The Use of Nile Blue in the Study of Tetrathionase Activity

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SUMMARY: Experiments by the Thunberg tube technique have shown that tetrathionate can oxidize reduced Nile blue in the presence of tetrathionate-adapted cells of a Gram-negative coliform organism but not in the presence of cells lacking a developed tetrathionase system, viz. unadapted cells of the same organism and cells of *Shigella sonnei*. Concentrations of tetrathionate of the order of $m/3200$ and slight tetrathionase activity can be detected by the Thunberg tube technique, which may be useful also in investigating the effect of certain physical and chemical agents on the tetrathionase system. It appears that tetrathionate can act as a hydrogen acceptor for organisms capable of reducing it.

Knox, Gell & Pollock (1943) showed that certain organisms can reduce tetrathionate in the presence of a hydrogen donator, and it has been suggested that tetrathionate acts as a hydrogen acceptor alternative to oxygen for those organisms that are able to reduce it (Knox *et al.* 1943; Pollock & Knox, 1943; Knox, 1945). Quastel & Whetham (1924) showed that under anaerobic conditions, in the presence of a washed suspension of *Bacterium coli*, fumarate inhibited the reduction of methylene blue and reoxidized reduced methylene blue. Quastel, Stephenson & Whetham (1925) showed that nitrate acted in a similar way, and Green, Stickland & Tarr (1934) performed similar experiments with other redox indicators. It is accepted that the reoxidation, of a reduced indicator under anaerobic conditions, is evidence of the presence of some substance or system capable of acting as a hydrogen acceptor alternative to oxygen. The extent to which a reduced redox indicator is reoxidized, however, depends on the relative rates at which it is being oxidized and reduced by the systems present in the preparation. The 'tetrathionase' system, which is an adaptive enzyme system (Knox & Pollock, 1944), transfers hydrogen from a suitable hydrogen donator to tetrathionate and almost certainly consists of a complex series of enzymes. In this investigation only that part of the system which transfers hydrogen from reduced Nile blue to tetrathionate has been studied. The experiments described here show that tetrathionate will reoxidize reduced Nile blue in the presence of a suspension of cells adapted to reduce tetrathionate, but not in the presence of cells which do not reduce tetrathionate.

EXPERIMENTAL

Methods

Organisms. The organisms used were: (1) a non-pathogenic Gram-negative coliform organism of the intermediate type I group, labelled '1433'; this organism was obtained from Dr M. R. Pollock and had been used by Knox *et al.* (1943) in their work on the selective action of tetrathionate, and by

Pollock (1946) in his work on nitratase; (2) a strain of *Shigella sonnei*, as an example of a non-reducer of tetrathionate.

Preparation of suspensions. Normal (unadapted) cell suspensions of '1433' and the suspension of Sonne cells were prepared by inoculating 3 ml. of 6 hr. tryptic heart-broth cultures on to 200 ml. of tryptic heart agar in Roux bottles. The Roux bottles were incubated at 37° for 16 hr., and the cells were washed off in quarter-strength Ringer solution. The cells from each Roux bottle were centrifuged, washed once, and made into a thick suspension in 10 ml. quarter-strength Ringer solution.

Adapted cell suspensions of '1433' were prepared by growing the organisms in Roux bottles containing M/50 tetrathionate, M/50 mannitol and M/5 phosphate buffer (pH 7·6) in tryptic heart agar and treating in the same way (Knox & Pollock, 1944). The dry weight of all these suspensions was of the order of 15–20 mg./ml.

Preparation of Thunberg tubes. The experiments were performed in evacuated Thunberg tubes placed in a thermostatically controlled water-bath at 37°. The following mixture was placed in the main part of each tube: 0·5 ml. Nile blue M/1600; 0·5 ml. sodium lactate M/2; 0·5 ml. phosphate buffer (pH 6·4) M/7·5; 0·5 ml. bacterial suspension. The mixture was buffered at pH 6·4 because Nile blue was more readily decolorized at this pH than at pH 7·6, and the activity of tetrathionase falls off rapidly at pH levels lower than 6·4 (Pollock & Knox, 1943). Volumes (0·5 ml.) of various concentrations of tetrathionate were placed in the hollow stoppers of the Thunberg tubes and the tubes closed and evacuated by water pump. After the Nile blue had been decolorized by the action of the cell suspension the tetrathionate was tipped into the mixture. Sodium tetrathionate prepared by the method described by Sander (1915) was used; it contained two molecules of water of crystallization and no detectable iodide, thiosulphate, sulphate or sulphite (Knox, 1945). In a few experiments tetrathionate was added to the mixture in the Thunberg tube before the Nile blue was reduced, to see if it would inhibit the reduction of the dye. A few similar experiments were performed with methylene blue as an indicator.

Results

Reoxidation of reduced Nile blue. Table 1 shows the results of an experiment using adapted and unadapted cells of '1433' and a Sonne suspension. It can be seen that tetrathionate caused an immediate reoxidation of the Nile blue in the presence of the adapted cells of '1433', while with the other two suspensions the addition of tetrathionate produced only a pale blue colour. The time taken for this colour to disappear was, however, slightly longer with the unadapted cells of '1433' than with the Sonne suspension.

Experiments to find the smallest concentration of tetrathionate that would reoxidize reduced Nile blue completely in the presence of adapted cells of '1433' are recorded in Table 2. It can be seen that tetrathionate down to a final concentration of M/3200 completely reoxidized the Nile blue used, and that an even lower concentration of tetrathionate delayed the disappearance

of the pale blue colour which appeared on adding the contents of the stopper.

The effect of diluting a suspension of adapted cells of '1433' is shown in Table 3. Twofold dilutions of adapted cells were added to 0.3 ml. of a Sonne suspension in a Thunberg tube containing the usual mixture, the Sonne suspension being used to ensure rapid reduction of the Nile blue. The tetrathionate was added when the Nile blue was reduced, and the resulting colour

Table 1. *Effect of adding tetrathionate to reaction mixtures containing various washed cell suspensions*

The main part of each Thunberg tube contained: 0.5 ml. M/2 sodium lactate; 0.5 ml. M/7.5 phosphate buffer pH 6.4; 0.5 ml. M/1600 Nile blue; 0.5 ml. of cell suspension.

Cell suspension	Final concentration of tetrathionate after mixing	Time taken to decolorize Nile blue (min.)	Deepest colour reached after adding contents of stopper	Time taken for colour to disappear again completely (min.)
'1433' adapted	Nil	6½	Pale blue	1-1½
'1433' adapted	M/100	6½	Deep blue	> 240
'1433' unadapted	M/100	7½	Pale blue	3½
Sonne	M/100	6	Pale blue	1½

Table 2. *Effect of adding various concentrations of tetrathionate to reaction mixtures containing tetrathionate-adapted cells*

The main part of each Thunberg tube contained: 0.5 ml. M/2 sodium lactate; 0.5 ml. M/7.5 phosphate buffer pH 6.4; 0.5 ml. M/1600 Nile blue; 0.5 ml. of adapted '1433' cells.

Final concentration of tetrathionate after mixing	Time taken to decolorize Nile blue (min.)	Deepest colour reached after adding contents of stopper	Time taken for colour to disappear again completely
Nil	6½	Pale blue	1-1½ min.
M/100	6½	Deep blue	Still deep blue at 4 hr.
M/200	6	Deep blue	1 hr.
M/400	6	Deep blue	30 min.
M/800	6	Deep blue	11 min.
M/3200	6	Deep blue	6½ min.
M/6400	6½	Pale blue	5 min.

and the time for it to disappear were noted. It can be seen from Table 3 that as the adapted cell suspension of '1433' was diluted the Nile blue was not fully reoxidized, and the time taken for the colour to disappear again completely was lengthened. On further dilution the time taken for the dye to be reduced completely again became shorter until in the last tube it was very little longer than in tube 2 which contained distilled water in place of adapted '1433' cells.

Experiments were performed with unadapted cells of '1433' and with a Sonne suspension, using various concentrations of tetrathionate in the stopper. Complete reoxidation of the Nile blue did not occur with either of these suspensions; only the usual pale blue colour appeared when the contents of the stopper were added to the mixture after initial reduction of the dye. With

the Sonne suspension the time taken (c. 1.5 min.) for this pale colour to disappear again was the same whether water or tetrathionate was added from the stopper. With the unadapted suspension of '1433', however, the time for the colour to disappear was slightly longer (c. 3.5 min.) when tetrathionate was added than when water was added (1.5 min.). These differences are very small

Table 3. *Effect of decreasing quantity of adapted cells in the reaction mixture*

Each tube contained: 0.5 ml. M/2 sodium lactate; 0.5 ml. M/7.5 phosphate buffer pH 6.4; 0.5 ml. M/1600 Nile blue; 0.5 ml. suspension; in the stopper 0.5 ml. M/80 tetrathionate. Tube 1 contained 0.5 ml. Sonne suspension, the others 0.3 ml. Sonne suspension and 0.2 ml. suspension of adapted cells of '1433' in the dilutions indicated.

Tube no.	Cell suspension			Deepest colour reached after adding contents of stopper	Time taken to recolorize Nile blue	Time taken for colour to disappear again completely
	Sonne (ml.)	Dilution of adapted '1433' of which 0.2 ml. was added	Time taken to decolorize Nile blue (min.)			
1	0.5	—	7	Pale blue	—	2½ min.
2	0.3	Distilled water only	9	Pale blue	—	3½–4 min.
3	0.3	1/1 (undiluted)	6	Deep blue	1 min. approx.	65 min.
4	0.3	1/2	7	Blue ?deep	?not quite fully; > 5 min. to reach maximum	c. 2 hr.
5	0.3	1/4	7½	Blue	Not fully; long time to reach maximum	2–3 hr.
6	0.3	1/8	9	Blue	—	Very pale after 25 min.; fully decolorized 40 min.
7	0.3	1/16	8	Pale blue	—	18 min.
8	0.3	1/32	8	Pale blue	—	8 min.
9	0.3	1/64	8½	Pale blue	—	5 min.

but may indicate partial reoxidation of the reduced Nile blue by the slight tetrathionase activity of the unadapted cells. Table 3 shows that such tetrathionase activity must be very small when compared to that of adapted cells.

Enzymic nature of process. The system responsible for the reoxidation of Nile blue by tetrathionate in the presence of adapted cells of '1433' can be destroyed by heat. The contents of the main part of a Thunberg tube were heated to 65° for 40 min. and allowed to cool after the Nile blue had been reduced and before tetrathionate was added from the stopper. The deep blue colour which appeared when tetrathionate was added to the unheated mixture did not appear when the same concentration of tetrathionate was added to the heated and cooled mixture.

Delay in reduction of redox indicators. Experiments were performed in which various concentrations of tetrathionate were added to the mixture of suspension, lactate, buffer and Nile blue in the main part of a Thunberg tube before the tube was evacuated and placed in the water-bath. It was found that even M/1600 (final concentration) tetrathionate gave a perceptible delay in the

reduction of Nile blue by adapted cells of '1433' but not by unadapted cells or by a Sonne suspension. Tetrathionate in quite small concentrations also inhibited the reduction of methylene blue by adapted but not unadapted cells of '1433'. Attempts to reoxidize reduced methylene blue with tetrathionate in the presence of adapted cells were not successful.

DISCUSSION

The experiments performed show that under suitable anaerobic conditions tetrathionate can oxidize reduced Nile blue in the presence of cells adapted to reduce tetrathionate, but not in the presence of cells which cannot reduce tetrathionate. The technique used was capable of detecting tetrathionate in concentrations as low as $m/3200$, and of detecting slight tetrathionase activity. The tetrathionase activity of the unadapted cells used here must be very small.

Attempts to inhibit the dehydrogenase systems of the adapted cells while leaving the tetrathionase system intact have so far been unsuccessful. Substances that inhibit the dehydrogenase systems appear to affect the tetrathionase system also, though perhaps rather more slowly. By means of the Nile blue reduction-oxidation technique, however, it may be possible to study the effect of various chemical and physical agents on the tetrathionase system.

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