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The effects of the addition of manganese dioxide to media on the viability of bacteria damaged by x-rays, phenol and radiomimetic agents

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Suspensions of two strains of *Escherichia coli* were counted before and after damage by phenol, X-rays and radiomimetic substances on nutrient agar which had been treated in various ways with manganese dioxide. The medium after some treatments gave increased counts with cells damaged by phenol and dimethyl busulphan and with undamaged cells, the strains responding rather differently. The beneficial action of manganese dioxide was not due to the breakdown of peroxides in the medium and may be due to the adsorption of toxic substances from the medium.

IT is known that manganese dioxide is capable of initiating the breakdown of peroxide in media and its inclusion therefore affords protection towards catalase negative organisms, such as *Shigella dysenteriae* (Proom, Woiwod, Barnes & Orbell, 1950). Richards (1959) claimed that untreated and phenol treated *Escherichia coli* were not influenced significantly by the addition of manganese dioxide to media, and concluded that the inhibitory effects of agar on damaged bacteria were not due to the presence of peroxides therein. However it has now been shown by Board (personal communication) that with phenol-treated *E. coli* toxic effects similar to those reported by Jacobs & Harris (1960, 1961) and by Richards (1959) are due to the presence of peroxides. Board demonstrated a protective effect by including manganese dioxide in a layer of non-nutrient gel over the nutrient agar; the protective effect was not present when manganese dioxide was included in the nutrient agar.

It has been reported that irradiation of cells sensitises them to peroxide produced in the extracellular fluid (Wyss, Clark, Haas & Stone, 1948; Ogg, Adler & Zelle, 1956) and it seemed possible that such cells would also be affected by the presence of peroxides in nutrient media. It was therefore decided to see whether Board's technique would improve media for the recovery of phenol-treated organisms and also to determine what rôle might be played by peroxides in media in the inhibition of irradiated cells and those exposed to radiomimetic substances.

If there were any beneficial effects of including manganese dioxide in media, it would be necessary to ascertain that they were in fact due to decomposition of peroxides. Organisms known to be peroxide sensitive were therefore counted on the various media to determine if manganese dioxide had a favourable effect on their growth.

Experimental

Organisms. Two strains of *Escherichia coli* type 1 (44°+) were used routinely, namely B/r and strain II of Harris, Richards & Whitefield

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(1961). Three other organisms reputed to be sensitive to peroxides were chosen: *Shigella dysenteriae* strain CN 191, obtained from the Wellcome Bacterial collection and described by Proom & others (1950); *Pasteurella boviseptica* strain CN 1066, also obtained from the Wellcome Bacterial collection and recommended by Proom; and *Chromobacterium violaceum* strain B6 of the Department of Agriculture, University of Edinburgh.

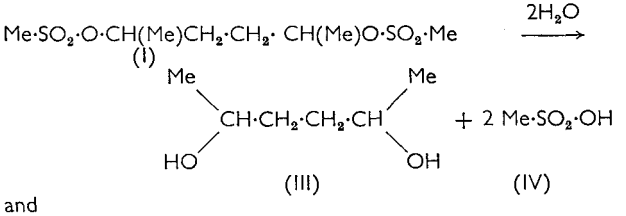
Suspensions. All the organisms were maintained on nutrient agar slopes. A large nutrient agar slope was inoculated from a 24 hr broth culture and the organisms were harvested with 10 ml of glass distilled water after 24 hr at 37°. This dense suspension was washed twice, resuspended in 10 ml of diluent, and stored at 8°. Such suspensions maintain their viability over long periods (Cook & Steel, 1955; Cook, Steel & Wills, 1956) and this was confirmed in the present instance. The suspensions were allowed to age for 5 to 6 days then used over 2 weeks, diluting before each trial, on the basis of an initial nephelometric measurement, to give a concentration of about 2×10^9 viable cells/ml.

BACTERICIDAL TREATMENTS

Phenol. One ml of standardised suspension in glass distilled water was added to 5 ml of phenol solution at 20° ± 0·1 and the mixture was shaken well. The final concentrations of phenol used were 0·65 and 0·90% (w/v) for strains B/r and II respectively, with corresponding reaction times of about 20 and 15 min. These conditions resulted in mortality levels of about 99%, the reaction times being varied to allow for changes in resistance between suspensions.

Irradiations. A Westinghouse Therapy X-ray machine was operated at 220 kV and 15 mA to give doses of about 2500 rad/min in the irradiation cell, as determined by ferrous sulphate dosimetry. Suspensions were diluted in 0·05M phosphate buffer at pH 7·0 before exposure, and were irradiated in shallow layers so that conditions were aerobic. The irradiation times for 99% mortality were 20 min (about 50 Krad) for strain B/r and 12 min (about 30 Krad) for strain II.

Radiomimetic agents. The substances used were dimethyl busulphan (I) and chlorambucil (II; Leukeran, Burroughs Wellcome Ltd.) both of which react with water according to the following equations:



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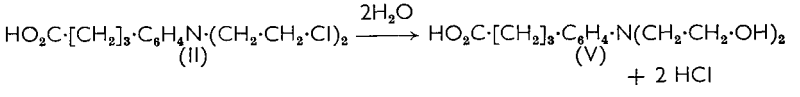

Treatments with dimethyl busulphan were at 37° to keep all the components of the reaction mixture in solution: at 20° insoluble material III

precipitated during the reaction creating difficulties in sampling and introducing indeterminate effects due to adsorption. Precipitation did not occur with chlorambucil and 20° was used with this substance, since it was the same temperature as was used for the phenol treatments. The reactions with water proceed exponentially with time and, under the conditions used, most of the substances had reacted in 1 hr; this was used as the arbitrary contact time to achieve 99% mortality. To obviate frequent weighings of small quantities, 6×10^{-2}M stock solutions in acetone were used. The fact that counts were only slightly lower after 2hr exposure than at 1 hr showed that there was no benefit in prolonging the contact time to permit more complete reaction of the substances and that the acetone itself had no bactericidal action. Since each reaction yields acid products i.e. IV, V and hydrochloric acid, the reactions were performed in buffer solution to prevent adverse pH effects.

Three ml quantities of reaction mixture were formulated as follows: acetone solution of radiomimetic agent, 0·3 to 0·4 ml; standardised bacterial suspensions in buffer, 0·5 ml; phosphate buffer (pH 7·0, 0·05M), to yield 3 ml. Both the suspension and the buffer solutions were equilibrated at 20 or 37° as necessary, before adding the acetone solution of the drug. After 60 min the reaction was quenched by diluting 100 times with buffer solution, further dilutions being made as necessary. The volume of acetone solution of the drug was varied slightly to obtain the required mortality level and this gave concentrations of about 6 to 8×10^{-3}M.

Media. Normal nutrient agar (unlayered control) was prepared from Oxoid CM15 granules (16 g/litre in distilled water) and solidified with 15 g/litre of New Zealand agar, 20 ml quantities being used for the over-dried plates. Four other media were also used. In one, 0·1% manganese dioxide was incorporated into the nutrient agar. The other three comprised normal nutrient agar with a layer of suitably formulated agar gel superimposed on it. These layered media consisted of 15 ml of control medium, but with a nutrient concentration 4/3 of that in the normal medium, covered by a 5 ml layer of gel containing no nutrients, so that the final concentration of nutrients, assuming complete diffusion, was the same as in the non-layered media. The upper layer of one of these three media was a plain agar gel (layered control), that of the second contained 0·1% manganese dioxide and the upper layer of the third contained 0·4% manganese dioxide. In this last medium the amount of manganese dioxide, relative to nutrients and agar, was the same as in the medium comprising normal nutrient agar plus 0·1% of manganese dioxide. The use of layered media was suggested to us by Board, who found them preferable to media containing manganese dioxide dispersed throughout.

To verify the status of the peroxide sensitive organisms, two of the species were also counted on these media in which peroxide formation had been induced by ultraviolet radiation. That such treatment does lead to the formation of peroxides may be inferred from the work of Bacq (1951) and of Latarjet, Caldas, Morenne & Chamaillard (1952) since the indirect lethal action of ultraviolet radiation was alleviated by the presence of catalase. The nutrient agar plates, with lids removed, were irradiated

about 12 inches distant from a Philips 30 W ultraviolet tube and the lids replaced immediately. Suitable exposure times were 15 and 30 min for *Past. boviseptica* and *Shigella dysenteriae* respectively.

Viable counts. Plate counts were made by the surface viable method using 5 replicate 0·017 ml drops and counting after 24 hr at 37°.

Results and discussion

The results of counting untreated cells of both strains of *E. coli* on the five media described above, and after treatment with each of the lethal agents, are given in Table 1. With untreated organisms, only strain B/r grown on the medium layered with 0·4% MnO_2 gel gave counts which the *t*-test showed to be significantly higher than the appropriate control, all other counts showing a non-significant difference from the appropriate control. The results of analyses of variance indicate that there were no significant differences in count between the five media, except in the case of strain II, where the counts were low on two of the modified media.

TABLE 1. THE EFFECT OF INCLUDING MANGANESE DIOXIDE IN THE NUTRIENT AGAR ON THE VIABLE COUNT OF *E. coli*

Treatment	Strain	Proportional count* on			
		Control +0·1% MnO_2	Layered control	Layered control with 0·1% MnO_2	Layered control with 0·4% MnO_2
None	II	82	82	99	85
	B/r	104	129	161	170†
Phenol	II	147†	84	175†	235†
	B/r	297†	39	158†	263†
X-rays	II	49	104	95	115
	B/r	70	100	97	92
Chlorambucil	II	62	90	99	105
	B/r	107	98	102	93
Dimethyl busulphan	II	43†	112	120	139†
	B/r	100	99	136†	168†

* Relative to unlayered control as 100. Mean of 5 trials in all cases except 4 trials with strains B/r and II with X-rays and 6 trials with strain II and dimethyl busulphan.
† Significantly different from the appropriate control (p. 5).

With phenol-treated organisms the strains varied markedly in their responsiveness, and analyses of variance showed that modification of the medium produced significant effects ($P < 0·001$ in all instances). Strain II showed significantly higher counts on all media containing manganese dioxide and the latter was more effective in the layered media than when added to normal medium. Strain B/r behaved in a similar way in that the inclusion of manganese dioxide was always favourable, except that 0·1% manganese dioxide in the medium was as effective as layering the nutrient medium with 0·4% MnO_2 gel. A further point of difference from strain II was that the layered control gave relatively much lower counts than the normal medium. When organisms had been exposed to X-irradiation, the results of *t*-tests indicate that there were no significant differences from the appropriate controls when manganese dioxide was present.

In the main, results with chlorambucil-treated *E. coli* showed that these organisms, like untreated ones, were insensitive to the inclusion of manganese dioxide in the media and this conclusion is supported by the results of *t*-tests.

After treatment with dimethyl busulphan, suspensions were influenced to a greater extent by media modified with manganese dioxide than were cells damaged by chlorambucil. Significant beneficial effects were observed with strain II counted on media layered with 0·4% MnO_2 gel and with strain B/r on media layered with either concentration of manganese dioxide in agar gel.

Thus the inclusion of manganese dioxide in media could result in increased counts of suspensions of *E. coli* after exposure to certain lethal agents. Since the dioxide is known to initiate the decomposition of peroxides, it remained to decide whether the favourable effects were due to this attribute or not. The peroxide-sensitive organisms were therefore counted on the various media to see whether normal medium contained peroxide and, if so, whether manganese dioxide had a favourable effect on the growth of these organisms. The results given in Table 2 showed that in no instance was there a significant improvement in the various media when manganese dioxide was present. Thus the medium employed here would be described by Proom & others (1950) as a "good" medium, i.e. one containing little or no peroxide.

TABLE 2. THE EFFECT OF INCLUDING MANGANESE DIOXIDE IN THE NUTRIENT AGAR ON THE VIABLE COUNTS OF UNTREATED PEROXIDE SENSITIVE BACTERIA

	Proportional count* on			
			Layered control with	
Organism	Control +0·1% MnO_2	Layered control	0·1% MnO_2	0·4% MnO_2
Chromobacterium violaceum	12	94	97	87
Shigella dysenteriae	31	108	143	110
Pasteurella boviseptica ..	76	109	105	102

* Relative to unlayered control as 100. Mean of 5 trials with *Chromobacterium violaceum* and 6 trials with the other two organisms. In no case did the *t*-test indicate that differences from the appropriate control were significant.

TABLE 3. THE EFFECT OF INCLUDING MANGANESE DIOXIDE IN NORMAL AND ULTRAVIOLET IRRADIATED MEDIA ON THE VIABLE COUNTS OF UNTREATED PEROXIDE SENSITIVE BACTERIA

		Proportional count* on				
					Layered control with	
Organism	Medium	Control	Control +0·1% MnO_2	Layered control	0·1% MnO_2	0·4% MnO_2
Shigella dysenteriae	Unirradiated	100	117	89	116	116
	Irradiated	28	117	2	111	117
Pasteurella boviseptica	Unirradiated	100	126	39	123	157
	Irradiated	<2	126	<1	115	132

* Relative to unirradiated normal control as 100, mean of 5 trials.

Finally, it was important to verify that these test organisms were in fact peroxide sensitive. Two of the species, *Shigella dysenteriae* and *Pasteurella boviseptica*, were therefore counted on the same range of media as before and also on these media in which peroxide formation had been induced by ultraviolet radiation. Table 3 presents the results of these experiments. It is obvious that the ultraviolet irradiation of the media resulted in low counts of both species, and that low counts did not occur when manganese dioxide was present, regardless of the manner of its presentation.

It must be concluded that although treatment of the media with manganese dioxide resulted in higher counts in some circumstances, notably after phenol treatment, this beneficial effect was not due to the removal of peroxide from the media. It is possible that the manganese dioxide adsorbed toxic substances, having effects similar to those noted with ferric chloride and activated charcoal (Jacobs & Harris, 1960, 1961).

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