there are two guanidino compounds (B_1 and B_2) that are likely to be equally rich in this element. Therefore, although the total free non-protein amino acid nitrogen may be very much less than the protein nitrogen stored in a seed, it may nevertheless constitute a small, highly concentrated reserve immediately available to the embryo on germination.

SUMMARY

1. The genus *Lathyrus* may be subdivided into groups of species characterized by associations of ninhydrin-reacting compounds in their seeds.

2. Lathyrine is a major constituent in the seeds of 12 species of *Lathyrus*.

3. β -(γ -Glutamylamino)propionitrile, the toxic factor of *L. odoratus* seeds, has been identified chromatographically in the seeds of *L. hirsutus* and *L. roseus*.

4. A new naturally occurring guanidino amino acid occurring in the seeds of 36 species of *Lathyrus* has been identified by paper chromatography and ionophoresis as homoarginine.

5. Seven unidentified ninhydrin-reacting compounds in concentrations of about 1% have been found in the seeds of one or more of the species

examined. Details of colour reactions, R_f values and ionic mobilities are given.

6. Non-protein amino acids may constitute a highly concentrated and readily available form of nitrogen storage in leguminous seeds.

The author thanks Professor W. Robson and Professor H. Harris for their many suggestions and encouragement, Professor C. E. Dent for a gift of homoarginine and Mr W. G. Mackenzie for help in obtaining seeds.

REFERENCES

- Bell, E. A. (1958). *Biochem. J.* **70**, 617.
 Bell, E. A. (1960). *Biochem. J.* **75**, 618.
 Bell, E. A. (1961). *Biochim. biophys. Acta*, **47**, 602.
 Birdsong, B. A., Alston, R. & Turner, B. L. (1960). *Canad. J. Bot.* **38**, 499.
 Fowden, L. (1959). *Rep. Progr. Chem.* **56**, 359.
 Gross, D. (1961). *J. Chromat.* **5**, 194.
 Johnstone, J. H. (1956). *Biochem. J.* **64**, 21 p.
 McKay, G. F., Lalich, J. J., Schilling, E. D. & Strong, F. M. (1954). *Arch. Biochem. Biophys.* **52**, 313.
 Schilling, E. D. & Strong, F. M. (1954). *J. Amer. chem. Soc.* **76**, 2848.
 Smith, I. (1960). *Chromatographic and Electrophoretic Techniques*, 2nd ed., vol. 1, p. 84. London: William Heinemann Medical Books Ltd.
 Tschiersch, B. (1959). *Flora, Jena*, **147**, 405.

Biochem. J. (1962) **83**, 229

Staphylococcal Penicillinase and the New Penicillins

By R. P. NOVICK*

National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 1 September 1961)

The isolation in quantity of 6-aminopenicillanic acid from penicillin fermentations (Batchelor, Doyle, Nayler & Rolinson, 1959) occasioned the synthesis of a large series of penicillins with different acyl side chains. A major objective was to prepare new drugs active against penicillin-resistant staphylococci. Since such organisms, as encountered clinically, invariably owe their resistance to their inducible penicillinase, it was evident that the success of a new compound depended upon its behaviour toward this enzyme; to be effective, it had either to be a worse substrate than penicillin G, or a worse inducer of the enzyme, or both.

The best of the new penicillins thus far reported is 2,6-dimethoxyphenylpenicillin (methicillin); all strains of staphylococci tested were inhibited by

it at concentrations ranging from 1 to 4 μ g./ml., whether they produced penicillinase or not (Rolinson, Stevens, Batchelor, Wood & Chain, 1960*b*; Stewart, 1960; Knox, 1960; Thompson, Harding & Simon, 1960; Branch, Rodger, Lee & Power, 1960), and almost all of a series of patients with severe penicillin-resistant staphylococcal infections were cured (Stewart, Nixon & Coles, 1960; Branch *et al.* 1960). It was reported that 2,6-dimethoxyphenylpenicillin was not hydrolysed (Rolinson *et al.* 1960*b*), or was only slightly hydrolysed (Stewart, 1960; Knox, 1960), by staphylococcal penicillinase, and that it was a good inducer of the enzyme (Branch *et al.* 1960; Knox, 1960; Rolinson *et al.* 1960*b*; Stewart, 1960). Its effectiveness was therefore considered to be due to its resistance to staphylococcal penicillinase. Two other new penicillins, α -phenoxyethyl- and α -phenoxypropyl-penicillin, were reported to be more

* Fellow of The National Foundation.

effective than penicillin G against penicillin G-resistant staphylococci on the basis of resistance to staphylococcal penicillinase (Garrod, 1960; Williamson, Morrison & Stevens, 1961; Jackson & Rao, 1961). However, both were found to be good substrates (Gourevitch, Hunt & Lein, 1960; Rolinson, 1961). In this paper, the last-mentioned results have been confirmed, and 2,6-dimethoxyphenylpenicillin has also been found to be hydrolysed at an appreciable rate by staphylococcal penicillinase.

The new compounds exhibit gradations in their effectiveness *in vitro* against penicillinase-producing staphylococci, and these studies were undertaken to determine whether the observed differences between them in this respect can be attributed to differences in affinity for staphylococcal penicillinase or to differences in ability to induce the enzyme.

In this paper, the expression 'penicillin resistance' refers to resistance due to penicillinase production and not to other types of resistance.

METHODS AND MATERIALS

Compounds. The sodium salt of penicillin G (benzylpenicillin) and the potassium salt of penicillin V (phenoxymethylpenicillin) were obtained from Glaxo Laboratories Ltd., Greenford, Middlesex; α -phenoxylethylpenicillin (phenethicillin) and α -phenoxypropylpenicillin (PA. 248) were gifts of Pfizer Ltd., Folkestone, Kent; 6-aminopenicillanic acid and 2,6-dimethoxyphenylpenicillin were gifts of Beecham Research Laboratories Ltd., Brockham Park, Betchworth, Surrey.

Organisms. *Staphylococcus aureus* 524SC (Rogers, 1953) was the source of penicillinase and was the test organism in penicillinase-induction experiments. It was obtained from Dr H. J. Rogers, and maintained on Hedley Wright agar slopes with monthly transfer. Every 6 months the strain was restarted from a freeze-dried preparation.

Media. The usual growth medium was Hedley Wright broth (Wright, 1933). In certain experiments, as specified, CHY medium was used. It was prepared by adding to minimal medium A of Davis & Mingioli (1950): yeast extract (Difco), final concn. 0.2%; acid-hydrolysed casein (Difco), final concn. 0.2%; glucose, final concn. 0.5%. Components were sterilized separately. Media were solidified when required by the addition of 1.5% (w/v) agar (British Drug Houses Ltd.).

Growth experiments. Bacteria were incubated in conical flasks with shaking at 35°. The volume of culture was always one-fifth of flask capacity. Growth was measured turbidimetrically with a Hilger Spekker absorptiometer and a neutral (Ilford no. 508) filter. Extinction measurements were converted into mg. bacterial dry wt./ml. with a standard curve.

Induction of penicillinase. Organisms from a 18 hr. broth or CHY culture were inoculated into fresh medium to give 0.01 mg. dry wt./ml. They were shaken at 35° until the extinction had doubled and 2,6-dimethoxyphenylpenicillin (final concn. 1.2 μ M) was then added. After a further 4 or 6 hr. of incubation, growth and induction were stopped by

the addition of 8-hydroxyquinoline (Pollock, 1950), final concn. 0.83 mM. In some experiments, the whole culture was used directly as a source of penicillinase. Alternatively, the culture was centrifuged and either supernatant or cells, resuspended in 0.2 M-potassium phosphate buffer, pH 5.8, were used. Like *Bacillus cereus* penicillinase (Kogut, Pollock & Tridgell, 1956), the staphylococcal enzyme was adsorbed by finely powdered glass, a process which was prevented by gelatin and by unknown constituents of broth. The glass-bound enzyme could be eluted with M-sodium chloride at pH 8.0. However, the affinity of staphylococcal penicillinase for glass was several hundred-fold lower than that of *B. cereus* penicillinase, and it could be handled safely with glass equipment in the absence of gelatin.

Measurement of penicillinase activity. Staphylococcal penicillinase activities are expressed as μ moles of penicillin hydrolysed/ml./hr. at 30° and at pH 5.8 [= units/ml. as defined by Pollock & Torriani (1953)].

The iodometric assay for *B. cereus* penicillinase (Perret, 1954) was found to be reliable for staphylococcal penicillinase as well. It was used to measure enzyme activities between 0.1 and 10 μ moles/ml./hr. at substrate concentrations between 1 and 10 mM, and will be referred to as the standard assay; unless otherwise stated this was the method used.

The micro-iodometric assay described in the following paper (Novick, 1962) was used for measuring enzyme activities between 0.001 and 0.2 μ mole/ml./hr. at substrate concentrations ranging from 1 μ M to 1 mM. It will be referred to as the micro-assay. Enzyme activity was about 40% lower when measured with the micro-assay than when measured with the standard assay.

RESULTS

Activity of staphylococcal penicillinase as a function of pH and of temperature. With a broth-culture supernatant as source of penicillinase and penicillin G as substrate, the pH optimum of the enzyme was 5.8 (Fig. 1A). All further assays were therefore carried out at this pH. With an incubation time of 10 min. this enzyme preparation had maximum activity at a temperature of 55° (Fig. 1B). Goldner & Wilson (1961) reported a temperature optimum of 30° for staphylococcal penicillinase. However, their observations were made with an incubation time of 1 hr. and a penicillinase prepared from homogenized cells of a different strain of *S. aureus*.

Induction by 2,6-dimethoxyphenylpenicillin: distribution and stability of induced penicillinase. Fig. 2 shows the results of an experiment in which *S. aureus* 524SC was induced in CHY medium, penicillinase activity of whole culture and supernatant being determined at 30 min. intervals. In this experiment a 50-fold increase in the specific activity of the cells was observed. This is a greater increase than has been observed with induction by other penicillins, with the exception of cephalosporin C (Swallow & Sneath, 1962) and certain of its

derivatives (Crompton, Jago, Crawford, Newton & Abraham, 1962) and with picramidopenicillanic acid, a new 6-aminopenicillanic acid derivative synthesized and studied by Swallow & Sneath (1962).

Fig. 2 shows that the enzyme is unstable in the culture supernatant and that gelatin protects it there (cf. Pollock & Perret, 1951). Enzyme in broth supernatants, however, was found to be stable. Under the conditions of this experiment, most of the penicillinase remained bound to the cells. In other experiments the bound fraction varied from 55 to 85 %, consistent with the findings of Swallow & Sneath (1962).

During induction in CHY medium the loss of supernatant activity could be due to the release of an inhibitor from the cells, to surface inactivation, or to destruction of penicillinase by a proteolytic enzyme. These possibilities have not been investigated.

Induction by four rapidly hydrolysed penicillins. Theoretically, a new penicillin might be effective by being a poorer inducer of staphylococcal penicillinase than penicillin G. A direct examination of this was not possible because induction of staphylococcal penicillinase, unlike that of *B. cereus* penicillinase (Pollock, 1953), appears to require the continuous presence of the inducer (Swallow & Sneath, 1962). Although induction with penicillin G has been reported (Geronimus & Cohen, 1957, 1958), rapid destruction of the compound probably

prevented observation of the maximal induction rate. The same has been found for α -phenoxy-methyl-, α -phenoxyethyl- and α -phenoxypropyl-penicillin, all of which are rapidly destroyed by staphylococcal penicillinase (see Table 3). Induction with these three as well as with penicillin G was attempted by a somewhat different technique. An electrometric titrator (type TTT1c, Radiometer, Copenhagen) was used to control the concentration of inducer during the growth of the organisms. It was intended by this means to take advantage of the acid group formed when penicillin is hydrolysed; the titrator operated two burettes simultaneously, one providing 0.01M-sodium hydroxide to maintain a constant pH between 6.2 and 7.2, the other supplying equimolar inducer to replace that hydrolysed. However, when penicillin G was supplied continuously, it was found to inhibit the organisms at a concentration of about 0.2 μ g./ml. The titrator was not sensitive enough to maintain this penicillin concentration continuously; because of the buffering capacity of the growth medium [hydrolysed casein

Fig. 1. A. Penicillinase activity as a function of pH. Incubations were carried out at 30° in 7 ml. total volume with 5 mM-penicillin G, 20 units of penicillinase activity (as measured at pH 5.8 and at 30°), and, for pH 3.2-6.2, 0.1M-sodium citrate buffer, or, for pH 5.8-9.0, 0.2M-sodium phosphate buffer. Incubation was for 10 min. and reaction rate was estimated iodometrically (Perret, 1954).

B. Penicillinase activity as a function of temperature. As in A, except that all incubation mixtures contained 0.2M-sodium phosphate buffer, pH 5.8.

Fig. 2. Penicillinase induction and distribution. Penicillinase induction was carried out in CHY medium and 10 ml. samples were removed at 30 min. intervals. The samples were chilled, 8-hydroxyquinoline was added, the extinctions were determined, and a 5 ml. portion of each was centrifuged to remove the cells. Penicillinase activity of whole culture (●) and of supernatant (■) was then estimated iodometrically (Perret, 1954). Point (+) represents supernatant activity in a parallel culture to which gelatin (0.5 %) was added at the start of induction to protect the enzyme. Growth of the culture (▲) is plotted semilogarithmically.

(0.1%), yeast extract (0.1%) and glucose (0.5%), in water], the hydrolysis of 0.2 μ g. of penicillin/ml. did not produce enough pH change to activate the magnetic valve of the titrator.

The ability of the four penicillins to induce penicillinase was examined indirectly by a comparison of the resistance of single induced and uninduced cells to each one. The results (Table 1) show that there is little, if any, difference between uninduced and 2,6-dimethoxyphenylpenicillin-induced single cells of *S. aureus* 524SC in ability to grow into colonies in the presence of any of the four penicillins, despite a 130-fold difference in initial enzyme content.

Similar results (for penicillin G only) were obtained in liquid culture in an experiment in which induced and uninduced exponentially-growing cells were inoculated at two different cell densities, 10^3 /ml. and 10^6 /ml., into sets of broth tubes containing penicillin G in threefold serial concentration increments. These were incubated with shaking for 24 hr., and scored for growth on the basis of the presence or absence of turbidity. As expected, the difference in inoculum size was correlated with a large difference in ability to grow in the presence of penicillin. However, whatever the inoculum size, the maximum concentration in

which the induced cells grew was about three times the maximum in which the uninduced cells grew. Again, the induced cells had 130 times more enzyme, initially, than had the uninduced. These results are consistent with the idea that the organisms are induced rapidly enough by any of the four penicillins to avail themselves of almost the maximum protective effect of penicillinase, and that differences in antimicrobial activity against penicillinase-producing staphylococci are not, among the penicillins studied here, a function of differences in ability to induce the enzyme.

Action of penicillinase. The term 'penicillinase' refers to enzymes which split the β -lactam bond of penicillins, as distinct from penicillin amidases (Rolinson *et al.* 1960a; Claredge, Gourevitch & Lein, 1960). All of the penicillins under investigation give, on alkaline or enzymic β -lactam cleavage, penicilloic acids which reduce approximately 8 equiv. of iodine/mole; on hydrolysis by amidase they give 6-aminopenicillanic acid, which is stable towards iodine.

For each of the penicillins, the rate of hydrolysis by a 2,6-dimethoxyphenylpenicillin-induced broth culture was compared with that by an uninduced culture. The results are expressed as ratios in the last column of Table 2. Table 2 also shows that

Table 1. *Resistance of single induced and uninduced organisms to four penicillins*

Uninduced (3.4 units of penicillinase/mg. dry wt.) and 2,6-dimethoxyphenylpenicillin-induced organisms (450 units/mg. dry wt.) in late exponential growth phase were diluted to give about 500 viable organisms/ml., and were plated in 0.1 ml. amounts with a glass spreader on broth-agar plates containing the indicated concentrations of the four penicillins. All platings were done in duplicate and the plates were counted after 20 and after 40 hr. of incubation at 35°. Results are given as sums of colonies on duplicate plates after incubation for 20 hr.; the counts at 40 hr. were not significantly different.

Concn. (μ g./ml.)	Penicillin G		α -Phenoxymethyl- penicillin		α -Phenoxyethyl- penicillin		α -Phenoxypropyl- penicillin	
	Induced	Uninduced	Induced	Uninduced	Induced	Uninduced	Induced	Uninduced
0.00	39	134	46	145	52	148	65	139
0.06	59	119	52	102	62	116	42	100
0.12	67	129	44	25	66	136	48	125
0.25	44	23	0	0	16	10	58	86
0.50	2	0	0	0	0	0	0	0

Table 2. *Hydrolysis by staphylococcal penicillinase*

Two 50 ml. broth samples, one containing 2,6-dimethoxyphenylpenicillin (1.2 μ M), were inoculated at the same time to a cell density of 0.01 mg. dry wt./ml. and shaken at 35°. After 6 hr. 8-hydroxyquinoline (final concn. 0.83 mM) was added to each culture, and the penicillinase activity of each was measured for the six penicillins, each at a concn. of 5 mM. The results are expressed as μ moles of substrate hydrolysed/hr./mg. of bacterial dry weight.

	Uninduced	Induced	Induced: uninduced
Penicillin G	5.8	350	60
Penicillin V	6.3	370	63
DL- α -Phenoxyethylpenicillin	5.4	330	58
DL- α -Phenoxypropylpenicillin	5.0	300	60
6-Aminopenicillanic acid	1.2	70	62
2,6-Dimethoxyphenylpenicillin	—	1.8	—

penicillins G and V, and α -phenoxyethyl- and α -phenoxypropyl-penicillins were all hydrolysed at comparable rates; 6-aminopenicillanic acid was hydrolysed at a considerably lower rate, and 2,6-dimethoxyphenylpenicillin very slowly indeed. Activity for 2,6-dimethoxyphenylpenicillin was too low in the uninduced culture to be measured, but the similarity of the ratios for the other five penicillins suggests that, for them at least, the same enzyme was responsible for hydrolysis in each case.

Two of the penicillins studied, α -phenoxyethyl- and α -phenoxypropyl-penicillin, are racemic mixtures. The two enantiomorphs of α -phenoxyethyl-penicillin were found by Gourevitch, Hunt & Lein (1960) to be hydrolysed at the same rate by staphylococcal penicillinase. In the present investigation, only the racemic mixture has been studied. The two optical isomers of α -phenoxypropylpenicillin have been found by Crompton *et al.* (1962) to be hydrolysed at different rates by staphylococcal penicillinase and to have different affinities for the enzyme. These results have been confirmed (with samples of D- and L- α -phenoxypropylpenicillin kindly provided by Dr E. P. Abraham, University of Oxford). Michaelis constants (K_m), determined by the micro-assay (discussed below) were also similar to those obtained by Crompton *et al.* (1962), by inhibitor analysis.

Measurement of Michaelis constants. To compare the affinity of staphylococcal penicillinase for each of the new penicillins with its affinity for penicillin G, K_m values were determined. α -Phenoxymethylpenicillin and 6-aminopenicillanic acid were included for further comparison. Because of the variability in distribution of staphylococcal penicillinase between the growth medium and the cells, separate measurements were carried out in most cases with cell-bound and extracellular enzyme. For 6-aminopenicillanic acid and 2,6-dimethoxyphenylpenicillin, the initial reaction velocities were measured at several different substrate concentrations with the standard assay. For penicillin G and for α -phenoxymethyl-, α -phenoxyethyl- and α -phenoxypropyl-penicillin, measurements were made with the micro-assay. The method of Lineweaver & Burk (1934) was used to calculate K_m values and maximal velocities, $V_{\max}$ (see Table 3). Also, the half-life of each compound was calculated at its minimal inhibitory concentration in the presence of 500 units of staphylococcal penicillinase/ml. The minimal inhibitory concentrations listed in Table 3 are for sensitive staphylococci (Williamson *et al.* 1961). The half-lives, $t_{\frac{1}{2}}$, were calculated as follows from the Michaelis-Menten equation:

$$-\frac{ds}{dt} = v = \frac{V_{\max} \cdot s}{K_m + s}$$

where v is initial reaction velocity, t is time, and s is substrate concentration.

$$\text{Thus} \quad \int_s^{1s} -\frac{ds}{s(K_m + s)} = \int_0^{t_{\frac{1}{2}}} \frac{V_{\max}}{s} dt$$

$$\text{and} \quad t_{\frac{1}{2}} = \frac{K_m}{V_{\max}} \ln 2 + \frac{s}{2V_{\max}}$$

For all the compounds studied, $s \ll K_m$ when s is the minimal inhibitory concentration, and the second term of this expression is negligible. Thus the half-lives are independent of substrate concentration:

$$t_{\frac{1}{2}} = \frac{K_m}{V_{\max}} \ln 2$$

All velocities and half-lives (Table 3) were calculated for the same penicillinase concentration, 500 units/ml., the approximate enzyme content of a maximally induced fully grown culture of *S. aureus* 524SC (containing about 1.5 mg. of dry bacteria/ml.). This gave a $V_{\max}$ for penicillin G of 500 μ moles/ml./hr.

From previous work (Garrod, 1960; Williamson *et al.* 1961), the penicillins can be ranked in order of effectiveness *in vitro* against penicillinase-producing staphylococci, as follows: 2,6-dimethoxyphenylpenicillin $\gg$ α -phenoxypropylpenicillin $\gg$ α -phenoxyethylpenicillin $>$ α -phenoxymethylpenicillin $\geq$ penicillin G. The K_m values (Table 3) are in the same order and thus correlate with data derived from testing *in vitro*. Half-lives at minimal inhibitory concentrations for sensitive staphylococci are also in the same order, but, though the half-lives of four of the penicillins are similar, that of 2,6-dimethoxyphenylpenicillin is many times longer, even though this drug must be used at 25–50 times the concentration of the others to inhibit sensitive staphylococci.

DISCUSSION AND CONCLUSIONS

The affinity of staphylococcal penicillinase for each of the penicillins studied is the major determinant of its half-life at the low concentration required for the inhibition of penicillin-sensitive staphylococci (see Table 3). This result supports the hypothesis that the greater the K_m for a particular penicillin, the less will that penicillin differ in its antibiotic action against sensitive and resistant staphylococci. Thus, because of its very high K_m , 2,6-dimethoxyphenylpenicillin has a half-life 5×10^5 times that of penicillin G (with extracellular penicillinase) when each is present at a concentration just sufficient to inhibit penicillin-sensitive staphylococci, whereas it is hydrolysed one-thirtieth as fast as penicillin G when both saturate the enzyme.

Table 3. *Kinetic data for hydrolysis by staphylococcal penicillinase of several penicillins*

The K_m was determined for each of the compounds with penicillinase in the supernatant of an induced broth culture (free enzyme), and with penicillinase bound to induced washed cells suspended in buffer (bound enzyme). Maximum velocities and velocities at minimal inhibitory concentrations were calculated from the Michaelis-Menten equation for each penicillin. The V_{max} values obtained represent rates of hydrolysis calculated for penicillinase at a concentration of 500 units/ml. Half-lives at the minimal inhibitory concentrations were also calculated (see text) for each of the penicillins in the presence of 500 units of penicillinase/ml. The minimal inhibitory concentrations listed are for sensitive staphylococci (Williamson *et al.* 1961).

Compound	K_m (μM)		$V_{max.}$ (μ moles/ml./hr.)		Minimal inhibitory concn. for sensitive staphylococci (μM)	Half-life at minimal inhibitory concn. (sec.)	
	Free enzyme	Bound enzyme	Free enzyme	Bound enzyme		Free enzyme	Bound enzyme
Penicillin G	2.5	19	500	500	0.03-0.1	0.012	0.09
Penicillin V	3.8	19	600	510	0.03-0.2	0.016	0.09
DL- α -Phenoxyethylpenicillin	10	49	450	410	0.05-0.3	0.053	0.31
DL- α -Phenoxypropylpenicillin	17	60	300	420	0.05-0.3	0.13	0.36
D- α -Phenoxypropylpenicillin	13	—	325	—	—	—	—
L- α -Phenoxypropylpenicillin	53	—	660	—	—	—	—
6-Aminopenicillanic acid	750	700	95	95	—	—	—
2,6-Dimethoxyphenylpenicillin (sodium salt)	28 000	32 000	16.5	16.5	2.4-5.0	1.2 hr.	1.3 hr.

On the basis of its K_m as well as on established clinical grounds, 2,6-dimethoxyphenylpenicillin is the most effective of the new penicillins against penicillinase-producing staphylococci, despite its being but one-fiftieth as active, mole for mole, as penicillin G against penicillin-sensitive organisms. By this line of reasoning, none of the other penicillins studied is likely to be very much more effective than penicillin G against penicillinase-producing staphylococci. This conclusion is likewise consistent with the results of the testing of the various drugs *in vitro*, which showed, in particular, that only 2,6-dimethoxyphenylpenicillin is as effective against penicillin-resistant staphylococci as against sensitive organisms (Rolinson *et al.* 1960b; Stewart, 1960; Knox, 1960; Thompson *et al.* 1960; Branch *et al.* 1960).

The K_m measured for staphylococcal penicillinase with penicillin G is extremely low, and is considerably lower than that for other penicillinases (Pollock, Torriani & Tridgell, 1956; Banfield, 1957). This is not surprising, since penicillin G is bactericidal for sensitive staphylococci at concentrations in the range 30-100 μM (Williamson *et al.* 1961). For resistant staphylococci which owe their resistance entirely to penicillinase, the enzyme must operate effectively in this range of substrate concentration if it is to protect the cells by preventing penicillin from reaching and maintaining such concentrations at some critical cellular locus. Clearly, the very high affinity of the enzyme for penicillin G accounts for this effectiveness. Even so, the enzyme is only just able to protect the cells and there is rapid killing of penicillinase-producing organisms when the inoculum is small or the penicillin concentration

high. 2,6-Dimethoxyphenylpenicillin, embodying a smaller change in the penicillin molecule than one which results in absolute stability to the enzyme, upsets the balance between drug concentration, penicillinase action and cell survival, with lethal consequences for the penicillinase-producing organism.

Whether, for any particular staphylococcal strain, most of the enzyme is free in the medium or bound to the cells, the conclusions reached concerning the efficacy of the various penicillins remain the same; they are based on comparative K_m values and on half-lives at minimal inhibitory concentrations, and these bear similar relationships to one another for both free and bound enzyme (see Table 3). The observation that K_m values for cell-bound enzyme are higher than for free enzyme (see Table 3) suggests that there may be an accessibility barrier between substrate and bound enzyme. The alternative of a difference in properties between the free and bound enzyme seems less likely because the difference in K_m is larger for the more rapidly hydrolysed penicillins and vanishes for the slowly hydrolysed ones, 6-aminopenicillanic acid and 2,6-dimethoxyphenylpenicillin (see Table 3).

Finally, investigation of the new penicillins has shown that the three properties of penicillin that determine its antimicrobial activity against penicillinase-producing staphylococci can be varied independently. Penicillin G seems to be a good inducer, has a very high affinity and is very potent as an antimicrobial. 2,6-Dimethoxyphenylpenicillin has almost lost its affinity for the enzyme while retaining the other two properties, and piperamidopenicillanic acid is a good inducer, has no antimicrobial activity (at a concentration of

40 $\mu\text{g./ml.}$) and is hydrolysed one-tenth as rapidly as penicillin G, each at a concentration of 2.7 mM (Swallow & Sneath, 1962). Thus far, no compound has demonstrably lost its ability to induce while yet retaining either antimicrobial activity or affinity for the enzyme, but such a compound might be therapeutically valuable. As regards enzyme induction, picramidopenicillanic acid and 2,6-dimethoxyphenylpenicillin provide further evidence that affinity for the free enzyme is not correlated with ability to act as an inducer (cf. Perrin, Jacob & Monod, 1960).

SUMMARY

1. The results of an investigation of staphylococcal penicillinase, the induction of the enzyme by several new penicillins, and its enzymic activity towards them are presented.

2. The enzyme has a pH optimum of 5.8 and a temperature optimum of 55°.

3. The continuous presence of inducer is required for the induction of this enzyme, unlike the penicillinase of *Bacillus cereus*. Induction by 2,6-dimethoxyphenylpenicillin is readily demonstrable because its hydrolysis by the enzyme is very slow. Induction by four rapidly hydrolysed penicillins is much more difficult to observe; it is probable that they are all good inducers because uninduced cells of *Staphylococcus aureus* are almost as resistant to each drug as are fully induced cells.

4. Measurement of Michaelis constants (K_m) revealed that effectiveness against penicillinase-producing staphylococci of the penicillins studied is an inverse function of their affinity for the enzyme. 2,6-Dimethoxyphenylpenicillin, with a K_m of 28 mM, is by far the most effective; four others, with K_m values of 2.5–17 μM , differ little from each other in being largely ineffective.

5. The K_m of staphylococcal penicillinase for 2.5 μM -penicillin G is considerably lower than that of other penicillinases.

6. Staphylococcal penicillinase is usually partly free and partly bound to the cells. Comparison of K_m values for free and bound enzyme revealed differences which might be accounted for by an accessibility barrier between bound enzyme and substrate.

7. Among the various new penicillins, three important biological properties (affinity for the enzyme, antimicrobial activity, and ability to induce the enzyme) all varied independently. In particular, high affinity for the active enzyme was not required of its inducer.

I wish to express my gratitude to Dr M. R. Pollock and to Dr J. Mandelstam for helpful criticism and advice, and to Mr D. J. McGillicuddy for expert technical assistance.

REFERENCES

- Banfield, J. E. (1957). *Experientia*, **13**, 403.
 Batchelor, F. R., Doyle, F. P., Nayler, J. H. C. & Rolinson, G. N. (1959). *Nature, Lond.*, **183**, 257.
 Branch, A., Rodger, K. C., Lee, R. W. & Power, E. E. (1960). *Canad. med. Ass. J.* **83**, 991.
 Claredge, C. A., Gourevitch, A. & Lein, J. (1960). *Nature, Lond.*, **187**, 237.
 Crompton, B., Jago, M., Crawford, K., Newton, G. G. F. & Abraham, E. P. (1962). *Biochem. J.* **83**, 52.
 Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.
 Garrod, L. P. (1960). *Brit. med. J.* **i**, 527.
 Geronimus, L. H. & Cohen, S. (1957). *J. Bact.* **73**, 28.
 Geronimus, L. H. & Cohen, S. (1958). *J. Bact.* **76**, 117.
 Goldner, M. & Wilson, R. J. (1961). *Canad. J. Microbiol.* **7**, 45.
 Gourevitch, A., Hunt, G. A. & Lein, J. (1960). *Antibiot. Ann.* 1959–1960, 111.
 Jackson, F. L. & Rao, K. K. (1961). *Lancet*, **i**, 850.
 Knox, R. (1960). *Brit. med. J.* **ii**, 690.
 Kogut, M., Pollock, M. R. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 391.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Novick, R. P. (1962). *Biochem. J.* **83**, 236.
 Perret, C. J. (1954). *Nature, Lond.*, **174**, 1012.
 Perrin, D., Jacob, F. & Monod, J. (1960). *C.R. Acad. Sci., Paris*, **251**, 155.
 Pollock, M. R. (1950). *Brit. J. exp. Path.* **31**, 739.
 Pollock, M. R. (1953). *Symp. Soc. gen. Microbiol.* **3**, 150.
 Pollock, M. R. & Perret, C. J. (1951). *Brit. J. exp. Path.* **32**, 387.
 Pollock, M. R. & Torriani, A.-M. (1953). *C.R. Acad. Sci., Paris*, **237**, 276.
 Pollock, M. R., Torriani, A.-M. & Tridgell, E. J. (1956). *Biochem. J.* **62**, 387.
 Rogers, H. J. (1953). *J. Path. Bact.* **66**, 545.
 Rolinson, G. N. (1961). *Lancet*, **i**, 941.
 Rolinson, G. N., Batchelor, F. R., Butterworth, D., Cameron-Wood, J., Cole, M., Eustace, G. C., Hart, M. V., Richards, M. & Chain, E. B. (1960a). *Nature, Lond.*, **187**, 236.
 Rolinson, G. N., Stevens, S., Batchelor, F. R., Wood, J. C. & Chain, E. B. (1960b). *Lancet*, **ii**, 564.
 Stewart, G. T. (1960). *Brit. med. J.* **ii**, 694.
 Stewart, G. T., Nixon, H. H. & Coles, H. M. T. (1960). *Brit. med. J.* **ii**, 703.
 Swallow, D. L. & Sneath, P. H. A. (1962). *J. gen. Microbiol.* (In the Press.)
 Thompson, R. E. M., Harding, J. W. & Simon, R. D. (1960). *Brit. med. J.* **ii**, 708.
 Williamson, G. M., Morrison, J. K. & Stevens, K. J. (1961). *Lancet*, **i**, 847.
 Wright, H. D. (1933). *J. Path. Bact.* **37**, 257.