THE STABILITY OF PENICILLIN SOLUTIONS AT NORMAL AND HIGHER TEMPERATURES

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Information published during the past five years on the stability of penicillin in aqueous solutions has shown that it is dependent on such factors as the purity of the penicillin salt used, the pH value of the solutions, the presence of buffering agents and the temperatures at which the solution is held. Progress in the manufacture of penicillin from the earlier relatively crude material to the pure crystalline salts has sometimes led to apparently contradictory statements by different workers concerning stability.

Thus, several authors have drawn attention to differences in stabilities of solutions of crystalline and of less pure penicillins, but not all have agreed that one is more stable than the other (Pratt[1], Halliwell[2], Macek, Hanus and Feller[3], Benedict, Schmidt, Coghill and Oleson[4]). Smith[5] stated that crude preparations often contained substances which diminished activity as well as others which enhanced it. Both he and Denston[6] observed differences according to the degree of purification of the penicillin used, and this probably accounts for the diverse opinions expressed by others. Smith also found some compounds capable of abolishing the inactivating effect, the most suitable of which was sodium hexametaphosphate, and this also enhanced considerably the stability of pure penicillin in solution. The stabilising influence of phosphate buffer was reported about the same time by Denston[6] and by Pulvertaft and Yudkin[7] and this has been confirmed by others in several subsequent papers. Although phosphate buffers have been employed by most workers, citrates have also been used with equal effect (Macek *et. al.*[3], Smith[5], Paul, Gaillot and Baget[8]), the present tendency being to favour citrate buffer instead of phosphate.

Since it had been shown that unbuffered solutions at pH 5·3 were much less stable than at pH 6·6 (Johnson and Lerrigo[9]), and that they became more acid during inactivation (pH values according to Macek *et. al.*[3] and Büchi and Gundersen[10] falling from 6 to 6·5 to about 4), the stabilising influence of the buffer solution was thought to be due simply to the prevention of the development of acidity. However, Pulvertaft and Yudkin[7], Pratt[11] and others observed that optimum amounts of buffer could be added according to the purity of the penicillin. These findings led to a revised opinion of the function of the buffer to include that of protection of the penicillin molecule as well as control of development of acidity. This opinion was further substantiated by work involving the heat stability of penicillin solutions.

Although it is now accepted that pure penicillin can retain its potency for several days at normal room temperatures and for much longer periods in the refrigerator, opinions are not in entire agreement on stabilities at higher temperatures. Denston[6] was able to boil a solution of pure penicillin in water containing 20,000 I.U./ml. with practically no loss after 5 minutes and only 27 per cent. loss after 30 minutes. She found on the other hand that high potency penicillin (1,400 I.U./mg.) lost about 20 per cent. of its activity after 5 minutes boiling and was practically inactive after 10 minutes. In contrast to this, Johnson and Lerrigo[9], working with solutions varying between 25,000 and 100,000 I.U./ml., were able to demonstrate only a small loss with a yellow amorphous penicillin after 5 minutes boiling, but substantial losses with purer materials. They attributed the apparent contradiction with Mrs. Denston to differences in quality of the penicillins used, and thought that her material might have carried buffer substances, since on adding buffer to their pure material they obtained only comparatively small losses. Pulvertaft and Yudkin[7] had earlier demonstrated the protective effect of phosphate buffer (*p*H 7·0), obtaining losses from solutions containing 5, 500 and 5,000 I.U./ml. of 5 to 50 per cent. in water, and only 3 to 15 per cent. in buffer, after boiling for 10 minutes. The amount of loss was dependent on the concentration and quality of the penicillin used. Similarly Watts and McLeod[12] had found losses of 50 per cent. and 75 per cent. in activity after boiling solutions for 30 minutes and 60 minutes respectively. Whilst agreeing that phosphate conferred a considerable degree of protection to penicillin solutions on heating, Hahn[13] recorded an even greater degree of protection by citrate buffer with saline in optimum concentration, there being practically no loss in potency after boiling for 1 hour. Hunter[14], on the other hand, working with very dilute solutions of penicillin containing only 4 I.U./ml., was unable to demonstrate the protective action of phosphate buffer, but found that milk exerted a considerable effect, losses of 98 to 100 per cent. in water and in buffer being reduced in milk to 45 per cent. after steaming for 1 hour and to 55 per cent. after autoclaving at 10 lb. steam pressure for 20 minutes.

In contrast to the work which has been reported on the heat stability of penicillin solutions, very little has been published in relation to the effect of bacteriostatics. Denston[6] examined the effect of phenol, chlorocresol, chlorbutol, phenoxetol and merthiolate and found that, in the refrigerator, phenol, chlorocresol and phenoxetol did not exert an adverse influence, but that at room temperature only phenol was without effect; solutions with chlorocresol tended to deposit on storage. Johnson and Lerrigo[9] obtained some evidence of improved stability with chlorocresol, but agreed that it readily gave rise to turbidity and was therefore unsuitable for use in penicillin solutions. They reported indications that phenol (0·5 per cent.) might confer greater stability on aqueous solutions of both crystalline and yellow amorphous penicillin, but also noted the development of a slight turbidity after seven days storage. These workers also examined the effect of phenoxetol and " Nipa " preservatives. Propyl *p*-hydroxybenzoate retarded the loss of potency (attributed to the high *p*H value of this substance) and the remainder of the solutions were without effect. Several of the solutions deposited crystals after a few days or became coloured. In view of these useful, but not unanimous, observations on the stabilising influence of certain compounds and also of the practical importance of being able to prepare solutions of penicillin which are stable to moderate heat, further investigations have been carried out on this aspect.

EXPERIMENTAL

Stability of Solutions to Storage and Heat. In the course of an investigation into various properties of penicillin solutions, we had occasion to use soil extracts, and these were found to exert a marked stabilising effect on the penicillin when heated for short periods or when kept at normal temperatures. This property was not confined to one particular soil, since 6 different soils all behaved in the same way. The soil extracts were prepared by autoclaving 1,000 g. lots in 3 l. of tap water for 1 hr. at 15 lb. steam pressure, and using the decanted supernatant liquid. They had normal *p*H values of 7·0 to 7·4, which fell to 6·8 when penicillin (3,000 I.U./ml.) was added; they possessed virtually no buffering power and exhibited no antibacterial properties. Solutions of yellow amorphous penicillin (sodium salt) of potency approximately 1,500 I.U./mg. and of the crystalline salt were made aseptically to a concentration of 3,000 I.U./ml. in these extracts and similar control solutions were made in distilled water and in phosphate buffer (*p*H 6·8). The potencies of these solutions were compared at intervals during storage at 25°C. with results as given in Table I. They illustrate the relative instability on storage of

TABLE I

STABILITY OF PENICILLIN SOLUTIONS AT 25°C.
PENICILLIN (SODIUM SALT) USED AT 3,000 I.U./ML

Period of Storage Days	Percentage Loss of Potency from Solutions					
	Distilled Water		Phosphate Buffer (*p*H 6·8)		Soil Extract	
	Crystalline	Amorphous	Crystalline	Amorphous	Crystalline	Amorphous
7	36	40	8	0	0	0
17	97	81	19	12	19	3
24	100	100	25	53	30	9
13	100	100	46	37	69	13

solutions in water and the marked stabilising effect of the soil extract and of phosphate buffer. In agreement with other workers already quoted, there is also some suggestion of the greater stability of impure penicillin.

Solutions in the same vehicles of crystalline potassium and crystalline and amorphous sodium salts of penicillin were also treated for various times at 60°C. and 80°C. Twelve groups of experiments were made (Table II) and in nearly every case the solutions in soil extract were more stable than those in phosphate buffer. The average losses of potency after heating for 12 hrs. at 60°C. were 23 per cent. (range 21 to 29 per cent.) in soil extract, and 54 per cent. in phosphate buffer; after heating for half an hour at 80°C. they were 34 per cent. (range 3 to 48 per cent.) and 51 per cent. (range 44 to 58 per cent.) respectively. Solutions in dis-

TABLE II

STABILITY OF PENICILLIN SOLUTIONS TOWARDS HEAT
PENICILLIN (POTASSIUM SALT) USED AT 3,000 I.U./ML.

Vehicle	Mean Percentage Loss in Potency after Heating			
	2 Hours at 60° C.	12 Hours at 60° C.	24 Hours at 60° C.	30 Mins. at 80° C.
Soil extract	5	23	27	34
Phosphate buffer	—	54	—	51
Distilled water	91	100	100	100

tilled water under the same conditions showed complete destruction of activity. It was observed that the stabilised solutions could be held for another 48 hrs. at 37°C. before any appreciable deterioration took place. In this group of experiments there was little to choose between the various penicillin salts used.

On occasion, the *p*H value of the solutions in soil extract fell below 5·0 during heating for reasons apparently associated with the glass container used, and this resulted in complete loss of activity. In order to prevent this occasional change in *p*H value, tests were carried out using equal mixtures of soil extract and buffer solutions. In every experiment the degree of stabilisation was of the same order as that of soil extract alone, and was superior to that of the buffer alone.

In a partial examination of the properties of this stabilising soil factor, samples of soil were extracted under acid and alkaline conditions (*p*H 2·5 and 10 respectively), the extract being subsequently neutralised. Normal soil extracts were also subjected to dialysis through cellophane against distilled water for 48 hrs. Three groups of experiments were carried out with penicillin solutions made from these extracts, the mean losses in potency after heating at 80°C. for 20 minutes being :—

Solution in normal soil extract (control) ... ... 17 per cent.
Solution in fraction dialysing through cellophane ... 25 „ „
Solution in fraction not dialysing through cellophane ... 13 „ „
Solution in acid soil extract ... ... 91 „ „
Solution in alkaline soil extract ... ... 24 „ „

The foregoing experimental results support the earlier findings of Johnson and Lerrigo[9] concerning the instability to heat of solutions of comparatively pure penicillin. The results also indicate, as suggested by the variations between tests under apparently identical conditions, that the mechanism of protection by both phosphate buffer and soil has not a simple explanation. It may be that the factors concerned are identical.

Stability with Bacteriostatics to Storage and Heat. Experiments have been carried out in these laboratories from time to time on the stability of penicillin solutions with a variety of bacteriostatics. Most of them were eliminated immediately either on account of inactivation of the penicillin, e.g., cresol, chlorbutol, phenoxetol and "Nipa" products, or of incompatibility at higher concentrations of penicillin, e.g., chlorocresol (cf. Denston[6], and Johnson and Lerrigo[9]). Only phenol (0·5 per cent.) and phenylmercuric nitrate (0·002 per cent.) appeared to justify any further detailed consideration, although Johnson and Lerrigo had already commented unfavourably on phenol on account of its tendency to produce slight turbidity after some days storage. Precipitation in the form of long needle crystals (phenyl phenaceturate) from citrate buffer solutions in a few days at room temperature and within 24 hrs. at 37°C. has also been reported by Kern, Terrill, Mann and Jones[15]. In our experience, solutions of penicillin containing up to 100,000 I.U./ml. with 0·5 per cent. of phenol remained clear in the refrigerator and at normal room temperature for over 4 weeks, with the exception of those of the amorphous sodium salt; these tended to become slightly turbid after 9 days or more at room temperature but remained clear in the refrigerator. At 37°C., solutions of all salts containing 30,000 I.U./ml. remained clear, but those containing 100,000 I.U./ml. in citrate buffer (0·1 M, *p*H 6·8), but not in water, developed a crystalline deposit in 3 days.

Potency tests have not always given the same stability responses, but in general they have confirmed the finding of earlier workers, already cited, that phenol did not adversely affect, and often enhanced, the stability of penicillin solutions. Table III gives the averaged results from

TABLE III

STABILITY OF PENICILLIN SOLUTIONS WITH PHENOL
INITIAL POTENCY OF SOLUTIONS 30,000 I.U./ML.

Penicillin Salt	Bacteriostatic	Vehicle	Percentage Loss in Potency After Storage											
			Room Temperature Days						4° C. Weeks					
			1	4	7	14	21	28	1	2	3	4	6	8
Sodium (crystalline)	Phenol	Water	0	0	5	15	20	35	0	5	10	15	25	40
	Nil	Water	7	20	40	75	90	100	15	—	15	15	20	20
Sodium (amorphous)	Phenol	Water	0	10	20	60	100	—	—	—	—	—	—	—
	,,	Citrate	7	20	30	35	35	40	12	20	20	25	30	30
	Nil	Water	2	10	30	100	—	—	20	25	30	30	40	45
Potassium (crystalline)	Phenol	Water	0	7	20	75	100	—	5	10	15	20	45	75
	,,	Citrate	2	10	20	25	30	35	20	25	25	25	25	25
	Nil	Water	15	40	90	100	—	—	30	60	70	75	85	95

several groups of experiments using 3 types of penicillin, all with an initial potency adjusted to 30,000 I.U./ml. These experiments did not show any significant differences between the responses of the types of penicillin, in spite of the comparative instability of the particular potassium salt used, but the effect was generally more marked at room temperature than in the refrigerator (4°C.).

Phenylmercuric nitrate behaved in a similar manner to phenol, although the responses were more variable. In some experiments the stabilising effect was most marked, for example, in one test the half-life of a solution of the potassium salt containing 3,500 I.U./ml. was extended from

2 days in water to 14 days with phenylmercuric nitrate added, and in another containing 30,000 I.U./ml. the half-life was extended from 4 days to 28 days. It is of some significance to note that solutions containing phenylmercuric nitrate or phenol with buffer tended to lose some 10 to 20 per cent. of their original potency fairly quickly, during the first 2 or 3 days, after which the rate of loss decreased considerably.

Phenol was also found to exert a protective influence, comparable with that already described with soil extract, towards penicillin solutions on heating to 80°C. On the other hand, phenylmercuric nitrate was completely devoid of such action, but it did not inhibit stabilisation by buffer solutions or soil extract. Typical results of such experiments, in which solutions of penicillin containing 3,000 I.U./ml. were heated at 80°C. for 20 minutes are given in Table IV. The stabilities of the solutions to further storage after heating with phenol were not appreciably affected.

TABLE IV

LOSS IN POTENCY OF PENICILLIN SOLUTIONS ON HEATING AT 80°C. FOR 20 MINUTES WITH BACTERIOSTATICS

INITIAL POTENCY OF SOLUTION 3,000 I.U./ML.

Bacteriostatic	Percentage Loss in Vehicle								
	Distilled Water			Phosphate Buffer pH 6·8			Phosphate Buffer + Soil Extract		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Phenol (ice cryst.) ...	11	11	0	3	7	9	14	8	17
Phenol (detached cryst.) ...	—	—	0	—	—	9	—	—	0
Phenylmercuric nitrate ...	100	—	—	17	—	—	0	—	—
Nil (control)	62	100	96	22	6	7	4	12	0

a, *b*, *c* = results from three separate tests.

DISCUSSION

There have been numerous references in the literature to the stabilising influence of buffer solutions on penicillin, and the use of these compounds in obtaining relatively heat-stable and sterile penicillin solutions has been advocated (Denston[6], Pulvertaft and Yudkin[7]). We have now shown that aqueous soil extract can fulfil the same function and may be even more effective as a stabilising agent. It has not been possible as yet to carry out any appreciable experimental work to investigate the fraction of the soil extract responsible for exerting this influence.

We have also confirmed the opinion expressed by Johnson and Lerrigo[9] that phenol does not adversely affect the stability of penicillin solution and may even enhance it. The enhancement was very definite when solutions were heated to 80°C., and again, as with soil extract, losses in activity were sometimes reduced to very small proportions. The results of these experiments may offer some contribution to the present need for a technique for dispensing sterile and stable aqueous penicillin solutions for injection. The results obtained with soil extract, which is virtually devoid of buffering power, and with phenol give considerable support to the concept, first put forward by Pratt[1], of the role of a stabiliser being one of protecting the penicillin molecule as well as controlling the *p*H value of the solution. This applied particularly to solutions subjected to heat. The mechanism of this protection has not been explained, but it evidently warrants further investigation.

SUMMARY

1. Earlier observations on the relative instability of solutions of the crystalline penicillins to heat have been confirmed, together with the stabilising influence of buffer solutions.

2. Aqueous soil extracts have been found to exert an even greater stabilising effect than phosphate buffer both at normal temperatures and at 80°C.

3. Phenol can also exert a high degree of stabilisation on storage at normal temperatures and towards heat at 80°C.

4. Phenylmercuric nitrate does not adversely affect the stability of penicillin solutions.

5. The practical significance of these stabilising agents is discussed.

We wish to record our thanks to Miss V. M. L. Howell and to Mrs. H. M. Payne for carrying out the many hundreds of assays involved in this work.

NOTE. Since this paper was written, Carr and Wing (*Pharm. J.*, 1951, **167,** 63) have published work confirming earlier opinions on the stabilising influence at normal temperatures of phenol and of sodium citrate.

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DISCUSSION

The paper was presented by MR. G. SYKES.

The CHAIRMAN inquired whether the authors had any idea as to the nature of the factor responsible and whether any soil extract would contain it. The statement that phenoxetol was incompatible with penicillin in solution was very interesting, and he wished to know whether the penicillin was pure or impure. Preliminary experiments which he had carried out with impure penicillin in the presence of phenoxetol had indicated that there was no inactivation.

MR. R. HOBBS (Greenford) considered that citrate buffers were superior in general to phosphate buffers and asked whether there was any reason for excluding citrate buffers from the comparisons, particularly in view of the fact that in most commercial preparations containing penicillin citrate buffers were used. In connection with the loss in potency when fractionated soil extracts were employed at 80°C. he noted that the fractions which had not been dialysed through cellophane retained their potency to an extent which was 12 per cent. greater than that of the fractions which had been so treated. Did that imply that the factor was either colloidal or sub-colloidal?

MRS. H. B. WILLIAMS (Greenford) asked whether the authors had experimented with concentrations such as 500,000 or 250,000 units per ml. which were in practical use. She had recently carried out some penicillin stability work at concentrations as high as 500,000 units per ml. using citrate buffers, in the presence of phenol. She noted, in agreement with the authors, that there was a 10 to 20 per cent. loss of potency in the first 2 to 3 days, but thereafter, in contrast to the authors' observations, the rate of loss increased considerably.

MR. A. G. FISHBURN (Manchester) said that, when solutions of crystalline penicillin were examined over a large range of concentrations in the presence of a citrate buffer, there was a marked break at 200,000 units per ml. The conventional sodium citrate buffer strength of 4·5 per cent. was effective up to concentrations of 200,000 units per ml. but above that ceased to have the same effect, and when concentrations of 500,000 units per ml. were reached, the stability appeared to be little different from that of unbuffered penicillin.

MR. C. J. EASTLAND (London) said that he was surprised that the addition of 2·5 per cent. of phenoxetol to an oily suspension of penicillin resulted in a 35 per cent. loss of activity in a matter of 5 hours. He had found that a solution of amorphous calcium penicillin (1,200 units per mg.) containing 2 per cent. of phenoxetol and kept under the same conditions as the oily suspension lost no activity in 24 hours. Possibly the purity of the phenoxetol might be involved. He asked whether it was possible that the soil extract factor was a heat-stable antibiotic which prevented the destruction of penicillin by influencing micro-organisms producing penicillinase.

MR. WILLIAMS (Cardiff) refuted the suggestion that phenoxetol might be impure and thought that the results might be explained by impurities in the penicillin rather than in the phenoxetol.

MR. SYKES, in reply, said that the type of soil did not appear to matter. The incompatibility of phenoxetol with penicillin was first announced by Mrs. Denston. In his own experiments the penicillin used was relatively impure (1,200 units per mg.) and the phenoxetol was as supplied. Concerning the relationship between penicillin concentration and stability, a few tests only had been done up to 300,000 units per ml.; but such little information as had been obtained indicated that concentration had little effect. The soil extract had no antibacterial effect.