SCIENCE PAPERS AND DISCUSSIONS

THE BASIS FOR "SUFFICIENT OF A SUITABLE BACTERIOSTATIC" IN INJECTIONS

By G. Sykes

From the Microbiology Division, Standards Department, Boots Pure Drug Co. Ltd., Nottingham

Received June 1, 1958

In the light of the relative lethal and inhibitory properties of several known bacteriostats, including those recommended in the B.P., along with some observations on their partitioning ratios between rubber and water, the requirements for "sufficient of a suitable bacteriostatic" are discussed, and various criteria are suggested. Attention is drawn particularly to the narrowness of the margin between effective lethal concentration and minimum inhibitory concentration of many bacteriostats and to the consequent need to prevent their loss by absorption into rubber, or through any other cause. Activity against mould spores as well as against resistant vegetative bacteria is also desirable.

The primary reason for including bacteriostatic substances in parenteral preparations dispensed in multidose containers is to ensure that contaminating organisms, introduced accidentally whilst withdrawing successive doses, shall not be able to proliferate and so cause subsequent damage to the medicament or, even worse, give rise to an infection in the patient. There are, of course, other possible sources of contamination, for instance during the preparation or storage of the injection, but adequate safeguards and controls are now available to prevent such occurrences. The British Pharmacopoeia requires that all such preparations in multidose containers shall contain "sufficient of a suitable bacteriostatic to prevent the growth of micro-organisms," and it cites as examples "Phenol, 0·5 per cent w/v; Cresol, 0·3 per cent w/v; Chlorbutol, 0·5 per cent w/v; Chlorocresol, 0·1 per cent w/v; Phenylmercuric nitrate, 0·001 per cent w/v." But these solutions vary considerably in their antimicrobial properties—they have substantial lethal as well as inhibitory properties—and so the questions arise: (1) what constitutes "a suitable bacteriostatic," and (2) what concentration is "sufficient"?

The position is further complicated by the fact that the B.P. states that the chosen substance shall also be compatible with the medicament, and it also draws attention to its possible loss by absorption into the rubber closure of the container. On these premises, therefore, the basic requirements for "a suitable bacteriostatic" may be summarised briefly under the three headings:

(1) ability to prevent the growth of, and preferably to kill, contaminating micro-organisms; (2) compatibility with the medicament, even on long storage and (3) low absorption rate into rubber; to which may be added (4) absence of toxicity to the patient in the quantities employed in the injection.

Each of these is probably equally important, but from the microbiological aspect items (1) and (3) are the most significant, and it is these which are discussed in this paper.

EXPERIMENTAL

Antimicrobial properties. Although five compounds are officially recommended in the B.P. as bacteriostats, phenol in 0·5 per cent concentration is the generally accepted standard against which any other suggested bacteriostat should be assessed. But this is not an easy matter, largely because phenol, as already stated, has considerable bactericidal as well as bacteriostatic properties and also because different types of compounds have quite different ranges of activities against the various groups of organisms. These points are illustrated in Tables IA and IIA, which show the relative lethal and inhibitory properties of the B.P. bacteriostats.

TABLE I

LETHAL PROPERTIES OF BACTERIOSTATS

Bacteriostat, per cent	Lethal times (in hours) for			
	Staph. aureus	*E. coli*	*Ps. pyocyanea*	Mould spores
A. B.P. BACTERIOSTATS				
Phenol 0·5	8–24	8	8	>24
Cresol 0·3	2–4	1	1	>24
0·15	>24	>24	>24	N.A.
Chlorbutol 0·5	8–24	1	2	>24
0·3	N.A.	>24	>24	N.A.
Chlorocresol 0·1	1	1	1	3
0·05	>24	2–4	2	N.A.
Phenylmercuric nitrate 0·001	4	1	1–4	3
B. OTHER BACTERIOSTATS				
Benzyl alcohol 2	1	1	1	>24
1	6–24	24	6	N.A.
Dichlorobenzyl alcohol 0·1	1–3	1	1	>24
Cetrimide 0·01	1	1	6–24	2
Methyl paraben 0·2	8	24	24	N.A.
Propyl paraben 0·02	4	>24	N.A.	N.A.
Mixed parabens 0·2 + 0·02	4–8	1–3	8–24	>24

>24 = substantial reduction in count, but less than 99 per cent, after 24 hours.
N.A. = no substantial reduction in count after 24 hours.

In each of these assessments, tests were made with (*a*) mixed cultures of several strains of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas pyocyanea* grown in nutrient broth, each group being treated separately, and (*b*) aqueous suspensions of spores from various species of *Penicillium*, *Cladosporium*, *Aspergillus* and *Mucor*. The bacterial cultures comprised as many as ten strains of each type, a number of which had been supplied earlier by Mr. J. W. Lightbown of the National Institute for Medical Research, London.

The lethal tests were made with solutions in distilled water. Each solution was inoculated with the chosen test organisms to give a final concentration of $1 \times 10^6 - 1 \times 10^7$ viable cells per ml. At intervals ranging between 1 hour and 24 hours, platings were made, and the minimum times recorded at which there were no survivors in 0·01 ml. of the solutions, that is, when there was a virtual kill of over 99·99 per cent.

The inhibitory tests were made with solutions in tryptic digest broth diluted tenfold with water and with 0·1 per cent of glucose added. The same organisms were used as in the lethal tests, but the amount of inoculum was much lower, in the range of 10,000 to 50,000 viable cells per ml., and the incubation was for 5 days at 25°. Diluted broth was used instead

of normal nutrient broth so that the organic content of the solution could be kept to a reasonable minimum; it was, of course, quite adequate to support the growth of the chosen test organisms.

The results reported in Tables IA and IIA serve to show (1) the different relative activities of the five B.P. bacteriostats against the different groups of organism examined, (2) the high resistance of *Staph. aureus* in comparison with the Gram-negative types, (3) the high resistance of *Ps. pyocyanea* in relation to that of *E. coli*, and (4) the inefficacy of phenol, cresol and chlorbutol as lethal agents for mould spores. These values must not, of course, be taken as absolute; they apply only to the particular conditions of the experiments made and the cultures used—if other cultures and conditions had been employed then different values might have been

TABLE II

INHIBITORY CONCENTRATIONS OF BACTERIOSTATS

Bacteriostat	Concentration per cent inhibitory to			
	Staph. aureus	*E. coli*	*Ps. pyocyanea*	Mould spores
A. B.P. BACTERIOSTATS				
Phenol	0·2	0·2	0·16	0·05
Cresol	0·16	0·1–0·16	0·2	0·05
Chlorbutol	0·25	0·16	0·16	0·2
Chlorocresol	0·025	0·02	0·05	0·007
Phenylmercuric nitrate	0·00013	0·0005 –0·0001	0·0002	0·00001
B. OTHER BACTERIOSTATS				
Benzyl alcohol	0·5	0·4–0·5	0·4	0·4
Dichlorobenzyl alcohol	0·025	0·04–0·05	0·04–0·05	0·016
Cetrimide	0·0004	0·001	0·04	0·0005
Methyl paraben	0·2	0·1	0·14	0·05
Propyl paraben	>0·02	>0·02	>0·02	0·015
Mixed parabens	0·1	0·1	0·1	0·025

obtained—but they illustrate clearly the points enumerated above. The Tables also indicate the narrowness of the margin, particularly with cresol, chlorocresol and chlorbutol, between the effective lethal concentration and the minimum inhibitive concentration, and this is further brought out in Table IA, which shows the profound effect of concentration on the lethal activities of these compounds, solutions at even only half their recommended strengths having considerably reduced, or sometimes practically no lethal powers.

Tables IB and IIB record the results obtained by similar tests with other bacteriostats. Of these, cetrimide and the parabens (esters of *p*-hydroxybenzoic acid) are well known, the latter often being used as mixtures in the ratios of 5 to 1 or 10 to 1 by weight; benzyl alcohol has been recommended at a concentration of 0·9 per cent or greater[1], particularly for the preservation of ophthalmic solutions[2], and 2:4-dichlorobenzyl alcohol is one of a series of derivatives of benzyl alcohol, the properties of which are described elsewhere[3]. The results again illustrate, as with the B.P. bacteriostats, the differing activities of the compounds against the various types of organisms, and also the narrowness of the margins in some cases between their effective lethal concentration and minimum inhibitory concentration.

Absorption by rubber. Attention has already been drawn to the loss by absorption of bacteriostats from injections in rubber closed containers[4], and in the same paper the partitioning ratios of the B.P. bacteriostats between rubber and water were given. By similar methods, the partitioning ratios for cetrimide, methyl and propyl parabens and 2:4-dichlorobenzyl alcohol have now been determined, and they are given in Table III; the values for the B.P. bacteriostats as found in the earlier paper[4] are also included for convenience. They show the unsuitability of

TABLE III

THE PARTITIONING RATIOS OF BACTERIOSTATS BETWEEN RUBBER AND WATER

Bacteriostat	Approximate distribution per cent between	
	Rubber	Water
Phenol	25	75
Cresol	33	67
Chlorocresol	85	15
Chlorbutol	80–90	10–20
Phenylmercuric nitrate	>95	<5
Benzyl alcohol	15	85
Dichlorobenzyl alcohol	90	10
Cetrimide	80–95	5–20
Methyl paraben	10	90
Propyl paraben	30–40	60–70

chlorocresol, chlorbutol, phenylmercuric nitrate, dichlorobenzyl alcohol and cetrimide in this context and the comparative acceptability of phenol, benzyl alcohol and methyl paraben.

DISCUSSION

Three statements are made in the B.P. about bacteriostats added to injections: (1) they must not interfere with the therapeutic efficiency of the drug or cause a turbidity, (2) they must be capable of preventing the growth of micro-organisms (in a similar context the Therapeutic Substances Regulations, 1952, use the phrase "the common contaminating organisms, both aerobic and anaerobic"), and (3) "A bacteriostatic need not be added if the medicament itself has sufficient bacteriostatic power". It also adds a warning about their absorption into rubber, but beyond these general statements it offers no guidance for the assessment of substances potentially useful as bacteriostats, neither does it indicate how bacteriostatic power can be measured. Attention has already been drawn to this[5], and in fact, various criteria have been suggested from time to time (*see*, for example, refs. 6–8).

In terms of antimicrobial activity, it is reasonable to expect that the preparation shall be effectively lethal as well as inhibitory to all types of organisms, including moulds (although it is too much to expect lethal action against the bacterial spores which have notoriously high resistances). The term "effectively lethal" is now generally accepted as implying the virtual sterilisation of a moderately heavy inoculum of suitably resistant vegetative bacteria within 24 hours, and this is, in fact, the performance level of the recognised yardstick for such assessments, namely, phenol in 0·5 per cent concentration. Such a standard has

already been put forward by the Ministry of Health Sub-Committee on Bacteriostatics[8], along with the suggestion that *Staph. aureus* and *Ps. pyocyanea* should be the organisms of choice. As representing the most resistant of the Gram-positive and Gram-negative groups of bacteria, they are admirably suitable for this purpose, but in order to obtain a satisfactory and reliable spread of resistance, several strains of each should be used, preferably of recent isolation. Even so, the values obtained cannot be taken as absolute, because one still encounters certain strains and types of organism, or particular conditions of culture, which exhibit unusually high resistance. Thus, some of the water-borne strains of *Pseudomonas* are more resistant to bacteriostats than is *Ps. pyocyanea*; likewise, although cetrimide in 0·02 per cent solution is normally effective, and a 0·01 per cent solution has been found satisfactory with procaine penicillin suspensions[8], there is a report of the growth of *Ps. pyocyanea* even in a 1 per cent solution[9].

In the opinion of the author, mould spores should always be included amongst the test organisms, because they will often grow where bacteria do not, and there is at least one finding on record of the growth of *Cladosporium* in insulin solution containing 0·17 per cent of cresol[5].

Further points for consideration are the conditions under which the bacteriostat is to be employed and their effect on its activity. In this respect, pH value, temperature and the presence of organic matter are probably the most significant. Organic matter in the form of amino acids, proteins, etc., always suppresses the activities of antimicrobial agents, but interference from this source is likely only to be small, because with a few exceptions the organic content of parenteral injections is low. The temperature effect can be variable depending on the type of substance being examined, but from general considerations of the conditions of storage and use, a test temperature of about 25° seems to be the most suitable. The effect of pH value can be more profound; most of the phenolic substances, for instance, tend to lose their activities as they move into the alkaline range. Fortunately in this respect most injections have either neutral or slightly acid reactions, and this in some cases allows the amount of added bacteriostat to be reduced. Such an example is found with insulin injections with pH values of about 3 in which as little as 0·2 per cent of phenol has been shown to be adequate, except possibly against mould spores[5].

Finally, there is the question of absorption by the rubber closure. If an injection is to contain sufficient of a bacteriostat, even after long periods of storage, it follows that the partitioning ratio of the bacteriostat between rubber and water should be low; failing this, either an adequate margin of concentration must be allowed in the initial solution, or some other precaution must be taken to conserve the concentration of the bacteriostat in the injection. In this context, suitable precautions, along with methods for assessing partitioning ratios, have already been described[4].

In conclusion, it may be said that although the five bacteriostats as recommended in the B.P. have proved satisfactory for most purposes, they have their limitations and disadvantages. In particular, they are

all readily absorbed, with the exception of phenol, into rubber, and so lost as effective agents in the preparation. There is room, therefore, for other suitable substances, and in fact many have already been proposed. Others will undoubtedly appear from time to time, but as yet there is no approved method for assessing their practical value. This is a matter needing careful consideration and controlled experimentation, and the criteria as outlined in the earlier part of this paper are put forward as a basis for discussion.

Acknowledgment. I wish to thank Mr. C. Bowler, Mr. D. V. Carter and Mr. A. Royce for the experimental data recorded in this paper.

REFERENCES

1. Gershenfeld, *Amer. J. Pharm.*, 1952, **124**, 399.
2. Klein, Millwood and Walther, *J. Pharm. Pharmacol.*, 1954, **6**, 725.
3. Carter, Charlton, Fenton, Housley and Lessel, *ibid.*, 1958, **10**, Supplement *149T*.
4. Royce and Sykes, *ibid.*, 1957, **9**, 814.
5. Sykes and Hooper, *ibid.*, 1954, **6**, 552.
6. Gershenfeld and Perlstein, *Amer. J. Pharm.*, 1947, **111**, 237.
7. Sokol, *Drug. Stds.*, 1952, **20**, 89.
8. *Bacteriostatics for Parenteral Injections of Procaine Penicillin:* a Report by the Ministry of Health Bacteriostatics Subcommittee of the Conference on the Control of Antibiotics, 1957; Ministry of Health, London.
9. Robinson, *Brit. med. J.*, 1957, **1**, 1242.

DISCUSSION

The paper was presented by the AUTHOR.

The CHAIRMAN. The B.P. might give further guidance by providing a testing procedure to ensure the bacteriostat was suitable.

MR. G. H. WRIGHT (Leeds). Has any research been done on polythene or other plastic closures?

DR. A. M. COOK (London). How would the Author recommend the test to be carried out, against a specific resistant strain of one organism or as a spectrum test against a number of organisms? He agreed that any test should include fungal spores.

DR. F. HARTLEY (London). The pharmacist must be satisfied that there was no danger of either mould or bacterial contamination. Tables I and II showed that the chosen concentration of bacteriostat inhibited mould spores below the lethal concentration. Unless there was a danger of pathogenic fungi, there was a wide margin of safety. Was there any point in having more than one substance?

MR. K. HOLLAND (Romford). Was it not time to stop using multi-dose containers?

MR. W. T. WING (Newcastle on Tyne). Tables I and II showed that the lethal and inhibiting effects of cetrimide were high, it was non-volatile but the partition ratio between rubber and water was high. If rubber closures were previously equilibrated with the chosen concentration of cetrimide, it might be satisfactory. Were the rubbers in Table III of different composition and did the contact time vary or were there defects in the assay?

DISCUSSION

DR. K. R. CAPPER (London). What media had been used in the tests. Did they contain inactivating substances?

The AUTHOR replied. Certain plastics were relatively non-absorbent but the majority were—some more so than rubber. There was no such thing as specific resistance amongst organisms. He had chosen staphylococci and coli and several strains of *Ps. pyocyanea.* A spectrum test was necessary, as a selective type of compound which was active against Gram-positive but not Gram-negative organisms was useless. The most commonly encountered contaminants were staphylococci and Gram-negative organisms, particularly water borne organisms growing at 25° rather than 37°. It was not unreasonable to aim for a single substance which would be effective against bacterial as well as common mould spore contamination. Moulds could gain access to a sterile room as readily as bacteria. As a bacteriologist he ought to condemn multi-dose containers, but there were other considerations. Although cetrimide was probably stable, difficulties arose because of its large absorption rate and reduced activity in presence of traces of organic matter. The lethal tests were made in aqueous solution and the media used included appropriate inactivators.