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# A study of tetrathionate enrichment techniques, with particular reference to two new tetrathionate modifications used in isolating salmonellae from sewer swabs

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## INTRODUCTION

Modern techniques for isolating salmonellae are efficient and simple when applied to faeces. With other materials, such as foodstuffs, bone meals and sewer swabs, complicated procedures, usually more costly in time and media, are used. The author set himself the task of looking for a less laborious, but not less efficient, procedure for isolating salmonellae from these other materials.

In pursuit of this aim, preliminary work (unpublished) was done on the growth of pure and mixed cultures of intestinal organisms in the presence of known and untried, selective agents, singly and in combination. Included among the pointers which emerged from this work were the observations that sodium lauryl sulphate in concentrations up to 20%, when incorporated in peptone media, apparently did not render the media inimical to the growth of salmonellae, *Shigella sonnei* and *Escherichia coli*; far lesser concentrations were, under certain conditions, highly inhibitory to Proteus strains. Conditions which favour the selective inhibition of Proteus by sodium lauryl sulphate are high-temperature incubation or the use of simple media containing only a small amount of peptone (0·2%). Liquid media which contained 1% sodium lauryl sulphate, 1% glucose, 1% Evans's peptone, and 0·5% NaCl at pH 7·5, permitted active multiplication of salmonellae at 44° C., but were *bactericidal* at this temperature to all strains of Proteus tested; these included *P. vulgaris P. morgani, P. mirabilis* and *P. rettgeri* strains. A high degree of selective inhibition of *P. mirabilis* strains was similarly obtained on a solid medium at 37° C., containing 4% sodium lauryl sulphate, 1% glucose, 0·2% Evans's peptone, and 0·5% NaCl at pH 7·5.

The concentration of NaCl is not critical in either of the two media, but when a different brand of peptone was used at higher concentrations, selective inhibition at 37° C., of Proteus occurred only between defined limits of electrolyte concentration somewhere within the range of M/10 to M/200: these limits depended on the concentration both of peptone and of sodium lauryl sulphate. The media described above would be improved by the addition of suitable buffers; in the case of the liquid medium, chalk has been found reasonably adequate.

When added to tetrathionate or to selenite media, sodium lauryl sulphate impaired the properties of neither, but considerably enhanced the inhibitory action of both media on Proteus strains. It was also observed that traces of

bismuth sulphite (in the form of 'Oxoid' Wilson and Blair Granules B) conferred on tetrathionate broth selective activity against paracolon bacilli.

As a result of various trials two modifications were evolved, both of which exploited a partnership between tetrathionate broth and ingredients of Wilson and Blair's medium. The difference between the two media was that the second medium contained only half the amount of the Wilson and Blair's ingredients present in the first but in addition it contained 1 % sodium lauryl sulphate which was absent from the other.

When these two media were compared with the standard tetrathionate enrichment medium in an examination of salmonella-contaminated bone meals it was found that both modifications consistently yielded purer growths of salmonellae than were obtained with the standard medium. No difficulty was experienced in isolating from a specimen of bone meal a salmonella organism from a blood agar plate inoculated after passing the specimen through the sodium lauryl sulphate enrichment medium.

Though these results were quite impressive, the sampling error inherent in the investigation of specimens of bone meal made it difficult to plan further experiments with such material which would be likely to convince the sceptically minded. It seemed likely that comparative examinations of salmonella-contaminated sewage by means of the sewer-swab sampling technique would be more suitable if such material were at hand.

Later there occurred in Wadhurst, a Sussex village served by this laboratory, an outbreak of *Salmonella heidelberg* infection. The investigation of the outbreak called for an examination of the sewer system by the aid of sewer swabs and provided exactly the type of sampling material which had been awaited.

This communication reports a detailed study of the comparative values of the three enrichment media as applied to bacteriological examination of the sewage in trunk, branch and tributary sewers.

## MATERIALS AND METHODS

The collection of specimens of sewage from trunk, branch and tributary sewers through selected manholes was arranged through the Medical Officer of Health and the Public Health Department responsible for the area concerned.

Sewer swabs (Moore, 1948) were prepared in the laboratory and sent to the Public Health Department in sterile 8 oz. wide-mouth screw-capped bottles (U.G.B. A 727) containing chalk. The swabs were allowed to remain 72 hr. in the various manholes at which they were put down. Three swabs from jars marked A, B and C respectively were put down at each of the forty-eight manholes sampled in the main investigation, but there were losses of one swab at two manholes and of two swabs from a third. Full sets of three swabs were thus received from forty-five sites.

In a subsidiary investigation of twelve neighbouring sites (numbers 49–60) only two swabs from jars marked A and C respectively were put down at each manhole.

On receipt at the laboratory each jar (with swab *in situ*) was topped up with

approximately 200 ml. of a tetrathionate medium. Jars marked C were topped up with the well-known modification of balanced tetrathionate broth described by Rolfe (1946) to 100 ml. of which is added 5·5 ml. of iodine solution and 11 ml. of thiosulphate solution. Throughout this paper this medium is referred to as medium *C*. Jars marked A and B were likewise topped up with media *A* and *B* respectively the composition of which is set out in the Appendix (p. 13). It will be noted that medium *A*, per topped-up jar, contains approximately 20 ml. of a 0·25% aqueous suspension of Wilson and Blair 'Oxoid Granules B', whereas medium *B* contains only 10 ml. of this suspension of 'Oxoid Granules B' per jar, but in addition this volume of medium contains approximately 10 ml. of a 20% aqueous solution of sodium lauryl sulphate (1%). One solid medium only was used for plating the enrichments. This was 'Difco' bismuth sulphite agar (dehydrated) with the incorporation, just before the plates were poured, of 6 ml. of 8% $Fe.SO_4.7H_2O$ per litre of reconstituted medium.

In the main investigation all jars were plated after approximately 24 hr. enrichment at 37° C. During plating special care was taken to establish a particularly wide inoculum gradient to avoid confluent or semi-confluent growth over an entire plate. Subsequent platings were at 48 hr. (sites 17–48) and 72 hr. (sites 1–16). In the subsidiary investigation platings were made after 24 and 48 hr. enrichment in medium *C* and once only after 48 hr. enrichment in medium *A*.

Probable or possible salmonella colonies were picked (in that order of priority) to sectors on a culture plate of Teepol agar (as described by Jameson & Emberley, 1956). At differing stages of the investigation differing arbitrary maxima were set of 8, 6, or 4 picks from particular plates. These maxima were always the same for all three enrichment media. The maximum permitted number of picks was made from every suitable plate.

Non-lactose-fermenting growth on Teepol agar sectors was tested by slide agglutination with polyvalent salmonella O serum. Sectors negative by this test were discarded: those giving equivocal agglutination were screened on Gillies's (1956) modification of Kohn's (1954) medium, but with Evans's peptone replacing the Difco products as far as possible, as suggested by Dr Joan Taylor (unpublished). Contaminated sectors were subcultured on whole plates, including, when necessary, bismuth sulphite agar.

At least one salmonella strain from each positive site was identified by single-tube agglutination at 2/3 titre with appropriate O and both phase H sera. When more than one salmonella type was isolated from a site, each type present was thus identified. Other salmonella isolations were identified by similar tube agglutination with one antiserum only, and by slide agglutination with the two remaining sera. Phase changes were induced by the filter-paper strip method described by Jameson (1961). All tetrathionate media and bismuth sulphite agar plates were freshly prepared before use.

## RESULTS AND DISCUSSION

In the main investigation *Salmonella heidelberg* was isolated from thirty-two sites, one of which also yielded *Salm. abony* and another *Salm. binza*. *Salm. paratyphi B* was isolated from one site. In the subsidiary investigation *Salm. typhimurium* was isolated from three sites, one of which also yielded *Salm. newport*, *Salm. anatum* was isolated from two sites and *Salm. muenchen* from one site. In a total of sixty sites salmonellae were isolated from thirty-nine. Double salmonella isolations were made from three sites (Table 1).

It had been observed in the bone meal experiments that progressively richer platings of salmonellae were often obtained from the tetrathionate enrichments with the passage of incubation time up to 72 or 96 hr. With medium *A*, in particular, sufficient multiplication of salmonellae frequently did not seem to occur during the first 24 hr. With these findings in mind, the sewer swab enrichments were initially plated after 24 and 72 hr. incubation respectively. Evidence soon accrued that with sewer swabs 72 hr. was in excess of the optimal enrichment period with

Table 1. *Salmonella isolations from sixty sewers of different sizes and localities*

| Site no. | Locality | Type of sewer | *Salmonella* types isolated | Media by which isolated: *A* | *B* | *C* |
|---|---|---|---|---|---|---|
| 1 | Woods Green | Branch | *heidelberg* | + | + | + |
| 2 | Woods Green | Trunk | *heidelberg* | + | + | + |
| 3 | Osmers Hill | Branch | *heidelberg* | + | + | + |
| 4 | Sparrows Green | Branch | *heidelberg* | + | + | N.T. |
| 5 | Wadhurst | Branch | *heidelberg* | + | + | + |
| 6 | Stone Cross | Branch | *heidelberg* | + | + | + |
| 7 | Stone Cross | Trunk | *heidelberg* | + | + | + |
| 8 | Stone Cross | Branch | *heidelberg* | + | + | + |
| 9 | Durgates | Tributary | . | − | − | − |
| 10 | Durgates | Tributary | *heidelberg* | − | + | + |
| 11 | Rotherfield | Trunk | . | − | − | − |
| 12 | Crowborough | Trunk | *paratyphi B* | − | + | − |
| 13 | Durgates | Tributary | . | − | − | − |
| 14 | Durgates | Tributary | . | − | − | − |
| 15 | Durgates | Tributary | . | − | − | − |
| 16 | Durgates | Tributary | . | − | − | − |
| 17 | Durgates | Trunk | . | − | − | − |
| 18 | Durgates | Trunk | *heidelberg* | + | + | + |
| 19 | Durgates | Tributary | *heidelberg* | + | − | − |
| 20 | Sparrows Green | Tributary | *heidelberg* | + | N.T. | + |
| 21 | Sparrows Green | Tributary | *heidelberg* | + | N.T. | N.T. |
| 21 | | | *abony* | + | N.T. | N.T. |
| 22 | Sparrows Green | Tributary | *heidelberg* | + | + | + |
| 23 | Pell green | Branch | . | − | − | − |
| 24 | Turners Green | Tributary | *heidelberg* | + | + | + |
| 25 | Wadhurst | Branch | *heidelberg* | + | + | + |
| 26 | Wadhurst | Branch | *heidelberg* | + | + | + |
| 27 | Wadhurst | Tributary | *heidelberg* | + | + | + |
| 28 | Wadhurst | Tributary | *heidelberg* | + | + | + |

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Table 1 (*cont.*)

| Site no. | Locality | Type of sewer | *Salmonella* types isolated | Media by which isolated: *A* | *B* | *C* |
|---|---|---|---|---|---|---|
| 29 | Wadhurst | Tributary | . | − | − | − |
| 30 | Wadhurst | Tributary | *heidelberg* | + | + | + |
| 31 | Wadhurst | Tributary | *heidelberg* | + | + | + |
| 32 | Turners Green | Tributary | *heidelberg* | + | + | + |
| 33 | Osmers Hill | Branch | *heidelberg* | + | + | + |
| 34 | Pell Green | Branch | . | − | − | − |
| 35 | Cousley Wood | Trunk | *heidelberg* | + | + | − |
| 36 | Durgates | Tributary | *heidelberg* | − | − | + |
| 37 | Sparrows Green | Tributary | *heidelberg* | + | − | + |
| 38 | Sparrows Green | Branch | . | − | − | − |
| 39 | Turners Green | Branch | *heidelberg* | + | + | + |
| 39 | | | *binza* | − | − | + |
| 40 | Pell Green | Branch | *heidelberg* | − | − | + |
| 41 | Wadhurst | Branch | . | − | − | − |
| 42 | Wadhurst | Tributary | *heidelberg* | + | + | + |
| 43 | Durgates | Tributary | *heidelberg* | + | − | + |
| 44 | Best Beech | Trunk | . | − | − | − |
| 45 | Lamberhurst | Private drain | . | − | − | − |
| 46 | Durgates | Tributary | *heidelberg* | + | + | + |
| 47 | Durgates | Tributary | . | − | − | − |
| 48 | Sparrows Green | Tributary | *heidelberg* | + | + | + |
| 49 | Frant | Trunk | . | − | N.T. | − |
| 50 | Bells Yew Green | Trunk | . | − | N.T. | − |
| 51 | Rotherfield | Trunk | *muenchen* | + | N.T. | − |
| 52 | Crowborough | Trunk | *typhimurium* | − | N.T. | − |
| 52 | | | *newport* | − | N.T. | − |
| 53 | East Grinstead | Trunk | . | − | N.T. | − |
| 54 | East Grinstead | Branch | . | − | N.T. | − |
| 55 | East Grinstead | Branch | *typhimurium* | − | N.T. | − |
| 56 | East Grinstead | Branch | *anatum* | + | N.T. | − |
| 57 | East Grinstead | Branch | *anatum* | − | N.T. | − |
| 58 | East Grinstead | Branch | . | − | N.T. | − |
| 59 | East Grinstead | Trunk | *typhimurium* | − | N.T. | − |
| 60 | East Grinstead | Branch | . | − | N.T. | − |

N.T. = not tested.

medium *A* and perhaps also with all the enrichment media used. From site 17 onwards, therefore, sewer swab enrichments were plated after 24 and 48 hr.

Table 2 shows that with medium *A* there was a well-defined peak enrichment period in the vicinity of 48 hr. Of twenty-one sites positive by medium *A* after 48 hr. enrichment, only thirteen were positive after 24 hr. No sites were positive by medium *A* at 24 hr. and negative at 48 hr., but three sites positive at 24 hr. were negative after 72 hr. enrichment. The table provides no evidence of any optimal enrichment time with either medium *B* or medium *C*.

The greater purity of salmonella enrichments in media *B* and *A* respectively over medium *C* is clearly shown in Table 3, although the system used for colony picking tended to minimize differences which might be present. For example, it

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Table 2. *Comparisons between salmonella isolations after plating at 24 and 48 hr. respectively, and after 24 and 72 hr. respectively from each of three enrichment media*

| | | Medium *A* | | | Medium *B* | | | Medium *C* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 hr. | | | 24 hr. | | | 24 hr. | | |
| | | + | − | | + | − | | + | − | |
| 24 and 48 hr. compared | 48 hr. + | 13 | 8 | 21 | 11 | 3 | 14 | 12 | 4 | 16 |
| | 48 hr. − | 0 | 11 | 11 | 2 | 14 | 16 | 4 | 23 | 27 |
| | | 13 | 19 | 32 | 13 | 17 | 30 | 16 | 27 | 43 |
| | | 24 hr. | | | 24 hr. | | | 24 hr. | | |
| | | + | − | | + | − | | + | − | |
| 24 and 72 hr. compared | 72 hr. + | 5 | 0 | 5 | 8 | 1 | 9 | 4 | 2 | 6 |
| | 72 hr. − | 3 | 8 | 11 | 1 | 6 | 7 | 2 | 7 | 9 |
| | | 8 | 8 | 16 | 9 | 7 | 16 | 6 | 9 | 15 |

The results obtained after differing periods of enrichment are set out for each of the three media separately. The three upper blocks of figures relate to comparisons between 24 and 48 hr. enrichment and the lower to 24 and 72 hr. In each of the six blocks, the right-hand column and the lowest line give totals.

A detailed analysis of the block referring to the 24–48 hr. comparison with medium *A* is as follows: Thirteen sites were positive after both periods of enrichment and eleven were negative after both periods. Eight sites negative after 24 hr. were positive after 48 hr. but no sites negative at 48 hr. were positive at 24 hr. A total of thirty-two comparisons were made comprising thirteen sites positive after 24 hr. and nineteen sites negative after this period. The corresponding figures for 48 hr. enrichment are 21 and 11 respectively.

was commonplace for platings from media *B* and *A* respectively to yield large numbers of pickable salmonella colonies apparently in pure, or almost pure, culture and it was nearly as commonplace for the corresponding platings from medium *C* to yield anything from a few likely pickable salmonella colonies to a dozen or two interspersed between much larger numbers of non-salmonella colonies. In such cases, though media *B* and *A* gained the maximum possible scores of 8/8, 6/6 or 4/4 according to the numbers of colonies pickable by the rules, selective colony picking aided by the plate microscope frequently obtained equally or almost equally high scores for medium *C*.

Table 3. *Percentages of colony picks that were salmonellae, made after enrichments in media A, B and C of swabs from forty-five sites cultured in all three media*

(Total numbers of colony picks are shown in parentheses.)

| | Medium *A* | Medium *B* | Medium *C* |
|---|---|---|---|
| Plating after 24 hr. (45 sites) | **51** % (168) | **77** % (166) | **34** % (248) |
| Plating after 48 hr. (30 sites) | **70** % (107) | **55** % (109) | **50** % (119) |
| Plating after 72 hr. (15 sites) | **48** % (42) | **82** % (44) | **17·5** % (40) |
| Combined platings (45 sites): 24 + 48 24 + 72 | **57** % (317) | **70** % (319) | **37** % (407) |

On account of the demonstrated peak enrichment time with medium *A*, column 2 of Table 4 is biased in its favour, whilst columns 1, 3 and 5 of the table carry a contrary bias. Here also the use of a plate microscope aided medium *C* on a number of occasions. These were when exceptionally difficult colony picks were successfully made from medium *C* platings, though only after at least one further plating and repicking (and occasionally two). It was never necessary to attempt these almost impossible picks on platings from the other two enrichment media. When the combined results in columns 4 and 5 of Table 4 are pooled it is seen that in the main investigation salmonellae were isolated from twenty-seven sites by medium *C* and from twenty-five sites by medium *B*. Both media were successful in twenty-three sites. Subdivision of the sites into trunk sewers, main branches and tributaries provides a key to the differing successes of the two media: medium *B* was not the more successful with tributary sewers in any column of the table, though it was more successful with trunk sewers in *all* the columns. As swabs in the trunk sewers presumably encountered larger numbers of salmonellae and certainly a wider range of competing organisms, this difference would be in keeping with what one would expect from media of differing selectivity when the latter is obtained at the price of a decrease in the viability of the salmonellae. No such decrease was however demonstrated experimentally. When 30-tube tests were used for making salmonella counts in fluid media *A*, *B*, *C* and meat infusion broth containing 1% peptone respectively, all the counts were equal within small margins of sampling probability. In an earlier experiment a similar result was obtained when

Table 4. *Success rates of media A, B and C at forty-five sites examined by all three enrichment methods*

| Numbers of sites positive | 1. Sites 1–48 excluding 4, 20, 21 | | | | 2. Sites 17–48 excluding 20, 21 | | | | 3. Sites 1–16 excluding 4 | | | | 4. Sites 17–48 excluding 20, 21 | | | | 5. Sites 1–16 excluding 4 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Plated after 24 hr. | | | | Plated after 48 hr. | | | | Plated after 72 hr. | | | | Combined 24 and 48 hr. platings | | | | Combined 24 and 72 hr. platings | | | |
| | *a* (8) | *b* (15) | *c* (22) | All sites (45) | *a* (4) | *b* (10) | *c* (16) | All sites (30) | *a* (4) | *b* (5) | *c* (6) | All sites (15) | *a* (4) | *b* (10) | *c* (16) | All sites (30) | *a* (4) | *b* (5) | *c* (6) | All sites (15) |
| (*a*) By at least one medium | 4 | 10 | 12 | **26** | 2 | 5 | 14 | **21** | 3 | 5 | 1 | **9** | 2 | 5 | 14 | **21** | 3 | 5 | 1 | **9** |
| (*b*) By all three media | 2 | 8 | 6 | **16** | 0 | 4 | 6 | **10** | 0 | 3 | 0 | **3** | 1 | 4 | 10 | **15** | 2 | 5 | 0 | **7** |
| (*c*) By medium *A* | 3 | 8 | 8 | **19** | 2 | 4 | 13 | **19** | 1 | 4 | 0 | **5** | 2 | 4 | 13 | **19** | 2 | 5 | 0 | **7** |
| (*d*) By medium *B* | 4 | 9 | 8 | **21** | 2 | 4 | 8 | **14** | 3 | 5 | 1 | **9** | 2 | 4 | 10 | **16** | 3 | 5 | 1 | **9** |
| (*e*) By medium *C* | 2 | 9 | 10 | **21** | 0 | 5 | 10 | **15** | 1 | 4 | 1 | **6** | 1 | 5 | 13 | **19** | 2 | 5 | 1 | **8** |

Key: *a* = trunk sewers; *b* = main branches; *c* = tributaries.

Note. Numerals in parentheses refer to the total number of sampling manholes in each category.

15-tube counts were made in Lemco broth and in medium *C* containing added salmonella-negative faeces (Table 6).

There can be little doubt that these tetrathionate media sustain the growth of minimal inocula of salmonellae. If so, apparent failures of tetrathionate enrichments must seem to be due either to sampling error or to failures by the salmonellae, before the nutrients necessary for multiplication have been exhausted by their competitors, to have *multiplied sufficiently* to make their presence known when a

Table 5. *Comparative results from enrichments in medium A plated after 48 hr. and in medium C plated both after 24 and 48 hr. (43 sites)*

| Numbers of sites positive | 1 Trunk sewers (10) | 2 Main branches (16) | 3 Tributaries (17) | 1+2+3 (sites 17–60 excluding 21) (43) |
|---|---|---|---|---|
| By medium *A* (48 hr. platings) | 3 | 5 | 14 | **22** |
| By medium *C* (combined 24 and 48 hr. platings) | 1 | 5 | 14 | **20** |
| By both media | 1 | 4 | 13 | **18** |

Table 6. *Showing relative numbers of tubes positive in salmonella counts made in various fluid media*

| | 30-tube tests (24 hr. incubation) | | | | 15-tube tests (48 hr. incubation) | |
|---|---|---|---|---|---|---|
| Relative sizes of inocula | Medium *A* | Medium *B* | Medium *C* | Meat infusion broth with 1% peptone | Medium *C* (with faeces) | Lemco broth |
| 10 | 10/10 | 10/10 | 10/10 | 10/10 | 4/5 | 4/5 |
| 1 | 7/10 | 6/10 | 5/10 | 9/10 | 3/5 | 3/5 |
| 0·1 | 1/10 | 1/10 | 1/10 | 1/10 | 1/5 | 0/5 |

single loopful of the enrichment is plated out. In order to illustrate this point it is convenient to over-simplify the position by making certain assumptions.

(1) That on a particular occasion a 0·5 ml. inoculum was added to 19·5 ml. of a tetrathionate medium.

(2) That the inoculum contained one salmonella and 10 million of a competitor which the salmonella was capable of outgrowing in the tetrathionate medium.

(3) That bacterial multiplication in the medium virtually ceased when the viable count reached 200 million/ml.

(4) That for successful detection by plating on the solid selective medium in use, at least 10 salmonellae had to be present in a 0·02 ml. loopful containing (by assumption 3) a total of four million organisms.

For successful isolation in this case it is therefore necessary for the count of

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salmonellae in the fluid medium at the time of plating to be not less than 500/ml.; that is to say there must be a 10,000-fold increase in the salmonella count within the time taken for a 400-fold increase in the competing organism. Expressed in terms of generations of multiplication, the salmonella must produce between thirteen and fourteen generations in the time taken for its competitor to produce eight or nine generations. Let us now assume either an eightfold increase in the original inoculum or an eightfold decrease in the volume of the enrichment medium. In either case for successful isolation the salmonella must now produce ten or eleven generations of multiplication within the time taken for its competitor to produce five or six generations—clearly a more difficult task for the salmonella.

McCoy (personal communication) has found that when differing quantities of samples of bone meal are enriched in a standard volume of fluid medium, salmonellae are frequently isolated from the smaller quantities although apparently absent from the larger. The thesis outlined above offers an explanation for this observation. A similar explanation might apply in the failures by medium *B*, when medium *C* succeeded, if the higher selectivity induced by the additives in medium *B* (which apparently do not reduce the viability of salmonellae) increases the generation times of organisms growing in it by amounts which vary proportionately less than the original variations in generation times in the basal tetrathionate medium. This would cause a modification in the *ratio* of generation times of salmonellae and their competitors in favour of the competitors. Further experimental evidence is required on this point.

In the hope of obtaining more information during the subsidiary investigation concerning the general concept which has been put forward, quantities which varied between 0·25 and 1 ml. were taken from twelve discarded 'negative' tetrathionate *C* enrichments and subcultured overnight in 25 ml. amounts of medium *B*, to allow any salmonellae which might have been present to increase their relative numbers during further generations of competitive multiplication. This secondary enrichment procedure, which is the subject of further investigation, was rewarded with an unexpected degree of success: salmonellae were isolated from no less than six of the secondary cultures. (Two of these six sites had already been positive by the medium *A* enrichments, Table 1.) On theoretical grounds a secondary enrichment should have an effect similar to that which would be produced by a large increase in the volume of the primary enrichment fluid, without a corresponding increase in the size of the initial inoculum. Though in this case the secondary enrichments were made in a different medium, it seems very probable that secondary enrichments in the primary enrichment medium would also have met with success.

Thomson (1954) has observed a dilution phenomenon when plating suspensions of faeces. Though numerous salmonella colonies grew from smaller inocula of faeces, he observed that they failed to grow from the relatively larger inocula. His full observation could be explained by a combination of two factors as follows:

(*a*) The concentrated faecal suspensions contained substances which were toxic under some of the conditions of his tests.

(*b*) The selectivity of his solid media was overwhelmed by the presence of very

large numbers of organisms: under these conditions they behaved as unselective media.

The further dilution, during the secondary enrichment procedure, of toxic substances which might have been present in the primary sewer swab enrichments cannot be wholly excluded as a possible factor also contributing to the success of the secondary enrichments. If, however, this were the case it would also be necessary to postulate that either these toxic substances were bacteriostatic in the presence of the solid medium but not in tetrathionate broth (medium *C*), or else that the salmonellae were initially present in the swabs in sufficiently large numbers to survive the dilution effect of subculturing 1 ml. or less from the original 200 ml. of fluid. Neither of these latter postulates seems very probable.

Additional observations made during the subsidiary investigation concerning 'straight' enrichments in media *A* and *C* respectively have been pooled with information gained during the main investigation, and set out in Table 5. Apart from the greater ease in picking salmonella colonies from the medium *A* enrichment platings, single platings from this medium at 48 hr. were positive in two sites more than from the combined 24 and 48 hr. platings from medium *C*. Tables 4 and 5 appear to show that the procedures used for isolating salmonellae during this investigation are less successful in trunk sewers than in the branches and tributaries. While it is quite probable that failures in isolation occurred, which during the main investigation might have been avoided by the use of secondary enrichments, and it is possible that such failures might occur more readily with swabs from the larger sewers, it is unwise to carry this inference far owing to the fact that the sampling points were not randomly selected. Thus it was usual to sample tributaries and branches only after salmonellae had been isolated from larger sewers below them.

One of the aims of the investigation was to ascertain the extent to which multiple picking of salmonella colonies might be rewarded by multiple salmonella isolations from sewer swabs. In this investigation double salmonella isolations were obtained from only three sites in spite of a total of 584 salmonellae picked from platings from thirty-nine positive sites, an average of fifteen per site. What is more significant is that if colony picking had been restricted to three picks per plate there would have been no fewer isolations of salmonellae. This was true not only of the aggregate of plates examined from a single sample, but also of each individual plate. That is to say, all the salmonella types isolated from any one plate were invariably represented in the first three colony picks from that plate. While this rather remarkable finding would not necessarily hold in other sewer swab investigations, it does provide an indication that with the techniques here used picking large numbers of colonies from individual plates is not likely to be very profitable. It would not be wise to apply the same inference to other materials, for example bone meal. The reason for the finding may not have been a lack of swabs containing multiple salmonella types. An alternative explanation could be that conditions prevailed in the fluid enrichment media which favoured the emergence of one salmonella strain, and sometimes two, in heavy numerical predominance over the remainder.

SUMMARY

Either triplicate or paired sewer swabs put down at sixty manholes were examined after 24, 48 or 72 hr. periods of enrichment at 37° C. in a standard tetrathionate medium (medium *C*) and in two new tetrathionate modifications—medium *A* being a combination of tetrathionate broth and ingredients of Wilson and Blair's medium, and medium *B* similar but containing only half the amount of the Wilson and Blair's ingredients, and in addition 1 % sodium lauryl sulphate.

Salmonellae were isolated from thirty-nine sites: there were three double isolations of salmonellae.

The richest growths of salmonellae were obtained from medium *B* and the poorest from medium *C*. Medium *C* however yielded more isolations from the smaller sewers than medium *B*: the converse held true in the trunk sewers.

An optimal 48 hr. enrichment time for sewer swabs was demonstrated in the case of medium *A*. No optimal time was found for either of the other two media. Platings at the optimal enrichment time from medium *A* yielded more salmonella isolations than the combined 24 and 48 hr. platings from medium *C*.

The dynamics of enrichment culture have been discussed.

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*Note.* After this paper was written an investigation was begun on the modes of action of media *A* and *B*. Pure cultures of salmonellae were inoculated into them and counts were made after various times. In both media the counts reached their peaks within 24 hr. and then declined fairly rapidly. It is at present a matter of speculation whether or not during the phase of salmonella decline competitors are eliminated from these media still more rapidly. If so, one should expect a short lived optimal time for plating out from each of the two media. When however organic material, for example 1 % sterilized bone meal, was previously added to the media, the decline in salmonella counts after 24 hr. was much more gradual. This might explain why, in contradiction with the earlier bone meal findings, platings from medium *A* after 72 hr. were frequently unsuccessful, and why medium *B* had its least success with the cleaner swabs, that is, with swabs received from the tributary sewers. The incorporation of sterilized bone meal with chalk in the jars might thus well have increased the value of media *A* and *B* during this investigation.

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## APPENDIX

### *Preparation of tetrathionate modifications* A *and* B

Both media are made from a common base prepared as follows:

| | | |
|---|---|---|
| Glucose | | 20 g. |
| Lab. Lemco | | 20 g. |
| Peptone (Evans) | | 20 g. |
| Sodium chloride | | 10 g. |
| Di-sodium-mono-hydrogen phosphate (anhydrous) | | 4·7 g. |
| Tap water | ad | 1000 ml. |
| Powdered chalk | | q.s. |

The basal medium is adjusted to pH 7·5 and sterilized by autoclaving at 10 lb. pressure for 10 min.

The following additional reagents are required:

*Lauryl sulphate solution.* 20% aqueous solution of sodium lauryl sulphate (Hopkin and Williams) dissolved with aid of heat. When cool the pH is adjusted with caustic soda to 7·5 by use of a pH meter. (Phenol red indicator may alternatively be used but other indicators are unsuitable.) Sterilized by steaming for 15 min.

*Bismuth sulphite suspension.* 0·25% aqueous suspension of 'Oxoid' Wilson and Blair granules B prepared freshly before use by bringing to boil for 1 or 2 min., then cooling.

Iodine solution
Thiosulphate solution } Prepared according to Rolfe (1946).

The various reagents are added to the basal medium aseptically in the proportions set out below:

| | Basal medium | Sterile Aq. dest. | Bismuth sulphite suspension | Iodine solution | Thio-sulphate solution | Lauryl sulphate solution |
|---|---|---|---|---|---|---|
| Medium *A* (ml.) | 50 | 40 | 10 | 5·5 | 11·0 | Nil |
| Medium *B* (ml.) | 50 | 35 | 5 | 5·5 | 11·0 | 5 |

*Notes*

(1) 'Oxoid' Wilson and Blair Granules B are stated by the makers to contain the following ingredients in 18 g.:

| | |
|---|---|
| Bismuth ammonium citrate | 3 g. |
| Sodium sulphite anhyd. | 5 g. |
| Sodium phosphate anhyd. | 5 g. |
| Glucose bact. grade | 5 g. |

(2) The tetrathionate concentration in medium *B* is slightly higher than in medium *A*.

(3) The bismuth sulphite suspension is an extremely fine one and settles out very slowly: the precaution of shaking should nevertheless not be omitted before adding and distributing.

(4) At room temperature a white precipitate, which rapidly disappears on warming, slowly occurs in the sodium lauryl sulphate solution. This solution should therefore be slightly warm when added. It should also be added last.