FACTORS AFFECTING TESTS FOR STERILITY OF DERIVATIVES OF BARBITURIC ACID

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Introduction

The Pharmacopœial Tests for Sterility break down in two well-known instances. The first, with bacteriostatic substances unless countermeasures are applicable, and the second in which the amount of bacterial contamination is small. The second effect, in addition, is enhanced by the first.

Davis and Fishburn[1] met both of these difficulties to some extent by filtration through a bacteria-proof filter pad, and its subsequent examination for contaminants. In adding support to this technique, Bang, Bowitz and Dalsgaard[2] suggested its application for certain bacteriostatic drugs.

Both papers made reference to the bacteriostatic activity of the barbiturate drugs. It was suggested that the pad technique was successful in overcoming this activity and suitable even when the degree of contamination was very small.

Alin and Diding[3] in their investigations into the 'bactericidal' action of solutions of phenobarbitone sodium showed added *Escherichia coli* and *Staphylococcus aureus* to become non-viable within 2 days, although *Bacillus subtilis* was not destroyed even after 30 days contact.

The bacteriostatic activity of the sodium salts of the barbiturates is a combination of two factors, namely, the inherent bacteriostatic properties of the drugs themselves, and the high alkalinity of their solutions.

Sterility tests conducted in a medium of about neutral *p*H are complicated by precipitate of barbituric acids, which may be confused with bacterial growth.

The present paper is the outcome of an investigation into the inhibitory power of some barbiturates with the intention of devising an adequate test for sterility.

Experimental

The Inhibiting Effect of High pH on Growth.

The optimal *p*H for the growth of the majority of bacteria has long been established at a value slightly on the alkaline side of *p*H 7·0. Since solutions of the soluble barbiturates are quite alkaline it was decided to investigate the growth of the test bacteria under alkaline conditions.

Increasing amounts of N sodium hydroxide were added to a series of flasks containing 100 ml. of sterile Difco aerobic broth (B3). This method of preparing broths of increasing *p*H was adopted because the more obvious method of preparing the broths and then autoclaving was

found to give rise at the higher *p*H values to a deposition of material in the broth together with a change of *p*H.

Small samples from each of the flasks were used to determine the final *p*H. A Cambridge portable glass electrode *p*H meter was used.

Ten ml. quantities were then distributed aseptically from each flask into each of 4 sterile culture tubes. Four series of tubes containing media of increasing *p*H were thus prepared. 24-hour cultures of the 4 organisms under test, *E. coli*, *Ps. æruginosa*, *Staph aureus* and *B. subtilis* were diluted 1 in 100. Inocula of 0·1 ml. were added to the series of tubes and at the same time a plate count of the inoculum was carried out. The tubes were incubated at 37° C. for 5 days. It was evident from the results shown in Tables I and II that the organisms were capable of survival and multiplication in media up to a *p*H of about 10·5.

TABLE I

Organism	Number per ml. in tubes	Initial *p*H in tubes of 10 ml. sterile broth				Count			
		6·88	9·98	10·62	11·37	Number counted	Dilution	Number in 0·1 ml. of inoculum	Number per ml. in tubes
E. coli ..	10,800	+	+	−	−	108	× 1000	108,000	10,800
Ps. æruginosa	38,000	+	+	+	−	380	× 1000	380,000	38,000
Staph. aureus	7900	+	+	+	−	79	× 1000	79,000	7900
S. subtilis ..	90	+	+	−	−	90	× 10	900	90

TABLE II

Organism	Number per ml. in tubes	Initial *p*H in tubes of 10 ml. sterile broth				Count			
		7·39	9·79	10·38	11·14	Number counted	Dilution	Number in 0·1 ml. of inoculum	Number per ml. in tubes
E. coli ..	1960	+	+	−	−	196	× 100	19,600	1960
Ps. æruginosa	19,100	+	+	+	−	191	× 1000	191,000	19,100
Staph. aureus	76	+	+	+	−	76	× 10	760	76
B. subtilis ..	120	+	+	−	−	120	× 10	1200	120

The inocula were large, however, and to show whether small inocula were still capable of initiating growth, similar series of tubes with broth of increasing *p*H were prepared. 10-fold dilutions of a 1 in 100, 24-hour culture of *E. coli* were used to inoculate the tubes, plate counts being again taken. The results shown in Table III clearly established that, neglecting a possible uneven distribution and consequent sampling errors, a very small inoculum was still capable of survival and growth up to about *p*H 9·0.

It was obvious that if the *p*H of solutions of the test barbiturates were higher than *p*H 9, it would be impossible to determine their bacteriostatic value and difficult to carry out an adequate and simple test for sterility. The *p*H of solutions of the 4 barbiturates under test both in freshly-distilled carbon dioxide-free water and in Difco broth were therefore determined, 100 mg. of the drugs being added to flasks containing increasing volumes of the media. It can be seen from Tables I, II and IV that for

a bacteriostatic value determination when the inoculum used may be large, concentrations of at least 2 per cent. of the barbiturate may be used with the complete confidence that the *p*H of such solutions will not be too high to interfere with the growth of the test organisms. On the other hand, in any test for sterility, where the amount of contamination would be expected to be small, concentrations of the barbiturates above about 0·2 per cent. should not be employed (Tables III and IV).

TABLE III

E. coli Number of organisms per ml. in each series of tubes	Time of incubation	*p*H				
		7·03	7·91	8·76	9·48	10·38
7500	24 hours	+	+	+	-	-
	48 "	+	+	+	−	-
	72 "	+	+	−	+	-
	5 days	+	+	+	+	-
750	24 hours	+	+	−	−	-
	48 "	+	+	+	-	−
	72 "	+	+	+	-	-
	5 days	+	+	+	-	-
75	24 hours	+	+	−	−	−
	48 "	+	+	+	−	−
	72 "	+	+	+	−	−
	5 days	+	+	+	-	-
8	24 hours	+	+	−	-	−
	48 "	+	+	+	-	-
	72 "	+	+	+	-	-
	5 days	+	+	+	-	−

TABLE IV

*p*H OF BROTH SOLUTIONS OF BARBITURATES

Broth ml.	Concentration per cent. w/v	*p*H			
		Phenobarbitone sodium	Barbitone sodium	Hexobarbitone sodium	Thiopentone sodium
5	2·00	9·04	9·62	9·88	9·70
10	1·00	8·92	9·40	9·63	9·50
20	0·50	8·72	9·18	9·28	9·32
30	0·33	8·56	9·02	9·23	9·15
40	0·25	8·46	8·98	9·15	9·01
50	0·20	8·40	8·78	8·99	8·90
75	0·13	8·33	8·59	8·80	8·61
100	0·10	8·16	8·53	8·45	8·43
Water for injection	1·0	9·45	9·90	10·52	10·76

The Growth-inhibiting Power of Sodium Salts of some Barbituric Acid Derivatives.

Phenobarbitone sodium. The bacteriostatic value of phenobarbitone sodium was tested against 4 organisms, *Staph. aureus*, *Ps. æruginosa*,

B. subtilis and *E. coli*. For each organism a series of tubes containing exactly 5 ml. of a sterile, freshly-prepared double strength Difco nutrient broth was used (Difco B3, 16 g./l. freshly-distilled water). To these tubes were added increasing amounts of a 10 per cent. w/v solution of phenobarbitone sodium in carbon dioxide-free, sterile water. The tubes were then adjusted with sterile water to give a final volume of 10 ml. in each tube. 2 drops of a 24-hour culture of the organism were added to each of the respective series of tubes and these were then incubated at 37° C. As previously shown, such inoculation is sufficiently heavy to initiate growth at *p*H as high as 10·5. Phenobarbitone was not precipitated, growth was easily observable and no difficulty in reading the end-point was encountered (Table V).

TABLE V

GROWTH AFTER 96 HOURS INCUBATION AT 37° C. IN SOLUTIONS OF PHENOBARBITONE SODIUM

Tube	1	2	3	4	5	6	7	8	9	10
Concentration of phenobarbitone sodium, per cent. w/v	0·1	0·5	1·0	1·5	2·0	2·5	3·0	4·0	4·5	5·0
Staph. aureus	+	+	+	+	+	+	+	+	+	±
Ps. æruginosa	+	+	+	+	+	+	+	+	+	−
B. subtilis	+	+	+	+	+	+	+	+	+	−
E. coli	+	+	±	−	−	−	−	−	−	−

The amount of drug required to produce bacteriostasis increased with incubation time up to 96 hours, after which prolonged incubation appeared to cause no appreciable change (Table VI).

TABLE VI

BACTERIOSTATIC CONCENTRATIONS (PER CENT. W/V) OF PHENOBARBITONE SODIUM AT VARIOUS INCUBATION TIMES

Incubation time	24 hours	48 hours	96 hours
Staph. aureus	4·5	5·0	> 5·0
Ps. æruginosa	4·5	5·0	5·0
B. subtilis	3·0	4·0	5·0
E. coli	1·0	1·5	1·5

The B.P. directs that at least 50 mg. (and the U.S.P. at least 100 mg.) of a solid should be used when testing for sterility, resulting in the production of at least a 0·5 per cent. solution when 10 ml. of medium is used. The above results suggest, in the case of *E. coli*, that a larger volume of broth be used to avoid bacteriostasis.

The alkalinity of solutions of phenobarbitone sodium in the broth media used, having a maximum of *p*H 8·72 with a 0·5 per cent. concentration and lower *p*H with decreasing concentration (see Table IV), is not sufficient to prevent the growth of the organism under test, even with a low degree of contamination.

Barbitone sodium. A determination of bacteriostatic concentration identical with that for phenobarbitone sodium was carried out with soluble barbitone using a 15 per cent. w/v solution in carbon dioxide-free,

sterile water. Again there was no growth-obscuring deposit of barbitone and sharp end-points were obtained (Tables VII and VIII). It is suggested that larger volumes of broth should be used to eliminate bacteriostasis when testing soluble barbitone for sterility.

TABLE VII

GROWTH AFTER 96 HOURS INCUBATION AT 37° C. IN SOLUTIONS OF BARBITONE SODIUM

Tube	1	2	3	4	5	6	7	8	9	10
Concentration of barbitone sodium, per cent. w/v	0·75	1·5	2·25	3·0	3·75	4·5	5·25	6·0	6·75	7·5
Staph. aureus	+	+	+	+	+	+	+	+	+	+
Ps. æruginosa	+	+	+	+	+	+	±	−	−	−
B. subtilis	+	+	+	+	−	−	−	−	−	−
E. coli	+	+	+	−	−	−	−	−	−	−

TABLE VIII

BACTERIOSTATIC CONCENTRATIONS (PER CENT. W/V) OF BARBITONE SODIUM AT VARIOUS INCUBATION TIMES

Incubation time	24 hours	48 hours	96 hours
Staph. aureus	6·0	7·5	> 7·5
Ps. æruginosa	5·25	5·25	6·0
B. subtilis	3·75	3·75	3·75
E. coli	3·0	3·0	3·0

Hexobarbitone sodium. The bacteriostatic concentration of hexobarbitone sodium was determined as previously described, using a 10 per cent. w/v solution. It was somewhat difficult to distinguish between turbidity due to bacterial growth and that due to precipitation of crystals of hexobarbitone. Long, needle-shaped crystals of the latter appeared in increasing amounts with increasing concentration of the hexobaribitone sodium and with prolonged incubation (Tables IX and X).

TABLE IX

GROWTH AFTER 6 DAYS INCUBATION AT 37° C. IN SOLUTIONS OF HEXOBARBITONE SODIUM

Tube	1	2	3	4	5	6	7	8	9	10	11	12	13
Concentration of hexobarbitone sodium, per cent. w/v	0·2	0·25	0·4	0·5	0·6	0·75	0·8	1·0	1·5	2·0	2·5	3·0	5·0
Staph. aureus	+	+	+	+	+	+	+	+	+	±	−	−	−
Ps. æruginosa	+	+	+	+	+	+	+	+	+	+	+	−	−
B. subtilis	+	+	+	+	+	+	+	+	−	−	−	−	−
E. coli	+	+	+	+	+	+	+	±	−	−	−	−	−

TABLE X

BACTERIOSTATIC CONCENTRATIONS (PER CENT. W/V) OF HEXOBARBITONE SODIUM AT VARIOUS INCUBATION TIMES

Incubation time	24 hours	48 hours	72 hours	96 hours	6 days
Staph. aureus	0·75	2·0	2·0	—	2·5
Ps. æruginosa	2·0	2·0	2·0	—	3·0
B. subtilis	0·75	1·5	1·5	—	1·5
E. coli	0·6	1·0	1·5	1·5	1·5

It is important to note that the "bacteriostatic values" given in the tables, do not necessarily represent the true values. The end-points were indeed obtained with the given concentrations, but those concentrations were a combination of an unknown amount of hexobarbitone sodium in solution together with precipitated hexobarbitone crystals.

It is suggested that for tests for sterility, larger volumes of broth than normal be used and that this will serve two purposes, viz., the possibility of bacteriostasis will be eliminated and there will be no difficulty in observing growth, precipitation of hexobarbitone and consequent subculture being avoided.

Thiopentone sodium. The results obtained in the case of thiopentone sodium were somewhat different from those previously given for the other barbiturates.

An attempt was made to carry out a ranging test for the bacteriostatic value, but on addition of a solution of the thiopentone sodium to broth, an immediate white, milky precipitate was thrown down. As the concentration of thiopentone sodium increased, the bulk of the precipitate increased to a maximum and then became less. This suggested that at small concentrations the *p*H was low enough to precipitate thiopentone, although the amount precipitated was necessarily small. At high concentrations the amount precipitated was again small because the *p*H was sufficiently alkaline to keep most of the thiopentone in solution. Between the two extremes of concentration, a maximum bulk of precipitate was therefore reached at the optimal conditions for *p*H and concentration.

It was shown, by adding graded amounts of 0·1 N hydrochloric acid from a microburette to 10-ml. quantities of a 1 per cent. w/v solution of thiopentone sodium, that thiopentone precipitated from an alkaline solution at *p*H lower than about 9·4.

In determining the bacteriostatic value, it was obvious that it would be impossible to avoid this precipitation of thiopentone. It was decided to proceed with the method previously adopted and, after a given period of incubation to redissolve the thiopentone by addition of a solution of sodium hydroxide. It was found that this method was quite successful and that little difficulty was experienced in reading results.

5 series of tubes containing 5 ml. of double strength Difco broth were prepared and increasing amounts of a sterile 10 per cent. thiopentone sodium solution added to the tubes of each series. The tubes were made up to 10 ml. with sterile water. Two drops of a 24-hour culture of the 4 test organisms were added to the series as previously described for phenobarbitone sodium. The fifth series was incubated, without inoculation, along with the others for 4 days at 37° C.

A further 6 tubes of double-strength broth were diluted to 10 ml. with sterile water. These were used as controls, 2 being incubated as they were and the other 4 infected with one of the test organisms respectively and also incubated.

After incubation, 2 ml. of N sodium hydroxide was pipetted into each of all the tubes. They were allowed to stand with intermittent

shaking for approximately 10 minutes and were then examined for turbidity and growth.

The series containing thiopentone sodium but which was not infected was used as a standard of clarity for determining absence of growth in the other tubes. The tubes of this series were as clear as the 2 tubes of single-strength broth incubated as controls. The 4 tubes containing no thiopentone sodium, but which were infected with the test organisms, were used to compare turbidity in the other tubes.

It was noticed that after standing for a few hours after addition of the 2 ml. of N sodium hydroxide, all the tubes showed a precipitate of a "woolly" nature at the bottom of the tubes. It was found essential therefore to read results as soon as the thiopentone had redissolved on addition of the sodium hydroxide (Table XI).

TABLE XI

GROWTH AFTER 96 HOURS INCUBATION AT 37° C. IN SOLUTIONS OF THIOPENTONE SODIUM

Concentration of thiopentone sodium, per cent. w/v ..	0·05	0·1	0·2	0·4	0·6	0·8	1·0	1·5	2·0	2·5
Staph. aureus	+	+	+	+	+	+	+	+	−	−
Ps. æruginosa	+	+	+	+	+	+	+	+	±	±
B. subtilis	+	+	+	+	+	+	−	−	−	−
E. coli	+	+	+	+	+	±	−	−	−	−

Again, as for hexobarbitone sodium, it may be pointed out that the true concentration of thiopentone sodium in solution during incubation was not known, some being precipitated as the free acid. The values given, therefore, represent the "bacteriostatic values" under the conditions used.

It can be seen that very low concentrations of thiopentone sodium were sufficient to inhibit growth, only 0·8 per cent. being necessary in the case of *E. coli*. Large volumes of broth should therefore be used in any test for sterility. To overcome the difficulty of determining whether turbidity is due to growth or to precipitated thiopentone, either subculture or addition of a suitable quantity of a freshly-prepared solution of sodium hydroxide should be employed. Concentrations of thiopentone sodium in broth greater than 0·2 per cent. should not be reached, since the *p*H of such solutions will be sufficient to inhibit the growth of small numbers of contaminating bacteria.

SUMMARY

1. The growth of bacteria under conditions of alkalinity has been demonstrated. It was shown that with fairly large inocula, growth was supported in Difco broth up to about *p*H 10·5. With small inocula of *E. coli*, the organism was still capable of initiating growth up to about *p*H 9.

2. The *p*H of various concentrations of 4 barbiturates, phenobarbitone sodium, barbitone sodium, hexobarbitone sodium and thiopentone sodium, in Difco broth were found and it was thereby shown that in

determination of their bacteriostatic values, any inhibition of growth, in concentrations less than 2 per cent., at the very least, could not directly be due to the alkalinity produced.

3. The bacteriostatic values of the 4 barbiturates were determined against 4 organisms, although in some cases, where the value found greatly exceeded a concentration of 2 per cent., it was not entirely established whether this value was due to the inherent bacteriostatic property of the drug itself, or to a combination of this together with the alkalinity produced (Table XII).

TABLE XII

BACTERIOSTATIC VALUES (PER CENT. W/V)

Organism	Phenobarbitone sodium	Barbitone sodium	Hexobarbitone sodium	Thiopentone sodium
Staph. aureus ..	5·0–>5·0	> 7·5	2·0–2·5	2·0
Ps. æruginosa ..	5·0	5·25–6·0	3·0	2·0–> 2·0
B. subtilis	5·0	3·75	1·5	1·0
E. coli	1·0–1·5	3·0	1·0–1·5	0·8–1·0

4. In any test for sterility on these barbiturates, it is suggested that large volumes of broth, sufficient to give a concentration less than 0·2 per cent. be used: (*a*) to avoid a high *p*H, and thus prevent growth, since the amount of contamination may probably be small; (*b*) to avoid a bacteriostatic concentration; (*c*) in the case of hexobarbitone sodium, to avoid difficulty in observation of growth due to precipitation of hexobarbitone.

5. When thiopentone sodium is being tested, the above observations equally apply, but, since the precipitation of thiopentone makes it difficult to discern growth, it is suggested that either the usual subculture method or the addition of sufficient solution of sodium hydroxide to redissolve the thiopentone, be employed. In the latter case, controls to compare turbidity should be used and the results observed soon after the addition of the sodium hydroxide.

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