THE INFLUENCE OF THE NATURE OF THE RECOVERY MEDIUM ON THE APPARENT VIABILITY OF PHENOL-TREATED BACTERIA

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Suspensions of *Escherichia coli* and *Staphylococcus aureus*, untreated and treated by exposure to phenol, *o*-cresol or *p*-chloro-*m*-cresol, were counted on various types of media. While the counts from untreated suspensions were not affected by the nature of the medium, those from treated suspensions were influenced markedly. There was no correlation between the nutritional status of a medium and its suitability for the cultivation of the treated organisms. Although both bacterial species gave similar patterns of response and ranked the media in roughly the same order of suitability, *E. coli* was the more responsive species. Generally, the order of suitability was: meat extracts, yeast extracts, meat extract-peptone broth, peptones (all the single ingredients 1 per cent, pH 7; media solidified with New Zealand agar). Although supplementation of the meat extract-peptone control medium, especially with yeast extracts, led to higher counts from treated suspensions, dilution of the extract broth also gave similar results. Further, a fresh infusion broth was superior both to the extract broth and to a digest medium, for counting treated bacteria. The deficiencies of the extract broth were due partly to the phosphate precipitation procedure used in its preparation. The results indicate that when media suitable for the growth of damaged bacteria are required, they should be selected without recourse to preconceived ideas about composition, concentration or method of preparation.

Many types of media are used for the growth of bacteria, numerous complex materials being employed as enrichments to promote the growth of exacting species (Knight, 1936; Oxoid Manual, 1961; Difco Manual, 1953) and Jacobs and Harris (1960) have drawn a parallel between such organisms and damaged bacteria. The importance of the nature of the medium used for the growth of damaged bacteria and bacterial spores has been emphasised by Nelson (1943) and the value of enrichments has also been stressed (Curran and Evans, 1937; Thompson, Mefferd and Wyss, 1951). In this connection, yeast extracts have been found to reverse inhibition by acriflavine (McIlwain, 1941) and penicillin (Chattaway, Hall, Happold and Holdsworth, 1949). Also, several authors observed that recoveries with frozen cells were higher in rich than in minimal media (Straka and Stokes, 1959; Nakamara and Dawson, 1962; Postgate and Hunter, 1963). Although Needham (1947) used a simple peptone water as a growth medium after disinfectant testing, the suitability of this was not determined using damaged cells.

However, the selection of media and their composition is primarily empirical, and it was thought useful to examine in detail the performance of a wide range of materials for the cultivation of damaged bacteria.

Materials and Methods

Throughout this paper, all percentages are given as weight in volume (w/v) unless otherwise stated.

Organisms and bactericidal treatments. Two species were used: a laboratory strain of *Escherichia coli* type I (44° +), described previously (Jacobs and Harris, 1960), and *Staphylococcus aureus* NCTC 4736. They were grown on nutrient agar slopes for 24 hr. at 37° and suspensions of washed cells containing about 10^9 viable cells/ml. were prepared.

To 4 ml. of bactericide at 20° ± 0·5 was added 1 ml. of suspension, and conditions were such that the final concentrations in the reaction mixtures were: phenol 1 per cent, *o*-cresol 0·4 per cent, *p*-chloro-*m*-cresol (PCMC) 0·075 per cent. The reaction was stopped when desired by diluting at least 200 times, and survivors were counted. A wide range of contact times and dilutions were used to allow for day to day variations in the responses of the suspensions, but conditions were such that mortalities were of the order of 90 to 99 per cent, and were often greater.

Media. The extract broth employed routinely and as control medium contained peptone (Oxoid no. L 37), 10 g.; Lab-Lemco, 10g.; sodium chloride, 5 g.; tap water to 1 litre: the normal method of phosphate precipitation was used.

Single medium constituents were used as simple 1 per cent solutions in tap water and were processed only by adjustment to pH 7·0, sterilisation and the addition of agar. For some experiments Hartley's broth and an infusion broth were prepared using established procedures (Mackie and McCartney, 1953), although the infusion broth was prepared both with and without the phosphate precipitation procedure.

Unless stated otherwise, media were solidified using 15 g./litre of New Zealand agar, though 25 g./litre of powdered Japanese agar was used for some experiments. All media were sterilised by autoclaving for 20 min. at 15 lb./in.^{2}

Colony counts and presentation of results. Counts were done by the surface viable method (Miles and Misra, 1938) using 5 replicate 0·02 ml. drops, and colonies were counted after 48 hr. or 24 hr. at 37°, using a lens, the 48 hr. count being used wherever possible since experience showed that numbers were then maximal. Generally at least 2 trials were done with untreated organisms, and at least 3 trials with suspensions exposed to all 3 bactericides, using each batch of media; 2 or 3 batches of media were often used. Thus the full results are too extensive for complete presentation and, for clarity, only mean values are given in the Tables. In each trial the counts were expressed as a percentage of the control count, and the mean relative responses computed as the means of these percentages. Since this work was completed the wide variation in response between individual trials, and between batches, has been explained (Harris, Richards and Whitefield, 1961): so too much emphasis should not be placed on the magnitudes of the observed responses, which indicate only the effects which it is possible

to obtain in such experiments. However the data rank the media reliably in order of suitability. The difficulties of applying conventional statistical procedures to such data have been discussed by Harris and Jacobs (1960).

Results

The Growth of Bacteria Damaged by Phenols on a Range of Medium Constituents

Preliminary trials. The results of initial experiments with a limited range of materials indicated clearly that, although untreated suspensions of both species gave similar counts on all the media, counts from phenol-treated suspensions varied widely and were highest on simple meat extract media, less on yeast extracts and lowest on peptone waters, the effects being more marked with *E. coli* than with *Staph. aureus*. Accordingly, the experiments were repeated using both species damaged by all three bactericides, and the results (Table I) confirmed the earlier findings. Although the two species behaved somewhat differently, in both cases Bacto-Beef Extract gave the highest counts and Bacto-Peptone the lowest, the control extract broth being only slightly better than the poorest medium.

TABLE I

THE COUNTS OF BACTERIA TREATED WITH PHENOLS ON A RANGE OF MEDIUM CONSTITUENTS

Medium†	Mean relative response* after treatment					
	E. coli			*Staph. aureus*		
	Phenol	*o*-Cresol	PCMC‡	Phenol	*o*-Cresol	PCMC‡
Oxoid Peptone	450	225	909	150	100	117
Bacto-Peptone	50	75	298	0	100	50
Lab-Lemco	2,800	3,450	14,500	375	225	300
Bacto-Beef Extract ..	26,450	36,150	32,700	850	1,030	917
Oxoid Yeast Extract ..	350	375	6,690	700	500	317
Bacto-Yeast Extract ..	375	1,150	14,390	1,250	600	500

* Relative to extract broth (Lab-Lemco, Oxoid Peptone) as 100,
† All 1 per cent in tap water, pH 7·0; media set with New Zealand agar.
‡ PCMC, *p*-chloro-*m*-cresol.

Results with a wider range of medium constituents. These are given in Table II. Once again, counts of untreated cells of both species were similar on all media, while counts from treated suspensions varied considerably. Bacto-Peptone was the least suitable medium, whatever the organism or treatment, and all the peptones, casein hydrolysate and malt extract gave low counts relative to the control. Materials giving the highest responses in this group were casein hydrolysate with *E. coli* and Bacto-Tryptone with *Staph. aureus*, and with these materials counts approached those of the control. In contrast to these results, counts on all the meat and yeast extracts were substantially higher than on the extract broth, except with *Staph. aureus* on Oxoid Liver Extract where results were rather variable. Results with *E. coli* were especially striking with this batch of media.

TABLE II

THE COUNT OF UNTREATED AND PHENOL-TREATED BACTERIA ON A WIDE RANGE OF MEDIUM CONSTITUENTS

Medium†	Mean relative response* after treatment							
	E. coli				*Staph. aureus*			
	Untreated	Phenol	*o*-Cresol	PCMC	Untreated	Phenol	*o*-Cresol	PCMC
Peptones, etc.								
Oxoid Peptone	87	5	10	5	101	46	28	39
Bacto-Peptone	91	< 1	1	< 1	100	16	19	17
Bacto-Tryptone	98	31	22	15	108	93	80	49
Eupepton No. 2	88	2	7	4	107	28	41	43
Casein Hydrolysate	93	47	105	74	107	52	74	45
Malt Extract	81	49	11	19	102	56	29	24
Meat and Yeast Extracts								
Lab-Lemco	106	49×10^4	45×10^4	16×10^4	106	650	746	411
Bacto-Beef Extract	106	50×10^4	40×10^4	13×10^4	118	660	623	440
Wilson's Beef Extract	93	41×10^4	36×10^4	12×10^4	114	736	763	367
Jardox	102	54×10^4	44×10^4	21×10^4	103	690	719	448
Oxoid Liver Extract	98	1750	688	2443	110	144	177	123
Bacto-Yeast Extract	93	38×10^4	35×10^4	16×10^4	100	245	359	203
Oxoid Yeast Extract	103	15×10^4	20×10^4	8×10^4	113	249	256	184
Yeastrel	92	28×10^4	34×10^4	14×10^4	108	618	599	353
Marmite	89	33×10^4	31×10^4	16×10^4	112	148	421	228

* Relative to extract broth as 100.

† All 1 per cent in tap water, pH 7; all media set with New Zealand agar. Sources as follows: Lab-Lemco and Oxoid products, Oxo Ltd., London, E.C.4; Bacto Products, Baird and Tatlock Ltd., Chadwell Heath, Essex; Eupepton No. 2, Casein Hydrolysate (vitamin free) and malt extract, Allen and Hanbury's Ltd., London, E.2; Wilson's Extract of Beef, Wilson's Meats Ltd., London, E.C.1; Jardox, Jardox Concentrated Products Ltd., London, S.E.20; Yeastrel, Brewer's Foods Supply Co. Ltd., Edinburgh, 3; Marmite, The Marmite Food Extract Co. Ltd., London, E.C.3.

Repetition of the above experiments using a second batch of media gave a similar pattern of results, though the magnitude of favourable responses was substantially less, especially with *E. coli* with which the meat extracts gave mean counts 3 to 4 times those on the control, while those with *Staph. aureus* were about twice the control count. The yeast extracts gave intermediate responses with both species.

Results with Japanese agar. It was known that media solidified with Japanese agar gave substantially higher counts than those prepared with New Zealand agar (Jacobs and Harris, 1961), and experiments were repeated using media solidified with the former material (Table III). This time, counts with treated *Staph. aureus* were lower than on the

TABLE III

THE COUNTS OF UNTREATED AND PHENOL-TREATED BACTERIA ON A RANGE OF MEDIUM CONSTITUENTS SOLIDIFIED WITH JAPANESE AGAR

Medium†	Mean relative response* after treatment							
	E. coli				*Staph. aureus*			
	Untreated	Phenol	*o*-Cresol	PCMC	Untreated	Phenol	*o*-Cresol	PCMC
Oxoid Peptone	100	18	10	5	100	13	46	25
Bacto-Peptone	93	13	64	62	93	16	34	27
Lab-Lemco	95	1,865	530	475	109	78	92	23
Bacto-Beef Extract	96	3,093	878	688	93	86	82	91
Oxoid Yeast Extract	100	935	376	311	93	71	59	78
Bacto-Yeast Extract	99	80	70	38	91	48	58	61

* Relative to extract broth as 100.

† All 1 per cent in tap water, pH 7.

control on all the single ingredient media, but with treated *E. coli*, Bacto-Beef Extract, Lab-Lemco and Oxoid Yeast Extract again gave mean counts higher than did the control extract broth, while Bacto-Yeast Extract, Bacto-Peptone and Oxoid peptone gave lower counts. Despite the smaller responses relative to the control, however, the materials were still graded in approximately the same order of suitability for revival.

The Influence of the Method of Medium Preparation on the Counts of Damaged Bacteria

The results reported above showed that a medium prepared from Oxoid peptone and Lab-Lemco almost always gave lower counts of damaged cells than one containing Lab-Lemco only, and this was sometimes true also with the simple peptone water medium. It seemed possible that the phosphate precipitation procedure used in the preparation of the extract broth could have been responsible for this phenomenon. Accordingly, counts of untreated and treated suspensions of both species were done on 3 media solidified with agar: viz., the standard extract broth; a simple solution of 1 per cent Lab-Lemco plus 1 per cent Oxoid peptone, with no pH adjustment; the latter adjusted to pH 7 before autoclaving, no phosphate precipitation being done.

TABLE IV

THE EFFECT OF PHOSPHATE PRECIPITATION TREATMENT OF A MEDIUM ON THE COUNTS OF UNTREATED AND PHENOL-TREATED BACTERIA

Treatment of medium† and batch number		Mean relative response* after treatment							
		E. coli				*Staph. aureus*			
		Untreated	Phenol	*o*-Cresol	PCMC	Untreated	Phenol	*o*-Cresol	PCMC
Phosphate precipitation ..	—	100	100	100	100	100	100	100	100
None‡	1	96	3,960	1,740	2,820	92	214	159	168
	2	100	71	81	110	90	38	55	63
	3	106	41	84	57	100	4	41	52
Adjust to pH 7·0	1	100	971	810	1,210	102	163	134	132
	2	103	183	148	192	104	127	142	137
	3	124	666	220	235	104	195	122	149

* Relative to standard extract broth, prepared with phosphate precipitation, as 100.
† All media 1 per cent each Lab-Lemco and Oxoid Peptone, 0·5 per cent NaCl; solidified with New Zealand agar.
‡ Batch 1, pH 6·2; batch 2, pH 5·9; batch 3, pH 5·5.

The mean results of trials with 3 batches of each medium are given in Table IV and show that the medium adjusted to pH 7, but on which no phosphate precipitation had been carried out, always gave higher counts of damaged cells than did the control. Once again, responses with *E. coli* were considerably larger than those with *Staph. aureus*. On the medium prepared without either phosphate precipitation or pH adjustment, higher counts than on the control were obtained with batch 1, but subsequent batches gave lower counts than the extract broth. This marked difference in response between batches could have been due to the variation in pH, which declined from 6·2 in batch 1 to 5·5 in batch 3. The reason for this pH variation is not known.

Digest and infusion media. Freshly prepared media, and especially those prepared by digestion, are generally accepted as superior to those containing prepared ingredients. The results of counting untreated and treated suspensions on extract, digest and infusion media are given in Table V. Since it was known that phosphate precipitation damaged the extract medium, the infusion broth was prepared both using and omitting this procedure, both types being adjusted to pH 7·2, to check whether damage resulted with this medium also. When phosphate precipitation was not used, a precipitate was produced during sterilisation, but this did not interfere with counting by the surface viable technique.

TABLE V

THE COUNTS OF UNTREATED AND PHENOL-TREATED BACTERIA ON EXTRACT, DIGEST AND INFUSION MEDIA

Type of broth and batch number		Mean relative response* after treatment							
		E. coli				*Staph. aureus*			
		Untreated	Phenol	*o*-Cresol	PCMC	Untreated	Phenol	*o*-Cresol	PCMC
Infusion, no phosphate precipitation	—	100	100	100	100	100	100	100	100
Infusion, with phosphate precipitation	1	—	—	—	—	—	—	—	—
	2	94	61	58	50	113	109	72	72
Hartley's Digest	1	104	<1	<1	<1	103	35	50	62
	2	96	7	13	2	97	89	50	29
Extract (standard)	1	99	<1	<1	<1	104	55	62	81
	2	97	22	29	21	103	91	95	69

* Relative to infusion medium, prepared without phosphate precipitation, as 100. All media set with New Zealand agar.

All four media gave similar counts with untreated suspensions but, with treated ones the infusion medium prepared without phosphate precipitation always gave substantially higher counts than the other media. Results are therefore expressed using this medium as a control in place of the standard extract broth. Previous results were confirmed, in that responses were more marked with treated *E. coli* than with *Staph. aureus* and phosphate precipitation usually impaired the infusion medium. Although the digest medium gave a substantially greater bulk of growth of both species than did the extract medium, counts after exposure to phenols were lower on the former than on the latter.

Supplementation. Routine media are often supplemented with yeast extracts and other materials to improve their growth capacity, especially for exacting species. Various supplements were added singly to the standard extract broth and the counts of phenol-treated suspensions on the resultant media, none of which affected the counts of untreated cells, are shown in Table VI. Treated suspensions however yielded counts higher on supplemented media than on the control. Yeastrel and Bacto-Yeast Extract were the most favourable additives, while responses to the addition of casein hydrolysate were small.

TABLE VI

THE INFUENCE OF SUPPLEMENTATION OF THE STANDARD EXTRACT BROTH ON THE COUNTS OF BACTERIA DAMAGED BY PHENOLS

Supplement†	Mean relative response* after treatment					
	E. coli			*Staph. aureus*		
	Phenol	*o*-Cresol	PCMC	Phenol	*o*-Cresol	PCMC
Bacto-Yeast Extract	482	522	333	170	160	160
Oxoid Yeast Extract	103	145	162	142	124	117
Marmite	182	370	256	162	149	128
Yeastrel	479	2,263	548	164	155	127
Casein Hydrolysate	109	110	134	129	114	125
Malt Extract	581	812	406	196	170	132
Oxoid Liver Extract	280	170	244	142	137	134

* Relative to unsupplemented extract broth as 100, mean of 3 batches of media, all set with New Zealand agar.

† 1 per cent of the substance was added to the extract broth after phosphate precipitation and adjusted to pH 7·0.

No favourable effects were observed when media were supplemented either with colloidal materials, e.g., 1 per cent horse serum, egg albumen, acacia or starch, or, despite the beneficial effect of Malt Extract, with a range of fermentable carbohydrates.

Medium concentration. Since all the supplemented media were more concentrated than the control, and the medium used in the standard Rideal-Walker test (B.S. 541, 1953) is twice the concentration used routinely here, the effects of medium concentration were investigated. Portions of a double strength extract broth were diluted with distilled water to give broths of normal strength and one third normal strength. The results with two batches of these media are given in Table VII.

TABLE VII

THE INFUENCE OF MEDIUM CONCENTRATION ON THE COUNTS OF UNTREATED AND PHENOL TREATED BACTERIA

Concentration of broth and batch number		Mean relative response* after treatment							
		E. coli				*Staph. aureus*			
		Un-treated	Phenol	*o*-Cresol	PCMC	Un-treated	Phenol	*o*-Cresol	PCMC
Double strength ..	1	100	<1	1	<1	102	71	60	49
	2	108	12	1	<1	105	50	67	66
One-third strength	1	103	688	3,935	671	105	328	243	174
	2	100	4,000	3,600	4,875	106	152	156	136

* Relative to normal strength extract broth as 100. All media set with New Zealand agar.

Contrary to expectation, counts of damaged organisms increased with increasing dilution; strikingly higher counts being obtained on the most dilute medium with treated *E. coli*, and although responses with treated *Staph. aureus* were smaller, they were still substantial.

DISCUSSION

Two general trends are clear from the above results; that untreated suspensions of *E. coli* and *Staph. aureus* always gave similar counts

whatever the type of medium employed, whereas treated cells were markedly sensitive to their environment, and that treated *E. coli* cells were considerably more responsive to revival than those of *Staph. aureus*. Both of these phenomena have been observed previously (Jacobs and Harris, 1960; 1961).

There are numerous reports on the restraining action which organic matter has on bactericidal action (e.g., Chick and Martin, 1905; Klarmann, Shternov and von Wowern, 1929; Garrod, 1935), and Winslow and Brooke (1927) attributed the abolition by dilute broth of the death of bacteria in distilled water to a protective action of colloidal matter. In this connection, the protection of bacteria by young, heat killed organisms (Lange, 1922) is also relevant. However, no evidence was obtained here that colloidal material favoured revival. However, if death from phenol is due partly to leakage of metabolites from cells (Gale and Taylor, 1947; Salton and Alexander, 1950), then replacement of leaked materials by nutrients from the medium may also be important. It is difficult to decide what substances may be most valuable for revival, but it is known that amino-acids, nucleic acid residues and growth factors may be liberated from damaged cells (Cook and Cronin, 1941; Gale and Taylor, 1947; Loofbourow, 1947; Loofbourow and others, 1947; Salton and Alexander, 1950). Therefore, it is not surprising that meat and yeast extracts, which are rich in such materials, were more favourable to revival than were peptones, which are relatively poor in them. The importance of yeast nucleotides in promoting the viability of cells other than bacteria has been demonstrated for tissue cultures (Lesfargues and Delaurey, 1947). However, media which contained abundant nutrients, and which gave excellent growth of untreated suspensions, often gave lower counts of damaged cells than did media of low nutrient content, so nutrient capacity alone cannot account for the observed differences. Also no support was found for the recommendations that fermentable carbohydrates should be included in the revival medium (Supfle and Dengler, 1916; Flesch, 1921).

One possibility is the occurrence in media of substances toxic to damaged cells, and this has been discussed by Jacobs and Harris (1960, 1961) who concluded that both extract broth and agar contain such substances. They showed that Japanese agar was less toxic than New Zealand, and this could explain the discrepancies between the results reported in Table III from those in Tables I and II. If the control medium prepared with Japanese agar was less toxic than the normal control with New Zealand agar, then favourable responses normally observed with other media could be masked.

Circumstances may be envisaged however, in which less rich media could give improved growth of damaged cells. The revival of such cells must depend on the relative rates of recovery and lethal processes, and there is some evidence that retardation of metabolism may result in recovery. When irradiated with X-rays, *E. coli* has been reported to recover better on less rich media (Alper and Gillies, 1958) and at temperatures below the normal optimum for the species (Hollaender,

Stapleton and Billen, 1953; Harris and Whitefield, 1964). If similar mechanisms operate with phenol treated organisms, the higher counts obtained on some media may reflect their lower nutritional status or greater toxicity.

The increased counts obtained with treated cells on the first batch of medium prepared without phosphate precipitation or pH adjustment (Table IV) indicates that acidity down to pH 6·2 was not actively harmful. However, the results must be viewed cautiously, since the improvement over the control could have resulted partly from hydrolysis of some constituents.

The main conclusion which may be drawn is that established ideas as to the suitability of a medium, and of what factors contribute to this, need reconsideration in relation to damaged cells, since existing information on media relates primarily to undamaged cells. Although there is a gradation from robust to exacting species of bacteria, it is clear that even those normally considered to be non-exacting become fastidious after damage, and situations in which damaged organisms occur are very common.

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