Table 6. *Activity of plasma and cells contained in 1 ml. of blood*

μl. CO_2 evolved in 20 min.

Exp.	Source of blood	Acetyl-β-methylcholine 0·03 M		Benzoylcholine 0·006 M		Acetylcholine		Ratio (a)/(b)
		Plasma	Cells	Plasma	Cells	(a) 0·06 M Plasma	(b) 0·0006 M Plasma	
1	Man: B.M. ♂	17	848	411	0	1116	472	2·4
	H.R. ♂	16	769	409	0	1075	414	2·6
	D.M. ♀	11	761	288	0	805	288	2·8
	D.M. 2 months later	12	—	289	—	801	275	2·9
2	Horse: (i) ♂	24	169	261	0	833	241	3·5
	(ii) ♀	15	160	231	0	844	227	3·7
3	Dog: (i) ♀	37	150	224	0	409	234	1·7
	(ii) ♂	33	92	485	0	868	348	2·5
4	Guinea-pig: (i) ♂	49	224	287	0	635	336	1·9·
	(ii) ♂	57	190	293	0	773	502	1·5
5	Rabbit	37	67	48	0	102	64	1·6
6	Ox	Trace	425	0	0	Trace	Trace	—
7	Sheep	31	149	0	0	32	94	0·3
8	Cat: (i) ♂	54	32	102	0	404	242	1·7
	(ii) ♀	27	16	83	0	365	183	2·0
9	Chicken	179	Trace	28	0	416	279	1·5
10	Duck	26	Trace	15	0	108	101	1·1
11	Pigeon: (i) ♀	37	0	—	—	503	361	1·4
	(ii) ♂	43	0	—	—	582	422	1·4

species, is apparently not indispensable in this respect; moreover, it could play only a minor rôle in controlling the level of acetylcholine in the blood, since it is unable to hydrolyse low concentrations of this substrate efficiently.

SUMMARY

1. Measurements of cholinesterase activity in which acetylcholine is used as substrate give an inaccurate picture of the levels of true cholinesterase and pseudo-cholinesterase in any mixture of the two enzymes.

2. Methods for quantitative estimations of true cholinesterase and pseudo-cholinesterase based on the use of acetyl-β-methylcholine and benzoylcholine as substrates are described.

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Bacterial Reduction of Tetrathionate

(A REPORT TO THE MEDICAL RESEARCH COUNCIL)

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We have previously reported that certain intestinal organisms have the power of reducing tetrathionate quantitatively to thiosulphate [Pollock, Knox & Gell, 1942]. The present paper gives the experimental evidence for this reduction and discusses some of the problems which it has suggested.

METHODS

The reaction is followed by direct iodometric titration—a method applicable to the study either of a culture growing in a tetrathionate medium, or of a washed suspension of organisms. Our work has been done mainly with washed suspensions of *Bacterium paratyphosum* B. At first these were prepared from the growth on plain nutrient agar, but the reaction then showed a long latent period before rapid reduction started, making the results difficult to analyse. When, however, the organisms were grown on agar containing a suitable concentration of tetrathionate or when a suspension prepared from the growth on plain agar was incubated with tetrathionate and mannitol or glucose for a few hours, the resulting suspension, even though repeatedly washed, was highly active, and reduction of tetrathionate was rapid and approximately linear. The significance of this difference between plain agar suspensions and tetrathionate-treated suspensions will be dealt with in a later paper.

Preparation of bacterial suspensions. 1 ml. of an 18 hr. broth culture of *Bact. paratyphosum* B was used to inoculate each of a series of flat bottles containing about 40 ml. of 2 % nutrient agar prepared from tryptic digest broth. After incubation at 37° for 18 hr., the growth was washed off with 6 ml. of $\frac{1}{4}$ strength Ringer's solution. The pooled suspensions were then centrifuged and the deposit resuspended in $\frac{1}{4}$ strength Ringer's solution to give a volume of about 2·5 ml. of suspension from each bottle. This suspension contained on an average about 12·5 mg. bacterial dry weight and 1·16 mg. bacterial N/ml. In a final dilution of 1 in 5 it was then incubated at 37° in a mixture containing 0·01M tetrathionate, 0·02M mannitol and 0·2M phosphate buffer (pH 7·7) until all the tetrathionate had been reduced to thiosulphate. This usually took from 2 to 3 hr. The mixture was then centrifuged and resuspended in $\frac{1}{4}$ strength Ringer's solution up to any convenient dilution.

Preparation of tetrathionate. Tetrathionate prepared in the usual way according to the equation

$$2Na_2S_2O_3 + I_2 = Na_2S_4O_6 + 2NaI, \qquad (1)$$
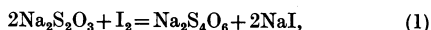
inevitably contains a high proportion of iodide. This has a slight but definite inhibitory effect on reduction of tetrathionate, though it does not appear to affect the reaction in any other way. We therefore generally used pure, iodide-free tetrathionate prepared by us according to the method described by Mellor [1930], although in a few experiments we used pure tetrathionate prepared by Messrs Baird and Tatlock. After drying over H_2SO_4 the tetrathionate was kept at 0° in the dark, and under these conditions, either in the solid form or as a 0·1M solution, it remained stable for many weeks.

Quantitative estimations. (1) The total value of I_2-reducing substances was estimated by titration with standard I_2 solution after acidification with CH_3COOH, using 0·5 % starch solution as indicator.

(2) Polythionates, thiosulphate, sulphite and sulphide were estimated by the methods of Kurtenacker & Wollack, and Kurtenacker & Bittner, as quoted by Starkey [1935 a].

(a) Tetrathionate: samples were added to excess Na_2SO_3 (2 ml. of a saturated solution) and made just alkaline to phenolphthalein. After 5 min. excess formalin was added to fix the sulphite, followed by excess CH_3COOH. The thiosulphate formed was titrated with standard I_2 and the amount of tetrathionate calculated from the equation

$$Na_2S_4O_6 + Na_2SO_3 = Na_2S_2O_3 + Na_2S_3O_6. \qquad (2)$$

(b) Thiosulphate: I_2 was added to convert this all into tetrathionate by equation (1), and the tetrathionate formed estimated by the excess sulphite method.

(c) Estimations for sulphite were made by fixation with formalin, for sulphide by precipitation with $ZnCO_3$ and filtration, and for pentathionate and trithionate, by treatment with KCN and $HgCl_2$ [Starkey, 1935 a].

(3) Bacterial N_2 was estimated by the Kjeldahl method.

(4) Dry weight of bacterial suspensions was determined by evaporation (after thorough washing with distilled H_2O) at 120° to constant weight.

Estimation of tetrathionate reduction

Unless otherwise stated the following final concentrations were used: 0·01M tetrathionate, 0·02M mannitol and 0·2M phosphate buffer (pH 7·7) containing phenol red as indicator. Mixtures containing substrates and buffer were prepared in test tubes ($\frac{3}{4}$ in. diam.) and warmed for 30 min. at 37°. After addition of the separately warmed bacterial suspension, the tubes were incubated at 37°. A control tube was set up at the same time, containing no bacterial suspension. Samples were removed at intervals and mixed immediately with an equal volume of 10 % CH_3COOH, thus stopping the reaction at once and acidifying the solution for the I_2 titration. 0·5 ml. of 0·5 % starch was added to each sample, which was titrated at once with standard I_2. The presence of the organisms did not interfere with accurate titration but ordinary bacteriological precautions were taken. pH was estimated colorimetrically. With 0·2M buffer and 0·01M tetrathionate the pH fell from 7·7 to about 7·5 by the time all the tetrathionate had been reduced, but this slight drop did not affect the velocity of the reaction appreciably (see Fig. 4).

RESULTS

Fig. 1 illustrates a typical experiment (done in duplicate). It can be seen that the I_2 absorbed increases linearly with time until the reaction is complete. That this increase in I_2 absorptive power is the result of the quantitative reduction of tetrathionate to thiosulphate is shown by the following facts, illustrated by an experiment shown in Table 1.

(1) Independent estimations, by method 2 (b) described above, of thiosulphate formed at each stage (column 3) show close agreement with the direct I_2 titrations (column 1). The direct I_2 titrations therefore can be taken to represent with a reasonable degree of accuracy the actual amounts of thiosulphate formed.

(2) It can be seen by comparing columns (4) and (6) (which are calculated directly from the actual estimations in columns (3) and (5) respectively) that at each stage in the reaction the molecular concentration of thiosulphate formed is approximately twice that of the tetrathionate which has disappeared.

These facts are in agreement with the following equation for the reduction of tetrathionate:

$$Na_2S_4O_6 + 2H = Na_2S_2O_3 + H_2S_2O_3, \qquad (3)$$

in which two molecules of thiosulphate are formed from every one of tetrathionate. Since thiosulphuric acid in solution is highly ionized this equa-

Fig. 1. Tetrathionate reduction by *Bact. paratyphosum* B. Suspension mixture contained $0.01 M$ tetrathionate, $0.2 M$ buffer and $0.02 M$ mannitol in a final volume of 12.5 ml.

The 'theoretical limit' line marks the titration value to be expected when all the tetrathionate has been converted into thiosulphate according to equation (3).

tion could also be expressed as an oxidation of H atoms to H ions:

$$2H \rightarrow 2H^+ + 2\epsilon. \qquad (3a)$$

This is consistent with the high degree of acidity developed during tetrathionate reduction, even in

the absence of glucose or mannitol, necessitating the use of high concentrations of buffer.

The close correspondence between the I_2 titrations and the amounts of thiosulphate estimated by method 2 (b) (Table 1, columns 1 and 3), is in itself evidence that not more than traces of other I_2-reducing substances are formed. It is true that if, for example, sulphite or sulphide were formed during the reaction, they would be undetectable because they would react chemically with the tetrathionate [Mellor, 1930; Mitchell & Ward, 1932]; but if this occurred to any appreciable extent the molecular ratio of thiosulphate formed to tetrathionate disappearing would inevitably be less than the figure of 2:1 which we have found experimentally. This can be seen by comparing equations (2) and (3). There was also the possibility of the formation of other polythionates (pentathionate and trithionate), but, if these were produced in any quantity, the 2:1 ratio of thiosulphate to tetrathionate would again be upset. Direct estimations of these substances (method 2 (c)) have failed to reveal their presence at any stage in the reaction. It is, however, possible that after complete reduction of tetrathionate small amounts of sulphite and sulphide are formed: indeed the 60 and 90 min. readings in columns (1) and (2), which show a slight decrease in the I_2 value after treatment with formalin, suggest that some traces of sulphite are present. Some further action of the organisms on the thiosulphate with evolution of H_2S is to be expected. Washed suspensions of *Bact. paratyphosum* B can certainly produce H_2S from thiosulphate, and H_2S can be readily detected after such suspensions have been incubated with tetrathionate—as soon as all the tetrathionate has been reduced to thiosulphate. Tarr [1933, 1934] has found that
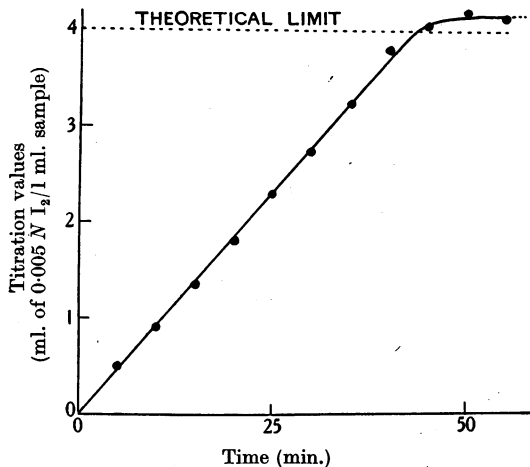
Table 1. *Formation of thiosulphate and disappearance of tetrathionate in tetrathionate reduction by* Bact. paratyphosum *B*

	Titration value of 5 ml. samples with 0·01 N iodine		Estimation of thiosulphate formed, expressed as		Estimation of tetrathionate concentration expressed as $M \times 10^{-3}$	
Time min.	(1) Direct	(2) After treatment with formalin	(3) ml. 0·01 M in 5 ml. samples	(4) Conc. $(M \times 10^{-3})$	(5) Still present	(6) Removed
			With bacterial suspension			
0	0·3	—	0	0	10·3	0
15	3·1	—	2·8	5·6	7·4	2·9
30	6·4	—	6·1	12·2	4·3	6·0
45	9·7	—	8·9	17·8	1·0	9·3
60	10·9	10·2	9·8	19·6	0	10·3
90	10·9	10·1	9·7	19·4	0	10·3
17 hr.	10·65	10·2	9·9	19·8	0	10·3
			Controls with no bacterial suspension			
0	0·15	—	—	—	10·5	0
90	0·15	—	—	—	10·7	0

The solutions, both in the control batch and in that with the bacterial suspension, were made up to a volume of 100 ml., containing $0.01 M$ tetrathionate, $0.2 M$ phosphate buffer (pH 7.7), and $0.02 M$ mannitol.

washed suspensions of *Proteus vulgaris* form H_2S from thiosulphate. Tarr's figures, however, showed that the rate of H_2S production was slow, while it can be seen from Table 1 that with *Bact. paratyphosum* B, even after 17 hr. incubation, there was no appreciable alteration in the amount of thiosulphate still present. This is still further evidence that in the reduction of tetrathionate to thiosulphate subsidiary reactions such as formation of sulphite or sulphide are quantitatively insignificant.

Toxicity of tetrathionate and thiosulphate

Tetrathionate, up to a concentration of $0.05\,M$, has no inhibitory action on its own reduction (Table 2). It is also clear from Fig. 1, where the reaction proceeds in a straight line practically to

Table 2. *Affinity of enzyme for tetrathionate*

Initial molarity of tetrathionate	Velocity of tetrathionate reduction (absorption of I_2, as ml. of $0.005\,N\ I_2$, by 2 ml. samples, after 30 min. incubation)
0.05	1.3
0.02	1.3
0.01	1.35
0.004	1.35
0.002	1.35

Each tube contained washed suspension of *Bact. paratyphosum* B, $0.2\,M$ buffer (pH 7.7), $0.02\,M$ mannitol and the appropriate concentration of tetrathionate. Total vol.: 5 ml. Incubation at 37°.

completion and where the thiosulphate formed approached a concentration of $0.02\,M$, that the thiosulphate likewise had no inhibitory effect within the limits used.

Hydrogen donators for tetrathionate reduction

Even in the absence of external H_2 donators, the rate of the reaction is rapid (Table 3). This residual

Table 3. *Effect of hydrogen donators on tetrathionate reduction by Bact. paratyphosum B*

Added H donator	Velocity of tetrathionate reduction (absorption of I_2, as ml. of $0.005\,N\ I_2$, by 2 ml. samples after 30 min. incubation)
Glucose	2.45
Mannitol	2.45
Lactate	1.95
Formate	2.35
No H_2 donator	1.4

Each tube was made up to contain bacterial suspension, $0.01\,M$ tetrathionate, $0.2\,M$ buffer (pH 7.7), and $0.02\,M$ H_2 donator. Total vol.: 5 ml. Incubation at 37°.

or endogenous reduction constitutes a high proportion of the total reduction in the presence of man-

nitol or glucose. We have been unable to decrease it even by repeated washings with $\frac{1}{4}$ strength Ringer's solution. For *Bact. paratyphosum* B, glucose, mannitol, formate, and, to a less extent, lactate can act as H_2 donators for tetrathionate reduction.

Affinity of enzyme for tetrathionate

It has not been possible to estimate the rate of tetrathionate reduction with concentrations of tetrathionate lower than $0.002\,M$, since the quantity of thiosulphate formed is then too small for accurate analysis. But Table 3 shows that there is no appreciable decrease in the initial velocity between $0.05\,M$ and $0.002\,M$, so that the affinity of the enzyme for tetrathionate must be fairly high; moreover, the reduction of tetrathionate is linear very nearly to the end of the reaction (Fig. 1). In the comparison of different velocities we have, however, confined our figures to the first half of the reaction, and not attached any significance to readings after the concentration of tetrathionate has fallen below $0.004\,M$.

Some physical factors affecting tetrathionate reduction

Effect of temperature. From Fig. 2 it can be calculated that the mean Q_{10} value between 18° and 37° is 1.94 and between 37° and 43° is 2.64. At
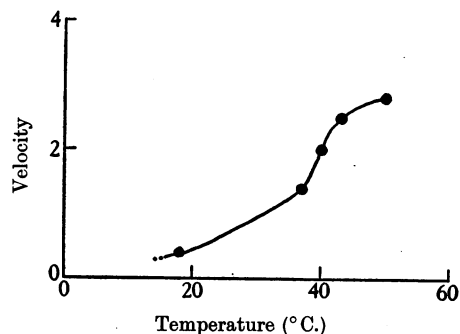

Fig. 2. Effect of temperature on rate of tetrathionate reduction. Velocity of tetrathionate reduction is expressed as the absorption of I_2 (as ml. of $0.005\,N\ I_2/1$ ml. sample) after incubation for 20 min. at the different temperatures. Each tube contained washed suspension of *Bact. paratyphosum* B, $0.01\,M$ tetrathionate, $0.2\,M$ buffer (pH 7.7) and $0.02\,M$ mannitol. Total vol.: 5 ml.

higher temperatures the Q_{10} falls again; and this may possibly be associated with enzyme destruction, which is rapid at temperatures above 50°. Fig. 3 shows the effect of preliminary heating of a bacterial suspension at 56°. It can be seen that the activity is reduced by over 50 % after only 10 min. treatment.

Effect of pH. The pH curve (Fig. 4) is of course a composite curve of at least two enzyme systems and corresponds to the pH curve of many known dehydrogenases [Cook & Alcock, 1931]. Accurate
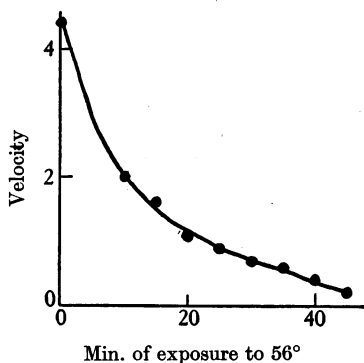

Fig. 3. Rate of inactivation of tetrathionate-reducing system at 56°. Initial velocity of tetrathionate reduction is expressed as absorption of I_2 (as ml. of $0.005 N$ $I_2/2$ ml. sample) after incubation for 30 min. at 37°.

Each tube contained washed suspension of *Bact. paratyphosum* B (exposed to 56° for varying periods from 0 to 45 min.), $0.01 M$ tetrathionate, $0.2 M$ buffer (pH 7.7) and $0.02 M$ mannitol. Total vol.: 5 ml.
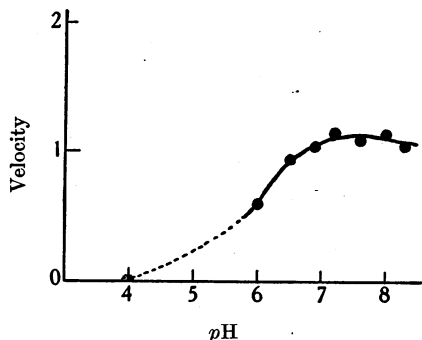

Fig. 4. Effect of pH on tetrathionate reduction by *Bact. paratyphosum* B. Velocity of tetrathionate reduction is expressed as absorption of I_2 (as ml. of $0.005 N$ $I_2/2$ ml. sample) after incubation for 30 min. at 37°.

Each tube contained bacterial suspension, $0.01 M$ tetrathionate, 0.2 phosphate buffer (pH 6.0–8.3) and $0.02 M$ mannitol. Total vol.: 10 ml.

results below pH 6.0 have not been obtainable, but when the reaction ceases in an unbuffered suspension mixture, the pH is about 4.0. It can therefore be assumed that the velocity below pH 4.0 is 0.

Effect of oxygen. Facilities were not available for testing the effect of known O_2 tensions, but Table 4 shows the relative velocities of tetrathionate reduction: (1) in evacuated Thunberg tubes, (2) in tubes open to the air, (3) in sealed tubes exposed to an atmosphere of O_2, and (4) in tubes through which

Table 4. *Effect of oxygen on tetrathionate reduction by* Bact. paratyphosum *B*

Treatment	Velocity of tetrathionate reduction (absorption of I_2, as ml. of $0.005 N$ I_2, by 2.5 ml. samples after 30 min. incubation)
(1) Anaerobic	7.2
(2) Open to air	6.85
(3) Exposed to O_2	5.75
(4) 'Oxygenated'	0.85

Each tube contained bacterial suspension, $0.01 M$ tetrathionate, $0.2 M$ buffer (pH 7.7) and $0.02 M$ mannitol. Total vol.: 2.5 ml. Incubation at 37°.

O_2 was bubbled continuously. The first three tubes showed little difference in velocity, probably because in all three cases the conditions were nearly anaerobic, since diffusion of O_2 from the atmosphere was not rapid enough to replace the O_2 in solution which was consumed [see Rahn & Richardson, 1941]. On the other hand, when O_2 was continuously bubbled through the mixture, tetrathionate reduction was decreased by about 90 %.

Specificity of tetrathionate reduction

The ability to reduce tetrathionate actively to thiosulphate is restricted to certain groups of organisms. For investigating the distribution of this property in different bacterial groups we used the following technique in order to provide conditions as near optimal as possible for a wide range of organisms: 18 hr. broth cultures of organisms to be tested were centrifuged and the deposit incubated with $0.01 M$ tetrathionate, $0.2 M$ phosphate buffer and 50 % tryptic digest broth as source of H_2 donators. Under these conditions the following organisms were found to reduce tetrathionate actively: *Bact. paratyphosum* B and many other *Salmonellas* (but excluding *Bact. paratyphosum* A), *Bact. typhosum* (*Eberthella typhosa*), *Proteus vulgaris* and *P. morganii*, and some intestinal non-pathogens of the coliform intermediate group. We were unable to detect any tetrathionate reduction by *Escherichia coli* I, many of the non-pathogens commonly met with in human faeces, dysentery bacilli, *Corynebacterium diphtheriae* and a few strains of streptococci and staphylococci. *Pseudomonas aeruginosa* (*Ps. pyocyanea*) occupies an interesting position, since it was found to reduce tetrathionate slowly and is reported by Starkey [1934, 1935a] to oxidize thiosulphate to tetrathionate.

DISCUSSION

The bacterial reduction of tetrathionate is of considerable biological interest and may have important implications in several different fields of

investigation. The relevance of tetrathionate reduction in the understanding of selective media for the diagnosis of suspected typhoid or Salmonella infections has been discussed elsewhere [Knox, Gell & Pollock, 1943]. We there suggested that tetrathionate might act as an alternative H_2 acceptor to O_2. If this is so we should expect that under anaerobic conditions tetrathionate would favour the growth of those organisms which can reduce it. In this way tetrathionate would be similar to other H_2 acceptors such as nitrate [Quastel, Stephenson & Whetham, 1925], but would be more specific and in that sense selective, because the power to reduce tetrathionate is more restricted. We have given some experimental results which suggest that this is so [Knox et al. 1943].

Tetrathionate reduction may be an important link in the natural cycle of synthesis and breakdown of S compounds by bacteria. Though this has not been studied so thoroughly as the N_2 cycle, there has accumulated in recent years some evidence indicating how H_2S is oxidized to sulphur and sulphate and converted to organic sulphur compounds by certain micro-organisms and then re-evolved by subsequent reduction of these substances by other bacteria [see summary by Bunker, 1936]. Beijerinck [1900, 1904] mentioned that bacteria in his group 'Aerobacter', which contained gas-forming intestinal organisms, were able to reduce tetrathionate, pentathionate, thiosulphate, sulphite and sulphur to H_2S. It is now clear that production of H_2S from tetrathionate by bacterial action occurs, at least with the organisms that we have investigated, only when all the tetrathionate has first been reduced to thiosulphate, and that this reduction is much more rapid than the subsequent reduction of thiosulphate to H_2S. $Na_2S_2O_3$ is known to be present in many soils [Starkey, 1935b] and the discovery that it can be rapidly formed from tetrathionate by bacterial action is an interesting counterpart to the already well-established fact that thiosulphate can be oxidized to tetrathionate by certain heterotrophic soil organisms, amongst which are members of the genus Pseudomonas [Starkey, 1935a]. The widespread occurrence in soils and inland waters of Ps. fluorescens [Waksman, 1931] may have some connexion with the long survival of Bact. typhosum and Salmonellas outside the human body, since their supply of an alternative H_2 acceptor to O_2 (i.e. tetrathionate) would then be continuously renewed.

The reduction of tetrathionate resembles in many ways the reduction of other H_2 acceptors (e.g. nitrate) by bacteria and the mechanism of H_2 transfer is probably similar. O_2 inhibits the reduction of nitrate [Stickland, 1931]: it has also a definite though less marked inhibitory effect on the reduction of tetrathionate. In both cases there appears to be a competition between H_2 acceptors for activated H_2. That the reduction of tetrathionate need be no subsidiary means of substrate oxidation is suggested by the speed of the reaction. In one experiment the rate of H_2 transfer was equivalent to an O_2 consumption of 68 mm./mg. dry bacterial wt./hr. (assuming all the O_2 to be reduced to H_2O)— a figure which is of the same order as that for the O_2 consumption of Esch. coli [Cook & Haldane, 1931].

It is suggested that the system responsible for the bacterial reduction of tetrathionate to thiosulphate be named provisionally 'tetrathionase'.

SUMMARY

1. Washed suspensions of Bacterium paratyphosum B can reduce tetrathionate rapidly and quantitatively to thiosulphate according to the equation

$$Na_2S_4O_6 + 2H = Na_2S_2O_3 + H_2S_2O_3.$$
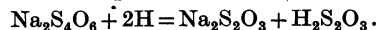
2. This reaction can be followed simply by direct iodometric titration.

3. The power of actively reducing tetrathionate appears to be restricted to certain intestinal organisms.

4. The effect of pH, temperature and O_2 tension on the reaction have been investigated.

5. Some biological implications of bacterial reduction of tetrathionate have been discussed.

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