REVIEW ARTICLE

THE ESTIMATION OF PENICILLINS AND PENICILLIN DESTRUCTION

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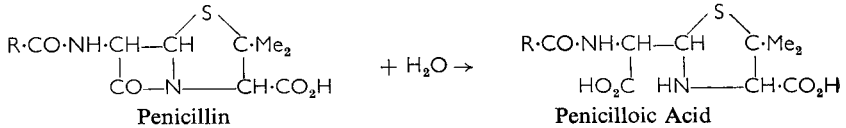
Research into the biological and chemical aspects of penicillin and its production has been actively pursued for almost 35 years; the announcement of the production of the penicillin "nucleus", 6-aminopenicillanic acid, on a commercially attractive scale (Batchelor, Doyle, Nayler and Rolinson, 1959) has given a tremendous stimulus to these studies in the past few years. With the increased range of penicillins now already available for clinical use, and the possibility of further compounds with new specificities in the future, there is a new need for a review of methods for estimating penicillins and penicillin-destroying enzymes. This review covers chemical, physical and microbiological methods and attempts to indicate which of these is most suitable for the varying needs of present day research in this field.

Penicillin-destroying enzymes

Penicillinase

An account of the distribution of these enzymes is given by Abraham (1951). The enzymes act by rupturing the β-lactam ring of penicillins, and perhaps should be more precisely called β-lactamases (Pollock, 1961), as shown in equation 1.



Penicillin — Penicilloic Acid

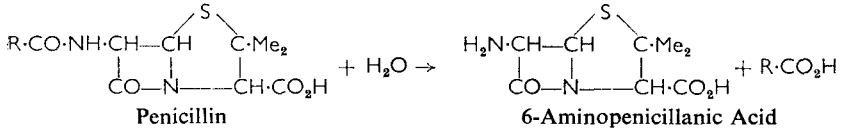
Equation 1

The antibacterial activity of the penicillin is for all practical purposes destroyed by this cleavage, which can also be brought about by alkali treatment. The nomenclature of the penicilloic acids, unlike that of the penicillins (Sheehan, Henery-Logan and Johnson, 1953) has not been strictly defined. "Penicilloic acid" is, strictly speaking, a general name and should be qualified, for example, "benzylpenicilloic acid", or "penicilloic acid G" derived from benzylpenicillin: however, the term has unfortunately been much used to refer specifically to the hydrolysis product of penicillin G. The use of trivial names, such as "methicilloic acid" from methicillin and "penicic acid" (Huang and others, 1960; Murao, 1955) from 6-aminopenicillanic acid, is hardly a satisfactory compromise. It would appear that the best solution to this problem of nomenclature of the penicilloic acids is to qualify the name of each individual compound by the full side-chain, for example, phenylacetamidopenicilloic acid from penicillin G, phenoxypropionamidopenicilloic

acid from phenethicillin, and aminopenicilloic acid from 6-aminopenicillanic acid.

Amidase

The properties and distribution of these enzymes, now of great commercial importance, are described by Batchelor and others (1959), Rolinson and others (1960), Claridge, Gourevitch and Lein (1960) and English, MacBride and Huang (1960). The latter authors call the enzymes "acylases". They act by removing the side-chain attached in the 6-amino-position of the penicillin nucleus.



Penicillin — 6-Aminopenicillanic Acid

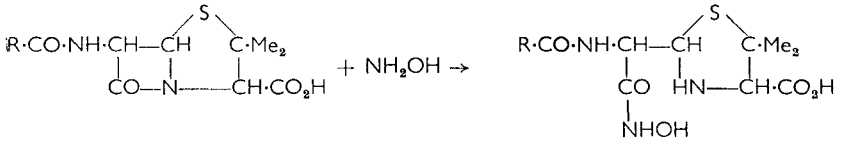
Equation 2

Unlike penicillinases, amidases catalyse both the forward and the backward reaction, and also do not completely destroy antibacterial potency, since the product of the reaction, 6-aminopenicillanic acid, also possesses some limited biological activity (Rolinson and Stevens, 1961).

Quantitative assays

Chemical

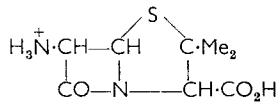
Hydroxylamine (Staab, Ragan and Binkley, 1946). This original method has been modified by Boxer and Everett (1949) and Ford (1947), amongst others. Hydroxylamine reacts with penicillins at pH 7·0 as shown by equation 3.



Equation 3

This reaction is complete within about 10 min. at room temperature and at pH values between 6 and 8. The product is a hydroxamic acid, stable for 2 hr., which forms a coloured internal salt with ferric ions, of unknown structure. This chromogen is stable for about 5 min., but can be further stabilised (Henstock, 1949) by extraction into n-butanol; its absorption is proportional to the concentration of penicillin, between 10–2,000 μg./ml., and can be measured colorimetrically at 500 mμ (Boxer and Everett, 1949; Batchelor, Chain, Hardy, Mansford and Rolinson, 1961). A spectrophotometer can be used, but this is inconvenient because of the short life of the chromogen, and because the method involves the mixing of alcoholic and aqueous solutions, and many gas bubbles are hence formed. The individual penicillins produce, mole for mole, differing colour intensities, and there does not seem to be any

simple relationship between chemical structure and chromogenicity of the hydroxamate-ferric complex. Hydroxylamine will react only with the molecular species having an intact β-lactam ring: hence during penicillinase assays, the substrate which remains rather than the product formed is being measured. Penicillin amidase activity cannot be measured directly by this assay, as the 6-aminopenicillanic acid produced by the hydrolysis still has an intact β-lactam ring. A method for the estimation of this enzyme is described by Batchelor, Chain and Rolinson (1961). At pH 2, 6-aminopenicillanic acid has a net positive charge,



while other penicillins are unionised, and exist as free acids which, being hydrophobic, are extractable into a suitable non-polar solvent, as for example, n-butyl acetate. The hydrophilic 6-aminopenicillanic acid remains in the aqueous phase, where it can be assayed by the hydroxylamine method.

Hydroxylamine does not react only with the penicillins. Hydroxamates are also formed with compounds containing a carbonyl group such as esters, when a purple chromogen is formed with the Fe^{3+}. For example, Lipmann and Tuttle (1945) describe the use of this assay for acyl phosphate determination at acid pH values. Despite this lack of specificity, however, the method is valuable for obtaining absolute rates of breakdown of penicillins, specificity patterns of different enzymes, or Michaelis constants (see Batchelor, Cameron-Wood, Chain and Rolinson, 1961; Knox and Smith, 1962, for examples). The chief disadvantage is the lack of sensitivity, as the lower limit for most penicillins is 20 μg./ml. even under the best conditions. This method has been adapted for automatic operation by Niedermayer, Russo-Alesi, Lendzian and Kelly (1960), for estimating up to 10,000 units/ml. (6 mg./ml.).

Iodometric (Alicino, 1946; Tucker, 1954; Perret, 1954). Penicilloic acids take up iodine; the stoichiometry of the reaction has not been worked out—it is known only that 8–9 atoms of iodine are taken up by each molecule of benzylpenicilloic acid. Penicillins which possess an unsaturated aliphatic side chain, such as penicillin O (allylmercaptomethyl) (Grove and Randall, 1955), and penicillin F (Δ^2-pentenyl) will reduce iodine while the β-lactam ring is intact; *p*-hydroxybenzylpenicillin also behaves in this way (Sneath and Collins, 1961). Iodine is absorbed by many other organic compounds, especially those that are unsaturated, and careful blank estimations must therefore be made. But as most unchanged penicillins do not reduce iodine, the amount of it taken up depends on the amount of penicilloic acid formed. Comparative assays using the hydroxylamine and iodometric methods give closely similar rates of hydrolysis by penicillinases (Boxer and Everett, 1949). The iodometric method cannot be used to follow hydrolysis by amidase, as 6-aminopenicillanic acid does not reduce iodine.

Perret's method estimates iodine uptake by sampling the reaction mixture into excess iodine, and back-titrating with standard thiosulphate. Citri (1958) uses a simplified version, in which the time taken for a standard amount of blue starch-iodine complex to be totally decolorized is used as a measure of reaction velocity. Weiss (1959) has reviewed the factors affecting the accuracy and sensitivity of the macro-iodometric assay.

Newer micro-iodometric assays, depending upon absorption by the I_3^- complex at 360 mμ (Goodall and Davies, 1961) and at 420 mμ (Ferrari, Russo-Alesi and Kelly, 1959), and on the absorption of the starch-iodine complex at 600 mμ, have been developed. The automatic method described by Goodall and Davies (1961), has been modified by Beecham Research Laboratories (Batchelor, F.R., personal communication). Novick (1962a,b) also used the starch-iodine complex as the chromogen, measuring absorption at 620 mμ with a spectrophotometer. He found that his micro-method gave 40 per cent lower activities for penicillinase than those obtained by the macro-method. The methods using the starch-iodine complex are very sensitive; about 1 μg./ml. of penicillin can be accurately estimated. Novick (1962a) has used his assay to determine the K_m of penicillin G for staphylococcal penicillinase, and it can obviously be extended to measure other K_m values for penicillins with especially high affinities for penicillinase.

Other methods. Penicilloic acids, unlike nearly all other organic compounds, will reduce arsenomolybdate at room temperature, in the presence of traces of mercuric chloride, to a chromogen "molybdenum blue" which is measurable colorimetrically (Pan, 1954). A semi-automatic modification has been described (Green and Monk, 1959).

Hiscox (1949) developed a method whereby the amount of ferricyanide reduced to ferrocyanide by penicillins is measured by back-titration with ceric sulphate. The assay can be used only for crystalline penicillins.

Scudi (1946) found that penicillins react with *N*-(1-naphthyl-4-azobenzene)ethylenediamine to form a red chromogen, which is measured at 540 mμ. The same author has also developed a very sensitive fluorimetric procedure (Scudi and Jellinek, 1946).

Acidimetric

When penicillins are hydrolysed by penicillinase a new carboxyl and a secondary amino-group are generated (equation 1). The latter, being weak, has little or no effect on the net production of hydrogen ions and hence the pH falls in proportion to the amount of hydrolysis. Benedict, Schmidt and Coghill (1945) found, by electrometric titration, that the pK of the newly-formed carboxyl group, in the case of benzylpenicillin, was 4·7 (compare the pK of the original carboxyl group, 2·16). Batchelor, Chain, Hardy, Mansford and Rolinson (1961) similarly found the pK of the penicilloic acid of 6-aminopenicillanic acid to be 3·7.

When the side chain is removed from a penicillin by hydrolysis with an amidase (equation 2), a new carboxyl and a primary amino-group are formed. Here the amino-group is sufficiently strong to absorb hydrogen

ions and hence the pH falls progressively more slowly as the reaction proceeds. Initial velocities can be taken by the usual method of drawing tangents, and hence reaction velocities, and Michaelis constants, could be calculated.

Comparison of rates of hydrolysis of different penicillins by penicillinase cannot be directly undertaken by these methods as different penicilloic acids are likely to have different pK values so that the fall in pH per molecule hydrolysed will differ for each penicillin.

The rate of production of hydrogen ions, and hence the rate of hydrolysis of penicillin, can be determined in the following ways.

Manometric assay (Foster, 1945). This method was standardised by Henry and Housewright (1947) and was modified further by Pollock (1952); it depends on the fact that the volume of CO_2 liberated from a bicarbonate buffer as penicillin is hydrolysed to penicilloic acid is proportional to the rate of hydrolysis. Manometric methods are time-consuming, especially where gassing is necessary; but many workers (Pollock, 1952; Pollock and Torriani, 1953; Steinman, 1961a, b; Crompton and others, 1962; Leitner and Cohen, 1962; and others) still use this assay.

Retention of either CO_2 or hydrogen ions will result in diminished gas evolution and thus cause misleading results. The results of Steinman (1961a) show that the hydrolysis of 6-aminopenicillanic acid by penicillinase cannot be estimated manometrically. Hydrolysis of 6-aminopenicillanic acid results in the formation of not only a carboxyl group but also of an α-amino group, and hence there is no net production of hydrogen ions. Furthermore Batchelor, Gazzard and Nayler (1961) showed that CO_2 reacts with 6-aminopenicillanic acid forming first a carbamate then a penillic acid.

The method is mainly used for the assay of penicillinases, although originally described also for penicillin assay. It appears that, at present, this assay method is in the process of being superseded by other methods.

Alkaline titrimetric methods. Patterson and Emery (1948) described a method for the estimation of benzylpenicillin in which the amount of standard alkali required to split the β-lactam ring was measured by back-titration with acid. Murtaugh and Levy (1945) used a pH stat for the same purpose, and Jeffrey, Abraham and Newton (1961) used this principle to follow the alkaline hydrolysis of cephalosporin C. The pH of the reaction mixture is kept constant by the automatic addition of sodium hydroxide solution, the rate of addition being proportional to the rate of hydrolysis. The latter method has also been used by Crompton and others (1962) to follow the course of alkaline hydrolysis of penicillin G, methicillin and deacetyl cephalosporin C.

Indicator method. The rate of change in pH is followed, in this method, by means of an indicator. The absorption of the alkaline form of the indicator at a certain wavelength will decrease as more penicilloic acid is formed. The indicator of choice is that whose pK coincides roughly with the pH at which the reaction is carried out (for example, phenol red pK = 7·8 at pH 7·6; bromothymol blue pK = 7·0 at pH 7·0; bromcresol

purple pK = 6·2 at pH 6·5, etc.). The method of Nirenberg (cited by Saz, Lowery and Jackson, 1961) measures the decrease in the red form of phenol red by determining the absorption at 558 mμ with a spectrophotometer. Work in this laboratory with bromothymol blue and bromcresol purple has shown the maximum absorption of the alkaline forms of these indicators to be at 645 mμ and 615 mμ respectively: the sensitivity of the method is shown by the fact that, in the presence of M/240 phosphate buffer and 0·0013 per cent (w/v) aqueous phenol red, a change in absorption of 0·001 unit (the smallest change that can be accurately detected) represents a change in pH of 0·0012 units. The absorption of the indicators changes in an almost linear fashion with pH over a reasonable range (for example between pH values of 7·8 and 7·1 for phenol red), and when following hydrolytic reactions, straight line progress plots are obtained. The method can also be used with a colorimeter to follow the reaction: this increases the flexibility of the assay.

Michaelis constants can be determined conveniently with this assay, but probably its greatest use is in detecting changes in reaction velocity (see Saz and others, 1961, for action of activators on staphylococcal penicillinase), particularly in relation to inhibition studies, which, up to now, have been studied mainly by the manometric method (Abraham and Newton, 1956). By virtue of its far greater convenience and increased ease of operation, it is possible that the indicator method may take the place of manometric methods in this respect.

Miscellaneous

Microbiological assay. The "Oxford cup method" was first described by Abraham and others (1941) and further developed by Heatley (1944). It has been modified by Schmidt and Moyer (1944), McKee, Rake and Menzel (1944), and Foster and Woodruff (1943b, 1944). The latter (1943a) also gave a carefully reasoned critique of various microbiological methods and finally recommend the Oxford cup method; in addition, Heatley (1948), Lees and Tootill (1955) and Kavanagh (1960) have reviewed this topic. The theory of diffusion of substances through agar has been worked out by Cooper and his associates (Cooper, 1955; Cooper and Linton, 1952; Cooper and Woodman, 1946).

The method is valuable for the determination of the antibacterial activity of clinical specimens and particularly useful for the investigation of the inactivation of penicillins by proteins, and it is more sensitive than any chemical method to date. Microbiological assay methods, however, are not particularly accurate, require many controls and are time-consuming. In addition, enzymic destruction of penicillins cannot be studied since there is no method available to stop the reaction which would not also interfere with the biological assay.

The choice of test organism is important when dealing with penicillins active against organisms which are relatively resistant to penicillin G. *Sarcina lutea* is widely used because of its high sensitivity to penicillin G and *B. subtilis* and *Staph. aureus* are also often used. But with ampicillin, which possesses activity against Gram-negative organisms, perhaps it

would be better to use a Gram-negative organism, and with penicillinase-resistant penicillins a penicillinase-producing organism may be a better choice as indicator strain. This could be vital when a penicillin is altered in the body (see Rollo, Somers and Burley, 1962) to become a compound which may be active against a badly chosen indicator organism but not active against the organism for which the penicillin was designed.

Spectrophotometric methods. Various direct spectrophotometric methods were described in the early days of large-scale penicillin production, mainly for estimating the amount of penicillin in fermentation liquors. Methods utilising absorption in the ultra-violet range have been reviewed by Colon, Herpich, Neuss and Frediani (1949) and Twigg (1949). The latter also discusses infra-red methods.

Polarimetric method. Abraham and Newton (1956) followed the alkaline hydrolysis of penicillin G by the change in optical rotation as sodium penicillin G was converted into sodium D (+)-α-benzylacetamidopenicilloate. The method is complicated by the fact that mutarotation of the product occurs.

Qualitative assays

Filter membrane method (Knox and Smith, 1961). This is a simple qualitative test for the production of acid by colonies when incubated for a short time with penicillin G and is a modification of the pour-plate method of Manson, Pollock and Tridgell (1954). Organisms grown on membranes are transferred to a solution of penicillin G containing Andrade's indicator: acid-producing colonies take up a red colour; controls can be tested without penicillin. Colonies producing penicillinase or amidase are hence positive. The results can be confirmed microbiologically as later described by Knox and Smith (1961). A modification has been described (Novick, R.P., personal communication) using *N*-phenyl-1-naphthylamine azocarboxybenzene as indicator; this compound precipitates as it changes to the purple coloured form at acid pH values, and thus diffusion of the dye does not occur; this modification is of use in the study of mixed populations.

Iodometric method. Foley and Perret (1962) have described a method based on the principle of the iodometric assay (Perret, 1954): penicillinase-producing colonies decolorise starch iodine impregnated filter paper, whilst other colonies do not.

Both these methods can be used to "screen" large numbers of organisms for penicillinase activity. In addition, the filter membrane method will indicate the amidase producers.

Haight-Finland (1952) *method.* This procedure, a modification of the method of Gots (1945), is recommended by Woolff and Hamburger (1962). An agar plate containing *Sarcina lutea*, and a just inhibitory amount of penicillin G, is inoculated with the organism under investigation and incubated. Satellite colonies of *S. lutea* grow in the region where penicillinase has destroyed the penicillin G.

The method is time-consuming and uneconomical as only five or six organisms can be investigated on each plate. However, by using a filter

membrane (Knox and Smith, 1961) many colonies of organisms can be investigated simultaneously using the same principle. The organisms are grown overnight on a filter membrane on nutrient agar containing 0·05 μg./ml. of penicillin G. The filter membrane is then discarded and the penicillin G-containing plate is inoculated with a confluent growth of *Sarcina lutea* and incubated. The *Sarcina lutea* then grows in the areas where penicillin G has been destroyed.

Chromatographic methods. Early workers using chromatographic procedures were concerned only with discovering the different number of penicillins present in fermentation liquors; chromatograms can be developed with biological systems (Goodall and Levi, 1946; Winsten and Spark, 1947); Baker, Dobson and Martin (1950) developed a colour reaction—they applied the hydroxamates of penicillins to the paper, ran in isopropyl ether + 15 per cent (w/v) isopropanol saturated with 0·1M potassium hydrogen phthalate, and sprayed with ferric chloride solution. The brown spots were eluted into n-butanol and the extinction read with an EEL colorimeter. Thus both quantitative and qualitative assays were performed simultaneously. The sensitivity of their method was 100 μg. Solvent systems that have been used are: water saturated diethyl ether (Glister and Grainger, 1950); n-butanol 4:ethanol 1:water 5; n-butanol 12:acetic acid 3:water 5; n-butanol 1:pyridine 1:water 1 (Cole and Rolinson, 1961).

Colour reactions, based on the iodometric method, have recently been developed (Thomas, 1961; Sneath and Collins, 1961). 6-Aminopenicillanic acid has been detected by phenylacetylation (Batchelor and others, 1959; Cole and Rolinson, 1961; Uri and Sztaricskai, 1961). Work in this laboratory has shown that ninhydrin used with the n-butanol 4:acetic acid 1:water 5 solvent, or the xylose-aniline spray of Saarnio Niskasaarki and Gustafsson (see Hulme, 1961) with the solvent system n-propanol 6:ammonia 3:water 1 (Hanes and Isherwood, 1949) will detect about 25 μg. of penicillins, penicilloic acids and 6-aminopenicillanic acid, in enzymic hydrolysis mixtures; excellent separation is obtained with both systems. Huang and others (1960) have devised a method for estimating aminopenicilloic acid by means of quantitative paper chromatography using ninhydrin as the colour developer.

Thus chromatographic methods have obvious applications for the differentiation between penicillinases and amidases.

Enzyme Units

Pollock and Torriani (1953) defined the unit of penicillinase as that amount of enzyme that hydrolyses 1 μmole of penicillin G per hr. at pH 7·0 and 30°. The figures were chosen because these workers were using the manometric assay. Other assay methods use different physical conditions, and so other definitions of the enzyme unit have appeared. A commonly used definition quotes pH 7·46 and 37°. (See for instance Leitner and Cohen (1962) who attribute this definition to Pollock and Torriani.) Perret (1954) defines his unit at pH 6·5 and 30°, and Novick (1962a) introduces another set of physical conditions, pH 5·8 and 30°,

and also attributes the definition to Pollock and Torriani. The position hence remains unsatisfactory; a relevant discussion of the experimental conditions and the units that should be used for enzymes is given by Thompson (1962).

CONCLUSIONS

Methods for Determining Penicillin Destruction

It has been emphasised that not all methods for estimating penicillin breakdown can be used with all penicillins. The hydrolysis of 6-aminopenicillanic acid by penicillinase cannot be followed either by the acidimetric or the manometric assays; it is better to avoid iodometric methods for penicillins that absorb iodine with their β-lactam ring intact.

By means of a screening technique, such as the filter membrane method, many organisms can be investigated simultaneously for penicillin destruction. In the light of modern developments, it is important to include a test for amidase activity on organisms positive in the filter membrane test, and for this purpose the butyl acetate extraction technique must be used. Furthermore, chromatography at an early stage in the investigation will also elucidate the precise nature of the hydrolytic cleavage of the penicillin. Quantitative amidase estimation can be carried out by the butyl acetate extraction technique or by chromatography, but neither is completely satisfactory for this purpose.

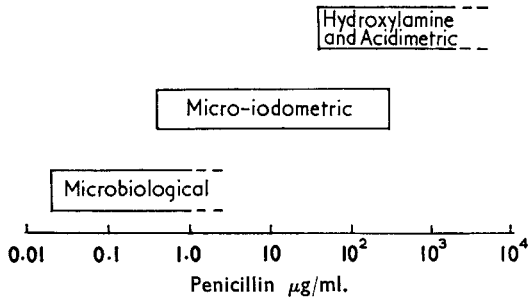


FIG. 1. The relation between the various methods and their use with varying concentrations of penicillins.

Methods for Estimating Penicillins

For assaying minute amounts of penicillins, microbiological methods are the only ones available, and by careful choice of experimental conditions and indicator organism, levels as low as 0·02 μg./ml. of penicillin G can be detected. For higher concentrations of penicillin (for example above 20 μg./ml.) the hydroxylamine assay is the most useful method because it gives a direct estimate of penicillin concentration. All the remaining methods are indirect, since it is necessary first to convert the penicillin to its corresponding penicilloic acid and then estimate this. However, when a chemical method is needed for concentrations less than 20 μg./ml. the iodometric method is the most suitable. Of all the methods

described above, the hydroxylamine and iodometric methods are the most useful for quantitative estimation of penicillins and penicillinase. The hydroxylamine method, being specific for the β-lactam ring, is the method of choice for relatively large concentrations of substrate and penicillinase. The micro-iodometric method is then available for measuring low levels of penicillinase, although it must be borne in mind that it gives results 40 per cent lower than those obtained with other assays. The relation between the various methods and their use with varying concentrations of penicillins is represented diagrammatically in Fig. 1.

Acknowledgement. We would like to thank Mr. F. R. Batchelor for a valuable discussion, and Dr. R. P. Novick for his kind cooperation.

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