USE OF A GLUCOSE BISMUTH SULPHITE IRON MEDIUM FOR THE ISOLATION OF *B. TYPHOSUS* AND *B. PROTEUS*.

By W. JAMES WILSON and E. M. McV. BLAIR.

(*From the Public Health Laboratories, Queen's University, Belfast.*)

(With Plate I.)

In previous papers we (1923 and 1926) have shown that (1) *B. typhosus* in the presence of a fermentable carbohydrate is able to reduce a sulphite to a sulphide, (2) a combination of bismuth and sodium sulphite affords an enrichment and selective medium for *B. typhosus* and at the same time partially or completely suppresses the growth of *B. coli*. In the present communication we describe further investigations with this medium and certain modifications and improvements which we have introduced.

In the examination of typhoid stools our results with this medium have been far more satisfactory than those hitherto obtained by us with the usual media containing lactose, bile salts, dyes, etc.

To avoid repetition and to allow of the use of abbreviations in the descriptions of the various combinations of ingredients in our media we may at the outset state that our stock medium consisted of a 3 per cent. nutrient agar prepared in the usual way and made faintly alkaline to litmus, *i.e.* in 1 litre there were 30 grm. powdered agar, 10 grm. peptone, 5 grm. Lemco, and 5 grm. sodium chloride. We had as stock solutions a 20 per cent. solution in water of anhydrous sodium sulphite, a 20 per cent. solution of commercial glucose and an 8 per cent. solution of ferrous sulphate crystals ($FeSO_4 7H_2O$). When we refer to liq. bis. we mean, unless otherwise stated, the liquor bismuthi et ammonii citratis of the *British Pharmacopoeia*.

The media were poured out into petri dishes and inoculations were made on the surface with watery suspensions of the bacilli. The medium which we used in our earlier work had the following composition: 100 c.c. agar, 5 c.c. glucose, 10 c.c. sodium sulphite, 2 c.c. liq. bis., and 1 c.c. ferrous sulphate solution.

The agar was melted and mixed with the above in the order mentioned, being boiled for 2 minutes before the addition of the iron solution. When the sulphite and bismuth solutions are boiled a flocculent white precipitate is formed and this in the presence of a certain excess of sulphite is the constituent which inhibits the growth of *B. coli*.

In previous work we have shown that the *B. coli* group may be subdivided into two varieties—one containing the majority of strains and unable to reduce sulphites, the other present in scanty numbers in faeces and capable like *B. typhosus* of reducing a sulphite to a sulphide in the presence of a

fermentable carbohydrate. One of our tasks was to discover a medium which would allow of a differentiation between reducing *B. coli* (R) strains and *B. typhosus*, and this we believe we have succeeded in effecting by the use of bismuth salts.

The combination of bismuth and sulphite does not interfere with the growth of *B. typhosus*, and the colonies of this microorganism are small, flat, dry, appear early and when isolated are black with a metallic lustre. For 20 hours the growth of *B. coli* is in most cases inhibited and when colonies ultimately develop they are large, raised, moist, sticky and mucoid, at first clear and later becoming brownish or even black. We find that in most cases almost complete permanent suppression of the *B. coli* occurs where the action of the bismuth and sulphite is supplemented by the addition of 0·5 c.c. of a 1 per cent. solution of brilliant green in distilled water to every 100 c.c. of the agar.

As an illustration of these statements we cite Exp. 1.

Exp. 1. Inoculations of watery suspension of *B. typhosus* and of *B. coli* reducers (R) and non-reducers (Non-R) were made on the surface of plates containing 100 c.c. agar, 5 c.c. glucose, 20 c.c. sulphite, 2 c.c. liq. bis. and 1 c.c. ferrous sulphate. The results are recorded in Table I.

Table I.

Microorganism	24 hours	48 hours	96 hours
B. typhosus	Numerous clear colonies, some brown, a few black	Mainly brown, a few with black centres	Mainly clear or brown
B. coli. R 1	No growth	1 brown colony raised and sticky	10 raised sticky black colonies
,, R 2	Good growth, clear colonies	Brown colonies	Dark brown sticky colonies
,, R 3	1 colony	3 colonies	3 dark sticky colonies
,, R 3A	2 colonies of fair size and a few minute colonies	1 dark and about 20 clear sticky colonies	Numerous dark raised sticky colonies
,, R 7	No growth	5 colonies—1 dark and 4 small raised colonies	Numerous dark sticky colonies
,, R 38	Fair growth brown colonies	Flat brown colonies	Dark sticky colonies
,, R 39	No growth	18 colonies, sticky, moist, raised, 5 with dark centres	Extensive growth, dark raised sticky colonies
,, Non-R 3	No growth	No growth	Numerous sticky brown colonies
,, Non-R 7	No growth	No growth	No growth

Similar results were obtained with R 19, R 38 A, and R H, the latter being obtained from the urine of a case of pyelitis, and also as regards the non-reducers with Non-R 1, 2, 4, 19, 21, 22, 23, 33, 37, 38 and 39 all obtained from typhoid stools, with 3 urinary strains, with 9 other faecal strains and with 12 strains obtained from contaminated water supplies.

We may sum up our work at this stage by saying that in the case of 10 reducing and 37 non-reducing strains of *B. coli* there was at least partial suppression of their growth for 24 hours and that in the case of *B. typhosus* no such suppression occurred. In the course of our work we have used at least 35 strains of *B. typhosus* and not once was inhibition encountered.

Table II illustrates the advantage of the use of brilliant green to supplement the action of bismuth.

Table II.

		Bismuth medium alone 48 hours	Bismuth medium and brilliant green 48–96 hours
Faecal emulsion		Numerous brown sticky colonies	No growth
B. coli from water supplies.	No. 1	No growth	,,
	No. 2	Moist brownish growth	,,
	No. 3	4 large moist brown colonies	,,

Through the kindness of the Director of the National Collection of Cultures we were able to test the action of the bismuth sulphite and the bismuth sulphite brilliant green media on some stock cultures. The media contained agar 100 c.c., glucose 5 c.c., sodium sulphite 10 c.c., liq. bis. 2 c.c., and ferrous sulphate solution 1 c.c.

Where brilliant green was used 0·5 c.c. of a 1 per cent. watery solution was added to 100 c.c. of the medium (see Table III).

With these bismuth sulphite media we examined stools from 15 cases of enteric fever in September and October 1926, and in all cases were successful in isolating the infecting microorganism. In 8 cases this proved to be the *B. typhosus*, in 6 *B. paratyphosus* B, and in 1 case both *B. typhosus* and *B. paratyphosus* B were present.

For the isolation of *B. typhosus* the media gave very good results but for *B. paratyphosus* B a brilliant green lactose bile salt agar was better. This medium was introduced by one of us (W. J. W.) in 1918 and had the following composition: Lemco 10 grm., agar 30 grm., peptone 20 grm., sodium taurocholate 5 grm., sodium chloride 5 grm. and lactose 5 grm. per litre. The reaction of the medium was made faintly alkaline to litmus and 0·3 c.c. of 1 per cent. solution of brilliant green was added to each 100 c.c. of melted medium which had been cooled to 50° C. before being poured out into plates.

At this stage we were satisfied that a medium containing 100 c.c. agar, 5 c.c. glucose, 10 c.c. sodium sulphite, 2 c.c. liq. bis. and 1 c.c. of 8 per cent. ferrous sulphate solution gave good results as regards the inhibition of *B. coli* and that 2 c.c. liq. bis. was about the optimum amount required, although we had found that 0·5 c.c., 1 c.c., 3 c.c., 4 c.c. and 6 c.c. were also efficient.

However the differentiation of the colonies of *B. typhosus* was not always as good as was desirable. At times the colonies of this microorganism were jet black, flat and dry but at other times they were clear or only brown. Our aim was then to discover the conditions under which black colonies of *B. typhosus* developed and to be able to obtain them constantly. It occurred to us that perhaps the use of sodium phosphate would absorb the acids produced in the fermentation of glucose and ensure the blackening of the colony with iron sulphide. We may say at once that this proved to be the case and that by the addition of sodium phosphate good black colonies could be secured in the presence of iron. Unfortunately the use of iron salts under these

conditions tended to remove the specific inhibitory action of the medium on the growth of *B. coli*.

After numerous trials we found that the addition of 0·5–1 grm. of anhydrous sodium phosphate (Na_2HPO_4) to each 100 c.c. agar gave good results

Table III.

	Bismuth 24–38 hours	Bismuth and brilliant green 24–38 hours
B. coli Escherich	No growth	No growth
,, Gratia	Tiny brown colonies and 6 large flat clear colonies	,,
,, *communior*	A few moist brown raised colonies	,,
,, Group I	Very mucoid confluent brown growth	,,
,, ,, II	,, ,,	,,
,, ,, III	,, ,,	,,
,, ,, IV	At first no growth, afterwards a few small raised brown dry colonies	,,
B. aerogenes	Raised brown sticky growth	A few sticky colonies
B. morgan	Fine clear colonies	Fine greenish colonies
B. alcaligenes	A few fine clear or brown colonies, dry	No growth
M. melitensis	No growth	,,
B. dysenteriae Flexner, V, W, X, Y Z	,,	,,
,,	A few fine clear colonies	,,
,, Shiga. Wynne	No growth	,,
,, Rough	,,	,,
,, Smooth	,,	,,
B. schmitz	,,	,,
B. cloacae	Some sticky brown colonies	,,
V. cholerae	No growth	,,
B. paratyph. A	Fine discrete clear colonies, dry	Clear dry colonies
,, B	Dark brown, somewhat moist colonies	Large blackish colonies
,, C Hirschfeld (90)	Slightly brownish sticky	Small colonies, some black
,, C Shutter	Brownish sticky. Metallic lustre	Black edged growth
B. enteritidis Gärtner (*Limerick*)	A few small brown flat colonies	No growth
Food poisoning strains. *Derby*	Moist sticky brown growth	Moist sticky black green growth
Newport	,, ,,	,, ,,
Binns	Small brown well defined colonies, not sticky	At first no growth, later small blackish
B. of Schweinpest-Schnürer	Brown flat dry growth	No growth
B. voldagsen Wegener	At first no growth, later a few dry brown colonies	No growth, later minute greenish colonies
B. pyocyaneus	Fine clear dry colonies	Minute colonies; medium bleached
B. proteus X 19	Clear dry colonies	At first no growth, later clear colonies
B. mallei	No growth	No growth
B. typhosus	Minute dry clear colonies, an occasional black colony	Clear colonies, one with black lustre

and that the inhibition of *B. coli* could be secured by increasing the amount of liq. bis. to 4 c.c. or 5 c.c. Even under these circumstances the addition of iron salts tended to reduce the inhibitory action. However we at last found a combination which gave us our desiderata, *i.e.* (1) black flat dry colonies of *B. typhosus*, (2) suppression more or less complete of *B. coli*. This medium was prepared in the following way:

100 c.c. agar were melted and 5 c.c. glucose, 10 c.c. sodium sulphite and 5 c.c. liq. bis. were added and the mixture boiled, then either 0·5 or 1 grm. of anhydrous sodium phosphate (Na_2HPO_4) was dissolved in 10 c.c. of distilled water and poured into the flask and finally 1 c.c. of an 8 per cent. ferrous sulphate.

Illustrations of the results of this medium alone and with the addition of 0·5 c.c. of a 1 per cent. solution of brilliant green per 100 c.c. are given below in Table IV.

Table IV.

		Glucose bismuth sulphite iron medium		Do. + brilliant green
		24 hours	40 hours	24 and 40 hours
B. typhosus		Good growth. Isolated colonies, flat, dry, black with metallic sheen	Good growth. Isolated colonies, flat, dry, black with metallic sheen	Black metallic colonies
B. coli.	R 1	A few clear raised moist sticky colonies	Colonies flatter and black, not unlike those of *B. typhosus*	No growth
,,	R 2	4 large sticky colonies and 8 minute colonies	Colonies flat and black	,,
,,	R 3	Several small sticky clear colonies	Colonies flatter and black	,,
,,	R 4	One clear colony	One dark flat colony	Not tested
,,	R 7	No growth	2 dark colonies	,,
,,	R 9	3 sticky clear colonies	3 dark sticky colonies	,,
,,	R 19	4 moist brown sticky colonies	4 moist black sticky colonies	No growth
,,	R 38	2 clear moist colonies	2 moist clear brown colonies	,,
,,	R 39	No growth	20 colonies black raised with clear margin	,,
,,	R H	1 brown black colony	2 black colonies	,,
,,	Non-R 4	1 small clear colony, no darkening	20 brownish sticky colonies, no blackening	,,
,,	Non-R 19	1 raised moist colony, brownish centre	1 raised moist colony, brownish centre	Not tested
,,	Non-R 22	Moist surface raised, sticky growth	Moist surface raised, sticky growth	,,
,,	Non-R 33	No growth	8 sticky brown colonies	No growth
,,	Non-R 37	1 colony raised, not dark, not very moist	1 colony raised, not dark, not very moist	,,
,,	Non-R 38	No growth	10 raised pale colonies	Not tested

These results indicate that for 24 hours on the glucose bismuth sulphite iron medium there is considerable suppression of *B. coli* and that where *B. coli* develops a non-reducing colony never has the appearance of a *B. typhosus* colony and that it is only after 24 hours that the *B. coli* reducing colonies simulate those of *B. typhosus*. The addition of brilliant green completely suppresses the growth of *B. coli* on the medium.

Action of the various constituents of the medium.

The effect of liq. bis. on a few microorganisms was determined with the results shown in Table V, where 2 c.c. were added to 100 c.c. agar.

It would appear that bismuth salts are not very favourable to the growth of *B. typhosus* but are made favourable by the addition of sodium sulphite. On *B. lactis aerogenes* and on *B. coli* Non-R 37 the bismuth alone in the

Table V.

	24 hours	48 hours
B. lactis aerogenes	Fair growth—yellowish brown	Fair growth—yellowish brown
B. coli: Non-R 37	Sticky yellowish brown growth	Sticky yellowish brown growth
B. typhosus	No growth	Very minute colonies
The usual bismuth sulphite iron medium yielded:		
B. lactis aerogenes	No growth	No growth
B. coli: Non-R 37	Minute colonies	Large brown colonies
B. typhosus	Numerous small colonies	Colonies larger and browner
Where iron was left out of the bismuth sulphite medium the results were:		
B. lactis aerogenes	No growth	No growth
B. coli: Non-R 37	,,	20 brown moist colonies
B. typhosus	Numerous minute clear colonies	Numerous clear colonies

amounts used has no inhibitory effect but this is produced by the addition of sodium sulphite.

Liq. bis. has an inhibitory action on the growth of *B. welchii* when sodium sulphite is either present or absent.

Quantities of 20 c.c. of glucose agar were taken and 0·2 c.c. undiluted liq. bismuthi and 2 c.c., 1 c.c. and 0·2 c.c. of liq. bismuthi diluted 1 in 10 were added to separate tubes and the mixtures poured out into petri dishes. No growth occurred when the surface of these plates was inoculated with *B. welchii* and they were incubated under anaerobic conditions, whilst excellent growths occurred on a control plate containing only glucose agar. It would appear that 1 in 1000 of liq. bismuthi is able to suppress the growth of *B. welchii*. It is possible that this action of bismuth may be of importance in connection with its use for therapeutic purposes.

In order to discover what constituents of the medium were responsible for the suppression of *B. coli* the following experiments were carried out.

Exp. 2. 4 c.c. liq. bis. were added to 100 c.c. of distilled water and then 20 c.c. of a 20 per cent. solution of sodium sulphite. No obvious reaction occurred at once but a flocculent precipitate separated out on boiling. The supernatant fluid which was found to contain excess of sodium sulphite was decanted and the remainder centrifugalised.

Table VI.

		24 hours	48 hours
A.	R 1, R 38 and faecal emulsion all yielded numerous flat colonies, clear or slightly brownish. No stickiness and no excessive moisture		R 1, R 38 and faecal emulsion all yielded numerous flat colonies, clear or slightly brownish. No stickiness and no excessive moisture
B.	R 1.	Only 3 large sticky	Fairly numerous sticky moist raised colonies
	R 38.	Confluent profuse moist sticky clear growth	Confluent profuse moist sticky clear growth
	F. E.	Mainly moist clear sticky growth	Mainly moist clear sticky growth
	T.	Small clear flat colonies. No darkening	Small clear flat colonies. No darkening
C	R 1.	Only 1 colony large and sticky and with brown centre	About 20 colonies
	R 38.	No growth	Numerous large sticky clear colonies
	F.E.	Numerous very fine almost invisible colonies	Numerous colonies, some sticky, some non-sticky
	T.	Good growth, clear flat colonies. Not sticky and not moist	Clear flat colonies
D.	R 1.	No growth	3 clear moist colonies
	R 38.	,,	Numerous sticky colonies
	R f.	,,	,, ,,
	T.	Good growth, clear flat colonies	Clear flat colonies

The deposit was suspended in 100 c.c. of distilled water, boiled and mixed with an equal volume of glucose agar. This medium was divided into four equal amounts of 50 c.c. designated A, B, C and D respectively.

These quantities were poured out into plates, A without alteration, but in the case of B, C and D there were previously added 1 c.c., 2 c.c. and 3 c.c. of a 20 per cent. solution of sodium sulphite. The plates were inoculated with emulsions of *B. typhosus*, *B. coli* R 1 and R 38, and of faeces. The results are shown in Table VI.

The inhibition is therefore not due to the bismuth sulphite precipitate but to the combined action of sodium sulphite and this precipitate, since the following experiment showed that sodium sulphite alone had not this action.

Exp. 3. Of a 20 per cent. sodium sulphite solution 2, 4, 6, 10, 15 and 20 c.c. were added each to 100 c.c. of glucose agar. Plates were poured out and inoculated with suspensions of *B. typhosus*, *B. coli* R 1, R 38, and a faecal emulsion.

At the end of 24 hours all the plates with the exception of the one to which 20 c.c. had been added showed good growth of clear colonies which were not excessively moist or sticky.

With 20 c.c. of the sulphite per 100 c.c. of medium no growth occurred for 24 hours, but at the end of 48 hours tiny colonies had appeared.

Exps. 2 and 3 were repeated several times with similar results and a further experiment (4) showed that the inhibition of the growth of *B. coli* was due to the combined action of the bismuth sulphite precipitate and sodium sulphite solution and not to the clear fluid.

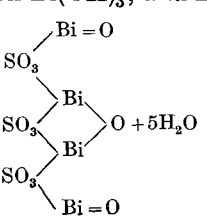
Exp. 4. 4 c.c. liq. bis. 80 c.c. dist. water and 20 c.c. of a 20 per cent. solution of sodium sulphite were boiled and the clear fluid removed from the precipitate by decantation and centrifugalisation. The precipitate was suspended in 100 c.c. of distilled water, boiled and mixed with an equal amount of glucose agar. Plates were poured out after the addition of 0, 2, 3 and 5 c.c. of sodium sulphite to each 50 c.c. The inhibition of *B. coli* on the plates containing the precipitate and sodium sulphite was found to be marked.

The clear liquor obtained after removal of the precipitate which had formed on boiling the mixture of liquor bismuthi and sodium sulphite was mixed with an equal volume of glucose agar and plates were poured out and inoculated with various strains of *B. coli* and *B. typhosus*. It was found that little or no inhibition of growth occurred.

A control experiment in which 100 c.c. water and 20 c.c. of 20 per cent. sodium sulphite had been added to 100 c.c. of glucose agar and plates poured out showed excellent growth of all the members of the colon-typhoid group examined.

What is the exact composition of the compound formed by the action of the sodium sulphite on the bismuth citrate dissolved in ammonia we are unable to state. It is probably a basic salt of bismuth sulphite.

Röhrig (1888) states that a basic salt $2Bi_2O_3.3SO_2.5Aq.$ is formed by the action of concentrated $SO_2Aq.$ on $Bi(OH)_3$, and might be represented thus:



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Reaction of Media.

Our stock nutrient agar is made faintly alkaline to litmus and has probably a hydrogen-ion concentration of about 7·4. When tested with bromo-thymol blue, phenol red and cresol red, the colours shown are respectively light green, yellow and yellow.

The solution of sodium sulphite is slightly alkaline to litmus, that of liq. bis. is strongly alkaline and that of ferrous sulphate strongly acid.

When to 100 c.c. agar there are added 5 c.c. glucose, 20 c.c. sodium sulphite and 5 c.c. liq. bis. and the mixture boiled for 1 or 2 minutes, it is found that the reaction is strongly alkaline to litmus, and that cresol red and thymol blue yield red and light yellow colours respectively. The *p*H is in the neighbourhood of 8·4 or 8·6.

The addition of 1 grm. of sodium phosphate and of 1 c.c. of an 8 per cent. solution of ferrous sulphate does not appear to alter the hydrogen-ion concentration to any extent, it remains approximately at 8·6.

The bismuth sulphite media have therefore a much greater alkalinity than those that are commonly employed.

COMPARISON OF RESULTS OBTAINED WITH MACCONKEY'S BILE SALT LACTOSE NEUTRAL RED AGAR MEDIUM WITH THE BISMUTH SULPHITE MEDIA.

On 21. I. 27 eight samples of faeces which had been sent from Enniskillen were tested for the presence of *B. typhosus*. We employed a medium consisting of 100 c.c. agar, 5 c.c. 20 per cent. glucose solution, 10 c.c. of a 20 per cent. sodium sulphite solution and 5 c.c. of liq. bis., adding to each 100 c.c. 0·5 grm. sodium phosphate and 1 c.c. of 8 per cent. ferrous sulphate solution. We also employed the same medium with the addition of 0·5 c.c. of a 1 per cent. watery solution of brilliant green.

The results obtained with the specimens were as follows:

Specimen C. On a MacConkey plate there were among numerous *B. coli* 11 non-lactose fermenting colonies all of which were proved by agglutination and cultural tests to be composed of typhoid bacilli. On the bismuth plates there were over 1000 colonies and all were composed of *B. typhosus*. The growth of *B. coli* was completely inhibited for over 24 hours.

Specimen D. On the MacConkey there were 6 typhoid colonies and numerous *B. coli* but on the bismuth plates there were several thousands of *B. typhosus*, and no *B. coli* colonies at the end of 24 hours.

Specimen E. On MacConkey medium besides numerous *B. coli* there were 24 colonies of *B. typhosus*. On both bismuth plates there were several thousand colonies of *B. typhosus* and none of *B. coli*.

Specimen H. On MacConkey plate numerous *B. coli* and 150 typhoid colonies. On the bismuth plates several thousands of *B. typhosus* in pure culture for 24 hours. At the end of 48 hours a fair number of *B. coli* developed on the bismuth plate but none on the plate containing brilliant green.

Specimen I. On MacConkey plate numerous *B. coli* and no typhoid colonies. On the bismuth plate there were numerous sticky raised *B. coli* colonies and no typhoid. On the

brilliant green bismuth plate there were 100 dark colonies all of which proved to be *B. typhosi.* After 48 hours there also developed some sticky *B. coli* on this medium.

Specimen K. On MacConkey plate there were numerous *B. coli* but no typhoid colonies. On the bismuth plates there were on each at least 400 colonies and all of them composed of *B. typhosus.* Even at the end of 80 hours no development of *B. coli* had occurred.

Specimen M. On MacConkey plate there were 7 *B. coli* and 41 typhoid colonies. On each of the bismuth plates there were 60 colonies of *B. typhosus* and no *B. coli.*

Specimen B. No *B. typhosus* isolated by means of any medium. From this stool we cultivated a *B. proteus* of the X 19 group.

The conclusion we draw from the results is that the bismuth sulphite media enormously increase the chances of isolating typhoid bacilli. In all cases where the *B. coli* are numerous in the stools far more typhoid bacilli developed on the bismuth than on the MacConkey plates. The explanation of this apparent enrichment is that the inhibition of the bismuth sulphite complex allows typhoid bacilli to grow in places where, without its action, they would have been crowded out and their presence masked by the development of *B. coli* colonies.

With bismuth media having the composition mentioned in the above tests we tested 16 typhoid stools and in 15 cases were successful.

The typhoid colonies were flat, black and dry where they were moderately discrete; where the growth was confluent the darkening was not so pronounced. At this stage we thought that we had standardised our media but we found that further work was necessary before this end was attained.

For many months we had employed the same stock liq. bis. et ammonii cit. and the results had been uniformly good, *i.e.* the typhoid colonies were black and the *B. coli* were suppressed. We now got a fresh supply from another firm and discovered that though the *B. coli* were suppressed, the typhoid colonies did not come up black even although varying amounts of the liquor were used in our media. With a view to establishing our work on a firm basis we obtained a precipitate from a known volume of the new liq. bismuthi by the addition of just slight excess of sodium sulphite. This precipitate was weighed and made up in the media with varying amounts of sodium sulphite. An alternative and more convenient method was to wash the precipitate and suspend it in a volume of water equal to the volume of the original liquor.

For example, 200 c.c. liq. bismuthi et ammonii cit. were boiled with 110 c.c. of a 20 per cent. solution of sodium sulphite. The precipitate was washed and suspended in 200 c.c. of distilled water. The following experiments are selected from many that were made with different amounts of the precipitate in the media.

Exp. 5. A medium consisting of 200 c.c. nutrient agar, 10 c.c. of a 20 per cent. solution of glucose, 20 c.c. of bismuth precipitate mixture and 10 c.c. of a 20 per cent. solution of sodium sulphite was prepared and divided into 2 equal parts designated A and B (Table VII).

In A no iron was added but in B 1 c.c. of an 8 per cent. solution of $FeSO_4 7H_2O$ was employed for every 100 c.c. of the agar. In both A and B varying amounts of sodium

phosphate were added to each 25 c.c. of the media. Four plates were poured out in each instance and one half of the plate was inoculated with *B. typhosus* and the other half with a *B. coli* (R 1).

Table VII.

A. *No iron.*

Amounts of sol. containing 1 grm. exsiccated sodium phosphate in 10 c.c. of water added to portions of medium	*B. typhosus*		*B. coli*	
	20 hours	40 hours	20 hours	40 hours
0 plate poured out	Clear colonies	Clear colonies	Nil	20 colonies
5 c.c. to remainder and plate poured out	,,	Clear flat colonies	,,	Nil
2·5 to remaining 50 c.c.	,,	Light brown	,,	10 large brown sticky colonies
2·5 to remaining 25 c.c.	,,	,,	1 moist	15 large moist raised colonies, clear edge

B. *With* 1 *c.c.* $FeSO_4$ 8 *per cent. solution to each* 100 *c.c.*

	Clear light brown flat	Clear light brown flat	Moist sticky light brown	Moist sticky light brown
5 to remaining 75 c.c.	Confluent light brown isolated black metallic	Confluent light brown isolated black metallic	Sticky moist brown	Sticky moist blackish
2·5 to remaining 50 c.c.	Brown and isolated flat black metallic	Brown and isolated flat black metallic	Raised moist brown	Blackish green
2·5 to remaining 25 c.c.	Confluent clear isolated black	Confluent clear isolated black	Moist clear sticky	Moist clear sticky

The conclusion to be drawn from these experiments as from many others that were performed is that (1) to obtain black colonies there is necessary the use of (*a*) phosphate, (*b*) iron; (2) the suppression of *B. coli* is not so marked in the presence of iron salts.

The addition of a solution of ferrous sulphate has an effect on the reaction of the medium and on the composition of various salts in it.

We may assume that reactions like these occur:

$$3FeSO_4 + 2Na_2HPO_4 = Fe_3(PO_4)_2 + 2Na_2SO_4 + H_2SO_4,$$
$$Na_2HPO_4 + H_2SO_4 = NaH_2PO_4 + NaHSO_4.$$

In our medium there is very great excess of sodium phosphate 1 grm. compared with the ·08 grm. of ferrous sulphate.

However, whatever the exact chemical explanation may be there is no doubt that iron salts so necessary for the production of black colonies of typhoid bacilli reduce the inhibitory action of the bismuth sulphite complex on *B. coli*.

Numerous experiments were carried out with different salts of iron and on the whole it was found that an iron phosphate mixture gave fair results.

Exp. 6. 100 c.c. agar, 5 c.c. 20 per cent. glucose, 10 c.c. bismuth precipitate mixture, 1 grm. Na_2HPO_4. This medium was divided into 4 equal portions; one portion was poured out into a petri dish and to the others 1, 3 and 5 c.c. of a phosphate iron mixture were added. This phosphate iron mixture was prepared by dissolving 5 grm. $FeSO_47H_2O$ in 500 c.c. of water and adding 8 grm. of Na_2HPO_4 to the solution (see Table VIII).

Table VIII.

Phosphate iron mixture	*B. typhosus*		*B. coli* R 1	
	20 hours	40 hours	20 hours	40 hours
1st 25 0	Clear colonies	Clear colonies	Nil	5 clear colonies
2nd 25 1 c.c.	,,	,,	,,	3 small clear cols.
3rd 25 3 c.c.	Clear colonies brown centre	Several black	,,	Nil
4th 25 5 c.c.	Clear colonies brown centre	Black colonies flat metallic	,,	1 clear colony

This experiment suggested that it is possible to get an iron preparation which will yield black colonies of typhoid and yet not annul the inhibition of *B. coli.*

Experiments were then made in which iron alum and ferri et ammonii cit. were added to the medium. It was found that the addition of 1 c.c. of an 8 per cent. solution of ferri et ammonii cit. and of 2·5 c.c. of an 8 per cent. solution of iron alum to every 100 c.c. of agar gave fair results, where 1 grm. of sodium phosphate was employed. With 2 grm. of phosphate 4·5 c.c. of iron alum solution were required.

At this stage fresh liq. bis. et ammonii cit. was obtained from the firm from which we had previously obtained a satisfactory supply. This new sample was found to be equally good. We found that a medium composed of 100 c.c. agar, 5 c.c. glucose 20 per cent. sol., 10 c.c. sodium sulphite 20 per cent. sol., 5 c.c. liq. bis. et ammonii. cit., 1 grm. Na_2HPO_4 and 1 c.c. of an 8 per cent. $FeSO_47H_2O$ sol., 1 c.c. of an 8 per cent. ferri et ammonii cit. or 2·5 c.c. of an 8 per cent. iron alum solution gave good black colonies of *B. typhosus.*

We now directed our efforts to the preparation of a solution of bismuth citrate in ammonia and water which would give constant results and would be free from the variableness of commercial products. In the *British Pharmacopoeia* the following quantities are specified for the manufacture of liq. bis. et ammonii cit.: 70 grm. bismuth oxynitrate, 52 grm. citric acid and sufficient ammonia to cause solution in 1000 c.c. water. It contains 5 grains of citrate = 3 grains of oxide of bismuth in 1 drachm, or expressed in the metric system 0·3 grm. citrate in 4 c.c. or 7·5 grm. citrate in 100 c.c.

In the *Extra Pharmacopoeia*, Martindale and Westcott (1925) state that "careful experiments by us showed that 1 molecular weight of commercial bismuth citrate required approximately 2 molecular weight of ammonia to dissolve to an alkaline solution." These data were useful to us in the preparation of a standard solution of bismuth.

Our endeavour was to obtain a solution of which 5 c.c. added to 100 c.c. glucose sulphite iron agar medium would ensure the development of black colonies of the typhoid bacillus and the complete or partial suppression of *B. coli.*

Our experience with commercial liq. bis. et ammonii cit. appeared to indicate that the amount of ammonia present was a most important and variable factor. An experiment was therefore carried out in which to constant

amounts of bismuth citrate gradually increasing amounts of strong liquor ammonii were added.

Exp. 7. To each of 10 test tubes 0·4 grm. of bismuth citrate was added and then to each 0·5 c.c. of distilled water and then 0·1, 0·2, 0·3, 0·4, 0·5, 0·6, 0·7, 0·8, 0·9 and 1 c.c. of strong ammonia solution; water was then added to make the volume in each tube 5 c.c. Each tube contained sufficient for 100 c.c. agar, but in the experiment we employed half the amount, *i.e.* 2·5 c.c. for 50 c.c. agar.

The medium contained for every 100 c.c. of 3 per cent. nutrient agar, 5 c.c. of a 20 per cent. solution of glucose and 10 c.c. of a 20 per cent. solution of sodium sulphite (anhydrous). To 50 c.c. of this medium 2·5 c.c. of the above solution of bismuth were added, the mixture was boiled and then 0·5 grm. sod. phosphate and 0·5 c.c. of 8 per cent. $FeSO_4 7H_2O$ sol. Two plates were poured out in each case and were inoculated with *B. typhosus* and with a R 1 *B. coli* (see Table IX).

Table IX.

Plates No.	*B. typhosus*		R 1 reducer	
	24 hours	48 hours	24 hours	48 hours
1	Black and metallic colonies	Black and metallic colonies	Black and metallic, somewhat similar to *B. typhosus*	Black and metallic, somewhat similar to *B. typhosus*
2	Similar to No. 1	Similar to No. 1	Similar to No. 1	Similar to No. 1
3	Black and metallic	Black and metallic	Scanty sticky brown growth	Scanty sticky brown growth
4	Nil	Clear and moist colonies	Nil	Nil
5–10	,,	Nil	,,	,,

We concluded that to 100 c.c. of the agar there should not be added greater amounts than 0·1, 0·2 and 0·3 c.c. strong liquor ammonii, *i.e.* the amounts present in the first 3 tubes. A similar experiment was then carried out in which varying amounts of bismuth citrate were added to 0·1, 0·2 and 0·3 c.c. of liq. ammonii contained in a series of test tubes.

Exp. 8. To each of 10 test tubes numbered from 1 to 10 were added 0·2, 0·2, 0·4, 0·6, 0·8, 0·2, 0·4, 0·6, 0·8 and 1 grm. of bismuth citrate, followed by 0·5 c.c. or 1 c.c. of dist. water.

To No. 1 was added 0·1 c.c. of strong liq. ammonii, to Nos. 2, 3, 4 and 5 were added 0·2 c.c. and to Nos. 6, 7, 8, 9 and 10, 0·3 c.c. liq. ammonii. The volume of each tube was

Table X.

No.	*B. typhosus*		*B. coli* R 1	
	24 hours	48 hours	24 hours	48 hours
1	Light brown colonies	Dark brown colonies	Light brown colonies	Dark brown colonies
2	,,	,,	,,	Light brown colonies
3	,,	,,	Brownish sticky	Brownish sticky, raised colonies
4	Isolated colonies black and metallic, others clear	Isolated black and metallic	Scanty sticky moist colonies	Scanty moist sticky colonies
5	All isolated colonies black and metallic	,,	Nil	Black raised moist clear
6	Nil	Clear colonies	,,	Nil
7	,,	,,	,,	,,
8	Brownish black	Greenish profuse growth	Moist brown sticky	Moist sticky profuse growth
9	Black and metallic	Flat and metallic	Clear moist raised sticky	Clear moist raised sticky
10	All isolated black	Black and metallic	Nil	Moist and brown

finally made up to 5 c.c. with distilled water. To 25 c.c. glucose sulphite agar was added 1·25 c.c. of each of the above solutions and then 0·25 grm. sod. phosphate and 0·25 c.c. of 8 per cent. $FeSO_4 7H_2O$ sol. A plate was poured out and half was inoculated with *B. typhosus* and the other with *B. coli* R 1. Readings were taken after 24 and 48 hours' incubation with results shown in Table X.

We concluded that tubes Nos. 4, 5 and 10 were probably most suitable for our purpose. With No. 4, although some growth of *B. coli* was permitted the typhoid colonies were larger than with Nos. 5 and 10. No. 4 bismuth solution had been prepared by adding to 6 grm. bismuth citrate 0·5 c.c. water, then 0·2 c.c. strong liq. ammonii and making up to 5 c.c.

No. 5 was prepared by adding to 0·8 grm. bismuth citrate 1 c.c. water, then 0·2 c.c. ammonia and making up to 5 c.c. and similarly No. 10 by adding 1 grm. to 1 c.c. water and 0·3 c.c. ammonia and making up to 5 c.c.

An experiment was next carried out to compare the action of solutions Nos. 4, 5 and 10. In it a five times greater amount of the solutions was prepared:

No. 4 = 3 grm. bis. citrate, 2·5 c.c. water, 1 c.c. strong ammonia solution and water up to 25 c.c.

No. 5 = 4 grm. bis. citrate, 5 c.c. water, 1 c.c. strong ammonia and water up to 25 c.c.

No. 10 = 5 grm. bis. citrate, 5 c.c. water, 1·5 c.c. strong ammonia and water up to 25 c.c.

It will be observed that the ratio of bismuth citrate to ammonia solution in preparation of Nos. 4, 5 and 10 was 9 : 3, 12 : 3 and 10 : 3 respectively. When the ammonia is added to the bismuth citrate suspended in the small quantity of water considerable heat is generated and almost complete solution occurs. The solutions are not clear, a small amount of the bismuth citrate remaining undissolved. We believe it is an advantage to have some undissolved bismuth citrate present. Before use the mixture is shaken. To each of three flasks containing 100 c.c. agar, 5 c.c. glucose 20 per cent. solution, 10 c.c. sodium sulphite (anhydrous) 20 per cent. were added 5 c.c. of solutions Nos. 4, 5 and 10 respectively. The flasks were boiled and then 1 grm. Na_2HPO_4 (anhydrous) and 1 c.c. of 8 per cent. $FeSO_4 7H_2O$ sol. added. Half of the contents of each flask were poured out into two petri dishes and to the remaining 50 c.c. was added 0·25 c.c. of a 1 per cent. watery solution of brilliant green and two more plates prepared from the mixture.

The plates were inoculated with emulsions of *B. typhosus* and of three reducing strains of *B. coli*, R 1, R 4 and R 7 (see Table XI).

It is obvious that the inhibition of *B. coli* reducers with these media is good and that black colonies are formed by *B. typhosus*, especially by that containing No. 4 solution. No. 4 solution was therefore taken as the standard and 500 c.c. of it prepared by mixing 60 grm. bis. citrate with 50 c.c. water and adding 20 c.c. liq. ammonii sp. gr. 0·880 and finally, when solution was almost completed, water up to 500 c.c.

Table XI.

		Medium alone				Medium + brilliant green			
			B. coli				*B. coli*		
		B. typhosus	R 1	R 4	R 7	*B. typhosus*	R 1	R 4	R 7
No. 4.	24 hrs.	Black metallic	Nil	1 col.	Nil	Black metallic	Nil	Nil	Nil
	48 ,,	,,	,,	,,	,,	,,	,,	,,	,,
No. 5.	24 ,,	,,	,,	,,	,,	Brown centred col.	,,	,,	,,
	48 ,,	,,	,,	,,	,,	,,	,,	,,	,,
No. 10.	24 ,,	Brown centred col.	,,	Nil	,,	Brown centres	,,	,,	,,
	48 ,,	A few black metallic	,,	,,	,,	Dark centres but not black	,,	,,	,,

We now take this No. 4 solution as our standard solution and have tested with it the stools of several cases of typhoid fever.

Examples of its use are as follows:

Stools of Miss H. A case of enteric fever. On the bismuth sulphite glucose iron agar plate eight typical flat black colonies developed and on the same medium + brilliant green two colonies. No growth of *B. coli* occurred. The colonies were proved by cultural and agglutination tests to be those of *B. typhosus*.

As no stools from patients suffering from enteric fever could be obtained, an examination was made of nine enteric stools which had been received on 20. I. 27 and which had remained in corked tubes until 23. III. 27 when they were tested on the standard sulphite media. At this date typhoid bacilli were cultivated from three out of the nine. Two other old enteric stools received on 1. II. 27 and 4. II. 27 were examined on 25. III. 27 and were found to contain living typhoid bacilli. In about one-third or more of the cases the viability of typhoid bacilli in stools kept at room temperature extends over a period of two months.

As our standard *liquor bismuthi* is slightly different in composition from the B.P. preparation with which most of our previous experiments had been carried out, we tested the effects of media containing it on certain stock cultures.

Twenty-two strains of *B. coli* were examined, 12 being reducers and 10 non-reducers. The results were as shown in Table XII.

Table XII.

		24 hours	36 hours
12 reducers	5 strains	Nil	Sticky brown or black growth
	7 ,,	A few moist sticky colonies	Raised sticky brown or black colonies
10 non-reducers	4 ,,	Nil	2 no growth, 2 a few sticky colonies
	4 ,,	A few brown colonies	A few brown sticky colonies
	2 ,,	Numerous brown colonies	Numerous brown colonies

With all the 22 strains of *B. coli* no growth whatever occurred on the bismuth sulphite plates containing brilliant green. Twenty-seven strains of *B. typhosus* rapidly developed on the bismuth media with or without brilliant green and within 24 hours formed flat dry black colonies.

Nine strains of *B. paratyphosus* B were tested and all showed absence of growth for 24 hours. Between 24 and 40 hours scanty growth of moist raised colonies mainly clear or brownish occurred on the bismuth plates. The same remark applies to the bismuth brilliant green plates with two exceptions in which the suppression of growth was permanent.

Similar in behaviour to *B. paratyphosus* B were certain organisms of the Salmonella group, *e.g.* types *Mutton*, *Newport*, *Derby*, *Schweinepest-Schnürer*, *B. voldagsen* Wegener, etc. On the other hand, *B. enteritidis* (Gaertner) Limerick was permanently suppressed and Binns type yielded dry brown or black colonies.

The four strains of *B. proteus* examined developed early, forming flat dry clear or slightly brownish colonies. After a delay of 24 hours *B. faecalis alcaligenes* produced clear flat dry colonies.

Bacilli of the proteus Group.

In our examination of faeces we frequently encountered bacilli forming clear dry colonies on the bismuth sulphite iron medium even where brilliant green had been incorporated in it. In the latter the colonies appeared dark green in colour, quite different from the jet black colonies of *B. typhosus*. On subculture their growth on agar had the spreading character of the *B. proteus* group; on the sulphite media this tendency for the growth to spread was completely suppressed. The relative proportion of *B. proteus* to *B. coli* was increased in samples of faeces that had been kept for some days or weeks.

A study of 14 strains showed that all liquefied gelatin, were gram negative, produced no pigment, formed acid and gas in glucose and were unable to ferment lactose and mannite. Ten fermented maltose and saccharose and formed indol; two formed indol but did not ferment maltose or saccharose and two neither formed indol nor fermented maltose or saccharose. None of these four fermented salicin and of the other ten it was found that six decomposed this glucoside with the production of acid and gas. Thirteen of these *B. proteus* strains were tested with a typhus serum and one was found to be more sensitive than our laboratory *B. proteus* X 19 strain to the agglutinins contained therein.

This strain was isolated—21. I. 27—from the stools of a case of enteric fever which was under the care of Dr McBrien at Enniskillen Fever Hospital. The patient, a young woman 25 years of age, never had had typhus fever and never had been in contact with the disease. On 5. IV. 27 the stools of this patient which had been kept at room temperature in a glass container were again planted out on the bismuth media and four colonies developed within 24 hours and all proved to be composed of *B. proteus*.

The four colonies were subcultured and were tested with a fresh typhus fever serum and all were found to be agglutinated in a dilution of 1 in 2560. Two control sera were negative in 1 in 20.

We then tested the bacillus with five old typhus sera, two from old Belfast

cases, one from Mexico, one from Galway and one from Newry. In all cases the titre of agglutination was higher with the Enniskillen strain than with our laboratory X 19 strain.

Culturally, *B. proteus* X 19 strain Enniskillen resembled the *indologenes* X 19 strains in that it did not ferment lactose and mannite and that it produced acid and gas in saccharose, maltose and salicin. Unlike other X 19 strains that we have studied it promptly produced acid and gas in laevulose broth.

A saturation experiment showed that the agglutinins in typhus serum for *proteus* X 19 and for our Enniskillen strain were identical.

Saturation Experiment.

Serum was obtained from a convalescent typhus case on 2. IV. 27. This had been kept and on 11. IV. 27 it was found to agglutinate completely the *B. proteus* Enniskillen strain and our laboratory X 19 strain in dilutions of 1 in 1280 and 1 in 640 respectively. Saturation of the serum diluted 1 in 80 by means of loopsful of growth taken from agar slopes of *B. proteus* Enniskillen strain and X 19 completely removed all the agglutinins for these micro-organisms from the serum.

Derry Typhus Serum.

	80	160	320	640	1280	2560	5120
B. proteus Enniskillen	× × ×	× × ×	× × ×	× × ×	× × ×	×	—
X 19 laboratory strain	× × ×	× × ×	× × ×	× × ×	—	—	—
Serum saturated with Enniskillen *proteus*	Enniskillen	—	—	—	—	—	—
	X 19	—	—	—	—	—	—
Serum saturated with *proteus* X 19	Enniskillen	—	—	—	—	—	—
	X 19	—	—	—	—	—	—

The isolation of an X 19 strain from a patient who was in no way associated with typhus fever is interesting in connection with the theories advanced to explain the Wilson-Weil-Felix reaction and is confirmatory of the work of Wolf (1922).

Fluid Media.

For the isolation of *B. proteus* from stools a fluid medium consisting of 100 c.c. bouillon, 5 c.c. of a 20 per cent. solution of glucose, 2·5 c.c. of a 20 per cent. solution of sodium sulphite (anhydrous) and 2 c.c. of liq. bis. et ammonii cit. (B.P.) or of our standard liquor bismuthi is exceedingly useful. The addition of 0·25 c.c. of a 1 per cent. watery solution of brilliant green to every 100 c.c. of bouillon is recommended. In this medium not only is *B. coli* inhibited in its growth but it is actually killed, whereas *B. proteus* multiplies, as does also in certain cases the *B. typhosus*.

An example of the success of this method is the following. The stools of the Enniskillen case which we had found to contain *B. proteus* which had been received on 21. I. 27 and had been stored, were planted out in the above medium on 5. IV. 27: on subculture on MacConkey plates on 6. IV. 27 *B. proteus*

was obtained in pure culture and all the colonies tested were agglutinated by typhus serum. The stools planted out on MacConkey plates without enrichment contained far more *B. coli* than *B. proteus*.

Summary.

1. The development and use of a medium which has selective properties for the growth of *B. typhosus* and *B. proteus* is described.

2. The principle of the method rests (1) on the positive property of the *B. typhosus* of being able to reduce a sulphite to a sulphide in the presence of glucose, (2) on the inhibitory action on the growth of *B. coli* of a bismuth sulphite in the presence of a certain excess of sodium sulphite.

3. The media finally developed are made in the following way:

A. To 100 c.c. of a melted 3 per cent. nutrient agar are added 5 c.c. of a 20 per cent. solution of glucose, 10 c.c. of a 20 per cent. solution of sodium sulphite (anhydrous), 5 c.c. of a standard bismuth solution. After boiling for two minutes an addition is made of 1 grm. of exsiccated sodium phosphate and 1 c.c. of an 8 per cent. solution of ferrous sulphate crystals.

Medium B is the same as above with the addition of 0·5 c.c. of a 1 per cent. watery solution of brilliant green. The standard liquor bismuthi is prepared by mixing 60 grm. bismuth citrate with 50 c.c. of distilled water and then with 20 c.c. liq. ammonii sp. gr. 0·880 and finally making the volume up to 500 c.c. with distilled water.

4. On these media the *B. typhosus* grows readily and forms flat black dry surface colonies. *B. proteus* grows on the medium in a non-spreading fashion but does not form black colonies. *B. coli* either fails to grow or after a period of inhibition forms brown sticky raised colonies.

5. Bismuth media were used in the examination of 31 enteric stools and in 30 instances the infecting microorganism was successfully isolated. Single examinations only were made and the material was usually 24 to 48 hours old at the time of examination.

6. Emulsions of enteric stools which as shown by the usual media contained only a dozen or so of typhoid bacilli were found by our bismuth media actually to contain several thousand.

7. The isolation from a case of typhoid fever of a *proteus* X 19 strain is recorded.

8. As regards its growth on bismuth sulphite media *B. paratyphosus* B behaves more like a reducing *B. coli* than a *B. typhosus* culture. For the isolation of *B. paratyphosus* B a lactose bile salt brilliant green medium is described.

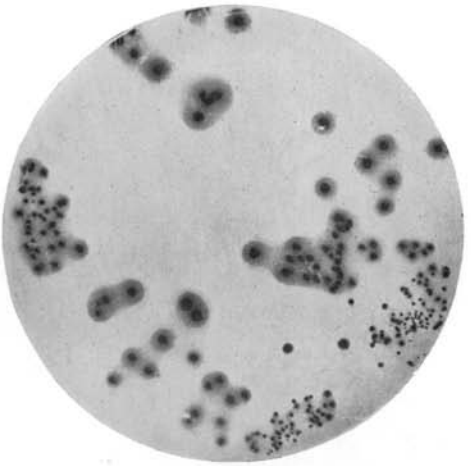

Fig. 1

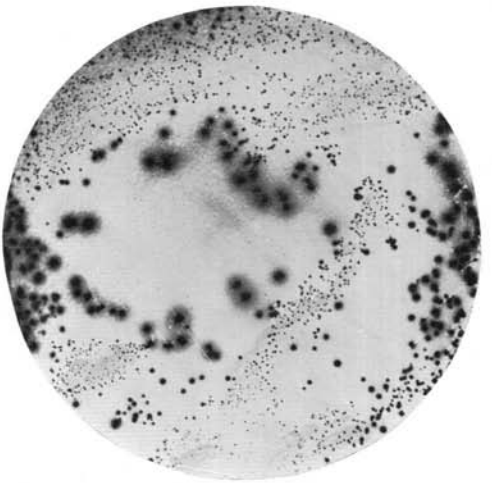

Fig. 2

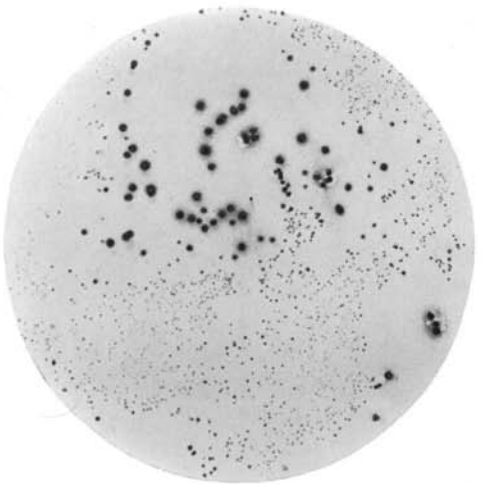

Fig. 3

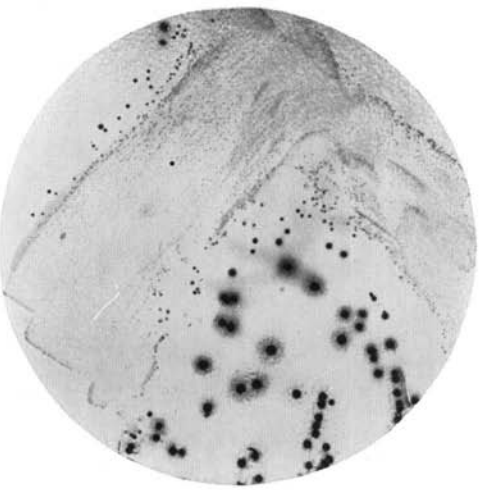

Fig. 4

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DESCRIPTION OF PLATE I.

Fig. 1. Colonies of *B. typhosus* developing on a bismuth sulphite medium containing for every 100 c.c. of agar 5 c.c. of liq. bismuthi et ammonii cit. (B.P.), when 1 drop of a watery emulsion of the stools of S.H. 18. I. 27 was plated out and incubated for 24 hours. The growth of *B. coli* was completely suppressed.

Fig. 2. Colonies of *B. typhosus* on medium containing our standard liquor bismuthi when inoculated with a faecal emulsion containing typhoid bacilli. No growth of *B. coli* has occurred.

Fig. 3. The same as Fig. 2, but the bismuth sulphite medium also contains brilliant green.

Fig. 4. Plate with medium containing our standard liquor bismuthi, heavily inoculated with a pure culture of *B. typhosus*. Note that the growth is clear or light brown where it is confluent but that the isolated colonies are typical, *i.e.* flat, black and surrounded with a metallic halo.

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