RESEARCH PAPERS

A STERILITY TEST FOR NEOARSPHENAMINE B.P. AND SULPHARSPHENAMINE B.P. FOR INJECTION

BY G. SYKES, A. ROYCE and W. B. HUGO*

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

with an Addendum on

THE SURVIVAL OF BACTERIAL SPORES IN ARSPHENAMINES

BY C. E. COULTHARD and B. H. CHANTRILL

From the Bacteriology Division, Boots Pure Drug Co., Ltd., Nottingham

Received March 17, 1952

THE British Pharmacopœia 1948 includes a large number of monographs on preparations for injection required to comply with tests for sterility, but this requirement has not so far been applied to neoarsphenamine or sulpharsphenamine used in making the respective injections. Neoarsphenamine and sulpharsphenamine are arsenobenzene derivatives which in most aqueous solutions are rapidly oxidised to the corresponding highly toxic arsenoxides, and it is probably on this account, and also on account of suggestions that the arsphenamines are self-sterilising, that sterility tests have been omitted. That these arsphenamines are not self-sterilising towards pathogenic bacteria is shown by Coulthard and Chantrill in the Addendum to this paper.

It is recognised that there are many difficulties in preparing batches of any compound used for injection which cannot be sterilised in its finished state. But, by applying appropriate aseptic methods during processing, bacterial infections can be adequately eliminated. Such methods are already used with a number of preparations, and they can be applied equally effectively to the arsphenamine derivatives. It is the purpose of this paper to present details of a method of testing for sterility which has proved satisfactory in detecting bacteria in various preparations of arsphenamine derivatives.

Any substance, toxic or potentially toxic to bacteria, can be tested for sterility provided it is adequately diluted in the test, or a suitable inactivating agent is included. The former method becomes impracticable, however, if dilutions of the preparation as great as 1 in 1000 or more have to be used. In the case of neoarsphenamine and sulpharsphenamine, the inhibitive concentrations to many of the commoner bacteria range between about 1 in 10,000 and 1 in 100,000 in nutrient broth, therefore, the alternative procedure of employing a suitable inactivating or neutralising agent is necessary.

Considerable work has been reported on the mode of action of arsenical drugs on living cells. As early as 1909, Ehrlich[1] theorised that these

* Present address: Department of Pharmacy, University of Nottingham.

compounds owed their toxicity to their affinity for thiol groups. Their mode of action against trypanosomes was studied by Voegtlin and colleagues in a series of papers published between 1920 and 1925. This work, summarised by Voegtlin,[2] showed that arsenobenzene derivatives were oxidised to the corresponding arsenoxides which then combined with essential thiol groups in biological systems, such compounds as glutathione (Rosenthal and Voegtlin[3]) or fixed thiol groups of proteins (Rosenthal[4]) being suggested. It also showed that arsenoxide derivatives were the biologically active ones and, furthermore, that their anti-trypanocidal activity could be removed by thiol compounds. The protective action of these compounds was confirmed by Eagle[5,6] and by Albert, Falk and Rubbo[7] in their respective studies on spirochætes and on trypanosomes and bacteria. This idea was again applied by Peters, Stocken and Thomson[8] when developing British anti-Lewisite (dimercaprol). Latterly, a large number of enzymes containing thiol groups have been described by Singer,[9] Gordon and Quastel[10] and Barron[11] the last of whom reviewed the importance of thiol compounds in biological processes generally.

It can thus be seen that arsenic interferes with some essential enzyme system containing a thiol group and that the action can be counteracted by adding to the system compounds themselves containing this group. Many such compounds have been used with greater or less success in reversing the toxic effects of arsenicals, typical examples being cysteine, thioglycollic acid, thiolactic acid, glutathione and dimercaptopropanol, and it was suggested by one of us (W. B. H.) that one of these compounds might be used in testing arsphenamines for sterility by incorporating it in the normal culture medium.

Experimental

As a result of work carried out many years ago on the sterility testing of arsphenamines considerable success was obtained in mitigating the toxicity developed, and so reducing inhibition of bacterial growth, by adding heat-coagulated muscle to the culture medium. This medium was, therefore, used in all subsequent routine batch testing. In such a meat medium thiol compounds occur naturally in variable amounts, and this could explain, at least in part, the results obtained. However, a more sensitive and reliable medium was required, and, taking into account other requisites for sterility test culture media, such as stability, ability to support growth of small inocula, availability and cost, a nutrient broth incorporating sodium thioglycollate appeared most promising. If used in sufficient concentration, this substance is inhibitory to bacterial growth, but experiments with a wide variety of organisms of the *Staphylococcus*, *Streptococcus*, *Bacillus*, *Bacterium* and *Clostridium* genera, showed that retardation of growth was apparent only in concentrations above 0·5 per cent.; hence 0·4 per cent. sodium thioglycollate was considered the maximum concentration which could be added.

In view of past experiences, two types of medium were examined: (*a*) a tryptic digest broth containing 0·4 per cent. of sodium thioglycollate,

and (*b*) the same medium with about a 1 cm. layer of heat-coagulated muscle added. They were filled in 50 ml. amounts into 4 oz. and 2 oz. screw-capped bottles, the former (with an air space above the medium) being designated for tests for ærobic bacteria and the latter for anærobic ones. The anærobic meat medium was always steamed for 30 minutes and cooled immediately before use. Although the oxidation-reduction potential of these media might appear to be held at too low a level for adequate ærobic growth, nevertheless, *Bacillus subtilis* grew very satisfactorily in both. Moreover, the anærobic organism, *Clostridium sporogenes*, grew luxuriantly in the meat medium (as might be expected), but sometimes failed to grow in the plain thioglycollate medium. The one disadvantage with this medium, namely, its susceptibility to oxidation if stored in plugged tubes, was readily overcome by preparing it in screw-capped bottles, a large air space being left in the ærobic test media. Under these conditions storage losses up to one month were negligible, but there was always a slight loss on sterilising in the autoclave usually representing about 0·05 per cent. in thioglycollic acid concentration. In all experiments reported here, freshly prepared media were used.

Sterile neoarsphenamine B.P. and sterile sulpharsphenamine B.P. (Boots Pure Drug Co., Ltd.) were used at final dilutions in the media of 1 in 100 or 1 in 200, these concentrations being found to be the most convenient in relation to the volumes of media employed and the size of dose in ampoules usually to be tested. The dilutions were made in each test in triplicate by first preparing a concentrated solution in distilled water and transferring appropriate amounts to bottles of medium as quickly as possible. These were then sown with 1 ml. of a highly attenuated dilution of a broth culture of the chosen test organism—in many cases less than 20 viable cells being inoculated per bottle, as shown by replicate plate counts. The test organisms chosen for detailed investigation were *Staphylococcus aureus* (F.D.A. strain), *Bacterium coli* (N.C.T.C. 86), *Bacterium prodigiosum*, a strain of *Bacillus subtilis*, and *Clostridium sporogenes* (N.C.T.C. 533), but similar responses were obtained with other unidentified species including a strictly ærobic spore-former and a strictly anærobic spore-former. All bottles, including controls without arsphenamines, were incubated at 37° C. for 10 days and plate counts were usually made daily; in the case of the anærobes, serial 10-fold dilutions were made in broth and the extinction point obtained.

Counts from typical experiments with neoarsphenamine at concentrations of 1 in 100 and 1 in 200 in thioglycollate broth and thioglycollate meat broth are given in Table I and illustrated in Figures 1 and 2. Results obtained with sulpharsphenamine (Table II) were not significantly different from those with neoarsphenamine. Control tubes without arsphenamine showed vigorous growths without exception within 48 hours, and at least 1×10^6 viable cells per ml. were present after 10 days incubation. It will be seen that, in general, growths from the very small inocula were initiated more readily and reached rather higher levels of viable cells in the weaker concentration of the arsphenamines. The

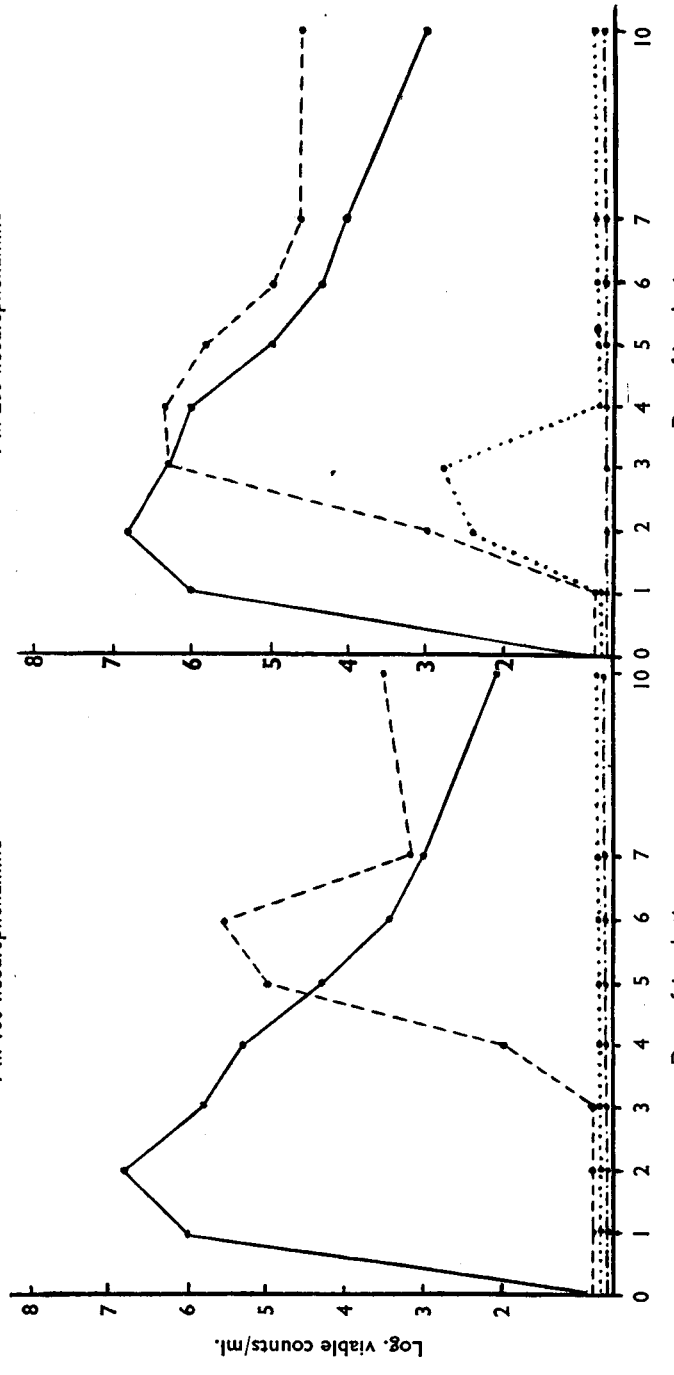

FIG. 1. Growth of bacteria with neoarsphenamine in 0·4 per cent. thioglycollate broth.

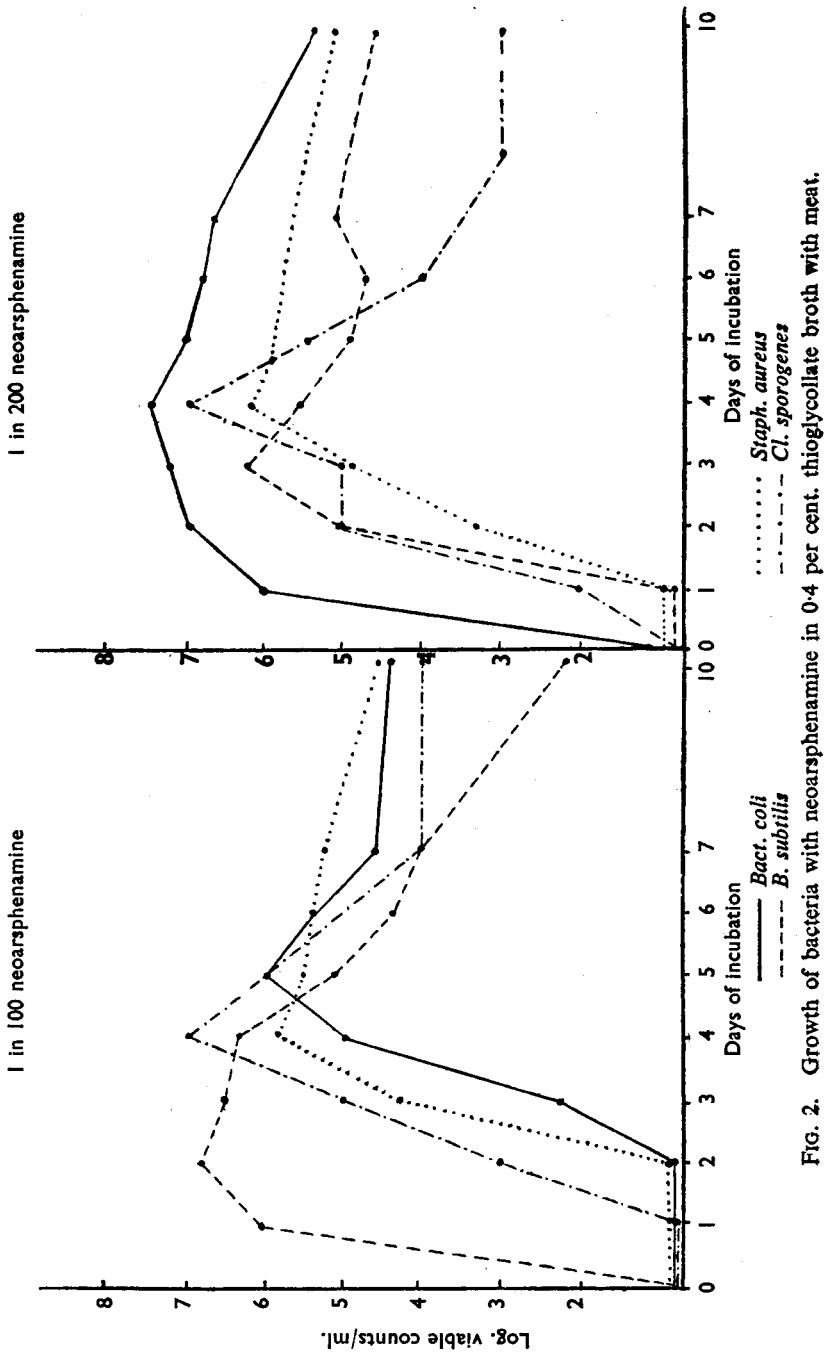

FIG. 2. Growth of bacteria with neoarsphenamine in 0·4 per cent. thioglycollate broth with meat.

TABLE I

GROWTHS OF BACTERIA IN STERILITY TEST MEDIA WITH NEOARSPHENAMINE B.P.

Concentration	Medium	Organism	Inoculum (organisms per 50-ml. bottle)	Logarithm of viable counts obtained per ml. after (days):— 1	2	3	4	5	7	10
1 in 100	Broth with 0·4 per cent. of sodium thioglycollate	*B. subtilis*	15	<2	<2	<2	2	5	3·2	3·6
		Staph. aureus	10	<2	<2	<2	<2	<2	<2	<2
		Bact. coli	8	6	6·7	6·2	5·6	4·1	2·6	2
		Bact. prodigiosum	10	6	7	6·3	5·8	4·5	2·5	<2
		Cl. sporogenes	26	<2	<2	<2	<2	<2	<2	<2
	Broth with 0·4 per cent. of sodium thioglycollate and heat-coagulated muscle	*B. subtilis*	15	<2	<2	2·5	5	6	4·3	4·3
		Staph. aureus	3	<2	<2	4·5	5·4	5·6	5·5	4·9
		Bact. coli	8	6	6·4	6·6	6·3	5·3	4·5	2·5
		Bact. prodigiosum	10	6	6·9	8	6·8	5·6	4·8	3·1
		Cl. sporogenes	20	<2	3	5	7	5	4	4
1 in 200	Broth with 0·4 per cent. of sodium thioglycollate	*B. subtilis*	15	<2	3·8	6·5	6·4	5·8	5·0	4·7
		Staph. aureus	10	<2	2·5	2·8	<2	<2	<2	<2
		Bact. coli	8	6	6·9	6·5	6·1	5·3	4·1	2·3
		Bact. prodigiosum	10	6	7	7·6	7·8	6·8	6	5·7
		Cl. sporogenes	26	<2	<2	<2	<2	<2	<2	<2
	Broth with 0·4 per cent. of sodium thioglycollate and heat-coagulated muscle	*B. subtilis*	15	<2	3·7	6·3	5·9	5·9	5·3	4·7
		Staph. aureus	3	<2	3	5	6·1	5·7	5·4	5·3
		Bact. coli	8	6	6·9	7·3	7·3	7·3	7·1	5
		Bact. prodigiosum	10	6	8	7·7	7·7	7	6	6
		Cl. sporogenes	20	2	5	5	7	4	3	<2

TABLE II

GROWTHS OF BACTERIA IN STERILITY TEST MEDIA WITH SULPHARSPHENAMINE B.P.

Concentration	Medium	Organism	Inoculum (organisms per 50-ml. bottle)	Logarithms of viable counts obtained per ml. after (days):— 1	2	3	4	5	7	10
1 in 100	Broth with 0·4 per cent. of sodium thioglycollate	*B. subtilis*	15	<2	4·6	6	5·4	5·4	5·1	2·6
		Staph. aureus	10	<2	<2	4	4·2	6·1	5·6	4·8
		Bact. coli	8	6	6·3	5	5	4	2	<2
		Bact. prodigiosum	13			5·7	7	5·6	2·3	<2
		Cl. sporogenes	26	<2	<2	<2	<2	<2	<2	<2
	Broth with 0·4 per cent. of sodium thioglycollate and heat-coagulated muscle	*B. subtilis*	15	<2	5·3	6	5·7	5·5	5·4	4·7
		Staph. aureus	3	2·3	5	6	5·7	5·3	<2	<2
		Bact. coli	8	6	6·3	5·8	5	4	<2	<2
		Bact. prodigiosum	13			6·7	7·5	7·7	6·6	5·2
		Cl. sporogenes	20	2	5	6	6	5	<2	<2
1 in 200	Broth with 0·4 per cent. of sodium thioglycollate	*B. subtilis*	15	<2	6·3	6	6·3	6	5·7	5
		Staph. aureus	10	<2	<2	5·3	7·4	6·2	5·8	4
		Bact. coli	8	6	6·8	6·8	5·5	3·5	<2	<2
		Bact. prodigiosum	13			6·7	6·9	7·6	6·9	5·2
		Cl. sporogenes	26	<2	<2	<2	<2	<2	<2	<2
	Broth with 0·4 per cent. of sodium thioglycollate and heat-coagulated muscle	*B. subtilis*	15	3·8	6·3	6	5·6	5·5	5	4·7
		Staph. aureus	3	2·3	4·3	6·7	6	5·5	4·9	5
		Bact. coli	8	6	6·9	7·3	7·3	6·5	4·7	<2
		Bact. prodigiosum	13			6·7	7·8	7·9	7·3	7·3
		Cl. sporogenes	20	2	7	7	7	5	4	4

Gram-negative organisms appeared to develop more easily than the Gram-positive ones. Maximum growths occurred between about the second and fourth days and beyond this period there was a decline in the number of viable cells. This decline was rather sharp with the higher concentration of arsphenamine. Nevertheless, easily detectable levels were present after 5 days incubation (the usual period for official tests for sterility), particularly when the meat medium was employed. Plain thioglycollate broth, whilst supporting growths of organisms in most experiments, failed to produce satisfactory growths of *Staph. aureus* in

the presence of either 1 in 100 or 1 in 200 neoarsphenamine, but was fairly good with sulpharsphenamine; it also yielded only delayed growths of *B. subtilis*. The foregoing results show that it is feasible to use a thioglycollate meat broth in testing for both ærobic and anærobic organisms. The tests could be carried out separately, the ærobic test in a container with a large volume of air above the medium, and the anærobic one in a filled bottle, steamed immediately before use; alternatively, they might be carried out simultaneously in the same container.

A Suggested Sterility Test Procedure

The following is an outline of a method for testing neoarsphenamine B.P. and sulpharsphenamine B.P. for sterility. It has been also applied to arsphenamine diglucoside B.P. and occasionally to other arsphenamine preparations.

The medium consists of a nutrient broth, made by the tryptic digest of heart muscle, with the addition of 0·4 per cent. of sodium thioglycollate (or the equivalent amount of the acid neutralised with sodium hydroxide) and a 1-cm. layer of heat-coagulated muscle. It is dispensed and sterilised in screw-capped containers, and should be relatively freshly prepared. The required amount of the test sample of the arsphenamine is dissolved in a sufficient quantity of this medium to give a final concentration not exceeding 1 in 200. The containers are incubated at 37° C. for 5 days. During incubation the medium will have become turbid, and subcultures are necessary to confirm presence or absence of growth. One loopful is inoculated into a fresh tube of the thioglycollate broth (ærobic test) or an agar-shake tube (anærobic test) and these are incubated for a further 24 to 48 hours at 37° C.

This medium has been applied successfully for many months in the detection of bacteria in routine tests for sterility of the arsphenamines.

The authors wish to express their thanks to Mr. D. V. Carter for his work on the stability of the thioglycollate media, and to Mr. C. Bowler for many hours of patient work at the bench.

Summary

1. A method is described for testing neoarsphenamine and sulpharsphenamine for sterility using a medium containing sodium thioglycollate and heat-coagulated muscle.

2. By this method very small inocula of a variety of bacteria can be detected.

References

1. Ehrlich, *Ber. dtsch. chem. Ges.*, 1909, **42**, 17.
2. Voegtlin, *Physiol. Rev.*, 1925, **5**, 63.
3. Rosenthal and Voegtlin, *J. Pharmacol.*, 1930, **39**, 347.
4. Rosenthal, *Pub. Hlth. Repts. Wash.*, 1932, **47**, 241.
5. Eagle, *J. Pharmacol.*, 1938, **64**, 364.
6. Eagle, *ibid.*, 1939, **66**, 436.
7. Albert, Falk and Rubbo, *Nature, Lond.*, 1944, **153**, 712.
8. Peters, Stocken and Thomson, *ibid.*, 1945, **156**, 616.
9. Singer, *Brewers Digest*, 1945, **20**, 85.
10. Gordon and Quastel, *Biochem. J.*, 1948, **42**, 337.
11. Barron, *Advances in Enzymology*, 1951, **11**, 201.