BILE SALT MEDIA AND THEIR ADVANTAGES IN SOME BACTERIOLOGICAL EXAMINATIONS.

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THE addition of bile salts to media was first employed by Ph. Limbourg (1889), who added the sodium salt of cholalic acid to a mixture of peptone and pancreas extract. He inoculated this medium with dog-faeces and investigated certain of the chemical changes which took place. Leubuscher (1890) used pure bile as a medium for the growth of *B. anthracis*, *B. typhosus*, and other bacteria and found it a good culture medium. Corrado (1891) grew certain organisms in pure bile and came to the conclusion

I. that it behaves
   (*a*) indifferently towards *B. typhosus* and *B. pneumoniae*,
   (*b*) as a stimulant to the growth of *B. mallei*,
   (*c*) bactericidally towards *B. anthracis*, which dies after 48 hours in bile.

II. that only in the case of the anthrax bacillus was a weakening of virulence noted. After 18 hours sojourn in bile this bacillus became non-pathogenic to animals.

Fraenkel and Krause (1899) concluded that *B. typhosus* suffered at any rate no loss of virulence during a 24 hours exposure to the influence of bile. They cite Mosse and also Falck as having used bile media. Matzuschita (1902) used a decoction of liver in preparing an agar medium and found it the best solid medium for faeces bacteria. Conradi (1906) strongly advocated the use of pure bile as a help towards the isolation of *B. typhosus* from the blood in cases of typhoid fever. Meyerstein (1907) added the salts of bile to a nutrient medium and grew various organisms in it. His results led him to the conclusion that:

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(1) *B. pyocyaneus* would grow well in a simple solution of bile salt,

(2) the same solution, with a small amount of nutrient substance added to it, formed a good medium for the growth of *B. coli*, and

(3) even the addition of large amounts of nutrient substances did not prevent it having an inhibitory action on the growth of *Staphylococcus pyogenes aureus*.

My first experiments were made in 1897 with a potato juice medium containing commercial bile salts; and these led to the suggestion in 1900 of bile salt lactose agar as a medium for *B. typhosus* and *B. coli communis*. The details of further experiments in which pure bile salts and bile acids were used were published in 1901 and in the same year Hill and I proposed bile salt glucose broth as a simple test for faecal contamination. A lactose broth had been previously used in our work for the Royal Commission on Sewage Disposal and glucose was substituted for the lactose merely so as to include such organisms as *B. enteritidis* (Gaertner). Of course, as stated by me (1904) in *Public Health*, if it is only a question of the presence of *B. coli communis* and its allies lactose broth is better than glucose broth.

The composition of the agar was as follows:—

Bile Salt	0·5%
Peptone	2·0%
Lactose	1·0%
Agar	1·5%
Tap Water	100 c.c.

and of the broth:

Bile Salt	0·5%
Peptone	2·0%
Glucose	0·5%
Litmus solution	q.s.
Tap Water	100 c.c.

On the agar, those organisms which decomposed the lactose produced acid in their immediate neighbourhood, and thus precipitated the bile salts with the consequent formation of a haze in the medium. This haze round the colonies differentiated them from those of non-lactose fermenters round which the medium remained quite clear.

Soon however it was observed that not every lactose-fermenting organism produced a haze when growing *on the surface* of the medium.

The reason for this will be apparent when Theobald Smith's work is referred to later on. At this time Grünbaum and Hume (1902) published their paper in which they proposed a modification of bile salt lactose agar. Their modification consists in the addition of 4 c.c. of $\frac{n}{1}$ NaHO to every litre of the agar and in the use of neutral-red as an indicator of the presence of acidity. They also say "the addition of Crystal Violet (1 in 100,000) permits of a striking double stain of the colonies." I had before this used neutral-red in bile salt broth for the "fluorescence test" but had not thought of it as an indicator. It seemed that the addition of this dye would do away with the difficulty occasioned by the absence of a haze round the surface colonies of some lactose fermenting organisms. It certainly acts very well as many colonies are pink and without a haze, but one still comes across surface colonies which, though colourless on neutral-red bile salt lactose agar, turn out to be colonies of lactose fermenting bacteria. The use of Crystal Violet is quite unnecessary as bile salt media alone inhibit most of the organisms found in air and soil (cf. MacConkey, 1900, p. 56) when the incubation temperature is 37° C. and over. The addition of 4 c.c. of $\frac{n}{1}$ NaOH per litre is to my mind a great disadvantage. The medium should be as neutral as possible to neutral-red, as otherwise organisms which attack the sugars, etc., only slowly and feebly may not be able to manufacture acid quickly enough and in sufficient quantity to overcome the alkalinity. Theobald Smith's observations are pertinent to this question. He says (1890) that in the absence of sugar both *B. coli* and the Hog Cholera bacillus are alkali producers. If a small quantity of sugar is present then, if the bacillus can act upon it, there is a rapid acid production and a slow alkali production. If the amount of acid is not too great it is neutralised by the alkali and the medium becomes alkaline. If the amount of acid is too great to be neutralised it tends to inhibit the growth of the bacillus. A similar result is obtained in the case of *B. typhosus*. Again in 1895 he states that more than 0·5% of glucose is harmful (cf. MacConkey, 1900, p. 48). The amount of acid produced is the same aerobically and anaerobically. There is a coincident production of alkali which is bound up with the growth of the organism, and which in the case of facultative anaerobes only goes on actively in the presence of oxygen. The acidity depends on the decomposition of sugar, etc.; the alkalinity, in the presence of oxygen, upon the growth

of the organism. He states further that if too little sugar be present the alkali production masks the acid production unless oxygen be removed.

My own observations entirely confirm these statements. An inoculated tube very rarely remains neutral; it almost always becomes either acid or alkaline. It may become acid and remain so; the initial acidity may be followed by alkalinity; or the appearance of alkalinity may be the first perceptible change. I have seen a red surface colony of *B. coli communis* on a litmus lactose agar plate gradually lose its redness and in the course of time become distinctly blue.

It is obvious therefore that the medium should be as neutral as possible to the indicator used[1]. I have used both litmus and neutral-red and have come to the conclusion that the latter is to be preferred. It gives a lighter coloured medium and much better differentiation. Grünbaum and Hume's suggestion has been adopted by me, but not in its entirety. The added alkali has been omitted and the neutral-red accepted to the extent of using half the quantity mentioned in their formula.

The media are now prepared as follows. First a stock solution is made consisting of:—

Sod. Taurochol. (commercial from ox bile)	0·5%
Peptone (Witte)	2·0%
Water (distilled or tap)	100·0 c.c.

It is absolutely necessary to use bile salts which are neutral to neutral-red. Some samples are acid to this indicator and their use causes the finished medium to have a tint which makes the recognition of slight acidity somewhat troublesome. If litmus is preferred as an indicator it is not necessary to be so particular about the reaction of the bile salts. All the samples of bile salts I have tested have been alkaline to litmus. In this respect litmus has the advantage over neutral-red. Bile salts obtained from pig's bile are as far as my experience goes much more troublesome to use than those from ox bile. Calcium in the proportion of 0·03% of $CaCl_2$ seems beneficial to the growth of some of these organisms (cf. MacConkey, 1905, p. 336), and therefore this salt should be added when distilled water is used to make up the medium. Whenever possible I use tap water on this account.

[1] The use of test tubes made of glass which yields an appreciable amount of alkali to the contained liquid may cause the medium to become too alkaline.

When a solution of bile salts is mixed with a solution of commercial peptone a precipitate is formed (cf. Maly and Emich, 1883). There may be some difficulty in getting rid of this. The best way to obtain a clear solution is to mix the ingredients together, steam them for 1—2 hours in a Koch's steamer, filter while hot and then allow to stand (24—48 hours if possible) until quite cold and sedimentation has occurred. Filtration through paper will then remove the precipitate and yield a clear liquid, which appears to keep indefinitely under proper conditions.

The various bile salt broths are prepared from this stock solution by adding glucose 0·5%, lactose 1%, cane sugar 1%, dulcit 0·5%, adonit 0·5% or inulin 1%, and neutral-red (1% solution) 0·25%, distributing into Durham's fermentation tubes and sterilising in the steamer for 15 minutes on each of three successive days. I have never experienced any disappointment from using a bile salt medium which had been steamed only twice, but it is best to be on the safe side and steam three times. Great care must be taken not to heat above 100° C. or for too long a time after the addition of the sugars, as otherwise the medium may be spoiled. The amount of dulcit and of adonit has been decreased on account of the great increase in the price of these substances.

Bile salt agar is made by dissolving 2% of agar in the stock fluid either in the steamer or in the autoclave. The mixture is cleared with the white or the whole of an egg, filtered, neutral-red added in the same proportion as for broth, and distributed into flasks in quantities of 80 c.c., which is enough to make three plates of the usual size. When required for use the fermentable substance is added to the agar in the flask and the whole placed in a water bath or steamer. When melted the agar is poured into Petri dishes, allowed to solidify, and then dried in the incubator or warm room, the plate being placed upside down with the bottom detached and propped up on the edge of the cover. It is necessary that the surface of the agar should not be wet, as in that case the colonies will most likely form a confluent mass; nor should it be too dry as then the colonies are stunted in their growth. Inoculations are made by placing a loopful of a liquid culture or emulsion of the material to be examined on the centre of the agar in one plate and rubbing it over the surface with a bent glass rod; the same rod, without recharging, being used to inoculate the surface of the other two plates (Drigalski and Conradi's method). The plates are incubated upside down.

As regards the temperature of incubation 42° C. has of course a more selective action than one of 37° C., but the latter is quite good enough for ordinary purposes and is the temperature at which I have done all my work during the last four years. Therefore a special incubator is not necessary (cf. W. H. C. Forster, 1905).

Nor are anaerobic conditions of incubation absolutely essential. I have used them in the way of experiment to see if thereby any great advantage was to be gained, but I could not satisfy myself that the gain was sufficiently great to warrant the introduction of this method into routine work. From the observations of Theobald Smith, to which I have referred, it is obvious that the absence of oxygen may be of use in the case of organisms with fermentative powers of a weak and slowly acting character. One would therefore like to make use of anaerobic conditions, but the various forms of apparatus designed up to the present are so cumbersome that the trouble involved in their use outweighs the gain due to anaerobiosis. Besides, if an organism which ferments, say lactose, does give a colourless colony on bile salt lactose agar it is easy to subculture into a lactose broth tube in which, if freshly steamed, we have sufficiently anaerobic conditions to clear up any doubtful point.

Of course if one desires to compare with regard to the production of acidity the growth of an organism in a broth tube with the growth of the same organism on the surface of an agar plate anaerobic conditions are essential, as in the presence of oxygen one might get evidence of acid formation in the broth tube and none on the plate.

It will have been noted that the sugars, etc., are not added to the agar until just before use. The object of this procedure is to allow that kind of agar to be used which is most suitable to the case in question. By using a lactose agar the lactose fermenters, may be separated from the non-lactose fermenters. A mannit agar will pick out the mannit fermenters; a dulcit agar the dulcit fermenters, and so on. By combining two or more sugars, etc., in one agar we can exclude the organisms which attack these substances and leave only the colourless colonies for investigation. The use of a combination of several fermentable substances in one agar, suggested by me in 1905 (p. 335), has been adopted by Dr Houston (1907, p. 45).

With the idea of stimulating the growth of *B. typhosus* I have tried the effect of adding various substances to bile salt agar but without success. Urea, asparagin, nutrose, somatose, roborat, plasmon, and Nährstoff Heyden were without effect.

Soon after Loeffler published his method I investigated the effect of the addition of malachite green (Höchst 120) in varying proportions to bile salt agar and came to the conclusion that this medium could not be relied upon to give satisfactory results.

Encouraging results have been obtained by combining caffein (Roth's method) with bile salt agar when the amount of caffein was 0·33%. But up to the present this combination has not proved satisfactory enough to induce me to recommend it for general use.

The method which has given me the best result in the isolation of lactose fermenters is as follows:

The material to be examined is inoculated, either diluted or undiluted, into bile salt lactose broth. In the case of a water, the amounts taken are 250 c.c., 100 c.c., 10 c.c., 1 c.c., 0·1 c.c. and 0·01 c.c.

Concentrated bile salt broth is added to the 250 c.c., and 100 c.c.; ordinary broth is added to the 10 c.c.; and the other quantities are added to the bile salt broth in tubes.

After 18—24 hours' incubation at 37° C. the highest dilution which shows any change is picked out and surface cultures are made from it on bile salt lactose agar. The unchanged tubes etc., together with the plates, are put in the incubator and examined next day. If any change in the direction of acidity has taken place in any of the tubes, plates are made from them.

If on examining the plates all the colonies appear alike, then only three colonies are subcultured; but if more than one kind of colony is present then two colonies of each variety are subcultured. I have repeatedly tried to distinguish one organism from another by the appearance of the colonies on bile salt neutral-red lactose agar but invariably without success. On the same plate an organism may give irregular filmy "coli-like" red colonies and also round raised "aerogenes-like" red colonies. The form of a colony on bile salt agar *is of no value in identifying a lactose fermenting organism.*

If bile salt agar plates inoculated with a mixture of lactose fermenters are left upside down at room temperature a peculiar change occurs in some of the colonies. They take on a mucoid character and increase so much in depth that portions of the growth may drop down on to the cover of the plate.

The best medium to use for making cultures from the colonies is slant agar; and the growth is rubbed over the whole of the surface of the medium. After 18—24 hours' incubation at 37° C. subcultures are made from the agar into the various media; a good loopful of culture

being put into each tube. The sloped agar is used in order to get a sufficiently large quantity of growth and a large loop is used for the inoculations, because the mass of growth used for inoculation seems to have an effect in determining the rapidity of fermentation. By this means results are often obtained in 48 hours which otherwise might not be obtained under a week.

The media used are:—

1. *Glucose broth*—for Voges and Proskauer's reaction[1].
2. *Peptone water*—for indole.

This test should be performed after seven days' incubation; four days is not always enough even when using the Benzaldehyde reaction. If preferred, the peptone water may be omitted and the test performed upon an alcoholic extract of the agar growth (cf. Böhme, 1905).

3. *Litmus milk*—up to the present I have always made use of litmus milk. The experience thus gained has forced me to the conclusion that the litmus milk test is not necessary in the case of lactose fermenters.

4. *Gelatin*—I do not think much weight can be attached to the appearance of growths on gelatin of those organisms which grow on bile salt media. I use gelatin simply for the purpose of observing the presence or absence of liquefaction. Liquefaction may take place rapidly or slowly. Some organisms take 6—9 months to liquefy half an inch of gelatin (e.g. *B. oxytocus perniciosus*). By appropriate means the time may be shortened somewhat (MacConkey, 1906), but even then liquefaction may require a month to be complete. Retardation of solidification after being melted may be noticeable 2—3 weeks before the completion of liquefaction. Now in routine work it is quite impossible to wait longer than about two weeks. So in practice we are at present forced to class with the non-liquefiers any organism which does not liquefy gelatin in a fortnight.

5. *Bile salt broths*—containing respectively lactose, cane-sugar, dulcit, adonit and inulin[2].

After inoculation, the tubes containing these media are kept under observation as long as there is no change in the reaction of the medium[3]. As long as the medium remains neutral there is a possibility of the

[1] Recently I have isolated organisms which sometimes gave this reaction and sometimes did not do so. It may therefore be necessary to modify one's opinion with regard to this test.

[2] Inosit also has a value for differentiation purposes.

[3] A week or 10 days is long enough in practice.

production of acid, but when once the reaction of alkalinity is visible in the inner tube the tubes may be discarded as acid production will not occur subsequently.

Gas may not be apparent for two or three days after the medium has turned acid.

Examinations are also made as to the presence or absence of motility and as to whether the organisms are Gram-positive or Gram-negative.

If the liquid media are inoculated in the morning, say between 10 and 11 A.M., they may be examined for motility the same afternoon between 4 and 5 P.M. It is not necessary to have visible growth for this purpose. A tube in which no growth is apparent to the naked eye may be fairly full of bacilli. To my mind the best idea of motility is gained by using a low power objective (half an inch), an eye-piece magnifying 8—10 times and dark ground illumination. This arrangement gives excellent results also in the case of agglutination reactions.

In the early stages of working with bile salt agar the results were somewhat conflicting and I gained the impression that this medium exercised a certain amount of inhibitory action on the growth of *B. coli communis* and *B. typhosus*.

As experience increased however it became apparent that, while some inhibitory effect might be exerted on organisms which had been grown on artificial media for a long time, freshly isolated organisms grew well.

As regards bile salt broth: Forster (1905) tested it against Drigalski and Conradi's nutrose agar. He used dilutions of a very thin emulsion of horse dung in sterile water for the inoculating material, with the following result:—

Nutrose agar aerobically at 37° C. for 24 hours.		MacConkey's medium anaerobically at 42° C. for 24 hours.	
Tube 1 = 0·1 c.c.	(growth)	Tube 1 = 0·1 c.c.	(growth)
2 = 0·01 c.c.	,,	2 = 0·01 c.c.	,,
3 = 0·001 c.c.	,,	3 = 0·001 c.c.	,,
4 = 0·0001 c.c.	,,	4 = 0·0001 c.c.	,,
5 = 0·00001 c.c.	,,	5 = 0·00001 c.c.	,,
6 = 0·000001 c.c.	(sterile)	6 = 0·000001 c.c.	(sterile)
7 = 0·0000001 c.c.	,,	7 = 0·0000001 c.c.	,,
8 = 0·00000001 c.c.	,,	8 = 0·00000001 c.c.	,,

By subculture *B. coli* was isolated from tube 5 of both series.

Thresh and Sowden (1904) compared phenol broth with bile salt broth in the examination of waters. They found that they obtained a

positive reaction in bile salt broth from a quantity of water which yielded no growth in phenol broth.

The following are experiments made by myself.

(I) A 1 in 100 emulsion of horse and calf faeces was put into a sterile Berkefeld filter (F 3) which was suspended in a jar of tap water. The water in the jar was changed every day. At the end of 12 weeks the contents of the filter were thoroughly mixed and a small quantity removed for examination. Dilutions were made up to 1 in 1000 millions. One cubic centimetre of each dilution from 1/1000 upwards was put into bile salt lactose broth and the same quantity also into ordinary alkaline nutrient bouillon. The tubes were incubated at 37° C. with the following result:—

Bile salt lactose bouillon 24 hours at 37° C.		Alkaline nutrient bouillon 24 hours at 37° C.	
Tube 1 = 1/1,000	A + G	Tube 1 = 1/1,000	Growth
2 = 1/10,000	A + G	2 = 1/10,000	,,
3 = 1/100,000	A + G	3 = 1/100,000	,,
4 = 1/1,000,000	No growth	4 = 1/1,000,000	No growth
5 = 1/10,000,000	,,	5 = 1/10,000,000	,,
6 = 1/100,000,000	,,	6 = 1/100,000,000	,,
7 = 1/1,000,000,000	,,	7 = 1/100,000,000	,,
48 hours at 37° C.		48 hours at 37° C.	
Tube 4	Growth, no acid	Tube 4	Growth
5	No growth	5	No growth
6	,,	6	,,
7	,,	7	,,

A = acid, G = gas.

Further investigation showed that there were lactose fermenters present in both kinds of broth in all dilutions up to 1/100,000 but not beyond; and that organisms were present in both media in the 1/1,000,000 dilution but there was no growth in any higher dilution.

In this case bile salt lactose broth proved just as good a medium as ordinary alkaline nutrient bouillon.

(II) On another occasion a similar experiment was performed with two other filters (F 4 and F 5) each containing a 1 in 100 emulsion of human faeces. After two months in tap water the contents were examined in exactly the same manner as in the case of F 3. The results were:—

F 4.

Bile salt lactose broth 24 hours at 37° C.		Alkaline nutrient bouillon 24 hours at 37° C.	
Tube 1 = 1/1,000	A + G	Tube 1 = 1/1,000	Growth
2 = 1/10,000	A + G	2 = 1/10,000	,,
3 = 1/100,000	A	3 = 1/100,000	,,
4 = 1/1,000,000	A	4 = 1/1,000,000	,,
5 = 1/10,000,000	Nil	5 = 1/10,000,000	Nil
6 = 1/100,000,000	,,	6 = 1/100,000,000	,,
7 = 1/1,000,000,000	,,	7 = 1/1,000,000,000	,,
48 hours at 37° C.		48 hours at 37° C.	
Tube 3 = 1/100,000	A + G	Tube 3 = 1/100,000	Growth
4 = 1/1,000,000	A	4 = 1/1,000,000	,,
5 = 1/10,000,000	? growth	5 = 1/10,000,000	,,
6 = 1/100,000,000	,,	6 – 1/100,000,000	? Growth
7 = 1/1,000,000,000	,,	7 = 1/1,000,000,000	,,

A = acid, G = gas.

Further investigation proved that there was growth in all tubes up to 1/10,000,000 but not beyond; and that lactose fermenters were present only up to 1/1,000,000.

F 5.

Bile salt lactose broth 24 hours at 37° C.		Alkaline bouillon 24 hours at 37° C.	
Tube 1 = 1/1,000	A + G	Tube 1 = 1/1,000	Growth
2 = 1/10,000	A + G	2 = 1/10,000	,,
3 = 1/100,000	A + G	3 = 1/100,000	,,
4 = 1/1,000,000	A + G	4 = 1/1,000,000	,,
5 = 1/10,000,000	A	5 = 1/10,000,000	,,
6 = 1/100,000,000	A	6 = 1/100,000,000	Nil
7 = 1/1,000,000,000	Nil	7 = 1/1,000,000,000	,,
48 hours at 37° C.		48 hours at 37° C.	
Tube 5 = 1/10,000,000	A + fluorescence	Tube 5 = 1/10,000,000	Growth
6 = 1/100,000,000	A + G	6 = 1/100,000,000	,,
7 = 1/1,000,000,000	?	7 = 1/1,000,000,000	?

A = acid, G = gas.

Subsequent inoculations showed growth in bile salt lactose broth up to 1/1,000,000,000 and in alkaline bouillon up to 1/100,000,000; but lactose fermenters were present only up to 1/100,000,000 in bile salt broth and up to 1/10,000,000 in alkaline bouillon.

The results of the examination of the contents of filters 4 and 5 after a sojourn of two months in tap water confirm previous experience and prove that bile salt broth is quite as good as any other medium for water analysis.

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Bile salt in a proportion of 0·5 % has no inhibiting effect upon *B. typhosus, B. coli communis* and allied organisms. As a matter of fact these bacilli will multiply in a broth containing a much larger percentage of bile salt. I have kept *B. typhosus*, etc. in 5 % bile salt broth at room temperature for six weeks, and at the end of that time found them alive and capable of giving an abundant growth when re-inoculated on to agar. Of course there were no fermentable substances in the bile salt broth of this particular strength as the production of acid would have vitiated the experiment.

I have also tried the effect of increasing the quantity of bile salt in bile salt agar. The percentages used were 4, 5, 6, 7, 8 and 9 %, and some 30 known organisms were tested. The results may be tabulated thus:—

B. prodigiosus *B. mesentericus fuscus* *B. mesentericus vulgatus*	did not grow.
B. cavicida (Brieger)	Growth in 4 % in 48 hrs. „ „ 6 % „ 4 days.
B. oxytocus perniciosus	„ „ „ „ „
B. lactis aerogenes *B. capsulatus* (Pfeiffer) *B. cloacae*	Poor growth on 9 % in 48 hrs.
B. typhosus *B. coli communis* (Escherich) *B. acidi lactici* (Hüppe) *B. neapolitanus* *B. pneumoniae* (Friedländer) *B. levans* *B. enteritidis* (Gaertner) *B. paratyphosus* etc., etc.	Appeared to be unaffected except that some members of the Gaertner group (e.g. Hog Cholera, Arkansas—Smith) grew slowly during the first 24 hours.

The following organisms will not grow on ordinary bile salt media.

B. anthracis,
B. anthracoides,
B. diphtheriae,
B. xerosis,
B. subtilis,
M. tetragenus,

B. faecalis alkaligenes grows well on bile salt media that have been made strongly alkaline; but not on the ordinary media.

Streptococci vary very much, some grow well but slowly, others scarcely at all.

Summary.

Bile salt media have been in use since 1900, and during the seven years which have elapsed it has been shown that *B. typhosus, B. enteritidis* (Gaertner), *B. coli communis* and similar organisms grow on these media just as well as on any other nutrient media. At 37° C. the growth of most of the organisms of the air and soil is inhibited by bile salt media.

These media can therefore be used with confidence for the isolation of *B. typhosus* and other intestinal organisms.

REFERENCES.

Böhme (1905). *Centralbl. f. Bakt.* Abt. I. Orig. XL. p. 129.
Conradi (1906). *Deutsche med. Wochenschr.* XXXII. p. 58.
Corrado (1891). *Atti d. R. acad. di Roma*, Anno XVI. Ser. II. Vol. I. [Ref. *Centralbl. f. Bakt.* XI. p. 696.]
Falck. *Arch. f. pathol. Anat. u. Physiol.* XCIII. p. 2. [Cited by Fraenkel and Krause.]
Fraenkel and Krause (1899). *Zeitschr. f. Hyg.* XXXII. p. 97.
Forster (1905). *Lancet*, Vol. II. p. 757.
Grünbaum and Hume (1902). *Brit. Med. Journ.* Vol. I. p. 1473.
Houston (1907). *Report on the condition of the Metropolitan Water Supply during the month of June* 1907.
Leubuscher (1890). *Zeitschr. f. klin. Med.* XVII.
Limbourg (1889). *Zeitschr. f. physiol. Chemie*, XIII. p. 196.
MacConkey (1900). *Thompson-Yates Laboratories Report*, III. pp. 41—57.
—— (1901). *Ibid.* III. pp. 151—154.
—— (1904). *Public Health*, May.
—— (1905). *Journ. of Hygiene*, V. pp. 333—379.
—— (1906). *Ibid.* VI. pp. 23—32.
—— and Hill (1901). *Thompson-Yates Laboratories Report*, IV. pp. 151—165.
Maly and Emich (1883). Ref. *Jahresber. f. Thierchem.* XIII. p. 289.
Matzuschita (1902). *Centralbl. f. Bakt.* Abt. I. Ref. XXXI. p. 505.
Meyerstein (1907). *Ibid.* Abt. I. Orig. XLIV. pp. 434—439.
Mosse. *Zeitschr. f. klin. Med.* XXXVI. p. 527. [Cited by Fraenkel and Krause.]
Smith, Theobald (1890). *Centralbl. f. Bakt.* Abt. I. VIII. p. 389.
—— (1895). *Ibid.* XVIII. p. 1.
—— (1897). *Ibid.* XXII. p. 45.
Thresh and Sowden (1904). *Public Health*, May.