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THE SELECTIVE ACTION OF TETRATHIONATE IN BACTERIOLOGICAL MEDIA

A REPORT TO THE MEDICAL RESEARCH COUNCIL

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(With Plate 2 and 1 Figure in the Text)

INTRODUCTION

Tetrathionate broth was first recommended by Muller (1923) as a selective medium for isolating *Bact. typhosum* and other organisms of the Salmonella group. In his original experiments, which were done with pure cultures, Muller used a medium containing sodium tetrathionate prepared by mixing solutions of iodine and sodium thiosulphate in the exact proportions required by the equation

$$2Na_2S_2O_3.5H_2O + I_2 = Na_2S_4O_6 + 2NaI + 5H_2O. \quad (1)$$

He found that whereas *Bact. typhosum* grew in a medium containing 1·2% (=0·044 *M*) of this 'balanced' tetrathionate, *Bact. coli* was inhibited by as little as 0·4% (=0·015 *M*). Finally, however, instead of a medium containing plain sodium tetrathionate with no excess thiosulphate, he recommended for practical use one containing an initial concentration of thiosulphate five times greater than that with which the iodine could react to form tetrathionate. The final concentration of thiosulphate ($Na_2S_2O_3.5H_2O$) in his medium is 4% (=0·16 *M*) and of tetrathionate approximately 0·5% (=0·018 *M*). He also added 5% chalk 'to fix small quantities of SO_2 which might be produced by decomposition of the tetrathionate'.

Muller did not test his media on a large scale with actual specimens, but subsequent workers have amply confirmed the value of his discovery (Schäfer, 1934–5; Seidenglanz, 1932; Schustowa, 1931; Holt, Vaughan & Wright, 1942; Hynes, 1942; Knox, Gell & Pollock, 1942; and others). It is generally recognized that the use of tetrathionate liquid enrichment media greatly increases the number of positive results as compared with direct plating on to MacConkey or Endo agar, and that in nearly all Salmonella infections it gives more positive results than either direct plating on to selective media or enrichment in liquid media containing plain brilliant green. With *Bact. typhosum*, however, it is not so satisfactory, and may even miss specimens found positive by direct plating on to Wilson & Blair's and other selective media. Another disadvantage is that members of the *Proteus* group, especially *Pr. morgani*, grow heavily in tetrathionate broth and frequently overgrow pathogens. Kauffmann (1930–1, 1935–6) devised a modification of Muller's tetrathionate broth which contained in addition to tetrathionate 5% ox bile and 1/100,000 brilliant green; but even this medium fails to inhibit *Proteus* effectively, though it is more inhibitory to most organisms than Muller's. It also gave only a 30% increase for *Bact. typhosum* over the number of positive results obtained by direct plating on Endo agar, and missed a certain proportion of specimens found positive by direct plating. Muller and subsequently Schustowa and Seidenglanz recommended tetrathionate agar for direct plating, and in this country Knox *et al.* have used a modified tetrathionate agar for both direct and indirect plating in conjunction with tetrathionate broth.

None of the modifications recommended by different workers has succeeded in overcoming the main defects of all tetrathionate media—the inadequate results with *Bact.*

typhosum and the liability to overgrowth with *Proteus*. It was partly for this reason that we became interested in the chemical and biological properties of tetrathionate.

Preliminary observations

(1) *Media of Muller's type*

Muller's tetrathionate broth when freshly prepared consists of a clear supernatant fluid with a heavy deposit of chalk. Its *p*H is about 8·0. On incubation at 37° C., or on prolonged standing at room temperature, it becomes opalescent and goes slowly acid. Kept in the ice-chest, however, it remains stable for weeks and the *p*H does not drop much below 8·0. Without chalk acidity develops more rapidly. When the medium is inoculated with a pure culture of *Bact. paratyphosum* B it is found after incubation for a few hours to have become strongly acid, and bubbles of CO_2 can be seen to be evolved from the chalk. The abundant H_2S production shown by *Bact. paratyphosum* B in plain broth is greatly delayed and may even be entirely suppressed. On prolonged incubation the medium becomes more alkaline and after 3 or 4 days may reach the original *p*H of about 8·0. The same changes occur with most other members of the genus *Salmonella* and with *Proteus*. A solid medium containing similar concentrations of sodium thiosulphate and tetrathionate is clear when freshly prepared, but on incubation or on prolonged storage at room temperature develops a uniform opacity, at the same time becoming increasingly acid. A confluent or semi-confluent growth of *Bact. paratyphosum* B on this medium usually shows a slight increase in alkalinity, but separate colonies give an acid reaction. The medium round the growth, after having originally gone opaque, becomes clear, and areas of 'lysis' extending for several millimetres round separate colonies make a sharp contrast to the increasing opacity of the surrounding medium (Pl. 2, fig. 2). 'Lysis' may be apparent after 18 hr. incubation but is usually not fully developed until after 40 hr. It is shown by *Bact. paratyphosum* B and by all the other common *Salmonella* strains we have met, but not by *Bact. typhosum* until after several days' incubation. Colonies of *Bact. typhosum* are clear and small, sometimes only pinpoint after 18 hr. incubation, but usually 1 mm. after 24 hr. *Proteus*, as well as *Salmonella*, shows good 'lysis', but the colonies are flatter and smoother and usually have a characteristic smell.

(2) *Media containing 'balanced' tetrathionate*

The changes described were striking enough to demand further investigation. The fact that no such changes occurred either in uninoculated tetrathionate media or in plain broth or thiosulphate broth inoculated with *Bact. paratyphosum* B strongly suggested that it was the tetrathionate in Muller's medium which was being acted upon during bacterial growth. Further positive evidence of this was provided by experiments with media containing balanced tetrathionate with no excess thiosulphate. Such media show a somewhat different picture. A liquid medium remains clear when not inoculated, even on prolonged incubation, unless the amount of tetrathionate is increased considerably above $0{\cdot}03M$ ($=0{\cdot}81\%$): if no chalk or buffer is included it rapidly becomes acid, in the same way as media of Muller's type in the absence of chalk. On inoculation with a culture of *Bact. paratyphosum* B or allied organisms the unbuffered medium becomes very rapidly acid as growth proceeds, and no H_2S is ever produced. If, however, a buffer is added, H_2S production can be demonstrated after 12–18 hr. incubation. A solid medium of the same formula is clear when freshly made and even after prolonged incubation never develops more than slight opacity. It does, however, become slowly acid. A confluent or semi-confluent growth of *Bact. paratyphosum* B shows a slight change to the alkaline side, though typical separate colonies give an acid reaction. In size these may reach 3 mm. in 18–24 hr. and they show crenated edges and irregular strands of opaque material. Colonies of *Bact. typhosum* are smaller than colonies of Salmonellas but much larger than on media containing excess thiosulphate. Instead of the 'lysis' described above, Salmonella colonies are surrounded by a zone of gradually increasing opacity, but usually this is first visible only after 40 hr. incubation.

Investigation of different constituents of Muller's broth

Muller's tetrathionate broth contains in addition to the basal medium four simple inorganic substances—thiosulphate, tetrathionate, iodide (potassium and sodium) and chalk. Either singly or together these must be responsible for the selective action of tetrathionate

media. The preliminary work outlined in the preceding descriptive account showed that media containing balanced tetrathionate differ considerably from media such as Muller's containing less tetrathionate with a large excess of thiosulphate, but it was still not clear what was the relative importance of the different constituents in determining the selective action.

(1) *Effect of thiosulphate and tetrathionate*

To investigate this, freshly isolated strains of faecal pathogens and of some common non-pathogens were used. Suitable dilutions of broth cultures were made and solid and liquid media inoculated. In liquid media growth was judged by the opacity developed, and on solid media by the number, size and rate of development of colonies. Sodium thiosulphate was made up as an accurate molar solution (24·8 g. $Na_2S_2O_3.5H_2O$ made up to a final volume of 100 c.c. with distilled water), and iodine as a 0·5 *M* or 'normal' solution (12·7 g. iodine with 20 g. potassium iodide made up to a final volume of 100 c.c. with distilled water). The inoculum usually consisted of about 100 organisms. Text-fig. 1 shows in graphic form the effect of different initial concentrations of thiosulphate and iodine in liquid media on a number of intestinal pathogens and non-pathogens. The approximate limits within which moderately good growth of the different organisms occurs (as judged by the opacity developed after 18–24 hr. incubation) are shown by the appropriately labelled sloping lines. The abscissa indicates concentrations of iodine, the ordinate concentrations of thiosulphate. The sloping line *A* represents media in which thiosulphate and iodine are present in the proportions required to produce balanced tetrathionate according to the chemical equation: below this line is the region of excess iodine and above it the region of excess thiosulphate. Text-fig. 1 represents the concentrations of thiosulphate and iodine which *would* be present if no reaction occurred: for example, Muller's medium (*M* in Text-fig. 1) would contain 0·2 *M* thiosulphate and 0·02 *M* iodine if the two substances did not react. It can be seen that the final concentrations, however, are 0·16 *M* thiosulphate and 0·02 *M* tetrathionate. To make clear exactly how the lines representing limiting concentrations for growth are arrived at we will give the results obtained in one experiment with a culture of *Bact. paratyphosum* B: an inoculum of 200 organisms grew well in 18 hr. in the following media: 7, 10, 11, 15, 16, 17, 20, 21, 22, 34, 35*a*, *M*, 37, 43, 44, 49*a*, 50, 55*a*, 55*b* and 55*c*, poorly in 8 and 38, and not at all in 1, 2, 3, 4, 5, 6, 9, 12, 23, 39, 45, 51, and 55*d*.

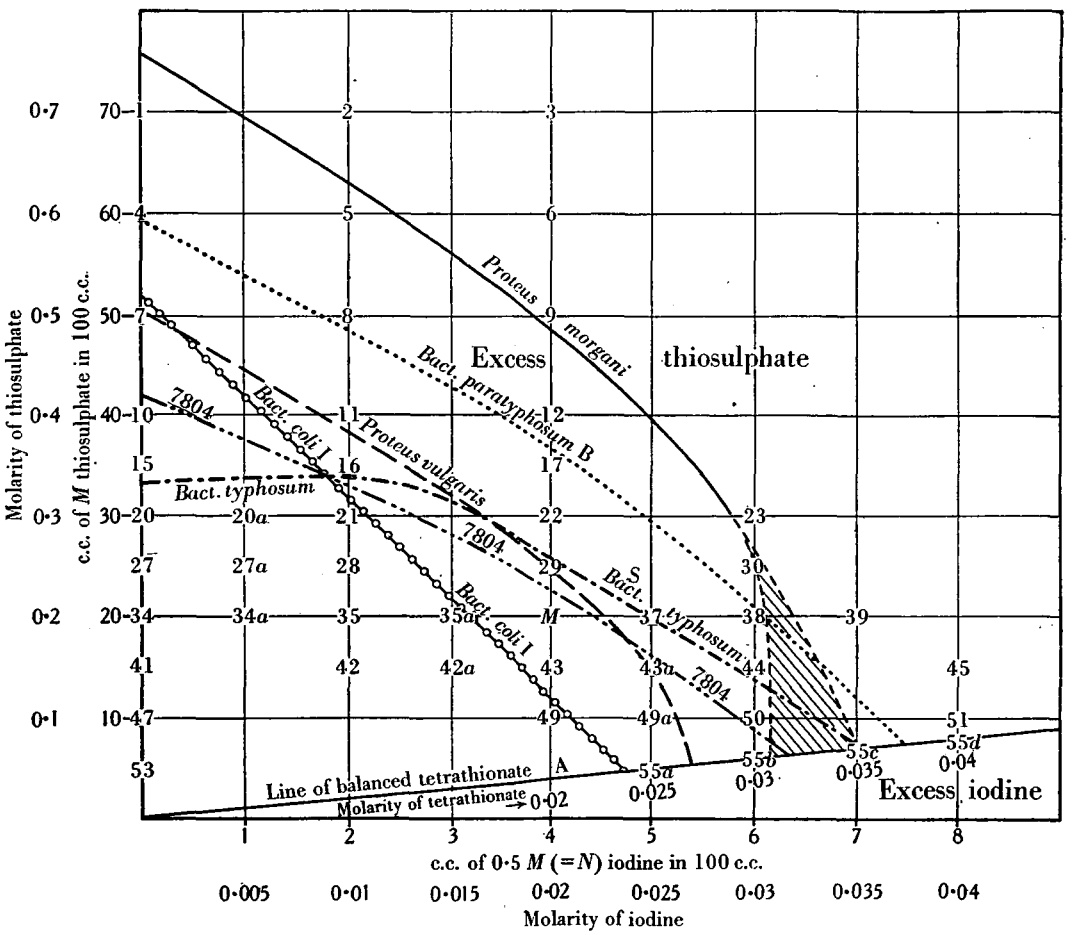
Text-fig. 1 is of considerable value in the construction of tetrathionate media in practice. Different results are to be expected when different basal media are used, and if a routine medium is found to be too inhibitory or not inhibitory enough it is easy by consulting Text-fig. 1 to gauge how much it is safe or desirable to alter the concentration of thiosulphate or iodine. It can be seen also that comparatively small alterations in either of these, but particularly in the iodine, may make critical changes in the selectivity of the medium. A study of the methods used by different workers reveals considerable variations even in what is supposedly the same medium: the method of preparing the thiosulphate and iodine solutions is often ambiguously stated or wrongly quoted. For example, Kauffmann's medium is often quoted as though it were the same as Muller's medium with the addition of brilliant green and bile. Reference to Kauffmann's original papers shows that in fact the final concentration of iodine in his medium is very considerably less than in Muller's. A 50% solution of thiosulphate is sometimes stated as being one containing 50 g. of $Na_2S_2O_3.5H_2O$ in a final volume of 100 c.c. of water, whereas other workers have taken this to mean a solution made by adding 50 g. of $Na_2S_2O_3.5H_2O$ *to* 100 c.c. of water (producing a difference of about 23%). These facts, taken together with the variations to be expected when different basal media are used, may account for conflicting reports as to the efficacy, say, of Muller's medium (*M* in Text-fig. 1) and of Schäfer's medium (*S* in Text-fig. 1). Text-fig. 1 also makes it clear within what sort of limits it is permissible to vary the medium used according to the nature of the examination which is being made. For example, when the examination is specifically for a resistant organism such as *Bact. paratyphosum* B a much more inhibitory formula can be used than is advisable for routine purposes. A medium such as 44 can be constructed at short notice: this method gave successful results in the examination of a water supply for *Bact. paratyphosum* B (Jones, Gell & Knox, 1942).

It must be noted that the lines in Text-fig. 1 indicating limiting concentrations of thiosulphate and tetrathionate for different organisms represent the average of many different experiments. Media containing tetrathionate with high concentrations of thiosulphate (e.g. medium 11) are extremely unstable and spontaneously deposit sulphur and become acid: it is therefore difficult to make even the initial *p*H

of all media in an experiment the same. The precise limits within which growth of different organisms occurs vary with the strain, with the size of inoculum, with the period of incubation and with the basal medium used. Text-fig. 1 therefore does not give more than a very general picture of the sensitivity of different organisms to thiosulphate and tetrathionate. In spite of this, it throws considerable light on the part played by these two constituents of tetrathionate media. The main points may be summarized:

(1) Sodium thiosulphate by itself is not suitable as a selective agent for practical use, since *Proteus morgani* grows in high concentrations which inhibit even *Bact. paratyphosum* B, and *Bact. typhosum* especially is relatively sensitive to thiosulphate.

(2) Even a slight excess of iodine suppresses all the organisms tested.



Text-fig. 1. Effect of different mixtures of thiosulphate and tetrathionate on faecal organisms.
M = Muller's medium; *S* = Schäfer's medium.

(3) In general, small increases in iodine (i.e. tetrathionate) are more critical and more rapidly produce an inhibitory effect than equivalent increases in thiosulphate.

(4) A medium containing excess thiosulphate with a comparatively small amount of tetrathionate is highly selective but is rather inhibitory for *Bact. typhosum* and not sufficiently inhibitory for *Proteus*.

(5) A medium containing balanced tetrathionate in a concentration of about 0·03 *M* gives good growth of *Bact. typhosum* and all Salmonellas tested combined with inhibition of several resistant non-pathogens including *Proteus vulgaris*. *Pr. morgani*, however, is still not adequately suppressed, though sometimes heavy inocula have failed to grow in 18 hr. in 55*a* when *Bact. typhosum* grew well. This erratic growth of *Proteus morgani* is indicated by the broken lines in Text-fig. 1.

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With solid media similar results have been obtained. Agar plates containing varying initial amounts of thiosulphate and iodine were prepared. The media tested were 21, 35*a*, 43, 49*a* and 55*b*. As the concentration of thiosulphate diminished colonies of *Bact. typhosum* were found to increase in size, despite the increasing concentration of tetrathionate, and were largest in the balanced medium 55*b*. Colonies of *Proteus* and of organism 7804 (a paracolon bacillus in some respects similar to *Bact. asiaticum*), however, became progressively smaller, until in medium 55*b* *Proteus vulgaris* and 7804 were completely suppressed, and even *Pr. morgani* was partially inhibited.

(2) *Effect of iodide*

Muller recognized the importance of the iodides and in his final medium recommended a reduction in the amount of tetrathionate because iodide had a toxic effect upon *Bact. typhosum*. We have found that the amount of potassium iodide used in Muller's medium is not enough to dissolve the iodine completely. We have therefore nearly doubled the proportion of potassium iodide to iodine. Because it is necessary to add potassium iodide in order to dissolve the iodine and because two molecules of sodium iodide are formed for every molecule of tetrathionate produced by interaction of sodium thiosulphate and iodine, it is clear that every increase in tetrathionate is inevitably accompanied by a proportionate increase in total iodide, and this must be borne in mind in ascribing any particular effect to tetrathionate itself. We have in fact done a limited number of experiments using separately iodide and pure iodide-free tetrathionate. Columns (*a*) to (*d*) of Table 1 show the effects of the separate constituents of Muller's broth on different organisms. It can be seen that tetrathionate and iodide have a separate and different selective action, though only in concentrations a good deal higher than when they are combined in tetrathionate media prepared from thiosulphate and iodine. For instance *Bact. paratyphosum* B will grow well in 0·1 *M* pure tetrathionate and in 0·25 *M* plain iodide, but grows poorly, if at all, in any higher concentration than 0·035 *M* of tetrathionate prepared in the ordinary way (containing approximately 0·125 *M* iodide): similarly, organisms such as 7804, which are particularly sensitive to plain iodide and therefore to low concentrations (e.g. 0·025 *M*) of ordinary iodide-containing tetrathionate, grow well in 0·04 *M* pure tetrathionate (Table 1).

(3) *Effect of chalk*

Text-fig. 1 represents media containing no chalk. We have found that addition of chalk makes a considerable difference to the direction and position of the lines representing limits of growth for different organisms. With *Bact. typhosum* chalk makes any given medium more inhibitory, with *Proteus* it has the opposite effect. The reason for this will be discussed later.

CHEMICAL PROPERTIES OF THIOSULPHATE AND TETRATHIONATE

At this stage the relative importance of the different constituents had been broadly defined, and the conclusion reached that the tetrathionate was probably the most important single constituent. Further progress could clearly not be made without some knowledge of the chemical properties of tetrathionate and thiosulphate.

Sodium thiosulphate is relatively stable, but sodium tetrathionate, prepared in the ordinary way from thiosulphate and iodine, though stable in dilute solutions is extremely unstable in high concentrations or at high temperatures. A 0·2 *M* aqueous solution of sodium tetrathionate prepared from iodine and thiosulphate if left at room temperature for a few hours goes acid, deposits sulphur and smells strongly of SO_2. A molar solution decomposes similarly in a few minutes. This change is speeded up in the presence of thiosulphate which apparently acts as a catalyst. Iodide also accelerates this decomposition: we have found that iodide-free tetrathionate is very much more stable. Thiosulphate is oxidized to tetrathionate by oxidizing agents (e.g. iodine) or by electrolysis, while tetrathionate can be rapidly reduced to thiosulphate (e.g. by hydrogen in the presence of platinum or palladium, and by sodium). H_2S reacts with tetrathionate and the two substances cannot coexist. Some of the changes which have been described above as occurring in tetrathionate media can be explained with a little knowledge of the chemical properties of tetrathionate and thiosulphate. The development of acidity and opacity in some uninoculated media can be explained by the known instability of tetrathionate in high concentrations, especially in the presence of excess thiosulphate. The apparent inhibition of H_2S production with organisms known to produce H_2S

Table 1

Organism	Molarity of limiting concentration for good growth in 20 hr.				Comparative heaviness of 40 hr. growth aerobically in		
	Tetrathionate made from thiosulphate and iodine (*a*)	Iodide-free tetrathionate (*b*)	Iodide (*c*)	Thio-sulphate (*d*)	0·1 *M* buffer in broth (*e*)	0·1 *M* buffer plus 0·02 *M* tetrathionate in broth (*f*)	Tetra-thionate reduction (*g*)
Bact. paratyphosum B	0·035	0·105	0·215	0·5	+ +	+ + + +	+
Bact. typhosum	0·0325	0·1	0·22	0·25	+ +	+ + +	+
Proteus vulgaris	0·025	0·095	0·27	0·3	+ + +	+ + + +	+
Pr. morgani	0·03	0·105	0·3	0·7	+ + +	+ + + +	+
Bact. paratyphosum A	0·02	0·06	0·17	.	+ ±	+	−
GSM 7804 } species of 'paracolon' group	0·035	0·11	0·19	0·4	+ + + +	+ + ±	−
	0·03	.	0·16	0·4	+ + + +	+ +	−
1433 (lactose fermenter of 'intermediate' group)	0·03	0·075	0·25	0·5	+ +	+ + + +	+
Bact. coli I	0·02	0·065	0·175	.	+ + +	+ ±	−

In columns (*e*) and (*f*) + signs indicate relative heaviness of growth in 40 hr. as judged by opacity.

Table 2. *Reduction of tetrathionate by a growing culture of* Bact. paratyphosum *B*

	Time in hours											
	0	1	2	3	4	5	5½	6	6½	7	7½	
Tetrathionate buffer medium inoculated with *Bact. paratyphosum* B	0·15	0·2	.	0·3	0·95	3·75	6·1	8·3	10·0	10·3	10·4	Titration value of 5 c.c. with 0·01 *N* iodine
	0	0	tr.	+	+ +	+ + +	+ + + +	+ + + +	+ + + +	do.	do.	Growth
	−	−	−	−	−	−	−	−	tr.	+	+ + +	H_2S
Plain buffer medium inoculated with *Bact. paratyphosum* B	0	0	tr.	+	+ +	+ + +	+ + + ±	+ + + ±	+ + + +	do.	do.	Growth
	−	−	−	−	−	−	tr.	+ ±	+ +	+ + +	+ + +	H_2S
Uninoculated tetrathionate buffer medium	0·15	.	.	.	.	.	.	.	.	.	0·4	Titration value of 5 c.c. with 0·01 *N* iodine

freely in plain broth can also be explained by the known reaction between H_2S and tetrathionate. But it was already clear from the preliminary work described earlier that some further explanation must be sought for the changes which occur in tetrathionate media in the presence of growing organisms. To investigate these it was necessary to find out by quantitative analysis what happens to the tetrathionate and thiosulphate in both inoculated and uninoculated media.

Quantitative analyses of polythionates, thiosulphate and sulphite were made by the methods of Kurtenacker & Bittner and Kurtenacker & Wollack as quoted by Starkey (1935). In uninoculated media, it was found that solutions of tetrathionate are less stable than solutions of corresponding molarity in water, but in the concentrations used in bacteriological work, solutions of tetrathionate in broth are moderately stable. For example, a 0·01 *M* solution in broth undergoes no significant decomposition if kept for 6 days at 37° C.: even a 0·03 *M* solution (as in medium 55*b*) loses only about 5% of its tetrathionate in 48 hr. at 37° C.

BACTERIAL REDUCTION OF TETRATHIONATE

Our first experiments with inoculated media were done with cultures of *Bact. paratyphosum* B. It was found that in nutrient broth containing plain sodium thiosulphate there was very little change in the amount of thiosulphate after incubation for several days. In tetrathionate broth, however, it was discovered that the iodine value of the culture rapidly increased and the tetrathionate correspondingly disappeared.

Table 2 shows a typical experiment with *Bact. paratyphosum* B. Two 100 c.c. lots of media were prepared, one containing 0·01 *M* pure sodium tetrathionate with 0·1 *M* phosphate buffer *p*H 7·7 in broth, the other containing buffer in broth without tetrathionate. Each lot was inoculated with 0·5 c.c. of an 18 hr. culture of *Bact. paratyphosum* B and incubated at 37° C. 5 c.c. samples were removed at intervals and after the addition of 2 c.c. of 10% acetic acid and about 1 c.c. of 0·5% starch were titrated to a permanent blue end-point with 0·01 *N* iodine. The amount of growth was noted macroscopically and lead acetate papers in the mouths of the flasks indicated H_2S production. A control uninoculated tetrathionate medium incubated at the same time showed only an insignificant increase in iodine value over the whole period of the experiment. Further investigations confirmed that *Bact. paratyphosum* B and certain other organisms have the power to reduce tetrathionate rapidly and quantitatively to thiosulphate. The equation for this reaction appears to be

$$Na_2S_4O_6 + 2H = Na_2S_2O_3 + H_2S_2O_3. \qquad (2)$$

The reaction can be demonstrated either with cultures growing in a nutrient medium, or more precisely with thick washed suspensions. It can be seen from Table 2 that a limiting value (about 10·0 c.c. of 0·01 *N* iodine) is reached; this corresponds to the maximum theoretical amount of thiosulphate that could be formed in the reduction of 0·01 *M* tetrathionate according to equation (2). With washed suspensions it can be demonstrated that the hydrogen atoms in equation (2) are made available by dehydrogenation of hydrogen donators such as mannitol or glucose. A short note on this has already been published (Pollock, Knox & Gell, 1942) and a more detailed account will follow.

There is no doubt that other reactions may also occur; for instance, thiosulphate itself is reduced to H_2S, but this must be a very slow process since, as mentioned above, there is little detectable diminution in thiosulphate even after several days' incubation of a growing culture. Tetrathionate reduction, because of its speed and completeness, must be the most important reaction so far as bacterial metabolism in tetrathionate media is concerned. It is significant that not all bacteria are able to reduce tetrathionate and with few exceptions tetrathionate-reducing organisms can grow in higher concentrations of tetrathionate than non-reducers (Table 1, columns (*b*) and (*g*)). *Bact. paratyphosum* B and most other common Salmonellas and members of the *Proteus* group are powerful tetrathionate reducers, and *Bact. typhosum* a good deal less powerful, while *Bact. paratyphosum* A, *Bact. coli*, *Bact. aerogenes*, dysentery bacilli and most other faecal organisms are completely unable to reduce tetrathionate. Table 1, however, shows that one organism of the paracolon group (GSM), although unable to reduce tetrathionate, is capable of growing in high concentrations to which it is apparently as resistant as *Bact. paratyphosum* B and *Proteus morgani*.

Bacterial reduction of tetrathionate is similar in many ways to reduction of nitrate. Stickland (1931) showed that reduction of tetrathionate by *Bact. coli* was inhibited by oxygen. Tetrathionate reduction by washed suspensions of *Bact. paratyphosum* B is completely inhibited by oxygen provided it is continuously

bubbled through the solution, whereas mere exposure, even to an atmosphere of pure oxygen, inhibits tetrathionate reduction to only a slight extent. The same is true with a culture of *Bact. paratyphosum* B in buffered tetrathionate broth. The oxygen tension in an 'aerobic' broth culture of bacteria must in fact be very low. Rahn & Richardson (1941) showed that the rate of diffusion of oxygen into nutrient broth is not nearly rapid enough to replenish the oxygen utilized by bacteria. Now when oxygen is bubbled through plain broth cultures of coliform organisms the growth is considerably heavier than in similar unaerated cultures. Similarly it can be seen from Table 1, columns (*e*) and (*f*), that growth of tetrathionate reducers in a suitably buffered tetrathionate medium is considerably heavier than in the control medium without tetrathionate, whereas with non-reducers growth is heavier in the control. The simplest explanation of all these facts is that tetrathionate is capable of acting as an alternative hydrogen acceptor to oxygen for those organisms that are able to reduce it.

This view has received support from experiments both with synthetic media and with faecal specimens. Quastel, Stephenson & Whetham (1925) found that a synthetic medium in which *Bact. coli* would grow only aerobically gave anaerobic growth as well when an activatable hydrogen acceptor such as nitrate was added. Tetrathionate has been found to act in the same way. Using as basal medium that employed by Quastel *et al.* but with the addition of 0·0015% tryptophane and 0·1 *M* phosphate buffer we found that four different strains of *Bact. typhosum* could grow anaerobically in the presence of 0·02 *M* tetrathionate, but not in its absence. In fact two of these strains would not even grow aerobically in this medium; they grew well, however, both aerobically and anaerobically, when tetrathionate was added. With faecal specimens we have compared aerobic and anaerobic incubation of tetrathionate broth in parallel in a series of 179 specimens. On plating out it was found that tetrathionate-reducing pathogens were isolated more easily and more often in pure culture from the anaerobic than from the aerobic tetrathionate broth tubes.

A further interesting similarity to nitrate reduction is that the production of gas from a fermentable carbohydrate is inhibited in the presence of tetrathionate, but only in the case of tetrathionate-reducing organisms. Pakes & Jollyman (1901) noticed the same effect with nitrate. This fact has some diagnostic value in the identification of intestinal bacteria.

It is now clear that some of the reactions known to occur in tetrathionate media can be explained by the phenomenon of tetrathionate reduction. The intense acidity developed by certain organisms growing in tetrathionate media can be understood from equation (2), for the oxidation of hydrogen atoms to hydrogen ions (which necessarily accompanies tetrathionate reduction) involves a large drop in *p*H. With *Bact. paratyphosum* B the *p*H falls rapidly to below 4·0, and growth and further tetrathionate reduction cease. It is also clear why a suitable buffer is essential: in its presence growth is much heavier because a limiting *p*H is not reached so quickly, and the tetrathionate is all reduced to thiosulphate. Whether chalk is really suitable is doubtful. Unless the medium is constantly shaken the chalk sinks to the bottom and does not act as an efficient buffer. Jegorov & Vrtis (1937) recommended phosphate instead of chalk. High concentrations of phosphate are, however, necessary and these are inhibitory to the growth of *Bact. typhosum*. On the other hand, chalk and (even more) phosphate, greatly increase the growth of *Proteus vulgaris* and *Pr. morgani* both of which are vigorous tetrathionate reducers. The delayed production of H_2S in tetrathionate media can also be explained: H_2S is only liberated when all the tetrathionate has been reduced to thiosulphate. The organisms are then growing in thiosulphate-iodide broth and with continued growth the *p*H slowly increases, just as it does when *Bact. paratyphosum* B is inoculated directly into plain thiosulphate broth.

Changes observed in solid media. Explanation of 'lysis'

The changes which have been described above as occurring in inoculated solid tetrathionate media are less easily explained. The study of bacterial environment on the surface of agar plates is not easy though it is certain that the conditions must be very different from those existing in liquid media. It has already been mentioned that *Bact. paratyphosum* B and other Salmonellas on tetrathionate agar plates do not produce in 18 hr. the strongly acid reaction characteristic of their growth in tetrathionate broth. Some reduction of tetrathionate, however, does occur as can be shown by estimating the washings off tetrathionate agar on which there has been a heavy growth of *Bact. paratyphosum* B. We have also been able to show indirectly that the 'lysis' which is so striking a feature of the growth of many Salmonella strains on tetrathionate-thiosulphate plates is just what would be expected if tetrathionate reduction were in fact occurring. The shaded areas in Pl. 2, fig. 1, indicate roughly the relative degrees of opacity developed in uninoculated

media by different mixtures of thiosulphate and tetrathionate. The opacity, which results from the gradual separation of sulphur at first in the colloidal state and later as a heavy precipitate, is to some extent reversible. Reference to Pl. 2 will show exactly how changes in concentrations of thiosulphate and tetrathionate will produce either increased opacity or clarification. For example, it shows that a horizontal shift from right to left always involves the substitution of two molecules of thiosulphate for every one of tetrathionate removed, exactly as occurs in the bacterial reduction of tetrathionate (equation (2)). Quantitatively this reduction of tetrathionate is exactly the reverse of the oxidation of thiosulphate that occurs in the reaction with iodine (equation (1)). Apart from the iodide, medium 34*a*, for example, becomes medium 34 when all the tetrathionate has been reduced, and since the production of opacity is reversible we should expect a clearing of the medium. This does, in fact, occur. On the other hand, on medium 55*b* it will be remembered that *Salmonella* colonies may actually show a zone of increased *opacity* after 36 hr. growth. This is exactly what we should expect since in this region of the graph a horizontal movement to the left passes at first through a zone of *increasing* opacity. It can, however, be seen from Pl. 2, fig. 1, that in all media containing a large excess of thiosulphate and a relatively small amount of tetrathionate, reduction of tetrathionate would be expected to produce lysis, and that the extent of this lysis will vary with the position of the medium in the graph and the speed of tetrathionate reduction. Medium 20*a*, for example, shows better and more rapid lysis than 35, although the original opacities are approximately the same, since in 20*a* less tetrathionate has to be reduced before clearing occurs. On the other hand, in the aerobic conditions prevailing it is unlikely that the amount of tetrathionate reduced on the surface of tetrathionate-thiosulphate plates is large. For instance medium 43 allows only late and feeble lysis, and 49*a* none at all.

Biological lysis is accurately simulated by placing a drop of sodium sulphite solution on to a medium such as 35*a*, when the medium around the drop clears in a few minutes. Sodium sulphite reacts quantitatively with tetrathionate:

$$Na_2S_4O_6 + Na_2SO_3 = Na_2S_3O_6 + Na_2S_2O_3. \quad (3)$$

Reference to Pl. 2, fig. 1, shows that this conversion of one molecule of tetrathionate into *one* molecule of thiosulphate would be expected to produce lysis—by a chemical change similar to but quantitatively different from the bacterial reduction of tetrathionate.

PRACTICAL APPLICATIONS

It is now possible to offer a rational explanation for some of the defects and puzzling features of tetrathionate media in practice. It has often been noticed that a given concentration of tetrathionate and thiosulphate may show a much weaker selective action with faecal specimens than might be expected from experiments with pure cultures. This can be largely explained by the reduction of tetrathionate to thiosulphate, which is considerably less inhibitory to most faecal organisms than the tetrathionate from which it is formed. *Bact. paratyphosum* B, for instance, will not grow well in concentrations of iodide-free tetrathionate higher than 0·105 *M*; if this is all reduced to thiosulphate the concentration of thiosulphate will be 0·21 *M*, well below the highest concentration allowing good growth. The same is true of many other organisms, with the possible exception of *Bact. typhosum* (Table 1), columns (*b*) and (*d*). Now Muller's medium (*M* in Text-fig. 1) containing 0·2 *M* thiosulphate and 0·02 *M* tetrathionate is near the limiting concentrations for growth of *Bact. typhosum* and of *Proteus vulgaris* but completely inhibits the growth of *Bact. coli* I (see Text-fig. 1). When, however, a sample of faeces containing these three organisms is inoculated into the medium the first two organisms are capable after a few hours' growth of reducing some of the tetrathionate to thiosulphate. If the process continues, as it will in the presence of chalk, the medium becomes less and less inhibitory to *Proteus vulgaris* and *Bact. coli* I, either or both of which may now overgrow the thiosulphate-sensitive *Bact. typhosum*. It is clear that this progressive diminution in selectivity due to tetrathionate reduction may explain the varying results obtained by plating

out from tetrathionate broth at different intervals of time. On the other hand, it is also possible that a slight reduction in toxicity of the medium may in different circumstances give an advantage to organisms which are capable of reducing tetrathionate and give them a 'flying start' over non-reducers. Since, however, *Proteus* is one of the most vigorous tetrathionate reducers the reason for its frequent isolation in heavy growth from tetrathionate broth is plain.

The work described has led to several practical applications:

(1) The discovery of 'lysis' in solid media, such as 35*a*, containing small amounts of tetrathionate with a large excess of thiosulphate raised hopes that a good differential as well as a selective medium could be evolved and for some time this medium was used as a routine for direct and indirect plating of faecal specimens. Tetrathionate reducers were well differentiated on this medium from non-reducers by their areas of 'lysis' (Pl. 2, fig. 2), but the medium was abandoned because it gave excellent growth of both *Pr. vulgaris* and *Pr. morgani* and was rather inhibitory to *Bact. typhosum*.

(2) The discovery that *Bact. typhosum* is relatively sensitive to thiosulphate led to the use of a liquid enrichment medium containing 25 % less thiosulphate than Muller's, that is, medium 43 in Text-fig. 1. This, while somewhat less selective than Muller's, gave much more reliable growth of *Bact. typhosum* and has been used as a routine for nearly two years. Results obtained with it have been described in a previous paper (Knox *et al.* 1942).

(3) The experiments with pure cultures summarized in Text-fig. 1 suggested that a medium containing balanced tetrathionate in a concentration of about 0·03 *M* (55*b*) would be superior to media of Muller's type and would be worthy of practical trial with faecal specimens. As a liquid enrichment medium 55*b* gave satisfactory results but showed no consistent improvement on media such as 43 or Muller's. This was probably because, once quite a small proportion of the tetrathionate had been reduced by any tetrathionate reducers present in faeces, the medium more rapidly lost its selectivity than media containing larger amounts of thiosulphate. Even as a liquid medium, however, 55*b* sometimes gave much better suppression of *Proteus* than 43 or Muller's or even Kauffmann's media. As a solid medium it has been proved successful and has been used as a routine for many months (Knox *et al.* 1942). Differentiation is sometimes difficult: 'lysis', of course, cannot be used for differentiation in this type of medium and the slight zone of opacity which develops round tetrathionate reducers is too indefinite for practical use as a differentiating feature. The medium is, however, highly selective and with *Bact. typhosum* has given results similar to Wilson & Blair's medium. It has given excellent results with several strains of *Salmonella* and with *Bact. paratyphosum* B.

Discussion

We are now in a position to attempt some synthesis of these observations. Preliminary work showed that growth of many organisms in a tetrathionate medium is accompanied by obvious changes which can be clearly distinguished from the slight changes which occur in a control uninoculated medium. Chemical investigations showed that these changes are the result of the bacterial reduction of tetrathionate to thiosulphate. Text-fig. 1 and Table 1 show that although the tetrathionate, the thiosulphate, the iodide and the buffer are all important, the most important part in the selectivity is played by the tetrathionate. How the tetrathionate acts we cannot at present completely explain, but it appears to be

capable of acting as a selective agent in two ways. By itself, and especially when associated with iodide, it can undoubtedly act in concentrations such as 0·03 *M* as a selective *inhibitor* of growth, and moreover as one which allows good growth of *Bact. typhosum* in concentrations which completely suppress *Proteus vulgaris* and partially inhibit *Pr. morgani*. On the other hand, in lower concentrations it behaves as a selective *promoter* of growth by acting as an alternative hydrogen acceptor to oxygen for those organisms which are able to reduce it, and thereby, in the nearly anaerobic conditions prevailing in a broth culture, provides these with additional energy not available to non-reducers. These facts, quite apart from their intrinsic interest, may have important practical applications. They suggest two lines of approach in searching for a tetrathionate medium which will combine good growth of *Bact. typhosum* with reliable inhibition of *Proteus*. A medium containing a high enough concentration (about 0·03 *M*) of balanced tetrathionate has been shown to approach nearer to this ideal than media of Muller's type. It suffers, however, from the defect that if only a quite small proportion of the tetrathionate is reduced by bacteria present in faeces it becomes much less selective and *Proteus*, if present, is able to grow freely. This suggests that it might be desirable in a medium of this type actually to suppress tetrathionate reduction and to rely for the selective action on a direct toxic effect of unchanged tetrathionate acting differentially on certain groups of organisms. On the other hand, a more efficient medium might be devised by making use of tetrathionate as, in a sense, a selective growth-promoting substance; such a medium would contain a concentration of tetrathionate too low to have any appreciable inhibitory effect and the main inhibitory selective action would be provided by some other constituent. To some extent Muller's and Kauffmann's media fall into this class, since the concentration of tetrathionate in these is not great enough to be highly inhibitory even in association with iodide; the undoubtedly high selectivity of these media must be attributed to a combination of tetrathionate, iodide, thiosulphate and chalk. Unfortunately, however, the selectivity is not entirely in the direction required for isolation of pathogens from faeces: the large excess of thiosulphate and the chalk both operate in favour of the *Proteus* group and to the disadvantage of *Bact. typhosum*.

These observations may have interesting applications to other media and to other groups of organisms beside the Salmonella group. Many selective media contain a reducible substrate, for example, tellurite media for *C. diphtheriae*, and Wilson & Blair's glucose-sulphite media and Leifson's selenite media for the Salmonella group. In each case the reduction of a specific substrate by certain organisms is a salient feature of the medium; but, what is more important, the reducible substrate is itself the selective agent or at least one of the selective agents.

This general conception of selective action is at present put forward only tentatively and, except in the case of tetrathionate itself, lacks experimental evidence. We have not yet succeeded in devising a completely satisfactory tetrathionate media for practical use, but the work we have outlined has explained the reason for some of the disadvantages of tetrathionate media, especially the luxuriant growth of *Proteus* in media of Muller's type, and has defined some of the difficulties which remain to be overcome. It has also made it clear that the study of bacterial enzymes opens up an interesting field in the elucidation of selective action and cannot be ignored in any attempt to provide some theoretical basis for the study of selective media, which so far have been elaborated almost entirely by empirical methods. It is already clear that here is one example of the way in which a

chemical and biochemical approach may be expected to lead to a better understanding of a bacteriological problem.

SUMMARY

The chemical and biological properties of the constituents of tetrathionate media have been investigated.

(1) The selective properties of thiosulphate and tetrathionate are expressed in graphic form, a method which clearly illustrates their differential effect on various intestinal organisms and is of value in preparing media to suit particular needs.

(2) Thiosulphate, tetrathionate, iodide and, indirectly, chalk all have some selective action, but the most important single constituent is tetrathionate.

(3) The discovery that certain organisms, mainly of the Salmonella group, are capable of reducing tetrathionate rapidly to thiosulphate suggests that in tetrathionate media part at least of the selective action is due to the ability of the tetrathionate to act as an alternative hydrogen acceptor to oxygen.

(4) In the light of these facts many of the defects and puzzling features of tetrathionate media can be explained, especially the frequent heavy growth of *Proteus*.

(5) A medium containing balanced tetrathionate shows a different kind of selectivity from media of Muller's type and has given consistent results as a routine solid medium for plating of faecal specimens.

(6) A diminution in the amount of thiosulphate in media of Muller's type is desirable, since *Bact. typhosum* is relatively sensitive to thiosulphate.

(7) The possible value of a biochemical approach to the study of selective media is emphasized.

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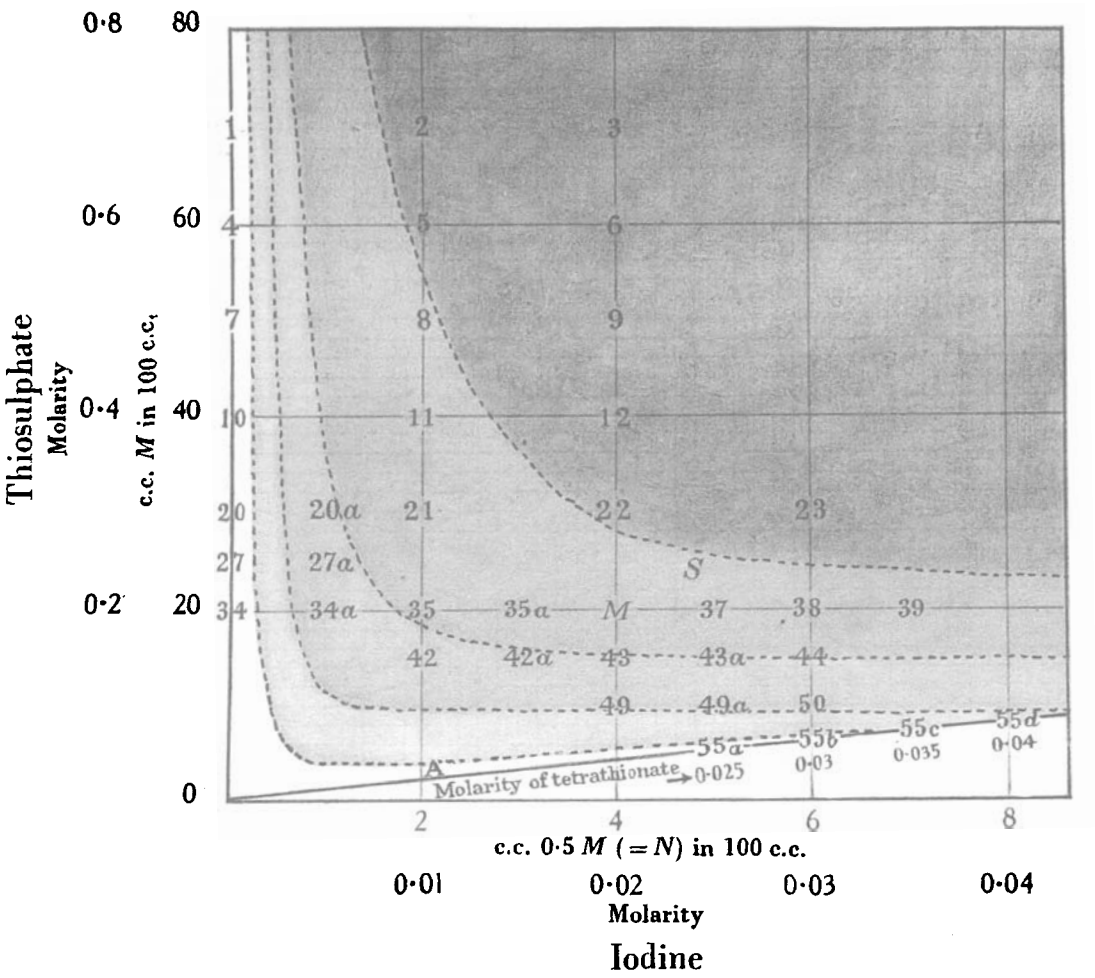

Fig. 1. Opacity developed in uninoculated media by different mixtures of thiosulphate and tetrathionate.

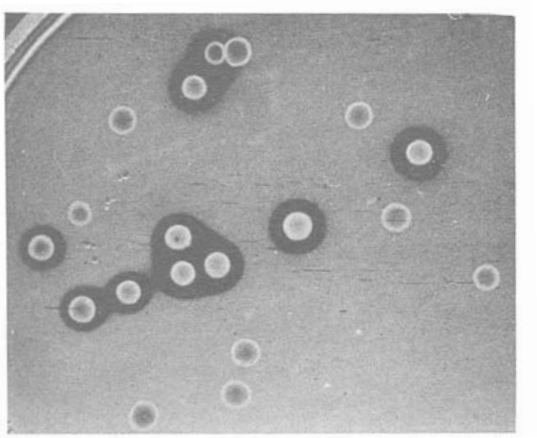

Fig. 2. Tetrathionate-thiosulphate agar (medium 35*a*: see text) showing 'lysis' produced around colonies of a tetrathionate-reducing organism (*Bact. paratyphosum* B) in contrast to absence of lysis around colonies of GSM (a paracolon bacillus which does not reduce tetrathionote: see text). 40 hours' incubation. Natural size.

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