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Laboratory Diagnosis of *Shigella* and *Salmonella* Infections *

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In recent years a great deal of work has been devoted to the study of enteric diseases and, in consequence, many methods have been developed for the isolation of their most common agents, *Shigella*, *Salmonella* and some pathogenic *Escherichia*, while at the same time new tests have been devised to differentiate the " groups " of the Enterobacteriaceae family, so that the beginner, especially, will be faced with the difficulty of selecting from among them those to be used.

Each author has his preferences according to his own experience, and it is regrettable that up to now no serious effort has been made to standardize techniques, which would save a lot of time and discouraging failures for the bacteriologist unexperienced in this field and also avoid contradictory results. It is not our intention to review the profuse literature on the subject, but rather to limit ourselves to describing such methods as have been used by us for many years in our laboratory in Montevideo, and which we can recommend as requiring a minimum of equipment and as saving time and labour. In some cases, though, we have thought it convenient to offer alternative methods which are known to be reliable and which are in use in many countries. Some tests are also described which are not generally needed but are of importance in certain particular cases.

We are aware that experienced bacteriologists will prefer some of the methods they are using to the ones we recommend. This study is not intended for them, however, but rather as a guide to people who are just starting work on enteric diseases diagnosis. For more detailed information, we recommend the books of Kauffmann (1954) and Edwards & Ewing (1955), of which we have made ample use.

Investigation of enteric pathogens will sometimes be the ultimate purpose, as for the public health officer, who is primarily interested in finding them for the potential danger they represent to the community. In clinical medicine, however, the role played by the bacteriologist does not end there. He will have to act as an adviser to the clinician, who needs more information in order to arrive at a correct diagnosis, follow the course of the case and indicate treatment, and for this purpose different materials must be examined (blood, urine, pharyngeal exudates, middle-ear discharges, etc.); certain other tests will also be needed, such as agglutination reactions and antibiograms of the isolated cultures.

Phage typing of *S. typhi* and some other *Salmonella* types has proved important from the epidemiological point of view to trace the source of infection in the course of outbreaks. Such a subject, though, does not properly fall within the scope of the present work.

Isolation and complete identification of all enteric pathogens require a lot of manual work, culture media and agglutinating sera, which make it difficult for the average hospital laboratories to cope with the task. They should aim, however, at allocating to their proper sub-groups all *Shigella* cultures, and at identifying most of the locally isolated *Salmonella* strains. Unidentifiable cultures should be sent to national centres, of which there should be at least one in every country, and newly described types should be forwarded to one of the three international centres in Copenhagen, Chamblee, Ga., USA, or London, for final verification.

* This is one of a series of studies on the laboratory diagnosis of various diseases which, it is hoped, will eventually be revised and published in monograph form. An effort is made to ensure that the diagnostic methods recommended in these studies are as internationally representative and acceptable as possible by securing the co-operation of a number of experts from different countries. A list of the reviewers of the study presented here is given in the Annex on page 276. To all of these, and to the two authors, the World Health Organization is greatly indebted.—ED.

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In our opinion all routine laboratories should endeavour gradually to prepare their own culture media, reagents for biochemical tests and, especially, agglutinating sera. When starting work, type cultures and sera should be obtained from specialized laboratories and used as standards, but the bacteriologist nevertheless needs personal experience in the preparation of all the tools in his hands, in order to obtain adequate knowledge of their proper use.

COLLECTION OF SPECIMENS

Stools

Fresh, recently evacuated stools obtained as early as possible after the onset of symptoms are the best for examination. When mucus and pus are present they should also be collected, as in acute cases they often yield a pure culture of the causative agent.

It is a common observation that antibiotic treatment of intestinal infections may prevent the growth of the causative agent on culture media, and therefore it is advisable to examine stools immediately after admission of patients and before treatment is started. Unfortunately, such treatment is frequently given at home and only when it fails are patients sent to hospitals.

When dealing with communities where many persons have to be examined, the rectal swab has proved to be especially useful in the search for carriers. Sterilized swabs are introduced into the rectum, gently rubbed on the surface of the mucosa, and used for the direct inoculation of media. As the introduction of swabs is sometimes painful, it is often advisable to submerge them in broth or some similar sterilized liquid, before use. In the case of infants, whose stools are sometimes not easy to collect, either because they are liquid or for some other reason, the use of rectal swabs is highly recommended. It is also excellent in cases of ulcerative chronic colitis, as generally the lesion on the mucosa can be reached in the course of rectosygmoidoscopy.

When examination has to be delayed for more than four hours, it is convenient to add a preservative to prevent the multiplication of normal intestinal bacteria which has a destructive effect on the pathogens, partly owing to bacterial competition and partly owing to pH changes. The simplest reliable preservative is Sachs' buffered glycerol solution (Sachs, 1939). Bangxang & Eliot's formula (1940) is also recommended. Stools, in a proportion of 1/10 should be well emulsified in the solutions to ensure preservation. Swabs can also be submerged in the same preservative. An excellent method for preserving stools, which is especially useful when samples have to be mailed to some distant laboratory, was recommended by Lie Kian Joe et al. (1954) from Djakarta University (see Varela, 1955) and consists in drying specimens on filter paper. The swab used for collection is rubbed gently on a small piece of filter paper, impregnating a surface of about 2 cm × 2 cm; this is allowed to dry at room temperature and then wrapped in sterile paper. For examination, the impregnated zone is cut, put into a tube with 3 ml of broth and macerated by means of a glass rod, and the liquid is collected for examination. (See A, p. 249).

Urine

Catheterization is needed to obtain aseptic urine from women, but with men, when examination is to be carried out immediately, it is sufficient to reject the first portions of the urine specimen. Urine should be centrifuged at high speed and the sediment used for examination. If the examination is not done at once, the alternative is either to keep the urine in the ice-box, or to add a preservative as when dealing with stools (particularly when the specimen is being sent to another laboratory).

Blood

Blood should be collected for culture as early as possible after the onset of symptoms and 1% sodium citrate or some other anticoagulant (e.g., heparin) added, as liquid blood is easier to handle than coagulated samples. If absolutely necessary, however, the clot can be used for culture.

Other materials

Pharyngeal and vaginal exudates and middle-ear discharges are best collected with a swab; bile by duodenal catheterization.

Any solid foods are cut into small pieces with sterile instruments and then ground with sand in a mortar. A few millilitres of saline are added and mixed thoroughly with the pestle, and the liquid is collected with a pipette.

As a general rule any specimens not to be examined at once should be kept in the ice-box, or, better still, in a deep-freeze, where they can be preserved for years.

CULTURE MEDIA AND BIOCHEMICAL TESTS

A. PRESERVATIVE SOLUTIONS

1. Sachs' (1939) buffered solution :

Glycerol	30 ml
Sodium chloride	0.42 g
Dipotassium phosphate (anhydrous)	0.31 g
Monopotassium phosphate (anhydrous)	0.1 g
Distilled water	70 ml

Add phenol-red solution to give a pink colour. Sterilize at 120°C for 15 minutes; pH should be 8; if the colour changes the solution should be discarded.

2. Bangxang & Eliot (1940) solution :

Sodium citrate	1 g
Sodium desoxycholate	0.50 g
Sodium chloride	0.90 g
Disodium phosphate solution (M/15)	0.30 ml
Distilled water	100 ml

Adjust reaction to neutrality to litmus, add 0.2 ml of N sodium hydroxide and phenol-red solution to give a pink colour. Sterilize at 120°C for 15 minutes.

B. PLATING MEDIA

Current Petri dishes, 10 cm in diameter, will take about 20 ml of medium, which should not form too thin a layer. They may be filled directly from the flask containing the medium if the usual precautions to prevent contamination are taken.

As it is essential in order to obtain isolated colonies that the surface of the medium be dry, the Petri dishes have to be left at room temperature for 24 hours, or left for a few hours in an open incubator at 37°C in an inverted position, leaning at an angle on the lid.

1. Kristensen, Lester & Jürgens (1925) brilliant-green agar (KLJ), modified :

Beef extract	0.50 g
Peptone (Bacto)	1 g
Sodium chloride	0.50 g
Phenol-red solution	4 ml
Agar	2.60 g
Distilled water	100 ml

Adjust pH to 7.2-7.4; sterilize at 120°C for 15 minutes.

To this basic, still-hot medium, add 0.2 ml of brilliant-green solution (0.5%), lactose 1.5 g (sterilized water solution) (see D, p. 251). Kauffmann (1954) has recently recommended the use of broth prepared with human placenta instead of beef extract.

Phenol-red solution :

Phenol red	0.2 g
NaOH (N/10)	8 ml
Distilled water	92 ml

2. Leifson's (1935) desoxycholate-citrate agar (DCA) :

Beef (or pork) infusion	100 ml
Peptone (Bacto, Proteose or Fairchild)	1 g
Lactose	1 g
Sodium citrate ($11H_2O$)	2.50 g
Sodium desoxycholate	0.50 g
Iron ammonium citrate (green scales)	0.20 g
Neutral red	0.002 (1/50 000)
Agar	2 g

To lean minced beef, add three times its weight of water. Macerate for one hour, stirring. Add 1 ml N HCl, for each 100 g beef; boil for one minute. Strain through cloth, filter through wet paper and add to initial volume. 100 g beef will produce 300 g infusion. Add peptone, adjust pH to 7.5, boil for 2-3 minutes, filter, add agar and 0.5 ml N NaOH, shake and allow the agar to be soaked for at least 15 minutes. Melt by boiling, or in the open steam sterilizer; when still hot, add lactose and citrate. If the medium is not to be used at once it can be kept as it is at this stage. Melt it before use and, when still at a temperature of between 80° and 90°C add iron ammonium citrate (20% water solution) and desoxycholate, adjust pH to 7.4, using phenol red as indicator, and add neutral red (1/100 water solution).

To avoid contamination, care should be taken that the temperature of the medium is kept sufficiently high, as it cannot be sterilized by autoclaving. Plates should be kept in the dark.

3. SS agar (Difco) :

Beef extract	0.5 g
Peptone (Proteose)	0.5 g
Lactose	1 g

Bile salts No. 3 (Difco)	0.85 g
Sodium citrate	0.85 g
Sodium thiosulfate	0.85 g
Iron citrate	0.1 g
Agar	1.7 g
Neutral red	0.0025 g
Brilliant green (1/10 000 water solution)	0.33 g
Water	100 ml

Dissolve the ingredients by quick boiling, adjust pH to 7.0-7.1: distribute in Petri dishes. If desired, the medium can be used without the addition of brilliant green, the purpose of which is to inhibit the growth of some cocci.

4. Wilson & Blair (1931) bismuth-sulfite agar (modified) (WB) :

Beef extract	0.05 g
Peptone (Bacto)	1 g
Glucose	0.5 g
Disodium phosphate ($12\,H_2O$)	0.5 g
Ferrous sulfate	0.05 g
Bismuth indicator	2.5 ml
Sodium sulfite	2 g
Agar	2 g
Brilliant green	0.0025 g
Distilled water	100 ml

Mix beef extract, peptone and water; adjust pH to 7.4; boil and filter. Add agar and sterilize. To this base, when still hot, add glucose, sodium sulfite (5 ml of a freshly prepared 20% solution), bismuth indicator, and heat in a water-bath at boiling point for 2-3 minutes. Add 5 ml of 10% anhydrous disodium phosphate, 0.5 ml of 8% solution of ferrous sulfate ($7H_2O$) and 0.25 ml of 1% brilliant green solution. Mix well and distribute in sterilized plates. The medium must not be heated at high temperature.

Bismuth indicator solution :

Bismuth citrate	12 g
Distilled water	10 ml
Ammoniacal water (28%)	4 ml

Dissolve and add 86 ml of distilled water. This solution must be kept in well-stoppered bottles.

Wilson & Blair's formula has proved excellent in our hands, but the following formula of Wilson, as recommended by Mackie & McCartney (1953), may be substituted for it with the advantage that its preparation is easier:

Nutrient agar 3% (melted and cooled to 60°C)	100 ml
Stock bismuth-sulfite/phosphate/glucose mixture	20 ml
Iron-citrate/brilliant-green mixture	4.5 ml

Pour into Petri dishes.

Bismuth-sulfite/phosphate/glucose mixture :

Dissolve 30 g bismuth-ammonium-citrate scales in 250 ml boiling distilled water. Add to this a solution obtained by boiling 100 g anhydrous sodium sulfite in 500 ml distilled water, and then while the mixture is boiling add 100 g disodium phosphate crystals (Na_2HPO_4, $12\,H_2O$). To the cooled bismuth-sulfite/phosphate mixture add a solution of glucose obtained by dissolving 50 g of commercial glucose in 250 ml of boiling distilled water. This mixture will keep for months.

Iron-citrate/brilliant-green mixture :

1% solution of iron-citrate scales (ferric citrate scales) in distilled water	200 ml
1% brilliant green in distilled water	25 ml

This mixture will keep for months.

Although Wilson & Blair's is an excellent medium, it has the inconvenience that different batches will give non-comparable results if great care is not taken in its preparation. Inexperienced persons should at the start buy it from commercial firms which prepare dehydrated uniform products giving good results. In the authors' experience, Difco medium is improved by the addition of 0.2 ml of 1% ferrous sulfate to 100 ml of the dehydrated medium. There are many modifications of the original medium (see Hajna & Perry, 1937-38; Tabet, 1938; Hobbs, 1943).

5. Lactose/bromothymol-blue agar (LBA) :

Beef extract	0.50 g
Peptone (Evans or Bacto)	1 g
NaCl	0.30 g
Disodium phosphate ($12H_2O$)	0.20 g
Lactose	2 g
Bromothymol-blue solution	1.2 ml
Water	100 ml

pH: 7.2-7.4

Bromothymol-blue solution:

Bromothymol blue	1 g
NaOH (N/10)	25 ml
Distilled water	475 ml

C. ENRICHMENT MEDIA

1. Combined Müller-Kauffmann enrichment medium (Kauffmann, 1930-31) (MKT) :

Infusion broth	90 ml
Calcium carbonate (precipitated)	5 g
Ox bile (filtered)	5 ml

Sterilize this mixture at 120°C for 30 minutes. Allow it to cool and add aseptically: (*a*) brilliant-green solution, 1 ml; (*b*) iodine solution, 2 ml; (*c*) thiosulfate solution, 10 ml. Distribute in sterilized 180 mm × 18 mm tubes while shaking, 10-15 ml in each. Incubate for several days at 37°C to control sterility.

Brilliant-green solution:

Brilliant green	1 g
Distilled water	1000 ml

Sterilize three days at 100°C.

Iodine solution:

Iodine	20 g
Potassium iodide	25 g
Distilled water	100 ml

This solution should not be heated.

Thiosulfate solution:

Sodium thiosulfate	50 g
Distilled water to	100 ml

Sterilize at 120°C for 15 minutes.

2. Leifson's (1936) selenite F (SF) :

Sodium hydrogen selenite (anhydrous)	0.6 g
Disodium phosphate ($12H_2O$)	0.5 g
Lactose	0.4 g
Peptone	0.5 g
Distilled water	100 ml

pH: 7

Boil for a few minutes. Distribute in sterile tubes, 10 ml in each.

The dehydrated medium can be obtained from commercial firms.

D. CARBOHYDRATE AND ALCOHOL FERMENTATION

Mannitol, adonitol, inositol, and salicin may be added to the medium prior to sterilization in a proportion of 0.5 %. Glucose and disaccharides such as lactose and saccharose can be sterilized either by filtering a 10% solution through Seitz membranes or by dissolving them in boiling water, allowing them to cool and adding an excess of chloroform. They can be kept in this way for years.

Carbohydrate media are distributed in tubes with gas bells, which are filled by heating in the water-bath at 100°C for a few minutes or the air evacuated by vacuum. It is advisable to use small tubes, not bigger than 120 mm × 12 mm, containing about 2 ml of medium, as some of the " sugars " are rather expensive. To prevent evaporation, these tubes, not bigger than 120 mm × 12 mm, containing as the routine laboratory, not needing all of them frequently, will have to keep these media for months. It has to be remembered that in hermetically closed tubes some fermentations will take place that are not normally seen in cotton-plugged tubes. Late fermenters of some sugars will bring about fermentation in a few days; fermentations will sometimes be observed that are not usual for the species.

A practical way of identifying cork-stoppered media consists in painting conventional marks (lines, crosses, etc.) on the tubes in different colours and combinations of colours, using cellulose-acetate paint which will remain unchanged for years and can be easily removed with warm water and a brush.

Broth base :

Beef extract	0.5 g
Peptone (Evans, Bacto or similar)	1 g
Sodium chloride	0.3 g
Disodium phosphate ($12H_2O$)	0.2 g
Distilled water	100 ml

pH: 7.4

Indicator:

Phenol red	1 g
Sodium hydroxide (N/10)	40 ml
Distilled water	460 ml

Add in the proportion of 1.25% to the broth base.

3. The starch test (Hormaeche & Munilla, 1957):

To the same basic medium as above, 1% of *non-soluble* starch is added. To avoid lumps and to assure uniformity of the medium, this substance is finely ground and added to a small amount of cold water, and the mixture is stirred and then boiled for 5 minutes. The resulting jelly is incorporated with the base, distributed in sterile tubes (120 mm × 12 mm) fitted with gas bells and sterilized at 110°C for 15 minutes. The tubes are inoculated with a loopful of a 24-hour broth culture and observed daily. If on the fourth day there is no visible gas in the bell, insert into the medium the red-hot wire loop, which will free any gas present.

E. INDOLE PRODUCTION

1. Peptone water :

Peptone	1 g
NaCl	0.5 g
Distilled water	100 ml

pH: 7.0-7.2

Any peptone rich in tryptophane may be used. Bacto tryptone is very suitable. If Bacto peptone is employed, the concentration should be double the indicated amount. Difco's casitone or BBL Trypticase can be used as substitutes. Trypsin digest casein broth of Kristensen et al. (1925) gives excellent results.

2. Indole detection

In many cases time will be saved by using Gnezda's method for indole detection. Impregnate filter paper with a saturated aqueous solution of oxalic acid, allow to dry and cut into strips. Place one of these between the stopper and the wall of each inoculated tube and incubate for 48 hours. A pink colour in the strip means indole production. When this test is negative, add Ehrlich's reagent to form a layer on the surface of the medium, and read after some minutes. A positive reaction will show a red ring between the two layers.

Ehrlich's reagent:

p-Dimethylamidobenzaldehyde	2 g
Ethyl alcohol (95%)	190 ml
Concentrated hydrochloric acid	40 ml

F. HYDROGEN SULFIDE PRODUCTION

1. Ferrous chloride gelatin (Kauffmann, 1954) :

Beef extract	0.75 g
Peptone (Parke Davis)	2.5 g
Sodium chloride	0.50 g
Gelatin	12 g
Ferrous chloride solution (10%)	0.5 ml
Distilled water	100 ml

Boil to dissolve the ingredients and then add the solution of ferrous chloride (freshly prepared).

To ensure sterility, it is convenient to heat the medium for 10 minutes in the open autoclave, and then distribute it aseptically in narrow sterilized tubes. These should be cooled by immersion in water to avoid sedimentation. Seal with paraffined cork stoppers. Inoculate by stabbing and incubate at room temperature, read after at least 7 days for H_2S production and after 60 days for gelatin liquefaction.

In the authors' experience this medium can be improved by adding 0.015 g sodium thiosulfate per 100 ml. This permits the recognition of some *Citrobacter* strains not producing H_2S in the medium prepared as above, and enhances weak positive reactions. Readings should take place after three days.

2. Peptone water :

Peptone (Bacto or Evans)	2 g
Sodium chloride	0.5 g
Distilled water	100 ml

Distribute in tubes, 3 ml in each, and sterilize at 120°C for 15 minutes. Place a dry strip of filter paper impregnated in a saturated solution of lead acetate between the wall and the stopper, incubate at 37°C, and read after 48 hours.

It is a well-known fact that most Enterobacteriaceae will produce H_2S under certain conditions—for instance, when cystine is added to the medium, as proved by Bürger as early as 1914. The method as described above is reliable and not over-sensitive for differentiation between *Salmonella* and related groups. *Shigella* and *Escherichia* are negative and so are some *Citrobacter*. Other peptones, such as Proteose or Parke Davis, cannot be substituted for Bacto or Evans in this liquid medium because, containing more organic sulfur, they will give undesirable positive reactions.

For a similar reason the sensitivity of the reaction increases with the volume of medium in the tube.

The addition of glucose without buffer may have an unfavourable effect, as H_2S production is stopped if the reaction of the medium goes below pH 6.

3. Triple-sugar/iron agar (Kliger's (1917) modified):

If this medium, which is described below (see R2, p. 257), is used for planting isolated colonies, H_2S production will be recognized.

It is not to be recommended for this purpose alone, as it has no advantages.

G. METHYL-RED AND VOGES-PROSKAUER REACTIONS

1. Buffered peptone-glucose solution :

K_2HPO_4	0.5 g
Peptone (Witte)	0.5 g
Glucose	0.5 g
Distilled water	100 ml

Dissolve phosphate and peptone in water, boil, filter and sterilize at 120°C for 15 minutes. Add glucose, Seitz-filtered (or see D, p. 251), and distribute in sterilized tubes (120 mm × 12 mm). To avoid contamination, it is convenient to heat for 15 minutes in the open autoclave. Do not sterilize at a higher temperature.

2. Methyl-red test :

After 4 days' incubation at 37°C, add 2 drops of methyl-red solution. A yellow colouring will indicate a negative reaction, red a positive one; there are doubtful reactions showing intermediate shades.

Reagent :

Methyl red	0.1 g
Ethyl alcohol (95%)	300 ml

To this solution, add distilled water to a volume of 500 ml.

3. Voges-Proskauer test for acetyl-methyl-carbinol (Shaw, 1956)

Inoculate two tubes of the same medium and incubate at 37°C. Test one of them after 2 days and the other after 4 days.

The Barritt (1936) method. Add 0.4 ml KOH water solution (40%), shake, and drop 0.5 ml of Barritt's reagent down the side of the tube, so that it forms a layer on the surface of the medium. After a few minutes a positive reaction will show a reddish-violet ring.

Barritt's reagent is prepared by dissolving 6 g of α-naphthol in 100 ml 96% alcohol. It should be kept in an amber-coloured flask wrapped in black paper, and a few crystals of sodium nitrite or sulfite should be added to prevent oxidation.

Blank tests should be performed with every new batch of reagent as impure alcohol may give a positive reaction.

When α-naphthol changes to a brownish colour it should be discarded.

The O'Meara (1931) method. As an alternative this method may be used.

Reagent :

Potassium hydroxide	40 g
Creatine	0.30 g
Distilled water	100 ml

Dissolve the alkali in the water and add creatine. To each 1 ml of culture, add 0.1 ml of reagent, mix and heat for 2 hours at 45°-50°C in the water-bath or for 4 hours at 37°C. Shake the tube; an eosin-pink colour indicates a positive reaction.

H. CITRATE UTILIZATION AS SOLE SOURCE OF CARBON

1. The Koser (1923) test :

Sodium chloride	0.5 g
Magnesium sulfate	0.02 g
Ammonium dihydrogen phosphate	0.1 g
Dipotassium phosphate	0.1 g
Sodium citrate	0.2 g
Distilled water	100 ml

pH: 6.7-6.9

Sterilize at 120°C for 20 minutes.

Inoculate with a loopful of a 24-hour broth culture, incubate at 37°C for 4 days. Different degrees of turbidity indicating growth mean a positive reaction.

2. Simmons (1926) citrate agar :

Sodium chloride	0.5 g
Magnesium sulfate	0.02 g
Ammonium dihydrogen phosphate	0.1 g
Dipotassium phosphate	0.1 g
Sodium citrate	0.5 g
Agar (washed for three days)	2 g
Bromothymol blue (see B5, p. 250)	4 ml

Adjust reaction to pH 7.1. Boil, distribute in tubes, sterilize at 120°C for 15 minutes, and incline so as to obtain a 2.5-cm butt and about a 4-cm slant.

Inoculate the surface of the slant from a saline suspension prepared from a young agar culture, and stab the butt. Incubate at 37°C for four days; a positive reaction will show growth and the medium turns to blue.

I. KCN INHIBITION TEST
(Moeller, 1954)

Peptone (Orthana special)	1 g
Sodium chloride	0.5 g
Monobasic potassium phosphate	0.0225 g
Dibasic sodium phosphate	0.564 g
Distilled water	100 ml

pH adjusted to 7.6

Sterilize at 120°C for 15 minutes. To this cold base add 1.5 ml of a 0.5% aqueous solution of potassium cyanide, aseptically prepared. The solution cannot be sterilized except by Seitz filtration, but this is not needed. Distribute the medium in 120 mm × 12 mm sterilized tubes, and seal carefully with sterile paraffined cork stoppers. This medium can be kept in the ice-box for several weeks.

Inoculate the medium with a loopful of a 24-hour broth culture and incubate at 37°C for two days. Growth is considered a positive reaction.

Proteose peptone No. 3 (0.3%) or Evans (0.6%) can be substituted for Orthana.

J. NITRATE REDUCTION

Peptone water base :

Potassium nitrate (nitrite free)	0.02 g
Peptone (Bacto)	0.5 g
Distilled water	100 ml

Distribute in tubes and sterilize at 120°C for 20 minutes. Incubate the inoculated tubes for 4 days at 37°C; add 2-3 drops of each of the two reagents described below. If nitrites are present, a red colour develops and often a precipitate is formed. If the reaction is negative, this may mean either that nitrites were not produced or that they were first formed and then further reduced. Add a minimum amount of zinc powder. When nitrates are present a red-pink colour will develop.

Reagents:

Sulfanilic acid	0.80 g
Acetic acid (5 N)	100 ml

To prepare the 5 N acetic acid solution, mix one part glacial acetic acid with 2.5 parts of water.

α-naphthylamine	0.50 g
Acetic acid (5 N)	100 ml

K. UREASE TEST

(Christensen modified; Hormaeche & Munilla, 1957)

Broth base :

Peptone (Bacto)	0.1 g
NaCl	0.5 g
KH_2PO_4	0.2 g
Cresol-red solution (0.4‰)	2.4 ml
Distilled water	100 ml

Boil the ingredients, adjust reaction carefully to pH 6.7, filter and sterilize at 115°C for 20 minutes. Add 2 g urea, Seitz-filtered or chloroform-treated (see D, p. 251). Distribute in sterilized tubes (120 mm × 12 mm). Inoculate from a 24-hour broth culture and observe for 4 days, when positive reactions will show a reddish-purple colour. Exact adjustment of the reaction of the medium is essential, as if it is higher than indicated many *Cloaca* cultures will be positive, and if lower some *Klebsiella* will be negative.

Cresol-red solution:

Cresol red	0.1 g
NaOH (N/50)	13.1 ml
Distilled water	237 ml

L. DECARBOXYLASES, MOELLER'S (1955) METHOD

Culture medium :

Peptone (Orthana special)	0.5 g
Beef extract	0.5 g
Bromocresol purple (1/500)	0.5 ml
Cresol red (1/500)	0.25 ml
Pyridoxal	0.5 mg
Glucose	0.05 g
Distilled water	100 ml

Mix the ingredients, boil for some minutes and filter through paper. Add one of the four amino-acids to be tested: *l*(+)-lysine dihydrochloride, *l*(+)-arginine monohydrochloride, *l*(+)-ornithine dihydrochloride, or *l*(+)-glutamic acid, 1%. Adjust reaction to pH 6 and distribute in 1-cm diameter tubes, about 1 ml in each. To ensure anaerobiosis, which is essential, pour 0.5 ml neutral liquid paraffin and sterilize at 115°C for 15 minutes. Inoculate from an agar culture and incubate for several days at 37°C. Intense positive reactions will show a colour change to reddish-purple in 1-2 days and weaker ones up to the 5th day; but as a colour shift may be produced by some cultures after several days in the absence of amino-acids, it is necessary in order to interpret these late reactions to add a control tube without amino-acids. In the case of glutamic acid, after 24 hours' incubation at 37°C add 0.2 ml of an 0.25 N HCl solution, incubate for a further 48 hours and add 0.2 ml 0.25 N NaOH solution. Shake well, allow to stand for some minutes, and read.

Instead of Orthana, either Evans or Proteose peptone 0.25 g can be used. Pyridoxal is not strictly necessary and pyridoxine may be used in its place.

Moeller mentions the fact that the medium can be used without cresol red. This makes it more sensitive, and after 2-3 days false positive reactions often appear. In the authors' experience the test performed in this way is quite reliable if readings are taken, at the latest, 48 hours after incubation, but not after this time.

Indicators:

Bromocresol purple	1 g
NaOH (N/5)	9.25 ml
Distilled water to	500 ml
Cresol red	1 g
NaOH (N/5)	13.1 ml
Distilled water to	500 ml

For lysine Carlqvist's (1956) ninhydrine test may be used instead of the one described above. Its results are equally good, and it has the advantage that it uses casitone or trypticase instead of lysine ; but, on the other hand, it involves too much work to be used routinely.

M. GELATIN LIQUEFACTION

1. Nutrient gelatin :

Beef extract	0.5 g
Peptone	0.5 g
Gelatin	12 g
Water	100 g

Boil the beef extract, peptone and water; filter and add gelatin; adjust pH to 7.4 with N NaOH; distribute aseptically in sterilized tubes and heat in the open autoclave for 20 minutes.

Any of the well-known bacteriological peptones may be used. A good-quality gelatin is needed. Difco's can be recommended for uniform results. Inoculate by stabbing and incubate at 20°C for one month. Incubation at 37°C and cooling periodically at 20°C for reading can be used as an alternative, but the results obtained with this method are not exactly comparable with those with the first method, which is more sensitive.

2. Iron gelatin :

As mentioned above (see F1, p. 252), this medium can be used for gelatin liquefaction. It should be incubated at 20°C for two months.

N. ORGANIC ACIDS

(Brown, Duncan & Henry, 1924)

Base medium :

Bacto peptone	1 g
NaOH (N/10)	0.85 ml
Bromothymol blue (see B5, p. 250)	1.2 ml
Distilled water	100 ml

Sterilize at 120°C for 15 minutes. Add 0.5 g sodium *meso-* or *laevo*-tartrate, 1 g sodium *dextro*-tartrate or mucic acid. Adjust pH to 7.4 with N NaOH. Distribute in sterilized tubes and heat in the open autoclave for 10 minutes.

Inoculate the tubes from a 24-hour broth culture, and incubate at 37°C for at least 15 days. Read daily. Positive reactions will change colour to yellow or will show discoloration. Control by adding 0.5 ml of a saturated solution of lead acetate in water, and read after allowing the precipitate to settle for 24 hours. Positive tests will only show a small precipitate at the bottom of the tube, while negative ones are recognized by an abundant precipitate (about two-thirds of the level of the medium). It is to be remarked that some reactions which appear to be negative because there is no change of colour will be found to be positive when lead acetate is added.

Mucic acid tests are read by colour change. Precipitate when lead acetate is added will be abundant in both positive and negative tests, but if an excess of acetic acid is added, it will be dissolved in positive reactions and not in negative.

O. STERN'S (1916) GLYCEROL-FUCHSIN BROTH

Basic medium :

Beef extract	2 g
Peptone	2 g
NaCl	0.5 g
Water	100 ml

Adjust pH to 7.4-7.6 and sterilize at 120°C for 20 minutes.

Basic fuchsin	10 g
Alcohol (95%)	100 ml

Leave in the incubator at 37°C until saturated. Filter before use.

Sodium sulfite	10 g
Distilled water	100 ml

To prepare the medium, add 0.2 ml of the above fuchsin solution, 2 ml of a freshly prepared sodium sulfite solution, and 1 g of pure neutral glycerol to 100 ml of basic medium. Distribute aseptically in sterile tubes and heat in the open autoclave for 10 minutes. The medium should be kept in the ice-box; if the colour changes to pink or red, the medium should be discarded.

Inoculate from a fresh culture and observe for three days. Intense positive reactions will show a reddish-violet colour, weaker reactions a red colour.

P. MALONATE-PHENYLALANINE TEST

(Shaw, 1956)

$(NH_4)_2SO_4$	0.2 g
K_2HPO_4	0.06 g
KH_2PO_4	0.04 g
NaCl	0.2 g
Sodium malonate	0.3 g
dl-phenylalanine	0.2 g
Yeastrel	0.1 g
Bromothymol blue (alcoholic solution, 0.5%)	0.5 ml
Water	100 ml

Distribute in tubes and sterilize at 10 p.s.i. (0.7 atm.) for 10 minutes. Inoculate, and incubate at 37°C for 24 hours. A change of colour to blue indicates a positive malonate reaction.

Add a few drops of 0.1 N HCl and 0.5 M ferric chloride. A deep green colour due to formation of phenylpyruvic acid means a positive phenylalanine test.

Ewing, Davis & Reavis (1957) have found that the formula of Shaw & Clarke, as recommended above, combining phenylalanine and malonate tests, is not as convenient as each of the two performed separately as follows:

1. Leifson's (1933) malonate test (modified):

$(NH_4)_2SO_4$	0.2 g
K_2HPO_4	0.06 g
KH_2PO_4	0.04 g
NaCl	0.2 g
Sodium malonate	0.3 g
Yeast extract	0.1 g
Bromothymol blue	0.5 ml
Distilled water	100 ml

Distribute in small tubes. Sterilize at 120°C for 10 minutes. Inoculate and incubate for 24 hours.

2. Phenylalanine test (Henriksen (1950), modified by Ewing, Davis & Reavis, 1957):

Yeast extract	0.3 g
l-phenylalanine	0.1 g
NaCl	0.5 g
Na_2HPO_4	0.1 g
Agar	1.2 g
Distilled water	100 ml

Distribute in small tubes, sterilize at 120°C for 20 minutes and allow to solidify so as to obtain long slants. Inoculate heavily, incubate for 4-24 hours, add 4-5 drops of a 10% ferric chloride solution and allow it to run over the surface of the slant. A green colour produced in a few minutes means a positive reaction.

Q. CHRISTENSEN'S (1949) CITRATE MEDIUM

Sodium citrate	0.3 g
Glucose	0.02 g
Yeast extract	0.05 g
Cysteine monohydrochloride	0.01 g
Ferric ammonium citrate	0.04 g
Monopotassium phosphate	0.1 h
NaCl	0.5 g
Sodium thiosulfate	0.008 g
Phenol red (0.2% solution)	0.6 ml
Agar	1.5 g
Distilled water	100 ml

Distribute, sterilize at 120°C for 20 minutes and allow to solidify so as to obtain a short slant. Inoculate by stabbing and streaking and incubate at 37°C for 5-7 days. Citrate utilization is recognized by alkalinization of the medium. When the medium is used to differentiate *Shigella* from *Escherichia*, the H_2S indicator (ferric ammonium citrate and sodium thiosulfate) may be omitted.

R. MEDIA FOR PLANTING COLONIES

1. SV medium (Surraco & de Pereyra, 1942):

Broth base:

Beef extract	0.5 g
Peptone	1 g
NaCl	0.5 g
Tap water	100 ml

Adjust reaction to pH 7.2-7.4 Boil for a few minutes, filter and sterilize at 120°C for 20 minutes. Add 1 g urea and 1 g lactose, Seitz-filtered, and 2.4 ml thymol-blue solution. Distribute in sterilized tubes fitted with gas bells. Extract the air by vacuum or by immersing the tubes for a few minutes in boiling water.

Indicator:

Thymol blue	1 g
NaOH (N/5)	10.75 ml
Distilled water to	500 ml

Several modifications of this medium have been described and some are widely used in Latin American countries.

2. TSI (Modified Kligler's (1917) triple-sugar/iron agar):

Beef extract	0.3 g
Yeast extract	0.3 g
Bacto peptone	1.5 g
Proteose peptone	0.5 g
Lactose	1 g
Saccharose	1 g
Glucose	0.1 g
Ferrous sulfate	0.02 g
Sodium chloride	0.5 g
Sodium thiosulfate	0.03 g
Phenol red	0.0024 g
Agar	1.2 g
Distilled water	100 ml

Sterilize at 120°C for 15 minutes, distribute and incline the tubes so as to have a 2.5-cm butt and 4-cm slant.

3. MIU medium (motility, indole, urea medium) (Gabrielli, 1956):

Peptone (rich in tryptophane)	3 g
KH_2PO_4	0.2 g
NaCl	0.5 g
Agar	0.3 g
Phenol red (alcoholic solution, 0.2%)	0.2 ml
Distilled water	100 ml

Boil to dissolve, filter through paper, sterilize at 120°C for 20 minutes and add 10 ml of a 20% sterile urea solution (see D, p. 251) to each 90 ml of medium. Distribute in sterilized tubes, 5 ml in each, and allow to solidify in an upright position.

S. MEDIA TO INVESTIGATE MOTILITY

1. Edwards & Bruner's (1942) semi-solid medium:

Beef extract	0.3 g
Peptone	1 g
Gelatin (Difco)	8 g
Sodium chloride	0.5 g
Agar	0.4 g
Water	100 ml

Dissolve beef extract, peptone and sodium chloride in water. Adjust reaction to pH 7.2-7.4, boil and filter. Add gelatin in small portions to avoid lumps, add agar, and boil. Distribute in 120 mm × 12 mm tubes, and sterilize at 110°C for 15 minutes. If other brands of gelatin are used instead of Difco's, it is important to know the amount to be employed in order to have a medium of appropriate consistency.

Semi-solid medium is inoculated by stabbing about 1 mm deep below the surface of the medium. Motile cultures will progress through the medium, while non-motile grow only along the stab.

2. Semi-solid medium for Gard (1938) plates:

Beef extract	0.5 g
Peptone	2 g
Sodium chloride	0.5 g
Agar	0.75 g
Water	100 ml

pH : 7.4

Distribute in 180 mm × 18 mm tubes, 25 ml in each, and sterilize at 120°C for 20 minutes.

This same medium can be used for the study of motility in tubes, and for this purpose a concentration of 0.5% agar is generally employed.

Several other methods have been recommended, such as U-tubes containing 0.1% nutrient agar which are seeded in one of the arms and the growth collected from the other; and tubes containing semi-solid agar inside which smaller ones open at both ends are inserted, emerging from the surface of the agar. In the latter case, cultures are seeded in the inner tube and the growth collected in the outer column of agar or *vice versa*. These methods are to be preferred when the purpose is to obtain motile variants, but they are not needed to investigate motility.

SEROLOGICAL METHODS AND REAGENTS

Although at the present time it is considered highly probable that most somatic antigens are associated with the cell wall, from a practical point of view the bacterial cell may be imagined as being constituted by successive layers of antigens of which the R antigen is situated deepest in the cell. Surrounding it there are one or, more often, several others characteristic of the S forms; these are called the O antigens, and are heat-resistant, not being destroyed by a temperature of 100°C sustained for 2 hours. Then comes the outer layer, consisting of what are called K antigens and of which two

different kinds are recognized—the capsular ones, constituting a visible capsule and which resist heating at 100°C or more, and the envelope or sheath antigens, destroyed below this temperature. These are generally not visible and there are several groups of them such as L and B in *Escherichia*, the Vi antigen in *Salmonella*, and some found in *Shigella*. Their importance resides in the fact that by their presence they inhibit agglutination of O antigens, a fact that, among others, has led to the view mentioned above as to the spatial position of these antigens.

Flagella have antigens of their own, different from those present in the cell body, and called H antigens. Flagellated types of *Salmonella* may present the phenomenon of the change of phase. In this type of variation, while the O antigens remain unmodified, the antigenic composition of the flagella is changed in such a way that new antigens appear to be substituted for the existing ones. Each of these " phases " is reversible to the other and such types as present this change are called " diphasic ". By convention these phases are called " 1 " and " 2 ".

TABLE 1

ANTIGENIC COMPOSITION OF *SHIGELLA FLEXNERI* (SUB-GROUP B)

Types (International Association of Microbiologists, 1954)	Antigenic composition (Wheeler, 1944)
1a	I: 1, 2, 4, 5, 9
1b	I: 1, 2, 4, 5, 6, 9
2a	II: 1, 3, 4
2b	II: 1, 7, 8, 9
3	III: 1, 6, 7, 8, 9
4a	IV: 1, 3, 4
4b	IV: 1, 6
5	V: 1, 5, 7, 9
6	VI: 1, 2, 4
X	—: 1, 7, 8, 9
Y	—: 1, 3, 4

ANTIGENIC COMPOSITION OF *SHIGELLA*

According to the 1953 report of the Enterobacteriaceae Subcommittee (International Association of Microbiologists, 1954), the *Shigella* group is divided into four sub-groups, designated by the letters A, B, C and D (see Table 12, p. 272).

The first, *Sh. dysenteriae*, is constituted by the mannitol-negative types. It contains seven of them, the first of which is the " Shiga type ", or *Sh. dysenteriae* as it was called in the past. Ewing et al. (1952) added an eighth type. Types of this sub-group are not serologically related by the presence of major common antigens, although there are such relations with other groups, as in the case of type 1 which gives cross-agglutinations with type O1 of the *Alkalescens-Dispar* (A-D) group. All of them are also related to the *Escherichia* group, and in some cases, as in types 3 and 5, their antigens are identical to *Escherichia* types (see Ewing, 1946).

Sub-group B, *Sh. flexneri*, is constituted by six mannitol-positive, serologically related types. Each of these has a main or type antigen of its own and another, complex in nature, shared by the whole sub-group. Table 1 shows the antigenic composition of sub-group B, type antigens being represented by Roman numerals and sub-group antigens by Arabic numerals. Types X and Y, corresponding to the original Andrewes & Inman (1919) types, contain only sub-group antigens.

Sub-group C, *Sh. boydi*, includes six types originally described by Boyd, to which five more have been added by different authors. With some rare exceptions they are mannitol-positive. They all contain type-specific antigens, but some of them have minor antigens shared by other types of the sub-group and by some A-D types (see Table 4, p. 263). In this sub-group as well there are types whose major antigens are identical with some *Escherichia* types.

Sub-group D only contains one type, *Sh. sonnei*. This can be found in two different forms, I and II, and it has different antigens in each of these. The antigens of the second are related to *Sh. boydi*, type 6.

Although in the report of the Enterobacteriaceae Subcommittee the A-D group has rightly been placed together with *Escherichia*, we think it convenient to mention it here in connexion with *Shigella*, as it is a common practice in clinical medicine to refer to this group as a separate one. It contains eight, mostly interrelated, types, which on the other hand share their major antigens with *Escherichia* and in some cases with *Shigella* sub-groups A, B and C (see Table 4, p. 263, and Table 8, p. 266).

ANTIGENIC COMPOSITION OF *SALMONELLA*

Salmonella are divided into primary somatic groups, designated by the letters A to I, and " further " groups (see Kauffmann, 1957). Within these sub-divisions, H antigens are taken into consideration to create serological types. The Kauffmann-White schema appended to the report of the Enterobacteriaceae Subcommittee mentions 275 of them, but by 1957 more than 500 were known. Table 2 shows the antigenic formulae of 28 of the most frequent types.

TABLE 2
ANTIGENIC COMPOSITION OF SOME *SALMONELLA* TYPES

Type	Somatic antigens	Flagellar antigens	
		Phase 1	Phase 2
S. paratyphi A	1, 2, 12	a	—
S. paratyphi B	1, 4, 5, 12	b	1, 2
S. reading	4, 12	e, h	1, 5
S. chester	4, 5, 12	e, h	e, n, x
S. san diego	4, 5, 12	e, h	e, n, z_{15}
S. derby	1, 4, 12	f, g	—
S. typhimurium	1, 4, 5, 12	i	1, 2
S. bredeney	1, 4, 12, 27	l, v	1, 7
S. paratyphi C	6, 7, Vi	c	1, 5
S. cholerae-suis	6, 7	c	1, 5
S. montevideo	6, 7	g, m, s	—
S. oranienburg	6, 7	m, t	—
S. thompson	6, 7	k	1, 5
S. bareilly	6, 7	y	1, 5
S. münchen	6, 8	d	1, 2
S. newport	6, 8	e, h	1, 2
S. sendai	1, 9, 12	a	1, 5
S. typhi	9, 12, Vi	d	—
S. enteritidis	1, 9, 12	g, m	—
S. dublin	1, 9, 12	g, p	—
S. panama	1, 9, 12	l, v	1, 5
S. gallinarum-pullorum	1, 9, 12	—	—
S. anatum	3, 10	e, h	1, 6
S. meleagridis	3, 10	e, h	1, w
S. london	3, 10	l, v	1, 6
S. give	3, 10	l, v	1, 7
S. newington	3, 15	e, h	1, 6
S. senftenberg	1, 3, 19	g, s, t	—

Salmonella antigens, both O and H, are found in other groups, especially *Arizona* and *Escherichia*, and in some cases they are identical.

T. PREPARATION OF AGGLUTINATING SERA

For serological diagnosis of both *Shigella* and *Salmonella*, only agglutination reactions are used.

Although several animal species have been used for the preparation of sera (especially horses), the only one employed in all specialized centres is the rabbit. These should be young, well-developed adults, weighing 1.5-2 kg or more, according to breed. Lighter animals yield too small an amount of serum, and for this reason rabbits of big, heavy breeds are to be preferred. Old animals are not good agglutinin producers.

Before starting immunization it is advisable to examine their sera for the presence of α agglutinins. This α antigen was described by Stamp & Stone in 1944. It is a somatic antigen similar to others, called K antigens by Kauffmann, and present in many bacteria of the Enterobacteriaceae family. They resist heat at 75°C for one hour, but are destroyed at 100°C for 15 minutes or, like H antigens, by alcohol treatment.

Francis & Buckland (1945) found α agglutinins in the sera of normal rabbits, sometimes with high titres, a finding confirmed by Kauffmann (1947). Immune sera should be tested for the presence of these agglutinins by slide tests, using as antigen a fresh living agar culture known to contain it. If a positive reaction is obtained, these agglutinins are absorbed with an excess of the same living culture.

The present authors have found some " normal " rabbits whose sera contained agglutinins for *S. typhimurium*; they were discarded.

Antigens for Shigella *sera*

Smooth strains have to be selected as antigens. Cultures are plated and colonies showing S characters are tested with a 1/500 trypaflavin water solution. Put on a slide a drop of saline, suspend in it the colony to be examined, place at its side another drop of trypaflavin solution, mix with the wire loop, shake gently and read after 2-3 minutes. R colonies produce a coarse flocculation, with big clumps; S colonies show no change. Intermediate stages give a fine flocculation, and although some of these may prove in the end to be excellent antigens, it is better to discard them so as to avoid confusing

results owing to the presence of R agglutinins giving cross-reactions with unrelated bacteria.

It has to be remembered that strains containing envelope antigens may give fine flocculation in the presence of trypaflavin; they may be easily differentiated from R flocculation, because the latter shows the presence of coarse floccules. It is advisable for inexperienced persons to test a Vi culture of *S. typhi* with trypaflavin to get acquainted with this type of flocculation.

Suspensions of the selected strains are tested before use with standard agglutinating sera, and they should be agglutinated to titre. Young broth cultures, 16-20 hours old, or saline suspensions, about 2×10^9 cells per ml, from agar cultures are used to inject the animals. It is advisable to heat them at 100°C for 2 hours to ensure the destruction of any K labile antigens which the strain may contain and which can cause confusing agglutinations. This is imperative in the case of cultures of the A-D group, in which, like *Escherichia* and *Providence*, such antigens are found more frequently than in other Enterobacteriaceae. These suspensions can be preserved by adding 0.5% phenol or formalin and keeping them in the ice-box.

Antigens for Salmonella *sera*

O suspensions to obtain somatic agglutinating sera are prepared in the same way as in the case of *Shigella*. Naturally non-motile O variants can be used, either killed by chemicals or by heat or living; if living, fresh cultures have to be prepared each time.

When the culture to be used is motile it has to be heated for 2½ hours at 100°C to destroy the H antigens completely. A lower temperature or shorter time, although affecting these antigens in such a way that there is no longer agglutination in the presence of specific sera, does not destroy the capacity of suspensions so treated to produce antibodies when injected.

Roschka's (1950) method is recommended for producing high-titre O sera; a saline suspension is prepared from a 16-20-hour agar culture and heated for 2½ hours in the open autoclave. The suspension is then centrifuged, the sediment suspended in 96% alcohol, heated at 37°C for four hours, recentrifuged and washed twice with acetone. It is then put in a Petri dish with a few millilitres of acetone and kept in the incubator until it is dry. The powder is finely ground, and used as antigen in a saline suspension, whose opacity corresponds to that of a 24-hour broth culture.

Vi sera are generally prepared using as antigen an original *ballerup* culture, now included in the *Citrobacter* group; this method is particularly convenient because, there being no antigen in common with the *Salmonella* group, such sera can be used unabsorbed. The culture is plated, and colonies containing Vi antigen which show an opaque appearance are selected and tested for agglutination with Vi serum before use. To prepare the antigen add to a saline suspension an equal volume of 96% alcohol, incubate at 37°C for 24 hours, centrifuge, wash with absolute alcohol or acetone, and dry in the incubator. The resulting powder is finely ground and suspended in saline before use.

Two cases have to be considered when preparing antigens for H sera. If the strain is monophasic, or a stable monophasic variant of a diphasic type, the only precaution to be taken is to have well-developed flagella. This is done by inoculating a tube of semi-solid agar and if the culture progresses to the bottom in 24 hours the upper two thirds are removed by gently heating the top of the column of medium on the pilot flame. Growth on the lower part is collected. This same procedure is repeated, if necessary, to obtain an actively motile culture. Monophasic types are to be used when possible.

If the desired antigen is contained only in diphasic types the best way to obtain it free of the opposite phase is by specific serum selection.

Methods using this principle are based on the fact that when H agglutinating sera are incorporated in a semi-solid medium, the homologous phase is immobilized while, when there is no relation between antigens and antibodies, the culture spreads freely, provided its flagella are well developed. For instance, if a culture of *S. typhimurium* which contains antigen i in phase 1 and 1,2 in phase 2 is grown on such a medium with addition of anti-i serum, phase 2 will progress while phase 1 will be immobilized. In this way it is generally easy to obtain a pure culture of the desired phase.

Either semi-solid tubes or Gard plates (see S1 and S2, p. 257) can be used with this method. For the first add about 0.1 ml of a 1/10 saline serum dilution to a tube of molten semi-solid agar at a temperature of 40°C, mix thoroughly, allow to cool, and inoculate the surface of the medium. Collect the growth at the bottoms as mentioned above. The principle of Gard plates is that *Salmonella*, when their flagella are well developed, swarm on the surface of this soft medium just as *Proteus* do on ordinary agar. For one plate incorporate 0.1 ml of undiluted serum in 25 ml of

molten medium, pour into a sterile Petri dish, and when solid inoculate at one side of the plate. Incubate for 24 hours and collect part of the outer fringe of the growth. Repeat the whole procedure a second time.

The serum has to be free of O agglutinins for the strain, as O agglutination may prevent swarming. This can be achieved either by using a serum prepared with a serological type containing the same H but different O antigens from the strain or by absorption (see U, below). To sterilize these sera add an excess of chloroform and use after 24 hours' contact.

Artificially induced phases are as good as monophasic types to prepare H sera, as they have no tendency to revert spontaneously to the original phase. These are obtained by similar methods to the selected natural phases, but they are not so easily produced. Such strains can be obtained from specialized laboratories.

In case of need it is possible to obtain the desired phase by plating the culture and selecting colonies which are tested by slide agglutination. With most strains this procedure has to be repeated several times in order to eliminate the other phase and even so, when injected, they always produce agglutinins for the opposite phase which have to be absorbed.

To inject rabbits, the use is recommended of a 0.5% formolized 16-20-hour broth culture. In the authors' laboratory, flagella suspensions free from bacterial bodies are used. They are obtained as follows:

Roux flasks of about 0.5-litre capacity containing agar are inoculated with a 3-4-hour broth culture. After 20 hours the growth is suspended in 10-15 ml saline and vigorously shaken with glass beads to detach the flagella. An excess of chloroform is added and left in contact for two hours. The chloroform-free suspension is centrifuged at high speed until the supernatant is limpid and free of bacterial bodies. This is verified by examining it in hanging drops; if necessary, centrifugation is repeated. Centrifugation must be done at once as, if the suspension is kept for a time, the flagella tend to form contact bodies which are sedimented when centrifuged. These antigens regularly give sera with a flagellar titre superior to 1/50 000 and O agglutinins inferior to 1/80.

Immunization of rabbits

Rabbits are injected in the marginal ear vein. Many immunization schedules have been advocated. In our experience a short course of four injections with brief intervals between each is to be preferred; we use 0.5, 1, 2 and 4 ml on the first, fourth, seventh and tenth days, respectively.

It must be remembered that the Kauffmann-White schema does not mention all antigens, but only the principal ones, important for classification. In many types, however, small amounts of both O and H antigens are present which may bring about cross agglutinations not explained by the antigens shown in the schema. Undesirable agglutinins for these minor factors are bound to be produced when long schedules for immunization are followed, either because more injections are given or because longer intervals are allowed between two of them.

Blood samples can be taken five days after the last injection and tested for agglutinins. If the titre is not satisfactory another 4-ml injection can be given although, as a rule, we bleed the rabbits on the fifth day without taking any samples since if rabbits are good and antigens well prepared, titres are regularly high enough.

Rabbits to be bled are slightly anaesthetized by inhalation of a mixture of 1 part alcohol and 3 parts ether. Blood can be collected from either the femoral or the carotid artery. After a long incision of the skin the artery is discovered and sectioned and blood collected in a flask through a funnel from the proximal end, which it is convenient to cut as long as possible. Cardiac puncture can also be used but it is not so convenient. Blood is allowed to clot, serum is pipetted off and centrifuged, and phenol is added to the serum in a proportion of 0.5% (10% of a phenol 5% water solution). If the serum is not to be absorbed, or after absorption, an equal volume of pure neutral glycerol is added as preservative. This addition of glycerol has the further advantage of preventing evaporation in the course of slide agglutination tests. Sera are kept in the ice-box to avoid loss of agglutinin titres.

Good sera should titre at least 1/2000 for O, 1/500 for Vi and 1/10 000 for H antigens when tested by the tube method.

U. AGGLUTININ ABSORPTION

When no antigenic cross-relations exist between serological types, sera can be used just as they are obtained from rabbits. This is illustrated by the sub-group A shigellae, as each type has its own antigens not shared by other types included in it or in other sub-groups; and the same is true of some O antigens in the *Salmonella* group.

Even when a serum has agglutinins for more than one antigen it can be sometimes be used as a serum. This is often the case when H sera for *Salmonella* monophasic types are prepared, as the difference in titre of O and H agglutinins is great enough to allow of their use at such a dilution that there is no longer any O agglutination. The use of flagellar suspensions as antigens to prepare H sera, as recommended above, is particularly suitable for obtaining H sera of low titre.

In most cases, though, sera have to be deprived of some undesirable agglutinins if they are to be used as specific reagents for the identification of only one antigen or group of antigens. This is done by agglutinin absorption, based on the fact that agglutinins are specifically fixed by their homologous antigens and can be completely removed by centrifugation when an excess of them has been added.

Before absorption, sera are titrated for agglutinins against all O or H antigens present in the type used to indicate whether they really need to be absorbed and also to give some idea of the amount of absorbing bacteria to be used.

These are grown on plain agar, Petri dishes being generally used. Edwards & Ewing recommend seeding them with 0.5 ml of a 24-hour broth culture, incubating them at 37°C for 24 hours, adding 1 ml of 0.5% phenolized saline and collecting the growth with a wire scraper. The resulting suspension is centrifuged and the supernatant discarded, and 1-2 ml of phenolized saline are added and mixed with the serum. This mixture is thoroughly shaken and put in the water-bath at 50°C for two hours, after which it is centrifuged and the supernatant collected.

As a result of long experience, since in our laboratory only specific sera for antigenic factors are used for routine work, we prefer to use for this purpose ordinary aluminium baking tins about 30 cm in diameter and 10 cm high which can be had very cheaply from any hardware store. A thick layer of agar about 1.5 cm high is put in them, and the tins are covered with cotton between a double layer of gauze and paper on top. The whole is tied with string and then autoclaved. 20 ml of a suspension of bacteria in water are used for seeding and the whole is incubated for 48 hours at 37°C to allow the surface of the agar to dry. Growth is collected with a blunt spatula and looks like peanut butter. A small amount of phenolized saline is added and the mixture is incorporated with the serum. This method has the double advantage of avoiding serum dilution and of simplicity, saving labour and material; although the surface of one of these tins is equivalent to about nine Petri dishes, each tin yields a bigger harvest of bacteria. This explains the small number of tins we use for absorption as compared with the large number of plates which would be required to get the same result.

In practice there are three cases in which sera have to be absorbed: (*a*) absorption of O antibodies from an H serum; (*b*) absorption of agglutinins for O and one of the H phases of a serum prepared with a diphasic type; and (*c*) preparation of pure specific agglutinins for O or H factors.

It is essential to remember that all sera after absorption have to be titrated again by the tube and the slide test.

Preparation of agglutinating sera for pure factors for Shigella

As a rule, as stated below, the average laboratory will not need to determine pure factors for *Shigella* identification which, in practice, is not carried so far as to type all strains beyond the sub-group to which they belong. Furthermore, in sub-group A there is no need of agglutinin absorption as there are no major antigens shared by types belonging to it,

TABLE 3

PREPARATION OF SPECIFIC SERA WITH *SHIGELLA FLEXNERI* (SUB-GROUP B)

Somatic factor	Serum prepared with *Sh. flexneri*	Absorbed by *Sh. flexneri*
I	1a (I : 1, 2, 4, 5, 9)	5 (V : 1, 5, 7, 9)+6(VI : 1, 2, 4)+X(− : 1, 7, 8, 9)+Y(− : 1, 3, 4)
II	2a (II : 1, 3, 4)	1a (I : 1, 2, 4, 5, 9)+Y(− : 1, 3, 4)
III	3 (III : 1, 6, 7, 8, 9)	1b (I : 1, 2, 4, 5, 6, 9)+X(− : 1, 7, 8, 9)+Y(− : 1, 3, 4)
IV	4a (IV : 1, 3, 4)	X (− : 1, 7, 8, 9)+Y(− : 1, 3, 4)
V	5 (V : 1, 5, 7, 9)	1a (I : 1, 2, 4, 5, 9)+X(− : 1, 7, 8, 9)
VI	6 (VI : 1, 2, 4)	1a (I : 1, 2, 4, 5, 9)
6	1b (I : 1, 2, 4, 5, 6, 9)	1a (I : 1, 2, 4, 5, 9)
3, 4	2a (II : 1, 3, 4)	2b (II : 1, 7, 8, 9)
7, 8	2b (II : 1, 7, 8, 9)	1a (I : 1, 2, 4, 5, 9)+2a(II : 1, 3, 4)

TABLE 4
PREPARATION OF SPECIFIC SERA WITH *SHIGELLA BOYDI* (SUB-GROUP C)

Factor	Serum prepared with	Absorbed by
1	*Sh. boydi 1*	*Sh. boydi 4*+A-D O1
2	*Sh. boydi 2*	Unabsorbed
3	*Sh. boydi 3*	Unabsorbed
4	*Sh. boydi 4*	*Sh. boydi 1*+A-D O1
5	*Sh. boydi 5*	*Sh. boydi 9*+A-D O3
6	*Sh. boydi 6*	*Sh. sonnei 2*
7	*Sh. boydi 7*	Unabsorbed
8	*Sh. boydi 8*	Unabsorbed
9	*Sh. boydi 9*	*Sh. boydi 425* (*sub-judice*) +A-D O2+A-D O3
10	*Sh. boydi 10*	*Sh. boydi 11*
11	*Sh. boydi 11*	*Sh. boydi 10*+A-D O1

with the possible exception of *Sh. dysenteriae* 1, which gives cross-agglutinations with the O1 type of the A-D group.

In sub-groups B, C and A-D, on the other hand, type determination has to be made by the use of pure factors as most types belonging to them share one or more antigens with the remaining types of the same sub-group; in some cases there are also relations between sub-groups. Tables 3, 4 and 5 show the types to be used for absorption when preparing these sera for pure factors containing only agglutinins for some specific antigen.

TABLE 5
PREPARATION OF A-D GROUP SPECIFIC SERA

Factor	Serum prepared with	Absorbed by
1	A-D O1	A-D O5
2	A-D O2	A-D O3+A-D O4
3	A-D O3	A-D O2+A-D O4
4	A-D O4	A-D O2+A-D O3
5	A-D O5	A-D O1
6	A-D O6	Unabsorbed
7	A-D O7	A-D O1+A-D O4
8	A-D O8	Unabsorbed

As to the number of plates of the absorbing cultures to be used no fixed rules can be given as this will vary with the serum contents of the agglutinins to be absorbed and the amount of the respective antigen contained in the absorbing culture. As a general rule to absorb 1 ml of serum 10 plates or less than one tin are needed. As the presence of superficial K antigens may inhibit absorption, it is convenient beforehand to test the serum in the presence of both unheated cultures and cultures heated to 100°C for 1 hour and to use one or the other for absorption according to results.

Sub-group D contains only *Sh. sonnei*, which may occur in either of the forms I or II. If the proper strains are used to prepare sera, neither of these will contain agglutinins for the other and they can be used without absorption. If they should prove not to be specific enough they can also be absorbed with the opposite form, as needed. It is well to remember that there are cross-relations between *Sh. sonnei* form II and *Sh. boydi* type 6.

Preparation of pure factors for Salmonella

O factors are prepared according to the absorption list in Table 6. Unheated cultures are used, 5-10 plates of each for 1 ml of serum. The growth of one tin (see U, p. 262) is usually enough for 5-10 ml of serum.

TABLE 6
PREPARATION OF *SALMONELLA* SPECIFIC SOMATIC SERA

Somatic factor	Serum prepared with	Absorbed by
2	*S. paratyphi* A var. *durazzo* (2, 12)	*S. typhi* (9, 12) (strain T 2)
3, 19[a]	*S. senftenberg* (1, 3, 19)	*S. paratyphi* A (1, 2, 12)
4	*S. reading* (4, 12)	*S. typhi* (9, 12) (strain T 4)
7	*S. thompson* (6, 7)	*S. newport* (6, 8)
8	*S. amherstiana* (8)	Unabsorbed
9	*S. gallinarum* (1, 9, 12)	*S. typhimurium* (1, 4, 5, 12)
10	*S. anatum* (3, 10, 26)	*S. newington* (3, 15)+ *S. minnesota* (21, 26)
15	*S. newington* (3, 15)	*S. anatum* (3, 10, 26)

[a] A pure 19 factor serum can be prepared, according to Kauffmann (1954), by absorbing an O *S. senftenberg* serum (1, 3, 19) by *S. paratyphi* A (1, 2, 12) plus *S. london* (3, 10, 26).

With respect to H factors, three cases have to be considered. First, when the serum has been prepared with a monophasic strain, only O agglutinins have to be removed. This can be done by using either the same type heated at 100°C for one hour or an O form, or living cultures of some other type with the same O antigenic composition but different H antigens. About five plates are needed in this case for 1 ml but only one tin for 5-10 ml of serum. Secondly, when the serum has been prepared with one phase of a diphasic type, not only O agglutinins but a variable amount, always present, of agglutinins of the opposite phase have to be removed. In this case it is convenient to use for absorption a type containing the same O and one of the H phase antigens. As an example, if " e, h " serum is desired, an *S. newport* culture

TABLE 7
PREPARATION OF SPECIFIC *SALMONELLA* H AGGLUTINATING SERA [a]

H Factor	Serum prepared with	Absorbed by
a	*S. paratyphi* A (1, 2, 12 : a)	*S. paratyphi* A (boiled) (1, 2, 12)
b	*S. paratyphi* B (monophasic, phase 1) (1, 4, 5, 12: b : -)	*S. typhimurium* (1, 4, 5, 12 : i : 1, 2)
c	*S. kunzendorf* (phase 1, induced, strain conn 1780) (6, 7 : c : -)	*S. thompson* (6, 7 : k : 1,5)
d	*S. typhi* (9, 12 : d)	*S. gallinarum* (1, 9, 12 : -)
e, h	*S. newport* (phase 1) (6, 8 : e, h : -)	*S. fayed* (phase 2) (6, 8 : - : 1,2)
f	*S. derby* (1, 4, 12 : f, g)	*S. budapest* (1, 4, 12 : g, t)+*S. essen* (4, 12 : g, m)
g, p	*S. dublin* (1, 9, 12 : g, p)	*S. gallinarum* (1, 9, 12)
h	*S. newport* (phase 1) (6, 8 : e, h : -)	*S. bonariensis* (phase 2) (6, 8 : - : e, n, x)+*S. fayed* (phase 2) (6, 8 : - : 1, 2)
i	*S. bonariensis* (phase 1) (6, 8 : i : -)	*S. narashino* (phase 2) (6, 8 : - : e, n, x)
k	*S. thompson* (phase 1, strain 94368) (6, 7 : k : -)	*S. kunzendorf* (6, 7 : - : 1, 5)
l, v	*S. london* (phase 1) (3, 10 : l, v: -)	*S. anatum* (phase 2) (3, 10 : - : 1, 6)+*S. schwarzengrund* (phase 2) (1, 4, 12, 27 : - : 1, 7)
m, t	*S. oranienburg* (6, 7 : m, t)	*S. thompson* (6, 7 : k : 1,5)
n, x	*S. abortus equi* (phase 2) (4, 12 : - : e, n, x)	*S. reading* (phase 1) (4, 12 : e, h : -)
p	*S. dublin* (1, 9, 12 : g, p)	*S. enteritidis* (1, 9, 12 : g, m)
s	*S. montevideo* (6, 7 : g, m, s)	*S. oranienburg* (6, 7 : m, t)+ *S. enteritidis* (1, 9, 12 : g, m)
t	*S. oranienburg* (6, 7 : m, t)	*S. montevideo* (6, 7 : g, m, s)+*S. enteritidis* (1, 9, 12 : g, m)
v	*S. london* (phase 1) (3, 10 : l, v : -)	*S. dar-es-salaam* (phase 1) (1, 9, 12 : l, w : -)+*S. uganda* (3, 10 : l, z_{13} : -1, 5)
w	*S. worthington* (phase 1, strain III.61) monophasic (1, 13, 23 : l, w : -)	*S. london* (phase 1) (3, 10 : l, v : -)+*S. uganda* (phase 1) (3, 10 : l, z_{13} : -)+*S. wichita* (1, 13, 23 : d, z_{37})
y	*S. bareilly* (phase 1) (6, 7 : y : -)	*S. thompson* (phase 2) (6, 7 : - : 1, 5)
1, 2	*S. paratyphi B* (phase 2) (1, 4, 5, 12 : - : 1, 2)	*S. abony* (phase 1) (1, 4, 5, 12 : b : -)
2	*S. paratyphi B* (phase 2) (1, 4, 5, 12 : - : 1, 2)	*S. bredeney* (phase 2) (1, 4, 12, 27 : - : 1, 7)+*S. thompson* (phase 2) (6, 7 : - : 1, 5)+*S. schleissheim* (4, 12, 27 : b : -)+*S. panama* (phase 2) (1, 9, 12 : - : 1, 5)
5	*S. thompson* (phase 2) (6, 7 : - : 1, 5)	*S. anatum* (phase 2) (3, 10 : - : 1, 6)+*S. newport* (phase 2) (6, 8 : - : 1, 2)+*S. daytona* (phase 1) (6, 7 : k : -)
6	*S. anatum* (phase 2) (3, 10 : - : 1, 6)	*S. münster* (3, 10 : e, h: 1, 5)+*S. thompson* (phase 2) (6, 7 : - : 1, 5)
7	*S. bredeney* (phase 2) (1, 4, 12, 27 : -1, 7)	*S. newport* (phase 2) (6, 8 : - : 1, 2)+*S. panama* (1, 9, 12: l, v : 1, 5)+*S. münster* (phase 2) (3, 10 : - : 1, 5)+*S. schleissheim* (4, 12, 27 : b : -)

[a] Phase 1 or phase 2 are obtained from diphasic strains by specific serum selection (see p. 260).

(6,8 : e,h:1,2) is used to prepare it and it is absorbed with *S. fayed* (6,8 : 1,w : 1,2) in phase 2. This example illustrates the advantage of carefully selecting the types to be used as antigens to prepare sera, and choosing such of them as will provide sera whose undesired agglutinins can be absorbed with a single type. For this type of absorption we use one tin of the absorbing culture for 5-10 ml of serum. Thirdly, when an H antigen is not found alone but together with one or more flagellar antigens, such as h, m, w, and so on, so that the serum has to be deprived not only of its O but also of part of its H agglutinins for a complex antigen, absorption is performed in a similar way to that for the second case, just mentioned. For instance, to prepare " p " factor, *S. dublin* serum (1,9,12 : g, p) is absorbed with *S. enteritidis* (1, 9, 12 : g, m). In this case a larger number of bacteria is needed, one tin for 2-5 ml or 10-20 plates for 1 ml of serum. Table 7 gives a list of the H specific sera needed and the types to be used to prepare them.

Cross-absorption test

The final proof of complete identity of a culture is provided by comparing it with a standard strain. This is done by the cross-absorption test.

O and H sera are prepared with the culture under test and with the type strain, and then each of the four is absorbed with an excess of bacteria, on the one hand with the culture used as antigen and on the other with the one to be tested. If absorption of agglutinins is complete in all cases it can be deduced that the two cultures are antigenically identical. If some residue remains it has to be deduced that the culture used to prepare the serum has some antigen not contained in the strain used for absorption.

V. PREPARATION OF POLYVALENT SERA

In routine work it is convenient to have polyvalent sera for two different purposes. One of them is to decide whether some newly isolated culture with the general biochemical characters of the *Shigella* or *Salmonella* groups does in fact belong to one of those groups; this is easily proved by the use of sera containing agglutinins for the whole group. If the test is positive. it is convenient to have a second type or polyvalent sera so that using them typing is accomplished by progressive steps, avoiding the need to test cultures against all O and H sera.

These sera can be prepared either by pooling monotype sera or by immunizing rabbits with a mixture of antigens. This second method is to be preferred as higher titres are obtained against individual antigens since the sera are not diluted. The schedule to be followed for immunization is longer than when preparing sera against single types. Eight to ten injections are given, at intervals of 3-4 days; five days after the last injection a blood sample may be taken and the serum tested for agglutinins. If necessary, one or more injections may be given of some particular antigens for which titres are low.

Shigella *sera*

As the number of antigens in this group is relatively small and as biochemical characters allow a presumptive diagnosis to be made of the sub-group, it is practical to have four polyvalent sera, for A, B, C and Alkalescens-Dispar sub-groups. As for the Sonne type, cultures showing its biochemical behaviour are tested with sera for forms I and II,

Sera are prepared using as antigens either a broth or an agar culture suspension heated at 100°C for 2 hours. In sub-group A at least four of the serotypes are used, and, depending on the most prevalent in the country, others may be added. For sub-groups B and C all the types should be represented.

For the A-D group the first four types are generally used. In this case again the bacteriologist has to decide what sera are most needed in his own country. Antigens to prepare sera for this group should always be heated for 2 hours at 100°C as L antigens are not infrequently found in members of it.

As there are minor relationships between subgroups A, B, and C and some of the A-D types it is convenient to absorb sera for these three subgroups with cultures of the last to avoid confusing results. On the other hand, A-D sera as a rule do not need to be absorbed as their content in agglutinins for the other groups is small and produces only slight and late agglutinations which can be ignored. Even so, it is best to test such sera and absorb them if needed. (For polyvalent serum preparation, see Table 8.)

Salmonella *sera*

A polyvalent serum for all O and H antigens of this group is prepared by injecting formolized broth cultures as mentioned above. Table 9 gives a list of types which can be used, but others can be substituted for them. Cultures used as antigens need to have well-developed flagella, and the correct

TABLE 8
PREPARATION OF POLYVALENT *SHIGELLA* SERA

Sub-group	Serum prepared with	Absorbed by
A	*Sh. dysenteriae* types 1 to 8	A-D O1
B	*Sh. flexneri* types 1 to 6	A-D O1+O3+O4
C	*Sh. boydi* types 1 to 11	A-D O1+O2+O3 +O4+*Sh. sonnei* II
A-D group	A-D types 1 to 4	Unabsorbed

phase has to be selected, although not so much care is needed as when preparing sera for H *Salmonella* antigens.

A second polyvalent serum should only contain agglutinins for the O antigens of the most prevalent types, 1 to 10, 12 and 15. It is prepared with a pool of broth cultures heated for 2 hours at 100°C. Any types of groups A, B, C, D or E can be used, but it is essential that they be smooth forms. Roschka's method (see p. 260) can be used with advantage.

Polyvalent O sera are controlled in the presence of all antigens used to prepare them to ascertain whether their agglutinating titres are high enough for each antigen.

W. AGGLUTINATION TESTS

Agglutination is usually carried out by one of two methods: the quick slide test and the slow tube test. As each of them has its own advantages and inconveniences they are used for different purposes. The first is very convenient for typing newly isolated strains, while the second is to be preferred for titrating sera and for evaluating the remaining agglutinins after cross-absorption, as it gives more reliable results. When looking for agglutinins in a patient's serum, the slide test is useful since, if it is negative, there is no need to perform the more complicated and time-consuming tube test. If it is positive, however, the tube test has to be used to measure the agglutinin content of the serum.

The microscopic test can also be used and has the advantage that it requires very small amounts of sera and bacteria while allowing differentiation between O and H agglutination. Haemoagglutination has also been used because of its greater sensitivity but it is not needed for practical purposes.

Bacterial suspensions may be used: (*a*) live, as in the case of titrating a pure O serum, with the exception mentioned below; (*b*) boiled for 1 hour to destroy H and some K antigens which prevent O agglutination; (*c*) alcohol-treated as an alternative for O titration of an OH serum (White's method (1926) is recommended; growth of an agar tube is suspended in 2-3 ml of 96% alcohol, heated

TABLE 9
TYPES TO BE USED TO PREPARE POLYVALENT *SALMONELLA* SERA

Type	Antigenic composition
S. paratyphi A	1, 2, 12 : a
S. paratyphi B (phase 1)	4, 5, 12 : b
S. montevideo	6, 7 : g, m, s
S. newport (phase 1)	6, 8 : e, h
S. typhi	9, 12 (Vi) : d
S. cairina (phase 1)	3, 10, 26 : z_{35}
S. illinois (phase 1)	(3), (15), 34 : z_{10}
S. simsbury	1, 3, 19 : z_{27}
S. aberdeen (phase 1)	11 : i
S. fanti	13, 23 : z_{38}
S. sundsvall (phase 1)	(1), 6, 14, 25 : z
S. jacksonville	16 : z_{29}
S. kirkee (phase 2)	17 : 1, 2
S. cerro	18 : z_4, z_{23}
S. kentucky (phase 2)	(8), 20 : z_6
S. minnesota (phase 2)	21 : e, n, x
S. tel-aviv (phase 1)	28 : y
S. donna (phase 1)	30 : l, v
S. adelaide	35 : f, g
S. lindi (phase 1)	38 : r
S. champaign (phase 1)	39 : k
S. karamoja (phase 1)	40 : z_{41}
S. waycross	41 : z_4, z_{23}
S. weslaco	42 : z_{36}
S. berkeley (phase 2)	43 : 1, 5
S. niarembe (phase 2)	44 : l, w
S. deversoir (phase 1)	45 : c
S. strasbourg (phase 2)	(9), 46 : 1, 7
S. bergen (phase 2)	47 : e, n, z_{15}
S. dahlem	48 : k : e, n, z_{15}
S. bulawayo	(1), 49 : z : 1, 5
S. greenside	50 : z : e, n

at 60°C for 4 hours and centrifuged, and the sediment is suspended in phenolized saline); (*d*) formol-treated (0.3%) broth cultures for H agglutinins.

Slide test

This test is usually performed with well-cleaned common microscope slides. A loopful of a heavy suspension of about 2×10^{10} cells per ml is deposited on the slide and an equivalent volume of serum at its side. They are mixed with the wire loop or a toothpick, following a straight line across the slide, which is then tilted several times back and forth. When the slide is viewed against a dark background agglutination is recognized by the production of big lumps.

When typing cultures we prefer to use a big glass plate about 30 cm × 20 cm on which 2 cm × 2 cm squares are drawn with paint or frosted lines. The symbols of antigens are written in the upper line of squares with a wax pencil, and in the squares below drops of sera to be used and of bacterial suspension are deposited with Pasteur pipettes or glass droppers. The use of these plates has the advantage that the whole antigenic pattern of the culture is seen at a glance.

Tube test

In this method progressive dilutions of sera are tested in the presence of a constant and equal volume of bacterial suspension. 10 mm × 75 mm tubes containing 1 ml of mixture are incubated in the water-bath at 45°-50°C. H agglutination is read after 2-3 hours and appears as big, loose floccules which when allowed to settle form a voluminous deposit, easily disintegrated by shaking. O agglutination has to be read after 24 hours and it appears as a small, tight granular sediment at the bottom of the tube. Vi agglutination tubes are incubated for 2 hours at 37°C and then overnight at room temperature before reading. Their aspect is similar to that of O agglutination tubes but the granules are smaller and more cohesive.

For the tube agglutination tests, saline suspensions in a 2×10^9 concentration or broth cultures, if necessary diluted to the same concentration, are used as antigens.

EXAMINATION OF SPECIMENS

In practice diagnosis of *Salmonella* and *Shigella* infections is made by looking for the causative agent in stools or other material from the patient, but agglutination tests may occasionally be useful. Their use is limited by the fact that when the causative agent is unknown there is no way of deciding what antigen to try for agglutinins in the patient's serum and also by the fact that negative reactions are rather frequent even among known *Shigella* or *Salmonella* patients.

Agglutination tests are very useful for mixed infections, in which two or more *Shigella* and/or *Salmonella* types are simultaneously isolated from a single patient. It is often found that O and/or H agglutinins develop for one type or the other at the end of the first or the beginning of the second week, save in the case of infants when they take longer to appear. When dealing with *Shigella* patients, it should be remembered that if envelope antigens are present in the culture to be used as antigen it is necessary to use untreated suspensions and suspensions heated to 100°C. For *Salmonella*, formol-treated suspensions (H antigens) and suspensions treated with alcohol or heated to 100°C (O antigens) are to be used when testing for H and O agglutinins.

Tests are usually performed by the tube method at 50°C. Blood is allowed to coagulate and serum is collected by means of a pipette and centrifuged if not clear. If desired the slide method can be used as a screen. When the slide test is negative it can be assumed that there are no agglutinins in the serum; when it is positive the titre has to be determined by the tube method.

Specimens to be examined may have to be dealt with differently according to their different natures. Uncontaminated materials, such as blood, spinal fluid and very often pus from closed abscesses, where only the causative agent is expected to be present, do not need any prior treatment.

Bile broth, in the proportion of one part of ox-bile to three parts of broth, has been used in our laboratory for many years for blood culture with excellent results. It has the advantage over other liquid media that the proportion of blood to the medium can be rather high without inconvenience. We currently use tubes containing 6 ml of medium to which 1-2 ml of blood are added. When using broth

it is not advisable to add more than 5-10 ml blood to each flask containing 100-150 ml of medium, as ox-bile prevents the growth-inhibiting action of blood.

Uncoagulated blood is added to the medium with a pipette, and the clot, when formed, is broken with a sterile glass rod. Tubes are incubated for 24 hours, transfers are made to plain agar slants, and if Gram-negative rods are found after 24 hours' incubation the culture is identified as described below. Negative tubes have to be kept under observation in the incubator for at least 10 days, making transfers to agar every two days.

It is important to remember that, especially in the case of infants under one year old, septicaemias due to different Enterobacteriaceae and having a typhoid-like course are rather frequent, so that the bacteriologist has to be prepared for them and be able to arrive at a correct diagnosis.

Spinal fluid is first examined after centrifugation and Gram-staining of the sediment so as to decide what media should be used. Meningitis cases due to Enterobacteriaceae are frequent only with infants, the majority of such cases being produced by other bacteria which will require special media. When Gram-negative rods are found, the sediment is seeded on plain agar tubes or plates. Pus is treated the same way, but centrifugation is not needed.

Most frequently, however, the bacteriologist has to deal with highly contaminated materials, especially stools, and the problem is to devise media which will allow *Shigella* or *Salmonella* to be isolated with a minimum of material, time and labour. For this purpose selective solid and enrichment liquid media are used.

SELECTIVE MEDIA

This group includes such media as will promote the growth of *Salmonella* or *Shigella* while inhibiting most of the associated flora. They are at the same time to some extent differential, as they will allow the colonies which deserve to be further studied to be recognized by some fundamental characteristic.

To this group belong Kristensen, Lester & Jürgens (KLJ) agar, desoxycholate-citrate agar (DCA), SS agar and Wilson & Blair's (WB) medium (see B, p. 250). The first is excellent for the isolation of *Salmonella* which, as non-lactose fermenters, give on it red colonies, round, about 1 mm in diameter, translucent and convex. *Escherichia*, *Klebsiella* and *Cloaca* are mostly inhibited, and, when growing, produce yellow colonies turning to greenish. *Proteus* are inhibited. *Pseudomonas aeruginosa* may grow, producing red, round colonies similar to *Salmonella*, but generally giving flat colonies with irregular margins and a metallic sheen. *Shigella* do not grow on this medium.

DCA agar is used for the isolation of *Shigella*, which grow very well on it, giving round, convex, colourless colonies. *Escherichia* and other lactose fermenters are mostly inhibited and, when growing, produce red, opaque colonies. Some *Proteus* may grow, producing O forms which are easily confused with *Shigella*. Although *Salmonella* cultures from collections grow on this medium, it is not recommended for their primary isolation as its results are inferior to those of KLJ or SS agar. This last has some advantages, as both *Salmonella* and *Shigella* will grow on it, but, on the other hand, it is not as good as KLJ for *Salmonella* or as DCA for *Shigella*. *Proteus* grow on it, producing O colonies, which like those of *Salmonella* and *Shigella*, are colourless, round and translucent.

WB is recommended only for *S. typhi* isolation. Some workers have obtained good results with it for *Salmonella* isolation but in our experience KLJ is much better. *Shigella* do not grow on it. *S. typhi* produces black or greyish-black colonies when isolated but when growth is confluent the colour is greenish-grey. *Citrobacter* may grow, also producing black colonies which resemble *S. typhi*. *Escherichia* are mostly inhibited but not *Proteus*, which will grow, producing green O colonies.

For examination of contaminated materials such as stools, sewage, foods, etc., we recommend the use of three selective plates—KLJ, DCA, and SS—and if S. *typhi* has to be also investigated a fourth plate of WB should be used. A plate of lactose/bromo-thymol-blue agar (LBA) is added as the use of a plate without inhibitors gives an indication of the prevalent flora. In recent acute cases of shigellosis and salmonellosis a pure culture of these bacteria is sometimes obtained on LBA plates. KLJ, DCA and SS are heavily inoculated, and then without loading the wire loop again a plate of LBA is seeded. Some experience is needed to obtain isolated colonies on this last plate. All these plates are incubated for 24 hours at 37°C.

No selective medium is completely satisfactory, as none of them fulfils entirely the bacteriologist's ideal of eliminating all bacteria save the ones looked for. Even when many are inhibited they remain alive, and colonies obtained from selective media have to be re-isolated as they are very often impure; this is

imperative when colonies are close together. If this precaution is neglected it often happens that mixed cultures of two *Salmonella* types or a *Salmonella* or *Shigella* plus *Escherichia*, *Serratia* or *Proteus* are obtained, giving confusing results when the object is identification.

For re-isolation of colonies, media without inhibitors are to be preferred. Any lactose agar plates (Endo, MacConkey, Drigalski and others) can be used. We use LBA or lactose/phenol-red agar.

ENRICHMENT

For enrichment liquid media are used, whose effect is to change the original balance of species present in the specimens, increasing the number of *Salmonella* while the rest either are inhibited or remain unchanged or in any case do not grow as abundantly as *Salmonella*.

Two media are generally used, Müller-Kauffmann's tetrathionate (MKT) and Leifson's selenite F (SF). For *Salmonella* isolation better results are obtained by enrichment than by direct plating; in some cases, however, enrichment fails when direct plating is positive, and that is one of the reasons why in routine work both methods are used simultaneously. We prefer MKT to SF for *Salmonella* isolation, as in our hands it has given more positive results. SF is better than MKT for isolation of *S. typhi*, but for this purpose Wilson & Blair plates are to be preferred.

Enrichment media are very heavily inoculated with about 2 g of stools, or an equivalent volume of any fluid being tested. Tubes are incubated for one and five days in the case of MKT and for one day only for SF, and plates for isolation are then seeded. These have to be selective because after enrichment *Proteus* are generally found, so that LBA or similar plates are usually invaded by them. One plate of KLJ and another of SS are to be recommended, the second being inoculated without reloading the loop. Suspicious colonies are re-isolated on LBA. For reasons which are explained later, it is advisable to pick no less than 10 colonies, suspend them in about 1 ml of saline and re-isolate, using two plates so as to obtain well-separated colonies.

PRELIMINARY IDENTIFICATION

(Table 10)

In practice the best policy is rapidly to recognize colonies belonging to species that are not *Shigella* or *Salmonella* rather than to try to identify these. There are two groups of bacteria producing colonies similar to those of *Shigella* or *Salmonella*:O colonies of *Proteus* (as this group does not ferment lactose) and late lactose fermenters belonging to the *Escherichia* group. Together these two groups constitute about 60%-70% of all colonies discarded after enrichment and only a little less after direct plating. For this reason we prefer the use of a simple medium, SV (see R 1, p. 256), for planting isolated colonies as it permits the recognition of the urea-splitting *Proteus* and bacteria that do not ferment lactose on primary isolation on plates but that do so when transferred to liquid media.

It is advisable to pick at least 5-10 colonies from each plate and transfer them to SV. Tubes are incubated for 24 hours. *Proteus* usually turn the colour to blue in less than 20 hours and most frequently in 4-5 hours. *Salmonella* produce a change to greenish-yellow, and *Morganella* to green. *Shigella* does not change the colour of the medium. Gas production from lactose is recognized by its presence in the bells, and tubes showing it are eliminated.

All tubes showing a yellow or greenish colour are kept for study. Transfers are made to lactose, glucose, saccharose, mannitol, peptone water (for production of indole and H_2S), agar slants and semi-solid media; these are incubated for 24 hours.

Shigella ferment glucose without gas production, except some cultures of *S. flexneri* type 6 (Newcastle, Manchester) which may produce a small amount of gas. With the exception of some A-D strains they do not ferment lactose in 24 hours. They do not produce H_2S and are non-motile. Sub-group A does not ferment mannitol, while the remainder do so. The Sonne type ferments lactose slowly, generally in 3-4 days; A-D ferments both lactose and saccharose, seldom in 24 hours, generally later. A-D is positive for indole while Sonne is negative. These characters are sufficient to decide the conduct to be followed so as to complete the diagnosis.

Salmonella, with the exception of *S. typhi*, *S. gallinarum*, and some anaerogenic variants of other types rarely found, ferment glucose and mannitol with gas production, generally produce H_2S and are indole-negative. They are mostly motile and do not ferment lactose or saccharose.

As recommended by Gabrielli (1956) colonies may be planted simultaneously in two media, TSI and MIU (see R2 and R3, p. 257). The first is inoculated

TABLE 10

PROCEDURE FOR IDENTIFICATION OF *SALMONELLA* AND *SHIGELLA*

1st day	Direct isolation (DCA+KLJ+(WB)+LBA)	Enrichment (MKT or SF)
2nd day	Transfer 4-6 colonies from each plate to: SV	After 1 and 5 days from MKT After 1 day from SF Isolate on plates KLJ+SS
3rd day	Tubes with gas or turned blue: Discard Transfer remaining colonies to: Glucose, Lactose, Sucrose, Mannitol, Peptone water, Agar slant	Re-isolate 10-15 suspicious colonies SS+LBA ↓ Follow as for direct isolation
4th day	*Colonies showing:* Glucose, gas Mannitol+ Lactose –, sucrose – H_2S+, indole – *Polyvalent* Salmonella *sera* Agglutination positive: Probably: *Salmonella* Identify serologically. Complete biochemical tests. Agglutination negative: Probably: *Arizona* *Citrobacter.* Complete biochemical tests.	*Colonies showing:* Glucose, acid Lactose – H_2S – *Polyvalent* Shigella *sera* Agglutination positive: Probably: *Shigella.* Identify serologically. Complete biochemical tests. Agglutination negative: Probably: *Providence* or A-D group. Complete biochemical tests and A-D group serology.

by stabbing the butt and streaking the slant and the second by stabbing along the wall of the tube only.

Both are incubated at 37°C for 24 hours or less. Lactose and saccharose fermenters turn both the slant and the butt of TSI to yellow; *Salmonella* and *Shigella* turn the butt to yellow, and the former produces bubbles of gas but neither modifies the slant. No change indicates that the strain does not ferment any carbohydrate. Hydrogen sulfide when present produces a blackening of the medium.

When the culture attacks urea MIU medium turns red and such tubes can be discarded as the culture belongs either to the *Proteus* or to the *Klebsiella* group; this can sometimes be decided by observing the corresponding TSI tubes, as *Klebsiella* ferments both lactose and saccharose while *Proteus* may ferment saccharose but does not ferment lactose.

The use of MIU also makes it possible to recognize motility as the growth progresses through the medium, and indole production by adding Ehrlich or Kovacs reagent on the surface of the agar. The combination of these two media allows time to be saved in the isolation of *Shigella* and *Salmonella*, sometimes as much as 24 hours.

After 24 hours' incubation Enterobacteriaceae groups can be recognized as shown in Table 11.

Cultures showing the behaviour of *Shigella* or *Salmonella* have to be typed by serological methods. For this purpose the growth on the slant of TSI is collected with the wire loop and suspended in saline so as to give a heavy suspension. Irrespective of the result of the agglutination test the culture has to be examined for the most characteristic biochemical characters which will enable it to be identified.

IDENTIFICATION OF *SHIGELLA*

Cultures fermenting glucose without gas production but not fermenting mannitol probably belong to sub-group A, although some strains of sub-groups B and C may also be mannitol-negative. Such cultures are first tested by slide agglutination with polyvalent sub-group A *Shigella* serum and, if this is positive, again with each of the monotype A sera; this makes it possible to arrive at a final diagnosis. The serological typing is completed by the bio-

chemical tests, as mentioned in Table 12. In general only the Shiga type is completely identified in routine laboratories, as the remaining types of this sub-group are much less important from the point of view both of public health and of clinical medicine.

Any cultures showing the above-mentioned biochemical behaviour but not agglutinated by polyvalent A serum have to be tested again with polyvalent B and C sera as they may be mannitol-negative strains of these sub-groups.

Mannitol-positive cultures may belong to any of the remaining sub-groups and therefore have to be tested again with their polyvalent sera, save when they are indole-positive, when Sonne serum is not needed. If so desired, when the test is positive the same course can be followed as indicated above for sub-group A, but in general the identification is not carried further than determination of the sub-group to which the culture belongs.

When serologically testing *Shigella* cultures, it has to be remembered that a negative agglutination may be due to the presence of labile K antigens and, for this reason, cultures not agglutinated are tested again after being heated at 100°C for one hour.

Cultures showing the general characters of the group but not agglutinated by *Shigella* sera may belong to the *Providence* group, as anaerogenic variants. O variants are not uncommon. The phenylalanine, KCN and citrate tests are of great help in differentiating between the two groups (see Table 14, p. 275).

Anaerogenic lactose-negative or late positive O variants of *Escherichia*, and among these all the A-D strains, can also give rise to mistakes, and it is important to remember that there are even antigenic relations between the two groups. Christensen's citrate test (see Q, p. 256), positive with *Escherichia* and negative with *Shigella*, is sometimes the only biochemical test which will differentiate the two groups.

IDENTIFICATION OF *SALMONELLA*

At present the identification of *Salmonella* is fundamentally based on antigenic analysis and, as more than 500 serotypes are known which cannot be recognized by biochemical tests, it is not possible for the average laboratory to classify all *Salmonella* cultures with which it will have to deal.

Nevertheless, in several countries where *Salmonella* surveys have been done, both in America and in Europe, it has been found that the most prevalent type is *S. typhimurium*, and that more than 90% of all *Salmonella* infections are due to a few types only, about 10 in all, although these types are not

TABLE 11

RECOGNITION OF ENTEROBACTERIACEAE GROUPS WITH TSI AND MIU MEDIA

Group	TSI medium			MIU medium		
	Glucose	Lactose or sucrose	H_2S	Motility	Indole	Urea
Salmonella or *Arizona*	AG	—	+	+	—	—
Salmonella typhi	A	—	+	+	—	—
Citrobacter	AG	d	+	+	—	—
Shigella	A	—	—	—	d	—
Escherichia	AG	+	—	d	+	—
Klebsiella or *Cloaca*	AG	+	—	d	—	d
Serratia	A or AG	+	—	+	—	—
Proteus	AG	d	+	+	d	+
Retgerella	A	—	—	+	—	+
Morganella	AG	—	—	+	+	+
Providence	A or AG	—	—	+	+	—

AG = acid and gas; A = acid; + = positive in 1-2 days; — = negative for 30 days; d = different reactions (positive or negative).

TABLE 12
BIOCHEMICAL PROPERTIES OF *SHIGELLA* AND A-D CULTURES

Sub-group	Type	Glucose	Mannitol	Saccharose	Lactose	Rhamnose	Arabinose	Sorbitol	Indole
A. *Sh. dysenteriae*	1	+	−	×	−	−	−	−	−
	2	+	−	−	−	+	(+)	+	+
	3	+	−	−	−	−	+	+	−
	4	+	−	−	−	−	×	+	−
	5	+	−	−	−	+	+	+	−
	6	+	−	−	−	−	−	−	−
	7	+	−	−	−	+	+	−	+
	8	+	−	−	−	d	+	+	+
B. *Sh. flexneri*	1	+	+[a]	(+)	−	−	+	−	+
	2	+	+	−	−	−	+	−	d
	3	+	+	(+)	−	+	+	+	+
	4	+	+	×	−	−	d	d	+
	5	+	+	(+)	−	−	×	−	+
	6	+[b]	d	−	−	−	+	−	−
C. *Sh. boydi*	1	+	+[a]	−	−	−	+	+	−
	2	+	+	−	−	−	+	−	−
	3	+	+	−	−	−	+	+	−
	4	+	+	−	−	−	+	−	−
	5	+	+	−	−	−	+	+	+
	6	+	+	−	−	−	+	+	−
	7	+	+	−	−	−	+	+	+
	8	+	+	−	−	−	+	+	−
	9	+	+	−	−	(+)	d	+	+
	10	+	+	−	−	−	+	+	−
	11	+	+	−	−	+	+	×	+
D. *Sh. sonnei*	−	+	+	×	(+)	+	+	−	−
A-D group	O1 (Alk. 1)	+	+	−	−	(+)	+	+	+
	O2 (Alk. 2; *Sh. tieté*)	+	+	d	d	d	+	−	+
	O3 (Alk. 3; Disp. 2)	+	+	d	d	(+)	+	+	+
	O4 (Disp. 1)	+	+	(+)	(+)	+	+	+	+
	O5	+	+	d	d	+	+	+	+
	O6 (Disp. 3)	+	+	−	+	+	+	+	+
	O7	+	+	+	+	(+)	+	+	+
	O8	+	+	−	(+)	+	+	+	+

+ = positive in 1-2 days; − = negative for 30 days; (+) = delayed positive; × = negative or late and irregularly positive (mostly mutative); d = different reactions (positive or negative).

[a] Mannitol-negative variants of groups B and C are known.

[b] Some cultures of this type produce a small amount of gas.

the same for different geographical regions. This makes it possible for well-equipped hospitals or public health laboratories to do very useful work, as it is quite possible for them to have, and to prepare themselves, all necessary sera for identifying such a limited number of types.

In Table 2, altogether 28 types are included with their respective antigenic formulae. This has been done for two reasons. First, because, as stated above, the most prevalent types are not the same all over the world, and it therefore seems convenient to give information on some types which are not very frequent but have been repeatedly isolated in several countries. In the second place, we have considered of illustrative value the inclusion of some types whose identification will provide good examples of some of the difficulties found in this kind of serological work and of how they have to be solved.

Cultures behaving like *Salmonella* in the limited group of biochemical reactions are tested by the quick slide method, as described above, with polyvalent *Salmonella* serum. For this purpose a suspension is prepared by adding to the agar cultures 2-3 ml of saline and shaking well. If the reaction is

negative, it can be assumed that the culture is not a *Salmonella*.

Two Enterobacteriaceae groups, *Arizona* and *Citrobacter*, can easily be mistaken for *Salmonella*. The *Arizona* group is so closely related to *Salmonella* that not only do they share many antigens, O as well as H, but also their biochemical behaviour is very similar. The *Arizona* group slowly ferments lactose and liquefies gelatin, but there are also a few *Salmonella* types that do so too and these two tests are inconvenient in that they take several days. Dulcitol fermentation helps in this case, as *Arizona* does not generally ferment it while *Salmonella* rarely fails to do so. Organic acid tests are also of value as *Salmonella* are positive for *d*-tartrate, citrate and mucate while *Arizona* cultures are negative (Kauffmann & Petersen, 1956). The malonate test (see P, p. 256) is also very useful for differentiating these two groups, as it is positive with *Arizona* and negative with *Salmonella*. It is to be remembered, however, that some slow gelatin-liquefying *Salmonella* may be positive, a fact that suggests that they should probably be considered as belonging to the *Arizona* group. This case illustrates the difficulties found by the taxonomist in defining the Enterobacteriaceae groups and how artificial our classifications really are, since in nature there are no sharp divisions between them.

The *Citrobacter* group may also give rise to mistakes, especially lactose-negative cultures, to which belong the type called for a time *S. ballerup* and several others later described and now included in the same group. This can be differentiated from *Salmonella* because the first is negative for lysine decarboxylase and KCN-positive, while the second is the opposite. (For biochemical behaviour, see Table 14, p. 275.)

When the culture under test reacts positively in the presence of polyvalent *Salmonella* serum, the next step is to determine its somatic antigenic composition, and to this end it is tested with the specific O sera 2; 3,19; 4; 7; 8; 9; 10; and 15 (see Table 6). As seven of these eight sera are specific for some factor the interpretation of positive reactions obtained with them does not present any difficulties or make for mistakes. Cultures agglutinated by serum 3,19, however, may belong to the 3,19 somatic group or to the 3,10 or 3,15 types; to which type such cultures really belong may be ascertained by the use of specific 10 and 15 sera. Thus a culture of *S. senftenberg* (3,19) will be agglutinated only by 3,19 serum and not by sera 10 and 15, while *S. anatum* (3,10) and *S. newington* (3,15) will give a positive reaction in presence of 3,19 serum and 10 or 15 sera.

A pure 19 serum is recommended by Kauffmann (1954) and has been used by several workers (see footnote to Table 6) without difficulties. Although occasionally we have been able to obtain a good 19 factor, such sera are generally rather low-titred after absorption and for this reason we prefer to use a 3,19 serum for routine work.

Once the somatic antigens of the culture are known, it is tested with H sera corresponding to the serotypes included in the respective O group (see Table 2).

If agglutination with all these sera is negative—as when the culture is not agglutinated with polyvalent O serum—but the test is positive with polyvalent *Salmonella* serum, it may be deduced that it belongs either to the *Arizona* group or to some other *Salmonella* type not included in Table 2, and the culture should be sent for final typing to some laboratory specializing in this kind of work.

It should be remembered that H agglutination may fail because the strain is an O form and even when motile flagellar antigens may be insufficiently developed. For this reason only actively motile cultures as recognized by direct observation under the microscope and which quickly progress to the bottom of the tube in semi-solid media must be used.

If tests with H sera are positive, several possibilities have to be considered. Monophasic types which possess a single antigen, such as *S. paratyphi* A, are easily recognized as they are agglutinated only by their respective specific sera, but when they have two or more antigens several sera are sometimes needed. For instance, *S. montevideo* (g,m,s) is agglutinated by g,p and m,t sera and to decide to which of these factors agglutination is due, pure specific m, p and s sera are used. The test will be positive with the first and the last but not with p, proving that agglutination with serum g,p is due to factor g. When dealing with diphasic types it is necessary to analyse both phases. In most cases it is easy to obtain these by plating the culture and serologically examining 10-12 colonies by the slide test. Each colony is suspended in a drop of saline on the glass plate or slide and the test carried out as indicated above. If the desired phase is not found, the only way to obtain it is to induce it by growing the culture in semi-solid medium containing the homologous H serum (see p. 260), but this involves a risk that some artificial phase may be produced.

TABLE 13
CHARACTERISTIC BIOCHEMICAL BEHAVIOUR OF SOME *SALMONELLA* TYPES

	Arabinose	Dulcitol	Inositol	Rhamnose	Trehalose	Xylose	Stern's glycerol	H_2S	Gelatin	Tartrate			Sodium citrate	Mucate
										d	*l*	*dl*		
S. paratyphi A	+	(+)	—	+	+	—	—	d	—	—	—	—	—	—
S. paratyphi B[a]	+	d	d	d	+	+	d	+	—	—	+	d	d	+
S. paratyphi C	d	+	—	(+)	(+)	+	—	+	—	+	+	—	+	—
S. cholerae suis	—	×	—	+	—	+	—	d	—	+	—	×	—	×
S. kunzendor	—	×	—	+	—	+	—	+	—	d	×	×	+	×
S. sendai	+	×	—	(+)	+	(+)	—	—	—	—	—	—	—	—
S. miami	—	—	—	+	+	+	++	+	—	×	—	—	+	—
S. typh	d	×	—	—	+	d	—	d	—	+	—	—	d	×
S. enteritidis	+	+	—	+	+	+	++	+	—	+	+	+	+	+
S. enteritidis var. *danysz*	d	+	—	+	+	+	—	+	—	(+)	d	(+)	d	+
S. enteritidis var. *chaco*	+	(+)	—	+	+	+	+	+	—	+	+	+	+	+
S. enteritidis var. *essen*	+	(+)	—	+	+	+	++	+	—	+	—	—	+	+

+ = positive in 1-2 days; — = negative for 30 days; (+) = delayed positive; × = negative or late and irregularly positive (mostly mutative); d = different reactions (positive or negative).

[a]The characters of *S. paratyphi* B in the table correspond to the so-called " human " type frequently producing typhoid-like infections in man. The " animal " type, positive in *d*-tartrate, mostly produces in man infections localized to the intestinal tract (Kauffmann, 1954).

The exact analysis of phase 2 is of decisive importance when two diphasic types belonging to the same O group also have the same H antigen in phase 1 but differ in the composition of phase 2. *S. london* (3,10:1,v:1,6) and *S. give* (3,10:1,v:1,7)—two types frequently mentioned in literature on the subject —are good examples of this. They can be easily differentiated by the use of pure sera 6 and 7. For the same reason it is necessary to use special sera to type some *Salmonella* included in Table 2. *S. reading* (4,12:e,h:1,5) can be mistaken for *S. kaapstad* (4,12:e,h:1,7) and *S. saint-paul* (1,4,5,12:e,h:1,2), two types not included in the table.

A similar case where H factors are involved may be mentioned in reference to some types having factor 1 in one of their phases combined with some other, such as v, w, z_{13} and z_{28}. When any of these last types are found, some confusion may arise because these types will be agglutinated by 1,v serum and could be wrongly classified. As an example, *S. javiana* (9,12:1,z_{28}:1,5) might be mistaken for *S. panama* (9,12:1,v:1,5), as phase 1 of both will be agglutinated by 1,v serum, and their phase 2 is identical. It is clear, though, that if the culture belongs to the *S. javiana* type it will not be agglutinated by v serum.

It should be remembered that mixed infections with two or even more *Salmonella* types are not uncommon, which makes it necessary to examine several colonies from each plate. After one of them has been serologically typed, the rest are tested with the same sera that gave a positive agglutination with the first and then only if some of them fail to be agglutinated must the whole process of typing as described be repeated again.

In most cases there is no need, in routine work, to complete the biochemical characters of the culture, as serological typing allows of a more precise classification; but there are cases in which some complementary test will be useful to confirm or decide diagnosis. Table 13 lists some of them.

In the work of typing, *S. typhi* constitutes a particular case. As this type does not produce gas from any carbohydrate, only *S. gallinarum* and anaerogenic variants of some other type, which are not frequent, can be mistaken for it, so that there

TABLE 14

BIOCHEMICAL BEHAVIOUR OF ENTEROBACTERIACEAE GROUPS

	Salmonella	*Arizona*	*Citrobacter*	*Shigella*	A-D group	*Escherichia*	*Klebsiella*	*Cloaca*	*Hafnia* (at 22°C)	*Serratia*	*Proteus*	*Retgerella*	*Morganella*	*Providence*
Glucose (gas)	+	+	+	—	—	+	+	+	+	+	+	—	+	d
Lactose	—	+ or ×	d	×	×	+	+	+	—	×	—	—	—	—
Saccharose	—	—	d	d	d	d	+	+	×	+	d	—	—	d
Mannitol	+	+	+	d	+	+	+	+	+	+	—	+	—	d
Dulcitol	+	—	d	d	+	d	d	d	—	—	—	—	—	—
Salicin	—	—	d	—	d	d	+	+	d	+	d	d	—	—
Adonitol	—	—	—	—	—	—	d	d	—	d	—	+	—	d
Inositol	d	—	×	—	—	—	+	d	—	d	—	+	—	d
Cellobiose	×	×	d	—	×	—	+	+	×	—	×	—	—	—
Rhamnose	+	+	+	d	+	+	+	+	+	—	—	d	—	—
Raffinose	—	—	—	×	d	d	+	+	—	d	—	—	—	—
Arabinose	d	+	+	d	+	+	+	+	+	d	—	—	—	—
Xylose	+	+	+	×	+	+	+	+	+	d	+	—	—	—
Sorbitol	+	+	+	d	+	+	+	+	—	d	—	—	—	—
Starch (gas 4 days)	—	—	—	—	—	—	+	—	—	—	—	—	—	—
Indole	—	—	—	d	+	+	d	—	—	—	d	+	+	+
Methyl red	+	+	+	+	+	+	d	—	—	—	+	d	+	+
Voges-Proskauer	—	—	—	—	—	—	+	+	+	+	d	—	—	—
Citrate	+	+	+	—	—	—	+	+	+	+	d	+	—	+
H_2S	+	+	+	—	—	—	—	—	(+)	—	+	—	(+)	—
Urease [a]	—	—	—	—	—	—	+	—	—	—	+	+	+	—
Gelatin	—	(+)	—	—	—	—	d	d	—	+	+	—	—	—
Growth in KCN	—	—	+	—	—	—	+	+	+	+	+	+	+	+
Malonate	—	+	d	—	—	—	+	d	+	—	—	—	—	—
Phenylalanine	—	—	—	—	—	—	—	—	—	—	+	+	+	+
Arginine dihydrolase	+	+	d	d	d	d	—	d	×	—	—	—	—	—
Glutamic acid decarboxylase	—	—	—	+	+	+	—	—	—	—	×	d	+	d
Lysine decarboxylase	+	+	—	×	+	+	+	d	+	d	—	—	(+)	—
Ornithine decarboxylase	+	+	d	d	d	d	—	+	+	d	d	—	+	—
Motility	+	+	+	—	—	d	—	+	+	+	+	+	+	+

+ = positive in 1-2 days; — = negative for 30 days; (+) = delayed positive; × = negative or late and irregularly positive (mostly mutative); d = different reactions (positive or negative).

[a] Urease test performed as mentioned in the text (see K, p. 254).

is no need to follow the progressive method for typing described above. Cultures showing its biochemical behaviour are tested in the presence of somatic 9 and Vi and flagellar d sera. As newly-isolated strains are frequently agglutinated only by Vi serum, to confirm diagnosis it is convenient to heat the culture at 100°C for a few minutes, a treatment which, by destroying the Vi antigen, causes the 9 agglutination to become positive. This is a good precaution, for the Vi antigen can be found not only in *S. typhi* but also in some other *Salmonella* types, such as *S. paratyphi* C, and even in the groups *Escherichia* and *Citrobacter*.

Many *Salmonella* antigens, both somatic and flagellar, have been recognized in bacteria of other Enterobacteriaceae groups, especially, but not only, among *Escherichia*. As some cultures of this group may show aberrant characters which can make them resemble *Salmonella* in a superficial examination, to avoid mistakes all strains that cannot be completely typed should be tested for all their biochemical characters.

In Table 14 we have summed up the most frequent behaviour of the Enterobacteriaceae groups, but the reader has to remember that in all this family aberrant strains are very frequently found which deviate from the type by one or sometimes several characters. In this connexion the fact has to be stressed that the Enterobacteriaceae constitute in reality a long line of interrelated bacteria which cannot be sub-divided into precisely defined genera and also that variation is extremely frequent, producing mutants sometimes very different from the parent strain. It is a familiar fact for the experienced bacteriologist that when most cultures are plated and a sufficiently high number of colonies are investigated variants are obtained which may show deviations from type in relation to almost any characteristic, although some of them—glucose fermentation, for instance—are particularly stable.

Annex

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