up to 20 p/m the end point becomes progressively greyer.
Aluminium as Al	50	As the aluminium content increases up to 50 p/m the end point becomes progressively redder.
Manganese as Mn^{2+}	—	Causes no interference with end point but titrates as hardness. 1 p/m Mn^{2+} = 1·8 p/m $CaCo_3$.
Phosphate as PO_4^{3-}	Greater than 200	200 p/m causes no interference. As the concentration of PO_4 increases above 200 p/m the initial colour becomes more mauve but with care the end point can still be detected.
Sodium hexametaphosphate	—	Each 10 p/m will sequester about 1 p/m of hardness expressed as $CaCO_3$.

*B.S. 2690:1956 should be consulted for details of how to overcome the effect of these substances and for an explanatory note on hardness.

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