51. On the Chemical Reactions Occurring between Certain Substances which Inhibit Bacterial Growth and the Constituents of Bacteriological Media

By A. E. Oxford, *From the Division of Biochemistry, London School of Hygiene and Tropical Medicine*

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Before testing a substance for its bacteriostatic or growth-inhibiting power it is advantageous to find out if it reacts chemically with any constituent of the medium, at the temperature of incubation during the period of time allowed for the growth of the bacterium. If, for example, the substance reacts even in part with a constituent or constituents of the medium, say during the first day of incubation, to produce a compound or compounds which are less powerful growth inhibitors, then the results obtained after 2 days' incubation will not give a true picture of its bacteriostatic power. If the inhibitor is not a powerful germicide, an indication that such a reaction has taken place may be afforded by a rapid diminution of the apparent bacteriostatic power as the period of incubation is prolonged, but a surer proof that the substance undergoing test is being chemically destroyed will be provided by a preliminary incubation of the sterile medium containing the disinfectant for a day or two before sowing. An identical medium is then made up and sown immediately, and if the compound appears to be much less active in the medium which has undergone the preliminary incubation at the same temperature, it has clearly been chemically altered during this process. The above experiment will however not detect a chemical alteration leading to the production of substances which are as active as, or more active than, the substance being tested, but if the medium has undergone a marked colour change, or no longer responds, after the preliminary incubation, to chemical tests designed to reveal the presence of the original substance, it may reasonably be inferred that such a chemical alteration has taken place.

Compounds which appear to be chemically altered by the constituents of complex bacteriological media ought therefore to be tested in simpler media containing if possible a simple carbohydrate as sole source of carbon, and the NH_4 or NO_3 ion as sole source of nitrogen. Even then it is first necessary to show that the compound is stable at the pH of the medium. Not many bacterial species will grow well on such simple synthetic media, but certain strains of *Pseudomonas* and of *Bacillus* do show good growth at 25° and are valuable test organisms in this connexion. If, for example, it is desired to find out whether or not an antibacterial substance is chemically altered by a certain amino-acid at pH 7 at 37° , the amino-acid (in solution at pH 7) and the disinfectant are added to the simple synthetic medium and the resulting solution incubated unsown at 37° for a given time; then cooled to 25° and inoculated with the *Pseudomonas* together with suitable controls which have not undergone a preliminary incubation. If after incubation for 1 or 2 days at 25° no growth is observed in the controls containing disinfectant, but growth is observed in the corresponding tubes which have undergone a preliminary incubation at 37° , it may be inferred that the amino-acid and the disinfectant have reacted together at this temperature with partial or complete destruction of the antibacterial power of the latter. The fact that the temperature at which the *Pseudomonas* grows (25°) is considerably lower than the temperature of the preliminary incubation (37°) is an advantage, since a slow reaction at 37° will be very much slower at 25° , and if the greater part of the compound escaped unchanged from the 37° treatment it is not likely to be destroyed at 25° however long the incubation is continued at the lower temperature.

The above considerations were suggested by the results obtained in the testing of certain mould products and their derivatives, all containing the methoxyl group, for anti-bacterial action. The compounds in question included the mould product penicillic acid (I; for preparation and structural formula see Birkinshaw, Oxford & Raistrick [1936], and for preliminary testing of its anti-bacterial power see Oxford [1942a]), and 4-methoxytoluquinone (II), a substance related in structure to the mould pigment fumigatin [III; Anslow & Raistrick, 1938]. The relatively considerable anti-bacterial power of these quinones has already been reported on by Oxford & Raistrick [1942] and by Oxford [1942b]. In each instance the bacteriostatic power appeared to fall off very considerably after the first day of incubation and the work here reported is an attempt to discover the cause of this falling off.

I

II

III

EXPERIMENTAL

- (1) *Evidence that the falling off in bacteriostatic power of penicillic acid and 4-methoxytoluquinone in heart broth media on continued incubation is due to a chemical reaction between the disinfectants and constituents of the media*

The two media hitherto used by Oxford [1942 a, b] for the testing of these substances were the following:

- (a) standard heart broth at pH 7.2 containing 0.5% NaCl and 1% peptone (B.D.H. for bacteriological purposes);
 (b) as (a) but containing 2% glucose in addition which was sterilized with the other constituents. This medium was certainly browner in colour than (a) and its pH was a little lower (6.8).

The diminution in bacteriostatic power after the first day of incubation is illustrated in Table 1.

Table 1. *Bacteriostatic actions of penicillic acid and 4-methoxytoluquinone against various Staph. aureus strains at 37°*

Organism (N.C.T.C. No.) and compound	Medium (a)			Medium (b)					
	Limiting dilution for complete inhibition of growth			Limiting dilution for complete inhibition of growth			Limiting dilution for visible partial inhibition of growth		
	1 day	2 days	3 days	1 day	2 days	3 days	1 day	2 days	3 days
No. 3093 against penicillic acid	—	—	—	33,000	<20,000	—	50,000	33,000	—
No. 3750 against penicillic acid	—	—	—	33,000	<20,000	—	50,000	25,000	—
No. 3095 against 4-methoxytoluquinone	—	—	—	300,000	150,000	100,000	—	—	—
No. 3750 against 4-methoxytoluquinone	150,000	100,000	<50,000	—	—	—	—	—	—

The method of testing has already been described by Oxford [1942a]. Briefly, 1 ml. of sterile aqueous penicillic acid or 4-methoxytoluquinone was added to 4 ml. of sterile

medium, the dilutions given in the table being those of the disinfectant in the above mixture. To each tube was added one loopful of a 1000-fold diluted 1-day culture of the organism in the medium. If an undiluted culture was used for sowing the diminution in anti-bacterial power was even more striking. Similar results were obtained when penicillic acid was tested against *Bact. coli*.

The 'preliminary incubation' technique, outlined above, was applied thus. Glucose broth tubes containing 83 μ g. of penicillic acid per ml. were incubated unsown for 5 days at 37°, then inoculated in the usual way with a diluted culture of *Staph. aureus* no. 3750 and reincubated. Good growth occurred in 20 hr., whereas in a control containing 40 μ g. per ml., which was inoculated immediately after the addition of the penicillic acid, no growth occurred in 20 hr. at 37°. More than half, very probably nearly all, of the penicillic acid had therefore been destroyed in the first instance in 5 days at 37°. The experiment was repeated using both broth (medium (a)) and glucose broth (medium (b)) and limiting the duration of the preliminary incubation of the unsown media to 18 hr. at 37°. In the broth tube a considerable part of the penicillic acid had obviously been destroyed, since, after inoculation with *Staph. aureus*, good growth was obtained in 1 day in a tube which originally contained 83 μ g. per ml. The result with the glucose broth medium was less striking in that a tube containing originally 83 μ g. per ml. showed no growth whereas a tube containing originally 50 μ g. per ml. did show growth. This result has a direct bearing on the puzzling result previously noted by Oxford [1942a] viz. that penicillic acid is much more effective in glucose broth than in plain broth. A possible explanation, arising out of the results just described, is that penicillic acid is chemically decomposed much faster in medium (a) than in medium (b). The following experiment, summarized in Table 2, shows clearly that, as far as the action of penicillic acid is con-

Table 2. *Penicillic acid against Bact. coli*

A 1000-fold diluted culture was used for sowing. Each tube contained originally 4 ml. of basal medium. Sterile water added last of all to make volume up to 5 ml. if necessary.

Basal medium	Penicillic acid added, ml. of 0.025% aq. solution	Final conc. of penicillic acid μ g. per ml.	Growth at 37°		
			20 hr.	26 hr.	2 days
Broth (medium (a); 4 ml.)	2	83	0	0	0
	1	50	0	0	+
	0.5	25	+	+	+
	0.4	20	+	+	+
	0.3	15	+	+	+
	0.2	10	+	+	+
	0	0	+	+	+
Broth (4 ml.) plus 20% aq. glucose (0.5 ml.) sterilized separately	2	80	0	0	0
	1	45	0	0	+
	0.5	25	+	+	+
	0.4	20	+	+	+
	0.3	15	+	+	+
	0.2	10	+	+	+
	0	0	+	+	+
2% glucose broth sterilized together (medium (b); 4 ml.)	1	50	0	0	0
	0.5	25	0	0	+
	0.4	20	0	0	+
	0.3	15	0	0	+
	0.2	10	0	+	+
	0	0	+	+	+
	0	0	+	+	+

cerned, glucose broth autoclaved with the glucose in it is a very different medium from broth to which is added the same amount of glucose sterilized separately in strong aqueous solution; in point of fact, plain broth and glucose broth made in the second way are equivalent for testing penicillic acid. The experiment merely confirms the well-known fact that when glucose is sterilized with broth at pH 7 deep-seated chemical changes

occur. These changes evidently destroy certain compounds which otherwise would react with penicillic acid to the detriment of its anti-bacterial powers.

The chemical destruction of 4-methoxytoluquinone in broth at 37° was demonstrated likewise: a tube containing 10 µg. of the quinone per ml. was incubated unsown for 2 days at 37°, then sown with a 1000-fold diluted culture of *Staph. aureus* no. 3761 in plain broth. At the same time an exactly similar tube was put up and sown immediately. After 18 hr. incubation at 37° there was growth only in the tube which had previously been incubated unsown.

(2) Unimportance of the pH effect

By 'pH effect' is meant the hydrolytic decomposition of penicillic acid and 4-methoxytoluquinone, accompanied by loss of anti-bacterial power, occurring at pH 7.2 in any medium incubated at 37°. For the evaluation of this effect it was necessary to work with an organism which will grow well on synthetic media. Two strains of the plant pathogen *Pseudomonas mors-prunorum* Wormald [Wormald, 1932], both recent isolations, were obtained through the generosity of Mrs D. H. Oxford to whom I desire to express my sincere thanks. Biochemically the strains were practically indistinguishable. *Ps. mors-prunorum* will grow well at 25° (but not at 37°) and can be maintained indefinitely on the following synthetic media both of pH 7.2.

Medium (c). Czapek medium of the following composition:

NaNO ₃	2 g.	FeSO ₄ .7H ₂ O	0.01 g.
K ₂ HPO ₄	1 g.	Sucrose or glycerol	20 g.
KCl	0.5 g.	Distilled water	1000 ml.
MgSO ₄ .7H ₂ O	0.5 g.		

In this medium the only source of nitrogen is NO₃ and the only source of carbon is a simple carbohydrate or sugar alcohol which suffers no change on autoclaving at pH 7.2.

Medium (d). A modified form of the microcosmic salt medium of Birkinshaw, Charles & Clutterbuck [1931] of the following composition:

NaNH ₄ HPO ₄ .4H ₂ O	4.9 g.	FeSO ₄ .7H ₂ O	0.01 g.
K ₂ HPO ₄	1.0 g.	Sucrose or glycerol	20 g.
KCl	0.5 g.	Distilled water	1000 ml.
MgSO ₄ .7H ₂ O	0.5 g.		

The carbon source is the same as in medium (c) but the sole source of nitrogen is NH₄.

Varying amounts of penicillic acid, dissolved in 1 ml. of sterile water, were added to 4 ml. quantities of the synthetic media, and the tubes were incubated unsown for 5 days at 37°; then cooled, inoculated with a loopful of a 2- or 3-day culture of *Ps. mors-prunorum* on Czapek medium, and reincubated at 25°. Only the tubes containing 10 µg. of penicillic acid per ml. showed growth in 3 days, whereas those containing 30 µg. and 50 µg. per ml. showed no growth. In the corresponding control tubes which had received no preliminary incubation there was sometimes no growth in tubes containing 10 µg. per ml.; otherwise the results were the same. Although penicillic acid is therefore not absolutely stable at pH 7.2 at 37° it must be concluded that its hydrolytic decomposition is far too slow to account for its rapid rate of destruction in broth at this pH. These results also show that, sensitive though penicillic acid may be towards free ammonia (see Birkinshaw *et al.* [1936]), it is nevertheless stable enough to ammonium salts at pH 7.2 as in medium (d).

The corresponding experiment with 4-methoxytoluquinone was carried out only on medium (d). It was found that the limiting dilution of the quinone for complete suppression of the growth of *Ps. mors-prunorum* for 7 days at 25° was 1 in 20,000 irrespective of whether the medium containing the quinone had been incubated for 3 days at 37° before sowing. Further, there was not the slightest darkening in colour during the preliminary incubation.

(3) *Proof that peptone reacts with penicillic acid and with 4-methoxytoluquinone at 37° at pH 7.2 with destruction of anti-bacterial power*

The observation of Fleming [1940] that peptone added to media containing sulphanilamide inhibits the bacteriostatic action of the latter suggested that the peptone (1%) present in the broth media (a) and (b) was responsible for the effects described in Section 1. In the ordinary way *Ps. mors-prunorum* is completely inhibited for 1 day in 1% peptone water (containing also 0.5% of NaCl and adjusted to pH 7.2) when penicillic acid is present at a concentration of 30 µg. per ml., and for 5 days when the concentration of the acid is 83 µg. per ml. If however unsown 1% peptone water containing 83 µg. of penicillic acid per ml. is incubated for 3 days at 37°, the resulting medium will not inhibit the growth of the organism for even 1 day at 25°. The corresponding experiment with 4-methoxytoluquinone yielded much the same result. 1% peptone water tubes containing 100 µg. of the quinone per ml. were incubated unsown for 3 days at 37°, with the result that the medium, almost colourless at the start, became dark brown. After sowing with *Ps. mors-prunorum* it showed good growth after 1 day's incubation at 25°. Similar tubes put up and sown immediately showed no growth in 1 day at 25°. It may be concluded therefore that the presence of 1% of peptone in the broth media (a) and (b) will amply account for their power of destroying penicillic acid and 4-methoxytoluquinone.

(4) *Chemical reactions occurring between various amines and amino-acids and the disinfectants at 37° in dilute solution at pH 7*

Peptone water probably contains many simple amines and amino-acids which may be responsible for its effect on penicillic acid and methoxyquinones. To obtain further information on this point the following experiment was set up. Approximately *M/4* aqueous solutions of the following were sterilized by bringing to the boiling point: methylamine hydrochloride, dimethylamine hydrochloride, diethylamine hydrochloride, trimethylamine hydrochloride, glycine, *dl*-alanine and Na glutamate. A corresponding number of sets of three tubes each were then put up, each tube containing 4 ml. of sterile sucrose-microcosmic salt medium (d): tube 1, +0.5 ml. sterile H₂O + 0.5 ml. of 0.025% sterile aqueous penicillic acid or 0.05% sterile aqueous 4-methoxytoluquinone. Tube 2, +0.5 ml. of sterile amine solution + 0.5 ml. of sterile aqueous penicillic acid or 4-methoxytoluquinone as above. Tube 3, +0.5 ml. sterile H₂O + 0.5 ml. of sterile amine solution. All were incubated unsown for 3 days at 37°; then cooled, inoculated in the usual way with *Ps. mors-prunorum* and further incubated for 2 or 3 days at 25°. At the time of the sowing of these tubes exactly similar sets were put up, sown immediately and incubated at 25° with the others.

Results. All the controls (tube 3) naturally grew. In the sets which had not had the preliminary incubation, tubes 1 and 2 showed no growth as expected. They contained 25 µg. of penicillic acid or 50 µg. of the quinone per ml. In the quinone sets which had undergone the preliminary incubation there was likewise no growth in tubes 1 and 2, although certain tubes, particularly those containing methylamine hydrochloride and the quinone or glycine and the quinone, had greatly darkened in colour. In the penicillic acid sets there was growth in tube 2 only in those instances in which methylamine hydrochloride and the acid, and glycine and the acid had been heated together at 37° before sowing. Tube 1 always showed no growth as expected. The alanine set was put up again, given 4 days' preliminary incubation at 37°, and inoculated etc. as before. This time there was growth in tube 2 after 2 days' incubation at 25°.

It seems clear therefore that penicillic acid, in very dilute solution, does react chemically with certain simple primary amines and amino-acids when the latter are present in excess at a concentration of *M/40*, under the conditions obtaining in bacteriological media, viz. at pH 7, and at a reaction temperature of 37° prolonged for several days. This reaction

results in a sensible diminution in bacteriostatic activity. 4-Methoxytoluquinone also reacts with amines and amino-acids under the same conditions with production of more highly coloured compounds, but in this case the reaction products are at least as highly bacteriostatic as the parent quinone. The cause of the diminution in bacteriostatic power when 4-methoxytoluquinone is incubated with sterile peptone water therefore still remains to be discovered.

(5) *Chemical reactions occurring between penicillic acid, and 4-methoxytoluquinone and the amino-acid p-aminobenzoic acid in dilute solution at pH 7*

This is a particularly interesting case since *p*-aminobenzoic acid is known to be an essential growth factor for certain bacteria and is also known to be 'antagonistic' to sulphanilamide. A 3.2% (0.23 *M*) sterile, neutral solution of *p*-aminobenzoic acid was made as follows: the acid (8 g. B.D.H.) was dissolved in 60 ml. of sterile *N* NaOH, 150 ml. of sterile water added, and the whole heated to 80°. After cooling, the volume was made up to 250 ml. with sterile water. When this solution (0.5 ml.) together with sterile water (0.5 ml.) was mixed with 4 ml. of sterile sucrose-microcosmic salt medium (medium (*d*)) the resulting medium was at pH 7.0 but unfortunately would not support growth of *Ps. mors-prunorum* at 25° even when a large inoculum was given. Evidently at a concentration of 0.3% the acid has a powerful anti-bacterial action as might be expected since it is a derivative of aniline. A laboratory strain of an organism of the *Bacillus* group resembling *B. megatherium* did however grow on the above medium containing *p*-aminobenzoic acid at 37° but unfortunately not at 25°. The experiment set up to investigate the action of *p*-aminobenzoic acid on penicillic acid and the quinone was therefore the following. Tube 1, 0.5 ml. of sterile H₂O + 0.5 ml. of sterile 0.05% penicillic acid or 4-methoxytoluquinone added to 4 ml. of sterile medium (*d*). Tube 2, as tube 1 but 0.5 ml. of sterile 3.2% *p*-aminobenzoic acid in place of the sterile water. Tube 3, as tube 1 but 0.5 ml. of sterile 3.2% *p*-aminobenzoic acid in place of the solution of penicillic acid or quinone. All were incubated unsown for 3 days at 37°; then each was inoculated with one loopful of a 2-day culture of the above *Bacillus* species on medium (*d*). At the same time an identical set of tubes was put up and inoculated immediately. All were further incubated at 37° for 1 day. Tube 1 showed no growth in each instance; tube 2 showed growth in both of the *p*-aminobenzoic acid + penicillic acid cultures, but not in the *p*-aminobenzoic acid + quinone cultures, and tube 3 showed growth in all cases. The tube containing *p*-aminobenzoic acid and quinone which had undergone the preliminary incubation was also dark brown in colour at the end of 3 days at 37°. *p*-Aminobenzoic acid will therefore 'antagonize' the action of penicillic acid but there can be little doubt that in this instance the effect is due to a rapid chemical reaction between the two acids at 37° and not to any competition between them for a bacterial enzyme system. The behaviour of *p*-aminobenzoic acid towards penicillic acid is in fact exactly analogous to the behaviour of glycine previously discussed, but the chemical reaction is evidently more rapid in the first-named case.

SUMMARY

The bacteriostatic powers of penicillic acid, and of 4-methoxytoluquinone, are very considerably diminished by incubation with sterile broth, or with sterile 1% peptone water at pH 7 for 1-3 days at 37°. In the case of penicillic acid a similar effect is observed with certain simple amines and amino-acids such as methylamine, glycine, alanine and *p*-aminobenzoic acid. These compounds also react chemically with 4-methoxytoluquinone under the same conditions with production of highly coloured derivatives which are not less active as anti-bacterial agents than the parent quinone. When heart broth containing peptone is autoclaved with 2% glucose at pH 7.2, the concentration of substances which react chemically with penicillic acid in the above sense is considerably diminished.

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