THE VALUE OF ADSORBENTS FOR DETECTING SURVIVAL IN BACTERIAL POPULATIONS EXPOSED TO PHENOLS

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THAT reversal of the action of antibacterial agents may follow their removal from cells is well known. Süpfle and Muller[1] and Engelhardt[2] have shown that charcoal would remove mercuric ions from bacteria and that the cells were thereby revived. McCulloch[3] was apparently able to confirm this. Brewer[4] found simple washing to be similarly effective with spores of *Clostridium* and *Bacillus* species treated with metaphen and merthiolate: also, Baumgartner and Wallace[5] observed beneficial effects due to incubation in distilled water after treatment and attributed these to desorption. That mercury compounds are not the only antibacterial agents whose action can be so reversed is shown by the results of Rahn[6], who used charcoal to remove quaternary ammonium compounds from cells; it is also probable that adsorption is at least part of the mechanism by which agar antagonises the actions of dyes[7] and quaternary ammonium salts[8].

The position which phenol has long occupied as a standard of reference (even if not a wholly satisfactory one) for bactericidal activity, and the general belief that its action could not be reversed, gave special interest to the report of Flett, Haring, Guiteras and Shapiro[9] that the inclusion of activated charcoal (Darco G 60) or ferric chloride in the subculture medium led to increased extinction times with populations of *Staphylococcus aureus* and *Eberthella typhosa* exposed to phenol. They attributed this to the action of the charcoal in adsorbing phenol carried over with the inoculum and of the ferric chloride in removing it by chemical reaction. However, these results were not confirmed by Tilley[10] and the experiments described in this paper were undertaken in an attempt to resolve the discrepancy between the results of Flett *et al.*[9] and Tilley[10].

THE INFLUENCE OF ACTIVATED CHARCOAL AND FERRIC CHLORIDE ON EXTINCTION TIMES

Materials and Methods

The organisms used for most of the experiments were two strains of *Bacterium coli* Type I (44° positive). One, strain 2, had been recently isolated from water. The other, Strain 163, was used in the early experiments, but as it proved to be somewhat unstable in colonial form and in phenol resistance, its use was discontinued.

The control broth used routinely contained Oxoid peptone 1 g., Lab-Lemco 1 g., sodium chloride 0·5 g. and tap-water 100 ml.; *p*H 7·4. It was solidified when necessary with 1·5 per cent. of New Zealand agar. To this

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broth was added, as required, 0·1 per cent. w/v of a finely-divided activated charcoal or sufficient of a 1 per cent. solution of ferric chloride (A.R.) to produce a final concentration of 0·03 per cent. w/v. The control broth, and that containing charcoal, both received distilled water equivalent in volume to the ferric chloride solution added.

It was noticed that on the addition of ferric chloride a flocculent light brown precipitate appeared which became darker and coagulated on autoclaving. To obviate this change, and since it was thought that the precipitate should be a better adsorbent in its original form, in experiments with a number of batches of standard medium a further subculture medium was included, consisting of autoclaved broth to which a filter-sterilised solution of ferric chloride had been added aseptically. In the early experiments B.D.H. acid-washed activated charcoal was used, but in later work Norit was employed because Tilley[10] had used it in his unsuccessful experiments. Subsequent tests showed that Norit gave similar results to Darco G 60, the material used successfully by Flett *et al.*[9]

All media were sterilised by autoclaving at 15 lb. per sq. in. for 20 minutes. All batches of media were numbered and the particular batch used in each experiment was noted. By batch of medium is meant an amount made up at one particular time and this does not signify a change in manufacturer's batch number.

Suspensions for exposure to the bactericides were prepared by washing the growth from a 24 hour $\pm \frac{1}{2}$ hour culture on solid medium at 37° C. with 0·02 M phosphate buffer (*p*H 7.0), to give a dense suspension which was diluted with buffer as required to a concentration of approximately 10^9 cells per ml., standardisation being carried out nephelometrically. 1 ml. of diluted suspension was then added to 4 ml. of a solution of the bactericide in buffer at 20° C. and transfers made at suitable intervals with an inclined loop (mean capacity 0·0225 ml.) into 5 ml. portions of the subculture media.

Since the failure of Tilley[10] to obtain positive results was possibly due to the responses being too small for detection with the $2\frac{1}{2}$ minute time intervals he used, experiments were initially arranged to permit of 1 minute intervals between successive subcultures into a particular medium; in later experiments 2 minute intervals were used.

Usually each experiment consisted of 3 replicate trials with a single batch of medium, but in some cases only 2 replicates were obtainable, while in others 6 or 9 replicates were performed to increase the reliability of the result, replicates usually being performed with different suspensions on different days. The full data obtained are far too extensive for reproduction here: they have been given by Harris[11], and only mean responses are given in this paper, the extinction times being expressed relative to the control times taken as 100.

Results

(*a*) *Experiments with* Bact. coli *exposed to* 1 *per cent. phenol.* The inclusion of the adsorbents did not affect the extinction times of Strain 163 in the experiments with the first 2 batches of media, but with batches 3, 4,

5A and 6A there were varying favourable (positive) mean responses, as shown in Figure 1. *Bact. coli* was tested in batches 5B, 6B, 7 and 8, again with varying results: only batch 5 gave a good response. Where the two strains were compared directly in the same batch of medium (batches 5 and 6) strain 2 was consistently the less responsive. It is clear, however, from Figure 1 that with *Bact. coli* and 1 per cent. phenol the response was much more often favourable than not and that negative responses were rare.

(*b*) *Experiments with* p-tert.-*butylphenol.* Alexander and Tomlinson[12] showed that the adsorption of phenol from solutions by bacteria is small, but Dagley, Freeman and Thompson[13] found *para*-substituted phenols to be more strongly adsorbed than phenol itself. It seemed, therefore, that *p-tert.*-butylphenol would be more likely than phenol to give consistently favourable results, so tests were done with that substance using *Bact. coli* strain 2. *Staphylococcus aureus* N.C.T.C. 4736 was included as a second test organism. For the former organism the concentration used was 0·045 per cent. and for the latter 0·040 per cent.

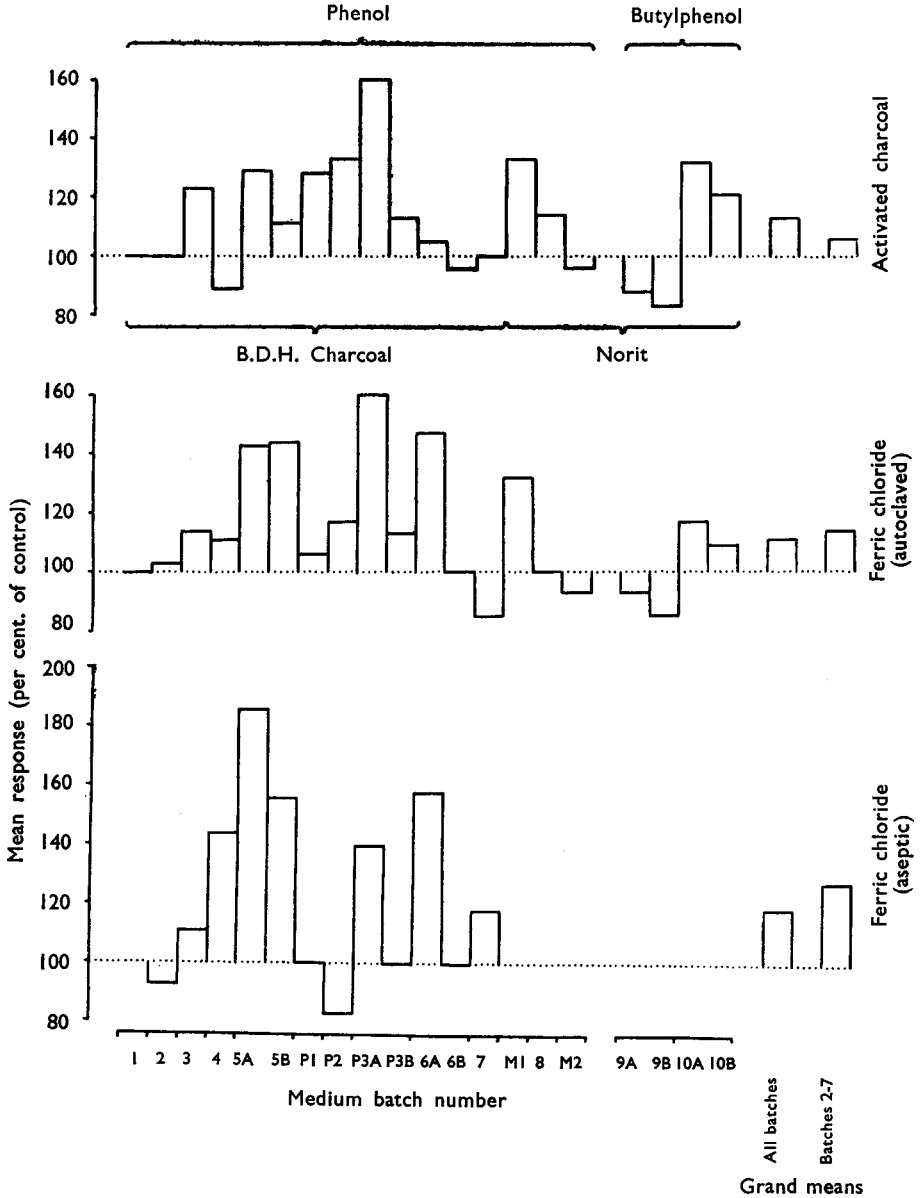
Two batches of media were used, nos. 9 and 10 (Fig. 1). With batch 9 there were no positive responses, but with batch 10 there were positive or null responses in individual trials, though the overall responses were positive. The results were qualitatively similar with both test organisms. The responses obtained with *Bact. coli* 2 were fully as variable as with phenol.

(c) *Experiments with other than standard media.*

On testing the separate ingredients of the standard medium, it was found that the formation of an immediate precipitate with ferric chloride was due to the meat extract. Trials were therefore carried out to discover whether the meat extract content of the subculture medium could influence the result. In all, experiments were done with 3 batches of 1 per cent. peptone water (P 1, P 2, P 3), and 2 batches (M 1, M 2) of a modified broth containing 0·5 per cent. of meat extract, half the standard amount, but the meat extract concentration did not appear to influence the mean response in any consistent way. Batch variation was very evident in both types of medium (Fig. 1).

The Adsorption of Phenol by Activated Charcoal and by the Ferric chloride-Broth Precipitate

It is evident from the grand means shown in Figure 1 that overall the responses were positive with all 3 methods of treatment, ferric chloride added aseptically tending to be most favourable. It should be pointed out here that when a batch of medium was tested on different occasions the results indicated that while the magnitude of the response varied considerably in different tests there was uniformity in its direction, positive or negative. This is not shown in Figure 1 where only mean responses are given, but is well illustrated by Table I, which gives the results of 9 trials on 3 days with batch M 1. However, it is clear that there was a marked variation in mean response from batch to batch of medium, despite the



Batches
1–10: 1 per cent. peptone, 1 per cent. meat extract.
M1, M2: 1 per cent. peptone, 0·5 per cent. meat extract.
P1–P3: 1 per cent. peptone.
1, 2, 3, 4, 5A, 6A, P1, P2, P3A—*Bact. coli* 163.
5B, 6B, 7, 8, 9A, 10A, M1, M2, P3B—*Bact. coli* 2.
9B, 10B—*Staph. aureus*.

Fig. 1. The influence of medium batch on the response of treated bacteria to charcoal or ferric chloride in the subculture medium.

constancy of the carry-over of bactericide in the inoculum. While one batch gave a strong positive response a similar batch was unresponsive or gave negative responses. This suggested that the occurrence of a response could not be ascribed wholly to adsorption or neutralisation, but depended, to some extent at least, on the condition of the medium. Before investigating that point, it was decided to determine whether the adsorption of phenol could possibly contribute to the response, by measuring the extent of its adsorption from solutions in nutrient broth and in tap-water at a concentration of 0·005 per cent., this being the approximate concentration obtained in the extinction time tests, where 1 loopful of a 1 per cent. solution was added to 5 ml. of medium. Tap-water was included to serve as a control.

TABLE I

THE INFLUENCE OF NORIT OR FERRIC CHLORIDE IN THE SUBCULTURE MEDIUM ON THE EXTINCTION TIME OF *Bact. coli* 2 IN 1 PER CENT. PHENOL

Medium	First day			Second day			Third day		
	Trial number	Extinction time, minutes	Mean response, percentage of control	Trial number	Extinction time, minutes	Mean response, percentage of control	Trial number	Extinction time, minutes	Mean response percentage of control
Control ..	1 2 3	20 18 12	100	1 2 3	12 14 12	100	1 2 3	20 16 18	100
Control medium plus 0·03 per cent. of $FeCl_3$, autoclaved	1 2 3	24 24 20	136	1 2 3	22 16 20	153	1 2 3	20 20 18	[107]
Control medium plus 0·1 per cent. of Norit	1 2 3	22 18 16	112	1 2 3	24 20 18	163	1 2 3	22 22 24	126

Medium batch M1:—1 per cent. of peptone, 0·5 per cent. of meat extract.

Choice of method.

Considerable difficulty was experienced in finding a reliable method for the determination of phenol in the presence of the constituents of broth. Using normal diazotisation methods colours are developed with broth which are sufficiently intense to mask any colour due to phenol. Finally a modification of the indophenol method[14] for the estimation of phenol in water was adopted. The advantages of this were that the phenol could be estimated directly in broth or the indophenol could be extracted from the broth with carbon tetrachloride and estimated in that solvent, thus eliminating any possible interference by broth constituents.

(a) *Determinations in water.* To 10 ml. of sample was added 2 ml. of 5 per cent. sodium bicarbonate solution, 2 ml. of freshly-prepared 0·1 per cent. *p*-amino-dimethylaniline hydrochloride solution, 6 ml. of sodium hypochlorite solution (0.05 per cent. of available chlorine), and 20 ml. of distilled water. These were then mixed and 20 ml. of the mixture was extracted 3 times with 5 ml. of carbon tetrachloride. The combined extracts were dried over anhydrous sodium sulphate, which was then filtered off

and washed with sufficient solvent to give a combined volume of 20 ml. of carbon tetrachloride extract.

(b) *Determinations in nutrient broth.* Samples of 5 ml. were treated as for water except that the sodium hypochlorite solution contained 0·65 per cent. of available chlorine. The total volume of reaction mixture was unchanged (40 ml.) and half was extracted with carbon tetrachloride.

The optimal amounts of sodium hypochlorite solution to give the maximum colour intensity and the minimum variation in colour intensity with time had to be determined for each set of conditions; this was especially so when determinations were carried out in broth. Under the conditions used the colour intensities reached a maximum in 5 minutes and were substantially unchanged up to 30 minutes. Determinations of indophenol in aqueous solution were made between 7 and 15 minutes, and primary carbon tetrachloride extractions were performed 25 minutes, after adding the sodium hypochlorite. No change in colour intensity occurred in the carbon tetrachloride on standing.

TABLE II

THE ADSORPTION OF PHENOL FROM NUTRIENT BROTH AND FROM WATER BY NORIT AND DARCO

Type of charcoal	Phenol in	Indophenol determined in	Adsorption per cent. Trial 1	Trial 2	Trial 3	Mean	Grand mean
Norit	Water	Water	83·8	83·3	84·5	83·8	82·6
		Carbon tetrachloride	81·1	81·4	82·5	81·3	
	Nutrient broth	Water	4·8	8·4	10·2	7·1	8·7
		Carbon tetrachloride	8·8	10·5	11·8	10·4	
Darco	Water	Water	84·5	84·4	85·0	83·6	81·3
		Carbon tetrachloride	83·4	68·9	84·4	78·9	
	Nutrient broth	Water	25·7	24·1	25·7	25·1	25·2
		Carbon tetrachloride	24·0	22·5	26·1	25·2	

(c) *The production of concentration-absorption curves.* Using a Unicam diffraction grating spectrophotometer, absorption curves were obtained for indophenol in water and in broth, both *in situ* and after extraction with carbon tetrachloride, in order to decide which wavelengths were optimal for the determination. They proved to be 675 mμ for indophenol in aqueous solution and 560 mμ in carbon tetrachloride. Concentration-absorption curves for phenol in broth and in water were then plotted from data obtained at intervals of 0·0005 per cent. up to a maximum of 0·0005 per cent. The reaction conditions had been adjusted to give rectilinear responses over the concentration ranges which trial experiments had indicated to be necessary.

The adsorption of phenol

(a) *By activated charcoal.* To 99 ml. of a 0·1 per cent. suspension of Norit or Darco autoclaved in nutrient broth or in tap-water 1 ml. of a

0·5 per cent. solution of phenol was added. After shaking for 30 minutes the charcoals were removed by filtration and the residual phenol in the filtrates was then estimated. Blanks and standards were provided by adding known amounts of phenol to the filtrates from charcoal treatments of broth and water. The results obtained (Table II) show that there was little adsorption of phenol in the presence of broth although over 80 per cent. was adsorbed from water.

The situation was shown to be similar in the case of dye adsorption. Using similar techniques, the adsorption of a basic dye (methylthionine hydrochloride) and an acid dye (*p*-rosaniline hydrochloride), both of which are strongly adsorbed from water, was markedly reduced if broth were present. Also, adsorption was reduced if the charcoal had first been saturated with broth constituents by autoclaving in broth followed by thorough washing with distilled water.

(*b*) *By ferric chloride.* The precipitate obtained by adding ferric chloride to nutrient broth was centrifuged off, washed thoroughly with distilled water and resuspended in water to give the same concentration of iron as was originally added to the broth. When the same technique that had been used for charcoal was employed with this suspension, and with ferric chloride in broth, no adsorption of phenol could be detected in either case.

The Influence of the Phenol Concentration in the Subculture Medium on Extinction Times

To determine whether the change in phenol concentration produced in nutrient broth by activated charcoal could possibly influence the survival of treated bacteria, extinction times for *Bact. coli* and *Staph. aureus* exposed to phenol were determined in 4 nutrient broth subculture media,

(i) 5 ml. portions, to serve as a control,

(ii) 20 ml. portions, to give a phenol concentration one quarter of that in the control,

(iii) 5 ml. portions, to which had been added 0·08 ml. of 1 per cent. phenol, to give a concentration 5 times that in the control.

(iv) 20 ml. portions, to which had been added 0·43 ml. of 1 per cent. phenol, to give a concentration 5 times that in the control. This acted as a control on the 20 ml. portions without added phenol to judge the possible effects of dilution of the inoculum.

The results (Table III) showed that neither the extinction times with the broth containing the higher concentration of phenol, nor the times using the larger bulk of medium for subculture, differed appreciably from those in the control. It therefore appeared that the small concentration change which could be effected by the activated charcoal was unlikely to have influenced the extinction times. It is concluded that removal of phenol is not a likely explanation of the increased extinction times which may result from the inclusion of adsorbents in subculture media. A preliminary communication of results which indicate that normal media may contain substances harmful to bacteria damaged by exposure to phenols is in the press.[15]

TABLE III

THE INFLUENCE OF THE PHENOL CONCENTRATION IN THE SUBCULTURE MEDIUM ON THE EXTINCTION TIMES OF BACTERIA EXPOSED TO 1 PER CENT. PHENOL

Organism	Volume of subculture medium, ml.	Phenol concentration, per cent.	Extinction time, minutes				Grand mean response, percentage of control
			Medium batch				
			11		12		
			A	B	A	B	
Bact. coli 2	5	0·004	12	14	10	14	100
	20	0·001	14	12	10	12	98
	5	0·020	12	10	14	16	106
	20	0·020	12	12	10	16	100
Staph. aureus 4736 ..	5	0·004	8	10	6	8	100
	20	0·001	8	6	6	8	90
	5	0·020	12	6	6	10	109
	20	0·020	12	12	6	8	118

SUMMARY

1. The inclusion of 0·1 per cent. of activated charcoal or 0·03 per cent. of ferric chloride in subculture media had no consistent influence on the extinction times of *Bact. coli* exposed to 1 per cent. phenol, but the overall responses in experiments made with several batches of media were positive.

2. On the whole, individual batches of medium consistently gave the same kind of response, positive or null.

3. Activated charcoal (0·1 per cent.) adsorbed about 80 per cent. of the phenol from solution in water at a concentration of 0·005 per cent. In the presence of broth much less was adsorbed; Norit adsorbed only about 9 per cent. and Darco G 60 about 25 per cent.

4. The ferric chloride-nutrient broth precipitate did not adsorb phenol from either water or broth.

5. In the concentration range 0·001 to 0·020 per cent., phenol in the subculture medium did not appear to influence the extinction times of bacterial populations exposed to that substance.

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DISCUSSION

The paper was presented by DR. N. D. HARRIS.

The CHAIRMAN, in introducing the discussion, referred to the danger that bacteriostatics in injections may be adsorbed on to materials in suspension.

Dr. K. BULLOCK (Manchester) asked for some information about the further paper to which the author had referred. Reference was made to substances in the broth being harmful to bacteria damaged by phenol. Were these substances harmful only to bacteria damaged by phenol? Would they not be harmful to other bacteria or to bacteria damaged by other bactericides?

MR. G. SYKES (Nottingham) said that the paper underlined the difficulties of testing disinfectants. They arose largely from the idea, which had persisted over the years, of trying to measure the 100 per cent. end-point instead of something less than 100 per cent. One of the significant features was the batch variations revealed. These variations existed despite the fact that the batches were made with the same materials and in the same manner. It illustrated the difficulty of obtaining reproducible results in work of this type. Although the actions of ferric chloride and charcoal differed the results might be of the right order namely, that ferric chloride was more effective than charcoal—because in the case of charcoal the action was particle-to-particle contact, whereas ferric chloride was more readily accessible as ions or molecules.

PROFESSOR H. BRINDLE (Manchester) asked what method had the authors used for filtering their materials? Adsorbent charcoal contained very small particles which were difficult to filter out, and this portion of the charcoal was very active and would readily adsorb phenol. Had they made any allowance for this possibility?

DR. HARRIS, in reply, said that a precipitate was obtained when ferric chloride was present in the broth. Even when ferric chloride was added aseptically, precipitation eventually occurred although the precipitate was more flocculent and more readily dispersed. He did not think ferric chloride could have reacted as molecules or ions under the conditions of their experiments. He had not experienced great difficulty in filtering out the charcoal, but if any haziness occurred he used centrifuging to remove the last traces of charcoal. He said that the results with the standards and blanks agreed on all occasions, and he thought there was little chance that phenol had been adsorbed. The second paper to which he had referred would probably be published in December. It was not true that substances in the broth were harmful only to bacteria damaged by phenol. He had reached the conclusion that there was a gradation of resistance with bacteria. Some bacteria were very sensitive while others—for example, *Bact. coli*—were relatively insensitive in their normal state. The difficulty of cultivating the Brucella species marked them as one of the sensitive types of organism. It was possible to take an organism such as *Bact. coli* and find that when treated with phenols

it became sensitive to substances in the broth to which it was formerly insensitive. With *Staph. aureus* it had been found that these substances in the broth tended to restrict growth, although there was no killing effect. Cresol and *p*-chloro-*m*-cresol had been used with similar results.