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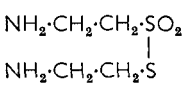
Radiation-protection of *Serratia marcescens* by a thiolsulphonate related to cysteamine

T. C. OWEN,* M. S. PARKER† AND G. M. STERN‡

A thiolsulphonate, 2-aminoethyl 2-aminoethanethiolsulphonate (also known as cystamine disulphoxide) affords significant protection to the micro-organism *Serratia marcescens* against the lethal effects of X-rays. The thiolsulphonate breaks down extensively at pH7, but the degree of protection given by it is much greater than can be accounted for by the action of decomposition products and is considered to be characteristic of the compound itself.

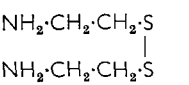
RECENTLY (Field, Ferretti, Crenshaw & Owen 1964), we have reported that a thiolsulphonate, 2-aminoethyl 2-aminoethanethiolsulphonate (I) related to cysteamine (II), and its *NN'*-diacetyl, *NN'*-didecyl, *NN'*-dimethyl and *NN'*-diureyl derivatives show significant radiation-protective activity in mice, comparable with the activity of cysteamine and its derivatives.

Among the many substances tested for radiation protective activity are amino-sulphur compounds closely related to cysteamine. Examples of these are cysteamine (II) itself (Bacq, Herve, Fischer, Lecomte, Blavier, Deschamps, LeBihan & Rayet, 1951), cystamine (III) (Bacq, 1956) and

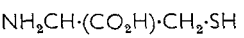


Thiolsulphonate (I)

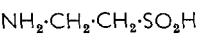
Cysteamine (II)



Cystamine (III)



Cysteine (IV)



Hypotaurine (V)

cysteine (IV) (Patt, Tyree, Straube & Smith, 1949). The rationale leading to development and testing of thiolsulphonates as potential radiation protective agents derives from their ability to convert thiols into mixed disulphides (Field, Owen, Crenshaw & Bryan, 1961). The capability of certain protective agents for forming disulphide linkages involving thiol groups of tissue constituents has been suggested as an important basis for protective action (Pihl & Eldjarn, 1958). Thiolsulphonates, however, are unstable in alkaline medium. The aminothiolsulphonates of interest here are no exception, being reasonably stable in aqueous solution only as their mineral acid salts (pH *ca* 4·5) and decomposing rapidly when the pH is raised to 5·5 or above by addition of bases (Field & others, 1964).

The work here described was carried out to determine whether the radiation protective action is an intrinsic property of the thiolsulphonate,

From the Department of Chemistry and the Department of Pharmacy, College of Technology, Liverpool, 3.

*Present address: Department of Chemistry, University of South Florida, Tampa, Florida, U.S.A.

†Present address: Department of Pharmacy, University of Strathclyde.

‡Present address: Department of Biochemistry, University of Birmingham.

or whether it could be ascribed to the cystamine (a known protective agent) and hypotaurine (V) which would result from its spontaneous decomposition at body pH. The stoichiometry of decomposition approximates to the rapid formation of four molecules of hypotaurine and one of cystamine from three of the thiolsulphonate.

Testing in organisms at a pH (4·5 or below) where the thiolsulphonate would be stable is manifestly impossible in higher organisms where close maintenance of neutrality (pH *ca* 7) is essential to survival; while quantitative comparison of the protection afforded by the thiolsulphonate and by its decomposition products requires large numbers of test animals to obtain statistically significant results. Accordingly, we chose as a test organism a micro-organism, the red pigmented bacterium *Serratia marcescens*. Use of this bacterium permitted examination of the thiolsulphonate at pH 4·5 where it is stable. Although the organism does not multiply at the lowered pH it remains viable and will grow upon readjustment to pH 7·3. Furthermore a statistically significant comparison of the activity at pH 7·3 of the thiolsulphonate and of its decomposition products was possible. The results have little or no direct bearing on the behaviour of this protective agent in other organisms, but are meaningful *per se*. Several factors influenced the choice of this particular bacterium. It is relatively easily cultured and counted, its radiation sensitivity is high (Dewey, 1960) and it is currently used in these departments in a continuing study of the effect of calcium ion concentration on its radiation sensitivity. Simultaneous determination of the protection afforded by a number of other sulphur compounds was planned as corroborative information, and it was felt that this could valuably be with a micro-organism other than *Escherichia coli* which had already been most extensively studied (Kohn & Gunter, 1959).

Experimental

MICRO-ORGANISM PREPARATIONS

Bacterial suspensions were derived from 18 hr cultures of *S. marcescens* (NCTC 1377) grown at 25° on nutrient agar slopes. Suspensions for irradiation were made by adding 6 ml of air-saturated sterile buffer (pH 7·3 or 4·0 as required) to a cultured slope, agitating gently 20 times, and diluting 1 ml of supernatant suspension with 49 ml of the same sterile buffer. 10 ml portions of the resulting suspension were irradiated.

SURVIVOR COUNTS

The irradiated and control suspensions were serially diluted three times, each 1 ml to 50 ml, with sterile pH 7·3 buffer, giving a total dilution of 1 to $1{\cdot}25 \times 10^5$. Agar plates were inoculated with 0·3 ml portions of the diluted suspension. Ten plates were prepared from each sample and each control (a freshly sterilised pipette for each inoculation) making a total of either 20 or 30 plates per experiment. The inoculated plates were incubated for 24 hr at 25° and colonies then counted. As a check on the reliability of counting, and as a precaution against late development

of colonies, a number of plates were selected at random intervals and counted on three separate occasions. No discrepancy greater than 3 in an average of 60–120 colonies occurred.

IRRADIATION

Cell suspensions (10 ml) were exposed to X-rays (230 KV, 15 mA, unfiltered) at a dose rate of 1,147 rads/min. The same radiolysis bottle (25 ml capacity; stoppered) was used for each exposure, the bottle having been well "browned" by irradiation before commencement of the work. Considerable care was taken to ensure reproducible geometry of exposure. Dose rates were determined by conventional ferrous (Fricke) dosimetry using the same radiolysis bottle. The LD50 was determined by carrying out a number of irradiations at various radiation doses (Table 1). Percentage kills were converted to probits of kill by probit transformation

TABLE 1. IRRADIATION OF *S. marcescens*; LD50 DETERMINATION*

Irradiation time (sec)	Mean colony count	Standard deviation (σ)	χ^2	Probability %	Kill %	Probit of kill
0	63	2·6	0·22	>80		
30	56	2·5	0·23	>80	11·1	3·83
0	117	7·6	3·5	>80		
60	64	3·3	1·7	>99	45·3	4·87
0	149	4·0	0·44	>95		
90	74	3·6	1·1	>98	50·3	5·0
0	134	8·4	4·7	>80		
150	28	2·8	2·8	>98	79·1	5·82
0	167	3·8	0·43	>99		
180	29	3·5	2·1	>80	82·6	5·95

* Data used for construction of Fig. 1.
LD50 interpolated from probit regression line, 1,490 rads in 78 sec.

(Finney, 1962), the probits plotted against the logarithm of irradiation time and the time of irradiation for 50% kill determined from the resulting linear graph. A time of 78 sec, corresponding to an LD50 of 1490 rads was found. Direct plotting of log % survivors vs. dose indicates that an initial threshold dose is required before an exponential dose-survival relationship is established (cf. Alper, 1961 and Fig. 1).

Three 10 ml portions of bacterial suspension were used for each determination of protective activity and toxicity of each of the potentially protective substances. These were: (1) a control; (2) a toxicity check containing the protector but not irradiated; (3) a sample containing protector and exposed to an LD50 radiation dose. Ten plates were prepared from each. Duplicate experiments were made.

Oxygen consumption in air-saturated aqueous buffers exposed to this low dose is negligible, eliminating the need for aeration during irradiation and minimising any possible effects from endogenous anoxia.

MATERIALS

Cystamine (III) and the thiolsulphonate (I) were prepared by oxidation of cysteamine with hydrogen peroxide (Field & others 1961). The

resulting hydrochlorides were converted to sulphates by adding an equivalent amount of sulphuric acid to their aqueous solutions, and the water and hydrogen chloride were evaporated off under reduced pressure (Owen & Wilbraham, unpublished work). Hypotaurine (V) was prepared by a modification of Cavallini's procedure (Cavallini, de Marco & Mondovi, 1953). Cysteamine (II) was prepared by hydrolysis of thiazolidinethione. Glycerol was of B.P. quality and cysteine (IV) hydrochloride was B.D.H. laboratory chemical grade.

Buffer solutions contained (*a*) 2·0 g/litre KH_2PO_4(pH 4·0): (*b*) 1·0 g/litre KH_2PO_4 with 1·25 g/litre K_2HPO_4 (pH 7·3), and were air saturated.

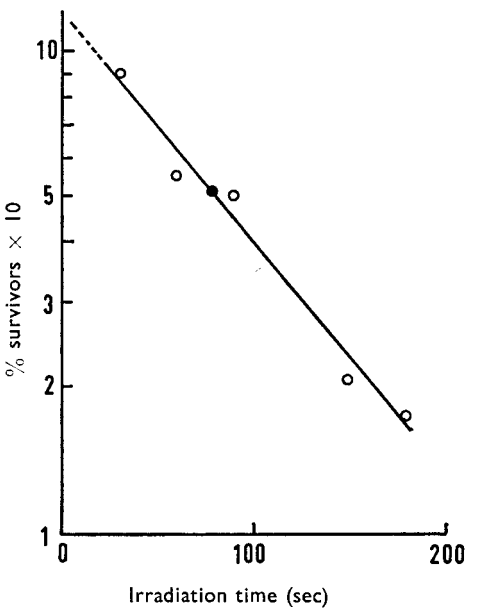


FIG. 1. Irradiation curve of *S. marcescens*.

PROTECTIVE ACTIVITIES

The protective activities and toxicities of these substances were examined by dissolving 10 mg in 10 ml suspensions of the bacteria. Preliminary work had indicated that higher concentrations were unduly toxic in certain instances and lower ones seriously impaired the significance of the protective activity observed.

Whilst the molecular weights of cystamine and the thiolsulphonate (sulphates) are roughly double those of cysteamine (hydrochloride) and hypotaurine, their equivalent weights are similar (125, 141, 113·5, 109 respectively) so that the equivalent concentrations (g equiv/litre) of the solutions used (10 mg in 10 ml) are comparable, ranging from 0·0071 for the thiolsulphonate to 0·0092 for hypotaurine. For the purposes of this

work, comparison of activities at similar equivalent concentrations is more pertinent than comparison at equimolar levels.

The protectors were added to the bacterial suspensions 5 min before irradiation, this time interval being indicated by preliminary studies. Kohn & Gunter (1959) had shown the importance of a "reactions period"

TABLE 2. IRRADIATION OF *S. marcescens*; PROTECTION AND TOXICITY. Radiation dose, 1,490 rads in 78 sec. Protector concentration, 10 mg/10 ml, pH 7·3*

Protective substance	Radiation dose (Rads)	Mean colony count	Standard deviation (σ)	χ^2	Probability %	Toxicity (% mortality)	Protection (*a*) (LD50 survival %)
None (control)	0	82	5·5	1·9	>80		
Thiolsulphonate (I) sulphate (*b*)	0	45	2·9	1·3	>98	45	
	1490	34	2·1	1·1	>99		76
None (control)	0	83	6·3	2·9	>80		
Cystamine (III) sulphate	0	69	5·4	3·8	>90	17	
	1490	35	2·4	0·97	>99		51
None (control)	0	114	5·5	1·6	>95		
Hypotaurine (V)	0	109	3·2	0·46	>99	4·4	
	1490	59	4·7	2·6	>90		54
None (control)	0	65	4·0	1·7	>95		
Cysteamine (II) hydrochloride	0	57	5·3	2·9	>80	12	
	1490	36	4·0	4·3	>80		63
None (control)	0	104	2·3	0·26	>99		
L-Cysteine (IV) hydrochloride	0	91	5·5	2·4	>90	12	
	1490	45	1·4	0·31	>99		50
None (control)	0	115	4·4	1·5	>99		
Thiolsulphonate (I) sulphate (*b*) pH 4·0	0	101	7·0	2·4	>70	12	
	1490	56	4·4	2·4	>90		55
None (control)	0	162	7·1	2·2	>90		
Dimethyl thiolsulphonate hydrochloride (*c*)	0	9	0·79	0·56	>99	94	
	1490	Not determinable due to high toxicity of compound					
None (control)	0	64	4·4	2·7	>95		
Glycerol	0	53	4·6	2·8	>90	17	
	1490	34	3·3	2·6	>95		64

(*a*) Number surviving irradiation in the presence of the protective substance, expressed as a percentage of the number surviving administration of the substance alone.
(*b*) 2-Aminoethyl 2-aminoethanethiolsulphonate sulphate.
(*c*) 2-Methylaminoethyl 2-methylaminoethanethiolsulphonate dihydrochloride. This compound protects mice (Field & others, 1964) but is toxic.
* Except where otherwise indicated.

(15 min) in chemical protection of *E. coli* B/r. With *S. marcescens* under our conditions no improvement of protection was observed after 5 min. The effects observed are reported in Table 2.

STATISTICS

Since the interpretation of the results depends heavily on quantitative estimation of toxicity and protection afforded, a reliable estimate of the statistical significance of the data is essential. Appropriate values are included in Tables 1 and 2.

Discussion

While the single point method adopted is of limited utility it is adequate for the objectives of this work; that is, to compare quantitatively the protective ability of the various substances under a given, arbitrarily selected, set of conditions. We would stress that different conditions might well lead to quite different results in certain instances.

The thiolsulphonate (I) is toxic to the bacteria so that investigation at low concentrations was necessary. Even so, a considerable measure of protection was observed under the conditions of these experiments at pH 7·3 but not at pH 4·0 when the compound is more stable chemically. Of those bacteria which survived the inherent toxicity of the substance (55% of a control sample) three quarters survived an LD50 radiation dose. While this may seem only a moderate measure of protection, it is much greater than that afforded by the other substances here examined all at pH 7·3 under similar conditions. The protection afforded by the thiolsulphonate is therefore considered significant.

The data obtained for cystamine indicate moderate toxicity and no protective ability while those for hypotaurine indicate very low toxicity and very slight protection. Thus the protective activity of the thiolsulphonate cannot be ascribed to action of these substances produced by spontaneous decomposition at pH 7·3.

Even complete metabolic reduction of the thiolsulphonate to cysteamine (II), a most improbable occurrence particularly in air-saturated buffer, would not account for the level of protection observed. Cysteamine does protect *S. marcescens* under our conditions, but to a markedly lesser extent than does the thiolsulphonate. It seems, therefore, that the protective ability is an intrinsic property of this latter substance.

Several possible mechanisms of radiation protection by aminothiols have been suggested. These include (*a*) trapping of primary radiolysis radicals ($H\cdot$, $HO\cdot$, $HO_2\cdot$) before these can interact with biochemically essential molecules (*b*) repair of damaged sites (produced by interaction of radicals with essential biochemicals) before oxygenative denaturation can occur, and (*c*) association of an essential sulphydryl group (*e.g.*, of enzyme or nucleoprotein) with the sulphydryl of the aminothiol, perhaps in a mixed disulphide linkage (see, e.g., Bacq, 1961). Quite probably all of these, and other, mechanisms may be involved.

Protection by the thiolsulphonate, seems to indicate that the mixed disulphide mechanism is at least involved here, although other mechanisms are by no means excluded. It is difficult to envisage its being a better radical trapping or repair agent than the less-oxidised cysteamine or cystamine, while its potentiality for rapid mixed-disulphide formation with thiols is well authenticated.

The observations with some of the other compounds studied merit mention. The protection afforded by glycerol, included here to give some basis for relating these studies to earlier work, was consistent with previous reports (Dewey, 1960). Surprisingly, L-cysteine showed no protective activity under our conditions; further investigation of this with different

concentrations and radiation doses is desirable. The thiolsulphonate exhibited low toxicity and negligible protection at pH 4 where it is reasonably stable, possibly because of non-absorption or metabolisation under these conditions. Finally, the rather high radiation-sensitivity of this micro-organism deserves comment. This is implicit in Dewey's observations and is confirmed by our finding of an LD50 of 1,490 rads, compared with about 3,500 rads for *E. coli* B/r (Kohn & Gunter). This makes for certain advantages (e.g. minimisation of endogenous anoxia) in studies at low doses such as those here described.

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