THE ASSAY OF PENICILLIN USING PENICILLINASE

BY A. ROYCE, C. BOWLER and G. SYKES

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

Received July 1, 1952

THE hydrolysis of the penicillins by penicillinase to penicilloic acid is a specific enzyme reaction. The acid produced is easily titratable with strong alkali, but so far no simple method has been described for regular and routine estimations by this means. As a result of recent investigations, we have now developed a technique which, in our hands, gives results in a much shorter time and is more accurate and more economical than the normal ring-plate or serial dilution methods of assay. It is also at least as accurate and more easily carried out than the chemical methods of assay in the fields where these are practicable.

The principle of estimating penicillin by treating with penicillinase and then titrating potentiometrically the penicilloic acid produced was first adopted by Murtaugh and Levy in 1945.[1] Later Henry and Housewright[2] used the same principle for estimating both penicillin and penicillinase activities but employed a manometric procedure with a Warburg apparatus. The potentiometric and manometric methods were also used by Wise and Twigg,[3] and by Pollock[4] for investigating some of the fundamental properties of penicillinase, but none appears to have considered a simple alkalimetric method using indicators, presumably because they were concerned largely with the dynamics of the reaction. We have also used these methods, but find that for quantitative estimations the indicator method gives equally satisfactory results.

Among the requirements for the successful operation of this method are (*a*) the penicillin-penicillinase reaction must be specific, (*b*) the penicillinase must be reasonably stable, (*c*) it must be highly active, and (*d*) the results must be reproducible.

PROPERTIES OF PENICILLINASE

The penicillin-penicillinase reaction. Henry and Housewright[2] examined the action of penicillinase prepared from *Bacillus cereus* on many substances of penicillin-like structure and on various degradation products of penicillin, and in no case did they record any reaction. They also calculated the molecular weights of the penicillins, on the assumption that one titratable carboxyl group was produced from each molecule of penicillin attacked. The value for benzylpenicillin (G) and *n*-heptylpenicillin (K) agreed closely with those already found and accepted, but those for the Δ^2-pentenyl- (F) and *p*-hydroxybenzyl-(X) compounds differed considerably. The discrepancies were thought to be due to the limited number of experiments which could be performed on the very small amount of material available. However, we have recently obtained some confirmation of this discrepancy with a sample of Δ^2-pentenylpenicillin. In our hands this had a potency of 1056 I.U./mg. by

the cylinder-plate assay, but only 550 I.U./mg. by the penicillinase method.

Since the penicillin now produced is practically all benzyl-penicillin, these discrepancies have virtually no significance in normal assays, but such considerations may well have detracted from earlier attempts to adapt the penicillinase method for routine use. Another factor of possibly greater significance may have been the low activity of the penicillinase then available.

Penicillinase is produced by a large number of organisms, mainly of the Gram-negative non-sporing or the Gram-positive aerobic sporing types, but many preparations from these are quite unstable. We have perhaps been fortunate in that our work has been carried out with a strain of *Bacillus subtilis*, which yields a very stable penicillinase. The strain was originally obtained from the National Collection of Type Cultures, but has since been subjected to careful selection towards greater penicillinase production. Highly potent material is obtained by methods based on the continued addition of penicillin to the growing culture, as advocated by Duthie.[5] Batches are obtained regularly of which 1 ml. is capable of inactivating 200,000 to 400,000 I.U. of penicillin per hour at *p*H 7·5 and normal room temperature. This potency is adequate for normal purposes but it can be further increased, if desired, by adsorption and elution methods. The material is very stable and does not lose more than 10 per cent. of its activity on storage for periods of 3 to 6 months at either 4° C. or at room temperature.

Rate of inactivation and effect of temperature and pH value. Provided there is excess of substrate (penicillin) present, the rate of reaction at any given *p*H and temperature remains constant and is dependent only on the enzyme (penicillinase) concentration. When the substrate is nearing exhaustion there is a retarding of the rate of reaction proportional to the concentration of the residual substrate at any moment. Pollock[6] has found with two penicillinase preparations that the enzyme continues to function at its maximum activity (at *p*H 7·2 and 30° C.) at penicillin concentration down to about 100 I.U./ml. Under our conditions of assay (see later), in which the reaction is allowed to proceed with relatively high concentrations of substrate and enzyme for 30 minutes, the process appears to go practically to completion. If the solution is back titrated continuously during the process of inactivation, the reaction continues at a uniform rate, then rapidly slows down and finally ceases abruptly. Beyond this point no further acid production is detectable. This is illustrated in Table I in which the course of inactivation of 50 mg. of benzylpenicillin (sodium salt) with 1 ml. of penicillinase was followed by titrating the acid produced during consecutive periods of 3 minutes each.

TABLE I

RATE OF ACID PRODUCTION DURING INACTIVATION OF PENICILLIN

Period (minutes)	Titration 0·01N sodium hydroxide ml.
0 to 3	2·25
3 „ 6	2·35
6 „ 9	2·35
9 „ 12	2·35
12 „ 15	2·25
15 „ 18	1·45
18 „ 21	0
21 „ 24	0
24 „ 27	0
27 „ 30	0

TABLE II

RATE OF INACTIVATION OF PENICILLIN BY PENICILLINASE AT DIFFERENT TEMPERATURES

Temperature ° C.	Relative rate of inactivation
1 to 2	30
12 „ 13	60
24	100
36 „ 38	150
46 „ 47	180
55 „ 60	220
65	enzyme destroyed

(Rate at 24° arbitrarily taken as 100.)

The rate of inactivation also depends very largely on temperature, and a short examination was made of this relationship under otherwise constant conditions. Details are given in Table II, from which it is seen that activity increases regularly, but not steeply, with temperature up to the point at which the enzyme is destroyed. Although there is some advantage to be obtained by working at elevated temperatures the reaction proceeds quite satisfactorily at room temperature, and, since it is also more convenient, this was adopted for all subsequent tests.

Henry and Housewright[2] investigated the rate of reaction in relation to *p*H value and found that the optimum for a temperature of 36° C. was at *p*H 7·2. Wise and Twigg[3] obtained an optimum at 25° C. at *p*H 7·8. Using approximately 50 mg. amounts of benzylpenicillin (potassium salt) dissolved in 10 ml. of water with 1 ml. of penicillinase added, and keeping the *p*H value constant to within 0·1 unit we found that maximum activity at 22° to 25° C. occurred at *p*H 7·5. Table III illustrates the relative rates of reaction at different *p*H values, the rate at *p*H 7·5 being arbitrarily fixed at 100. It is notable that the rate declines more slowly on the alkaline side than on the acid side, and that alkaline hydrolysis becomes increasingly significant with increase in *p*H value beyond about 9·0. Considerable buffering of the reacting solution also occurs due to the penicilloic acid formed preventing the *p*H value from falling below about 5·5.

TABLE III

RATE OF INACTIVATION OF PENICILLIN BY PENICILLINASE AT DIFFERENT *p*H VALUES

*p*H value	5·0	5·5	6·0	6·5	7·0	7·5	8·0	8·5	9·0	9·5
Relative rate of inactivation	23	36	45	82	86	100	95	91	75	61

METHODS OF ASSAY OF PENICILLIN PREPARATIONS AND RESULTS

Penicillin salts. The following is a procedure which is particularly suitable for the assay of the salts of penicillin, but which, by adjusting the amount of sample, can be used with many of the pharmaceutical preparations of penicillin.

Adjust the penicillinase to *p*H 7·5 using phenol red as indicator. Prepare a colour control by mixing 1 ml. of this with 10 ml. of distilled water containing 0·2 ml. of phenol red indicator. Weigh accurately 50 mg. of the penicillin sample, dissolve in 10 ml. of water also containing 0·2 ml. of phenol red indicator, and adjust the *p*H value to match that of the control. Add 1 ml. of penicillinase and allow to stand at room temperature for 30 minutes. Titrate with 0·01N sodium hydroxide until the colour of the solution again matches that of the control. Allow to stand

for some minutes longer to ensure that the reaction is completed, and titrate further if necessary. Calculate the potency of the preparation on the basis that each 1 ml. of 0·01N sodium hydroxide is equivalent to 6023 I.U. of penicillin. This value is obtained from the facts that the British Working Standard of Penicillin (ASC 2) has an agreed potency of 1690 I.U./mg., and that each molecule of benzylpenicillin gives rise to one of penicilloic acid. It is very close to the values of 6015 and 6021 found experimentally.

It is obvious that the time taken to complete the inactivation will be influenced by the activity of the penicillinase, weaker preparations requiring longer inactivation periods. It is not advisable, however, to use material of considerably weaker activity as the reaction time then becomes inconveniently long and the results less accurate. The accuracy of the assay can be increased by using larger amounts of penicillin, but results of quite a high order of accuracy can be obtained from amounts as low as 5 or 6 mg.

To demonstrate the reliability of the method, assays were carried out on a large number of production batches of the different penicillin salts and compared with cylinder-plate and iodimetric results. In each case the normal routine procedures were used. The iodimetric assays, and some of the penicillinase assays, were carried out by a colleague, Mr. A. S. Middleton of our Chemical Division, Standards Department. Table IV shows the values obtained on several of these batches by the 3 methods and illustrates their general concordancy. Replicate penicillinase values are included to show the reproducibility of the method. Of the 28 batches of sodium, potassium and calcium salt examined, there is a mean difference between the penicillinase and cylinder plate results of less than 0·1 per cent., and between the penicillinase and iodimetric results of 0·9 per cent. With the procaine salt the mean differences are 3·2 per cent. and 1·7 per cent. respectively, both values being positive. This is a reflection of our experience that cylinder plate assays on procaine benzylpenicillin are frequently rather higher than theory. The penicillinase method has also been applied successfully to other compounds of penicillin which are difficult to assay by the iodimetric method.

Pharmaceutical preparations. Solution tablets and oral tablets are readily assayed by this method, although they may occasionally be formulated with buffer substances which are liable to impair the accuracy of the estimations. Since, however, they are usually of high potency the inaccuracies tend to be minimised. Determinations in lozenges present difficulties owing to the presence of only small quantities of penicillin in a large amount of base. However, difficulties have also been reported by other workers[7,8] using the plate assay due to the sugars in the preparations tending to yield abnormally high values. Assays on penicillin mixtures with insoluble substances, e.g., sulphonamide powders and snuffs, can be carried out on the mixture directly by simple suspension in water, but generally it is advisable to separate the insoluble matter and complete the assay on an aliquot of the clarified aqueous extract.

The various preparations of penicillin in oily bases, oil-wax suspension,

TABLE IV

COMPARATIVE ASSAYS OF PENICILLIN SALTS

Salt and batch number		Assay values (I.U./mg.)			Percentage difference between penicillinase and	
		Penicillinase	Cylinder plate	Iodimetric	Cylinder plate	Iodimetric
Sodium	786	1576:1575	1627		+3·2	
	780	1634:1629	1616		−1·0	
	2285	1648:1631	1624	1648	−1·0	+0·5
	2286	1642:1635	1604	1671	−2·1	+2·0
	2287	1646:1651	1565	1628	−5·0	−1·2
	10918	1569:1566	1603		+2·2	
			Mean difference, per cent.		−0·6	+0·4
Potassium	1144	1559	1579	1544	+1·3	−1·0
	1145	1576	1551	1584	−1·6	−0·8
	1146	1558	1583	1555	+1·6	−0·2
	1147	1578	1545	1553	−2·1	−1·6
	1148	1557	1518	1540	−2·5	−1·1
	1149	1547	1533	1547	−1·0	0·0
	1205	1549:1541	1520	1537	−1·6	−0·5
	1206	1571:1560	1529	1596	−2·0	+2·0
	1208	1568:1581	1563	1553	−0·8	−1·4
	1221	1570:1579	1592		+1·4	
	1222	(1590:1577) (1578:1579)	1530	1554	−3·2	−1·7
	1229	1603:1607	1599	1562	−0·4	−2·7
	1230	1607:1604	1616	1578	+0·7	−1·7
	1231	1602:1601	1627		+1·6	
	1468	1602:1602	1649	1555	+2·9	−2·9
	1469	1605:1611	1658	1547	+3·1	−3·8
			Mean difference, per cent.		−0·2	−1·2
Calcium	771	1477:1477	1557		+5·3	
	1309	1525:1519	1592		+4·6	
	1320	1533:1543	1512		−1·7	
	1347	1568:1552	1587		+1·7	
	1348	1546:1537	1501		−2·7	
	1387	1517:1532	1498		−1·7	
			Mean difference, per cent.		+0·7	
Procaine	1487	1031:1025	1048		+2·0	
	2231	1006:992	1037	1024	+3·7	+2·4
	2238	993:1005	1042	1025	+4·4	+2·7
	2239	983:1008	1075	1007	+7·5	+0·7
	2240	997:1017	1000	1025	−0·7	+1·8
	2241	1007:1007	1015	1021	+0·7	+1·4
			Mean difference, per cent.		+3·1	+1·7

ointments, etc., can also be assayed without prior extraction of the penicillin. For these preparations, a weighed quantity (depending on its potency) is suspended in water with indicator and an equal volume of chloroform or other suitable organic solvent is added. The mixture is shaken gently, adjusted to *p*H 7·5 and 1 ml. of penicillinase added. It must be titrated continuously during the inactivation period to keep the penicillin in the aqueous phase. If excess acidity is allowed to develop the free penicillin will migrate into the organic solvent phase, and thus retard the process of inactivation. The colour control in this case is the adjusted mixture of reagents without penicillinase. This procedure yields very satisfactory results when high potency oil-wax suspensions are assayed, but with ointments the accuracy is lowered owing to the initial low potency of the preparation. Penicillin fermentation liquors cannot be assayed directly by this method due to the buffering capacity and high

colour of the solution. It is the frequent practice to assay these liquors by chemical or physico-chemical methods, usually after extraction with an organic solvent; the penicillinase method can be introduced at this stage giving results of the same order of accuracy.

Summary

1. A simple routine method is described of carrying out accurate assays of penicillin and its preparations, by inactivating with penicillinase and titrating the acid formed. The method is readily applicable for use in pharmacies, hospital dispensaries or small laboratories.

2. The accuracy of the method depends on (*a*) the use of a potent penicillinase, (*b*) the absence of buffering substances, and (*c*) the amount of penicillin present to be estimated.

3. Comparative assays of the penicillinase with the cylinder plate and iodimetric methods are given.

References

1. Murtaugh and Levy, *J. Amer. chem. Soc.*, 1945, **67**, 1042.
2. Henry and Housewright, *J. biol. Chem.*, 1947, **167**, 559.
3. Wise and Twigg, *Analyst*, 1950, **75**, 106.
4. Pollock, *J. gen. Microbiol.*, 1951, **5**, ii.
5. Duthie, *ibid.*, 1947, **1**, 370.
6. Pollock, 1952, private communication.
7. Sykes, Lewis and Goshawk, *Proc. Soc. appl. Bact.*, 1951, **14**, 40.
8. Bond, *Analyst*, 1952, **77**, 118.

DISCUSSION

The paper was presented by MR. A. ROYCE.

MR. G. F. HALL (Nottingham) said that, unlike benzylpenicillin, for procaine benzylpenicillin the iodimetric method was not entirely satisfactory. In the U.S.A. the Federal Food and Drug Administration specified the bioassay with the iodimetric method as an alternative. The British Pharmacopœia used the biological assay. He himself had suggested an extraction method which did not find favour. A further suggestion in which the procaine was precipitated with silicotungstic acid had not been favourably received.

DR. K. BULLOCK (Manchester) asked whether the control was just a colour standard. Was there no non-enzymic hydrolysis?

MR. A. ROYCE, in reply, said that there were difficulties with the iodimetric method and also with the ring plate method. It was found in routine determinations of procaine penicillin that results tended to be a little too high. The control tube contained no substrate, only water and enzyme being present. The *p*H and the buffering capacity varied with different batches of penicillinase. When penicillin broke down some acid was produced, but none was produced within the time limit of the test. Although the reaction was stated to be over in 30 minutes in the paper, in practice in most cases the reaction was concluded in 10 minutes. There would be no change in that time in the colour of the indicator added to the solution, but if it were left a change was detected later in the day.

The CHAIRMAN remarked that no one had suggested that Mr. Royce should have a standard strain of *B. subtilis*.

MR. A. ROYCE replied that there was a great deal of literature on penicillinase and many organisms were used. The organism he used was originally a N.C.T.C. culture of *B. subtilis*, but it had been in the laboratory for a number of years and had been selectively cultured with a view to greater penicillinase production. It was to be doubted whether the same strain would be available in the N.C.T.C. at the present time. He would be happy to supply the culture to anyone interested.