COMPARATIVE STUDIES OF METHODS OF EVALUATING ANTIBACTERIAL SUBSTANCES

PART I. EVALUATION OF BACTERIOSTATIC ACTION

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METHODS of evaluating antibacterial substances have been reviewed by Reddish[1,2] and Berry,[3] and the established methods justly criticised. Until more is known of the actual chemical and/or physical nature of the bacteria-bactericide reactions the evaluation of antibacterial substances is likely to remain empirical, but so diverse are the natures of different antibacterial substances that their modes of action must be different. It would seem then, that the study of the dynamics of a particular disinfecting system by two or more of the available methods would indicate whether the different methods are in fact measuring the same phenomenon. This series of papers presents the results of attempts to do this.

INTRODUCTION

The distinction between bacteriostatic and bactericidal action is confused. Marshall and Krenoff[4] defined bacteriostasis as "A concept of those conditions in which living bacteria, under the influence of a definite chemical agent, are induced to multiply at any rate less than normal." Parkinson[5] postulated two types of bacteriostasis, a true bacteriostasis in which there is an absolute arrest of growth and the bacteria die of senescence, and a dynamic bacteriostasis in which the organisms are killed at a rate equal to or greater than that at which they reproduce. Price[6] maintained that it was futile and unrealistic to try to differentiate between bacteriostatic and bactericidal agents, the two apparent effects being in fact due to the differences in resistance of individual bacteria, and instanced the use of antagonists to bactericidal agents to prove this point.

Since this work is an initial attempt at the comparison of techniques of measuring bacteriostatic action, the bacteriostatic strength of a substance will be taken as that strength which prevents further growth of the organism, and the investigation of antagonists will be left to a future study.

The traditional methods of ascertaining bacteriostatic strength are of three types, a liquid culture method and two plate methods. In the first the bacteriostatic agent is diluted with a nutrient broth and the liquid inoculated with a test organism and the culture examined for growth turbidity after incubation. In the second method an agar plate is seeded with a test organism and the size of a zone of inhibition of growth, caused by diffusion of the bacteriostatic from a cup or cylinder placed on the

plate, is measured. In the third, the bacteriostatic agent can be incorporated in the solid medium and growth or no growth of a culture streaked on the surface is recorded.

The second method has been used in the assay of antibiotics and the factors affecting the results have been examined and discussed by Cooper and Woodman.[7]

The third method has been used mainly in a qualitative way to ascertain the bacteriostatic "spectra" of various agents. The first method is the method of choice for ascertaining the strengths of bacteriostatics to be incorporated in injections, in multidose containers, and other pharmaceutical preparations to prevent the growth of micro-organisms in the preparations. This method cannot be easily used with bacteriostatic agents which cause a precipitate or cloudiness when diluted with the nutrient medium as is the case with some quaternary ammonium compounds and formulated disinfectants of the lysol and black and white fluid types. Nor has the existence of any correlation between the results of the first and third methods been examined.

Experimental

Organisms

1. *Pseudomonas æroginosa* (Syn. *Pseudomonas pyocyanea*) N.C.T.C. 8203.
2. *Echerichia coli* I (Syn. *Bacterium coli*) N.C.T.C. 8196.
3. *Salmonella typhi* N.C.T.C. 160.
4. *Staphylococcus aureus* N.C.T.C. 4163.
5. *Shigella dysenteriæ* type 1. N.C.T.C. 8217.
6. *Bacillus anthracis* N.C.T.C. 8234.
7. *Streptococcus fæcalis* N.C.T.C. 370.
8. *Mycobacterium smegmatis* N.C.T.C. 8159.
9. *Neisseria catarrhalis* N.C.T.C. 5483.
10. *Bordetella bronchiseptica* (Syn. *Hæmophilus bronchisepticus*) N.C.T.C. 452.
11. *Corynebacterium diphtheriæ, mitis.* N.C.T.C. 3989.

Bacteriostatic Substances

(*a*) Solids

Phenol, A.R. quality.
6-Chloro-3-hydroxytoluene (chlorocresol B.P.).
Phenylmercuric acetate B.P.C.
Cetyltrimethylammonium bromide (Cetrimide B.P.C.).

(*b*) Formulated preparations.

Solution of cresol with soap. B.P. (Lysol).
Solution of chloroxylenol. B.P. (Roxenol).
A commercial "black fluid."
A. 10. 39.* (A formulated quaternary ammonium compound.)

* Supplied by Messrs. Airkem Ltd.

Medium

The medium was peptone 1 per cent., Lab. Lemco 1 per cent., sodium chloride 0·5 per cent. in distilled water. For the solid medium this was gelled with 2 per cent. of Davis agar. In the majority of cases the medium was prepared double strength and diluted with an equal quantity of the bacteriostatic diluted with sterile water.

Apparatus

In all experiments the inoculum consisted of 1 drop from a No. 22 gauge needle on the special pipette designed by Cook and Yousef.[8] In order to check on the variability of the volume of 1 drop the following experiment was performed.

Experiment. Using the same needle the weight of one drop of distilled water was estimated by weighing ten lots of 10 drops, 10 lots of 5 drops, 10 lots of 2 drops and 10 lots of 1 drop. A series of 10 lots of 1 drop of distilled water from each of 6 different needles was then weighed.

Results. The results are summarised in Tables I and II.

TABLE I

VARIABILITY OF WEIGHT OF 1 DROP OF DISTILLED WATER FROM 1 PIPETTE

	Number of drops weighed			
	1	2	5	10
Mean weight of 1 drop in mg. ..	16·70	16·74	16·80	16·79
Standard deviation	0·19	0·21	0·05	0·05
95 per cent. fiducial limits	±0·14	±0·15	±0·04	±0·03

TABLE II

ANALYSIS OF VARIANCE OF WEIGHT OF 1 DROP OF DISTILLED WATER ESTIMATED USING 6 DIFFERENT PIPETTES

Source of variance	d.f.	Sum of squares	Mean square
Between pipettes	5	2·9135	0·5827
Between weighings	9	0·5308	0·0590
Residual	45	4·1282	0·0917
Total	59	7·5725	

Table II shows that the greatest source of variance is the between-needle variance but the total variance is small and the 95 per cent. fiducial limits using all the 60 readings are 16·83 to 17·02 mg. and so the size of 1 drop from any one pipette can be taken as 1/59 ml.

METHODS

Liquid Dilution Method. 10 ml. of medium was made by aseptically diluting 5 ml. of double strength culture medium with 5 ml. of the bacteriostatic solution in sterile distilled water. One drop of a 24-hour culture of the test organism was added as inoculum. The tube was then incubated for 48 hours at 37° C. and examined for growth. Controls of uninoculated tubes were also set up.

TABLE III

Bacteriostatic		Phenol					Chlorocresol					Phenylmercuric acetate					Cetrimide				
Percentage strength		2	1	10^{-1}	10^{-2}	10^{-3}	0·2	10^{-1}	10^{-2}	10^{-3}	10^{-4}	0·05	10^{-2}	10^{-3}	10^{-4}	10^{-5}	0·5	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Organism	Liquid or solid method																				
Ps. pyocyanea ..	Liquid	−	−	+	+	+	−	−	+	+	+	P	−	+	+	+	P	P	P	+	+
	Solid	−	−	+	+	+	−	−	+	+	+		−	+	+	+	+	+	+	+	+
Bact. coli	Liquid	−	−	+	+	+	−	−	+	+	+	P	−	−	+	+	P	P	P	+	+
	Solid	−	−	+	+	+	−	−	+	+	+		−	−	+	+	−	−	+	+	+
Salm. typhi	Liquid	−	−	+	+	+	−	−	+	+	+	P	−	−	+	+	P	P	P	+	+
	Solid	−	−	+	+	+	−	−	+	+	+		−	−	+	+	−	−	+	+	+
Staph. aureus	Liquid	−	−	+	+	+	−	−	+	+	+	P	−	−	−	−	P	P	P	−	+
	Solid	−	−	+	+	+	−	−	+	+	+		−	−	−	−	−	−	−	+	+
Shig. dysenteriæ	Liquid	−	−	+	+	+	−	−	+	+	+	P	−	−	±	+	P	P	P	+	+
	Solid	−	−	+	+	+	−	−	+	+	+		−	−	+	+	−	−	−	+	+
B. anthracis	Liquid	−	−	+	+	+	−	−	+	+	+	P	−	−	−	−	P	P	P	−	+
	Solid	−	−	+	+	+	−	−	+	+	+		−	−	±	−	−	−	−	+	+
Strept. fæcalis	Liquid	−	−	+	+	+	−	−	+	+	+	P	−	−	−	−	P	P	P	−	+
	Solid	−	−	+	+	+	−	−	+	+	+		−	−	±	−	−	−	−	+	+
N. catarrhalis	Liquid	−	−	−	+	+	−	−	−	+	+	P	−	−	−	−	P	P	P	−	+
	Solid	−	−	−	+	+	−	−	−	+	+		−	−	±	−	−	−	−	+	+
H. bronchisepticus	Liquid	−	−	−	+	+	−	−	+	+	+	P	−	−	+	+	P	P	P	−	+
	Solid	−	−	±	±	+	−	−	−	+	+		−	−	±	±	−	−	−	+	+
Corynebact. diphtheriæ	Liquid	−	−	−	±	+	−	−	+	+	+	P	−	−	−	−	P	P	P	−	+
	Solid	−	−	±	±	+	−	−	+	+	+		−	−	−	−	−	−	−	+	+
Myco. smegmatis	Liquid	−	−	+	+	+	−	−	+	+	+	P	−	−	−	−	P	P	P	−	+
	Solid	−	−	+	+	+	−	−	+	+	+		−	−	−	−	−	−	−	+	+

Bacteriostatic		A.10.39					Roxenol					Black fluid					Lysol				
Dilution		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Organism	Liquid or solid method																				
Ps. pyocyanea	Liquid	P	+	+	+	+	P	P	+	+	+	P	P	P	+	+	P	P	+	+	+
	Solid	−	+	+	+	+	−	+	+	+	+	−	+	+	+	+	−	−	+	+	+
Bact. coli	Liquid	P	−	−	+	+	P	P	−	+	+	P	P	P	+	+	P	P	+	+	+
	Solid	−	−	−	+	+	−	−	−	+	+	−	−	−	+	+	−	−	+	+	+
Salm. typhi	Liquid	P	−	+	+	+	P	P	+	+	+	P	P	P	+	+	P	P	+	+	+
	Solid	−	−	+	+	+	−	−	+	+	+	−	−	+	+	+	−	−	+	+	+
Staph. aureus	Liquid	P	−	−	+	+	P	P	−	+	+	P	P	P	−	+	P	P	+	+	+
	Solid	−	−	−	+	+	−	−	−	+	+	−	−	−	−	+	−	−	+	+	+
Shig. dysenteriæ	Liquid	P	−	−	+	+	P	P	+	+	+	P	P	P	+	+	P	P	−	+	+
	Solid	−	−	+	+	+	−	−	+	+	+	−	−	+	+	+	−	−	+	+	+
B. anthracis	Liquid	P	−	−	+	+	P	P	−	+	+	P	P	P	−	+	P	P	−	+	+
	Solid	−	−	−	+	+	−	−	−	+	+	−	−	−	−	+	−	−	−	+	+
Strept. fæcalis	Liquid	P	−	−	+	+	P	P	−	+	+	P	P	P	−	+	P	P	−	+	+
	Solid	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	+	+
N. catarrhalis	Liquid																				
	Solid																				
H. bronchisepticus	Liquid	P	−	+	+	+	P	P	+	+	+	P	P	P	+	+	P	P	−	+	+
	Solid	−	+	+	+	+	−	−	+	+	+	−	−	+	+	+	−	−	−	+	+
Corynebact. diphtheriæ	Liquid	P	−	−	−	+	P	P	−	+	+	P	P	P	−	+	P	P	−	−	+
	Solid	−	−	−	+	+	−	−	−	−	+	−	−	−	−	+	−	−	−	+	+
Myco. smegmatis	Liquid	P	−	−	+	+	P	P	−	+	+	P	P	P	−	+	P	P	−	+	+
	Solid	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	−	+	+

Showing results of bacteriostatic screening tests using liquid dilution and solid dilution methods. − = no growth, + = growth, P = precipitate prevented reading after 48 hour incubation.

Solid Dilution Method. 20 ml. of medium was made by aseptically adding 10 ml. of bacteriostatic solution in distilled water to 10 ml. of double strength agar medium melted and cooled to 65° C. This medium was poured into a sterile petri dish and when solidified was dried for 1 hour in an incubator with the lid raised. One drop of a 24-hour culture of the test organism was dropped onto the dried plate as in a Miles and Misra count. When the drop had been absorbed into the medium the plate was incubated at 37° C. for 48 hours and then examined for colony development.

TABLE IV

		Phenol				A.10.39					Roxenol		
	Percentage strength	0·20	0·18	0·16	0·14	0·10	0·08	0·06	0·04	0·02	0·10	0·08	0·06
Bact. coli ..	Liquid	−	−	+	+	+	+	+	+	+	−	+	+
		−	−	+	+	−	−	+	+	+	−	−	+
		−	−	+	+	−	−	+	+	+	−	−	+
		−	−	+	+	−	−	−	+	+	−	−	+
		−	−	+	+	−	−	−	+	+	−	−	+
	Solid	−	−	+	+	+	+	+	+	+	−	+	+
		−	−	+	+	±	+	+	+	+	−	+	+
		−	−	+	+	±	+	+	+	+	−	+	+
		−	−	+	+	±	+	+	+	+	−	+	+
		−	−	+	+	−	+	+	+	+	−	+	+
	Percentage strength	0·16	0·14	0·12	0·10	0·14	0·12	0·10	0·08	0·06	0·16	0·14	0·12
Shig. dysenteriæ	Liquid	−	−	+	+	−	+	+	+	+	−	−	+
		−	−	+	+	−	+	+	+	+	−	−	+
		−	−	+	+	−	+	+	+	+	−	−	+
		−	−	+	+	−	−	−	−	+	−	−	−
		−	−	+	+	−	−	−	−	+	−	−	−
	Solid	−	−	+	+	+	+	+	+	+	−	−	+
		−	−	+	+	+	+	+	+	+	−	−	+
		−	−	+	+	+	+	+	+	+	−	−	+
		−	−	+	+	+	+	+	+	+	−	−	+
		−	−	+	+	+	+	+	+	+	−	−	+
	Percentage strength	0·16	0·14	0·12	0·10		15·0	10·0	4·0	2·0	9·0	8·0	7·0
Ps. pyocyanea ..	Liquid	−	−	+	+								
		−	−	+	+								
		−	−	+	+								
		−	−	−	+								
		−	−	−	−								
	Solid	−	−	+	+		−	+	+	+	−	+	+
		−	−	+	+		−	+	+	+	−	+	+
		−	−	−	+		−	+	+	+	−	+	+
		−	−	−	+		−	−	+	+	−	+	+
		−	−	−	+		−	−	+	+	−	+	+

Showing results of bacteriostatic tests using liquid dilution and solid dilution methods, − = no growth, + = growth, after 48 hours incubation.

The above two methods were carried out simultaneously. The initial ranging experiments were carried out using a tenfold dilution of the bacteriostatic agent with all the test organisms. The experiment was then repeated using a closer range of dilutions of some of the bacteriostatic solutions and each test carried out in triplicate. An even closer range of dilutions was used against *Bact. coli*, *Shig. dysenteriæ* and *Ps. pyocyanea* and carried out in quintuplicate against phenol, A. 10.39 and roxenol B.P. With the latter two bacteriostatic agents against *Ps. pyocyanea* it was only

possible to perform the test by the solid method since liquid dilutions were turbid.

Counting Method. This method was used with *Bact. coli, Shig. dysenteriæ and Ps. pyocyanea* against one strength of phenol, one strength of Roxenol and two strengths of A.10.39.

The method used was to set up 5 tubes for each test organism. The first tube contained 10 ml. of sterile water, the second 10 ml. of quarter strength Ringer's solution, the third tube 10 ml. of broth with added bacteriostatic, the fourth tube sterile water plus bacteriostatic and the fifth tube quarter strength Ringer's solution plus bacteriostatic. Viable

TABLE V

Tube	Inoculum	Counts after incubation for 0 hours	5 hours	10 hours	50 hours
Control (water)	*Bact. coli*	$8{\cdot}3\times10^{5}$	$1{\cdot}0\times10^{6}$	$1{\cdot}8\times10^{6}$	$2{\cdot}8\times10^{6}$
" (Ringer)		$7{\cdot}9\times10^{5}$	$2{\cdot}6\times10^{6}$	$3{\cdot}8\times10^{6}$	$3{\cdot}0\times10^{6}$
Phenol 0·18 per cent in broth ..		$8{\cdot}3\times10^{5}$	$6{\cdot}2\times10^{5}$	$5{\cdot}0\times10^{5}$	0
" " " water ..		$8{\cdot}0\times10^{5}$	$2{\cdot}1\times10^{5}$	$3{\cdot}5\times10^{5}$	59
" " " Ringer		$7{\cdot}1\times10^{5}$	$3{\cdot}5\times10^{5}$	$2{\cdot}4\times10^{5}$	637
Control (water)	*Shig. dysenteriæ*	$2{\cdot}2\times10^{5}$	$1{\cdot}9\times10^{5}$	$2{\cdot}2\times10^{4}$	$7{\cdot}3\times10^{3}$
" (Ringer)		$3{\cdot}2\times10^{5}$	$7{\cdot}0\times10^{5}$	$4{\cdot}3\times10^{5}$	$3{\cdot}2\times10^{5}$
Phenol 0·12 per cent. in broth ..		$2{\cdot}8\times10^{5}$	$1{\cdot}7\times10^{5}$	$4{\cdot}2\times10^{4}$	0
" " " water ..		$2{\cdot}1\times10^{5}$	$1{\cdot}1\times10^{5}$	$7{\cdot}0\times10^{3}$	0
" " " Ringer		$3{\cdot}1\times10^{5}$	$2{\cdot}1\times10^{5}$	$2{\cdot}6\times10^{5}$	$3{\cdot}9\times10^{4}$
Control (water)	*Ps. pyocyanea*	$1{\cdot}0\times10^{6}$	$3{\cdot}1\times10^{5}$	$5{\cdot}8\times10^{5}$	$7{\cdot}2\times10^{6}$
" (Ringer)		$1{\cdot}6\times10^{6}$	$1{\cdot}4\times10^{7}$	$1{\cdot}5\times10^{7}$	$1{\cdot}2\times10^{7}$
Phenol 0·14 per cent. in broth ..		$3{\cdot}1\times10^{6}$	$6{\cdot}5\times10^{6}$	$1{\cdot}1\times10^{7}$	$3{\cdot}0\times10^{7}$
" " " water ..		$2{\cdot}2\times10^{6}$	$3{\cdot}4\times10^{5}$	$8{\cdot}9\times10^{5}$	$5{\cdot}5\times10^{4}$
" " " Ringer		$8{\cdot}9\times10^{5}$	$3{\cdot}6\times10^{6}$	$2{\cdot}9\times10^{6}$	$6{\cdot}4\times10^{5}$
Control (water)	*Bact. coli*	$1{\cdot}3\times10^{6}$	$5{\cdot}0\times10^{5}$	$1{\cdot}6\times10^{6}$	$3{\cdot}4\times10^{6}$
" (Ringer)		$1{\cdot}3\times10^{6}$	$5{\cdot}7\times10^{5}$	$1{\cdot}0\times10^{7}$	$6{\cdot}5\times10^{6}$
Roxenol 0·08 per cent. in broth		$1{\cdot}7\times10^{6}$	$2{\cdot}5\times10^{5}$	$5{\cdot}5\times10^{5}$	$1{\cdot}3\times10^{7}$
" " " water ..		$1{\cdot}1\times10^{6}$	$1{\cdot}2\times10^{7}$	$1{\cdot}3\times10^{6}$	$6{\cdot}7\times10^{4}$
" " " Ringer		$1{\cdot}3\times10^{6}$	$1{\cdot}6\times10^{6}$	$9{\cdot}9\times10^{5}$	$5{\cdot}0\times10^{3}$
Control (water)	*Shig. dysenteriæ*	$2{\cdot}1\times10^{5}$	$3{\cdot}0\times10^{5}$	$2{\cdot}6\times10^{5}$	$7{\cdot}8\times10^{3}$
" (Ringer)		$3{\cdot}1\times10^{5}$	$9{\cdot}3\times10^{5}$	$1{\cdot}2\times10^{6}$	$1{\cdot}1\times10^{5}$
Roxenol 0·12 per cent. in broth		$3{\cdot}7\times10^{5}$	$3{\cdot}9\times10^{6}$		$1{\cdot}1\times10^{7}$
" " " water		$3{\cdot}0\times10^{5}$	$2{\cdot}3\times10^{5}$	$2{\cdot}2\times10^{5}$	$2{\cdot}0\times10^{6}$
" " " Ringer		$3{\cdot}2\times10^{5}$	$3{\cdot}3\times10^{5}$	$1{\cdot}5\times10^{6}$	$5{\cdot}9\times10^{5}$
Control (water)	*Ps. pyocyanea*	$4{\cdot}7\times10^{5}$	$8{\cdot}3\times10^{5}$	$1{\cdot}1\times10^{6}$	$1{\cdot}6\times10^{7}$
" (Ringer)		$1{\cdot}2\times10^{6}$	$9{\cdot}7\times10^{6}$	$1{\cdot}7\times10^{7}$	$2{\cdot}2\times10^{7}$
Roxenol 8·0 per cent. in broth		$1{\cdot}0\times10^{5}$	$1{\cdot}0\times10^{3}$	$3{\cdot}4\times10^{2}$	0
" " " water		0	0	0	0
" " " Ringer		0	0	0	0
Control (water)	*Bact. coli*	$1{\cdot}5\times10^{6}$	$1{\cdot}6\times10^{6}$	$6{\cdot}8\times10^{6}$	
" (Ringer)		$1{\cdot}4\times10^{6}$	$2{\cdot}9\times10^{6}$	$1{\cdot}4\times10^{7}$	
A.10.39 0·08 per cent in broth ..		$1{\cdot}4\times10^{6}$	0	0	
" " " water ..		$3{\cdot}9\times10^{4}$	0	0	
" " " Ringer		$9{\cdot}4\times10^{5}$	0	0	
Control (water)	*Shig. dysenteriæ*	Estimate	$3{\cdot}4\times10^{5}$	$3{\cdot}2\times10^{5}$	
" (Ringer)		$3{\cdot}0\times10^{5}$	$1{\cdot}6\times10^{6}$	$1{\cdot}8\times10^{6}$	
A.10.39 0·16 per cent in broth			0	0	
" " " water ..			0	0	
" " " Ringer			0	0	
Control (water)	*Ps. pyocyanea*	$1{\cdot}1\times10^{5}$	$2{\cdot}2\times10^{5}$	$4{\cdot}2\times10^{5}$	
" (Ringer)		$3{\cdot}5\times10^{4}$		$1{\cdot}6\times10^{7}$	
A.10.39 10 per cent. in broth ..		0	0	0	
" " " water ..		0	0	0	
" " " Ringer ..		0	0	0	

Showing results of counts performed on bacteriostatic test solutions after various times of incubation at 37° C.

counts were performed on each tube after 0, 5, 10 and 50 hours incubation at 37° C. The counts were performed in quintuplicate on tenfold dilutions by the Miles and Misra overdried plate method.

Results

Table III shows the results of the initial ranging tests. Table IV shows the results using closer ranges on 3 organisms with 3 bacteriostatic agents.

A viable count was performed in quintuplicate on 24-hour cultures of each of the 3 principal organisms using the Miles and Misra technique and gave the following results:—

Bact. coli	$9{\cdot}8 \times 10^8$ organisms per ml.	(p for $\chi^2 = 0{\cdot}7$).
Shig. dysenteriæ	$2{\cdot}2 \times 10^8$ „ „	(„ „ $= 0{\cdot}7$).
Ps. pyocyanea	$2{\cdot}4 \times 10^{10}$ „ „	(„ „ $= 0{\cdot}3$).

Using an inoculum of 1 drop per 10 ml. of test solution this was equivalent to inoculum levels of approximately

Bact. coli	$1{\cdot}5 \times 10^6$ organisms per ml.
Shig. dysenteriæ	$4{\cdot}0 \times 10^5$ „ „
Ps. pyocyanea	$4{\cdot}0 \times 10^7$ „ „

Table V summarises the results of the counting technique.

The results using A.10.39 were obtained using strengths which had shown no growth in the dilution methods and these results showed that for the counting technique these strengths were more than bacteriostatic and so the experiment was repeated using weaker solutions and also single drops from the reaction tube were plated out at intermediate times. The results are summarised in Table VI.

TABLE VI

	Inoculum	Count after incubating for							
		0	½ hour	1 hour	1½ hours	2 hours	2½ hours	5 hours	10 hours
Control	*Bact. coli*	$1{\cdot}5\times10^6$	—	—	—	—	—	$8{\cdot}5\times10^5$	$5{\cdot}3\times10^5$
0·06 per cent. A.10.39									
in broth		$1{\cdot}5\times10^6$	—	—	$<10^4$	—	$<10^4$	$6{\cdot}5\times10^3$	$2{\cdot}0\times10^3$
in water		$1{\cdot}1\times10^6$	—	—	$1{\cdot}3\times10^3$	—	0	0	0
in Ringer		$1{\cdot}4\times10^6$	—	—	$1{\cdot}8\times10^3$	—	0	0	0
Control	*Shig. dysenteriæ*	$3{\cdot}9\times10^5$	—	—	—	—	—	$2{\cdot}6\times10^5$	$1{\cdot}0\times10^5$
0·08 per cent. A.10.39									
in broth		$3{\cdot}7\times10^5$	—	$<10^4$	—	$<10^4$	—	$8{\cdot}1\times10^3$	$3{\cdot}0\times10^3$
in water		$3{\cdot}5\times10^5$	—	600	—	0	—	0	0
in Ringer		$3{\cdot}3\times10^5$	—	0	—	0	—	0	0
Control	*Ps. pyocyanea*	$3{\cdot}8\times10^6$	—	—		—	—	$1{\cdot}6\times10^6$	$4{\cdot}5\times10^5$
8·0 per cent. A.10.39									
in broth		$2{\cdot}1\times10^5$	1770	—	0		0	0	0
in water		0	0	—	0	—	0	0	0
in Ringer		0	0	—	0	—	0	0	0

Discussion

If the definition of bacteriostasis outlined in the introduction is adopted then results of preliminary tests using tenfold dilutions show that there is a definite correlation between the solid dilution and liquid dilution methods of evaluating the bacteriostatic values for the given agents

under the stated conditions. It is suggested that for preliminary screening of an antibacterial agent the solid dilution techniques can be recommended as being much more economical of medium and apparatus, since up to 12 different organisms can be tested on one 4-inch petri dish. The technique would need slight modification for use with organisms that tend to spread over the surface of the plate as for example *Proteus vulgaris.*

The extension of the methods for use with narrower ranges of bacteriostatic agent dilutions also shows the same correlation between the 2 methods in cases of 2 of the agents used, phenol and roxenol, where a range as close as 1 part in 5000 was used. The results with A.10.39, the formulated quaternary ammonium compound, however, were not so precise, a range of 1 part in 1000 being necessary to decide between growth and no growth, and in this case the solid dilution method shows growth in more concentrated mixtures than with the liquid dilution method. Two possible reasons can be suggested to account for this difference. If the quaternary compound exerts its bacteriostatic effect by a mechanism differing from that of the phenolic compounds, then it would be expected that the distribution of the resistances of the bacteria to these two mechanisms would be different and account for the scatter of the results with A.10.39. A second possible explanation is that the media used contain an antagonist to the quaternary ammonium compound and this antagonist is not uniformly distributed throughout the media and the solid medium contains more than the liquid medium.

Ps. pyocyanea has shown itself much more resistant to the bacteriostatics tested than all the other organisms, with the exception in the case of phenol. This resistance makes the evaluation of the activity against *Ps. pyocyanea* by the fluid method impossible with many of the formulated antibacterial substances and therefore the solid dilution method is selected as the technique for use.

In comparing the counting method with the dilution methods only 0·14 per cent. phenol against *Ps. pyocyanea*, 0·08 per cent. roxenol against *Bact. coli*, and 0·12 per cent. roxenol against *Shig. dysentariæ* can be said to be bacteriostatic. In all other cases the dilutions which were bacteriostatic by the dilution methods are bactericidal when tested by the counting method.

The suspensions of the 3 organisms in distilled water and quarter strength Ringer solution were relatively stable over the 50-hours period, in fact the latter permitted a small amount of growth.

Table VI shows that with 0·06 per cent. of A.10.39 against *Bact. coli* and 0·08 per cent. against *Shig. dysenteriæ* the rate of kill is much quicker in water and Ringer's solution than in broth, this lends weight to the assumption that the broth contains some substances which is antagonistic to the antibacterial action of A.10.39. This fact together with the bactericidal effects shown by the other substances supports Price's[6] hypothesis that there is no true bacteriostasis and Parkinson's definition of dynamic bacteriostasis. The differences in rate of kill shown by the counting methods results supports the theory that the different antibacterial substances act in different ways even against different organisms and that

the various methods used for evaluating are in all probability measuring different reactions. To clarify this situation some method should be evolved, for the counting technique, of ensuring that the effect of the bacteriostatic agent is completely suppressed in the dilutions taken from the reaction mixture before the actual count is made. This has not been done in the above work but is under consideration.

SUMMARY

1. Three methods of measuring bacteriostasis have been examined; dilution of the bacteriostatic agent with a liquid culture medium, incorporation of the agent in a solid medium, and a counting method.

2. A fair degree of correlation has been shown to exist between the first two methods.

3. The counting method has given support to Price's[6] hypothesis that there is no real difference between bacteriostatic and bactericidal actions.

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