there are two guanidino compounds (B₁ and B₂) 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 homoaarginine.
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, *Rf* 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 homoaarginine and Mr W. G. Mackenzie for help in obtaining seeds.

**REFERENCES**


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**Staphylococcal Penicillinase and the New Penicillins**

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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 μg./ml., whether they produced penicillinase or not (Rolinson, Stevens, Batchelor, Wood & Chain, 1960b; 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. 1960b), 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. 1960b; Stewart, 1960). Its effectiveness was therefore considered to be due to its resistance to staphylococcal penicillinase. Two other new penicillins, α-phenoxethyl- and α-phenoxypyropyl-penicillin, were reported to be more
effective than penicillin G against penicillin G-resistant staphylococci on the basis of resistance to staphylococcal penicillinase (Garrod, 1960; William son, 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-phenoxethylpenicillin (phenethicillin) and α-phenoxypyropylpenicillin (PA. 248) were gifts of Pfizer Ltd., Folkestone, Kent; 6-aminopenicillinic 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 15 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 μM) 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 1 M-sodium chloride at pH 9·0. 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 μmoles of penicillin hydrolysed/ml./hr. at 30° and at pH 5·8 (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 μmoles/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 μmole/ml./hr. at substrate concentrations ranging from 1 μM to 1 mM. It will be referred to as the micro-assay. Enzyme activity was about 40% higher 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 staphyloccocal penicillinase than penicillin G. A direct examination of this was not possible because induction of staphyloccocal 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-phenoxyethyl-, α-phenoxyethyl- and α-phenoxypropylpenicillin, all of which are rapidly destroyed by staphyloccocal 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 μg./mL. The titrator was not sensitive enough to maintain this penicillin concentration continuously; because of the buffering capacity of the growth medium [hydrolysed casein

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**Fig. 1.** A. Penicillinase activity as a function of pH. Incubations were carried out at 30° in 7 mL total volume with 5 ml-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.

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**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 μg. 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 524 SC 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, 10³/ml. and 10⁴/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

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

<table>
<thead>
<tr>
<th>Conc. (μg./ml.)</th>
<th>Penicillin G</th>
<th>α-Phenoxyethylpenicillin</th>
<th>α-Phenoxyethylpenicillin</th>
<th>α-Phenoxypropylpenicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Induced</td>
<td>Uninduced</td>
<td>Induced</td>
<td>Uninduced</td>
</tr>
<tr>
<td>0.00</td>
<td>39</td>
<td>134</td>
<td>46</td>
<td>145</td>
</tr>
<tr>
<td>0.06</td>
<td>59</td>
<td>119</td>
<td>52</td>
<td>102</td>
</tr>
<tr>
<td>0.12</td>
<td>67</td>
<td>129</td>
<td>44</td>
<td>25</td>
</tr>
<tr>
<td>0.25</td>
<td>44</td>
<td>23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.50</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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**Table 2. Hydrolysis by staphylococcal penicillinase**

Two 50 ml. broth samples, one containing 2,6-dimethoxyphenylpenicillin (1:2 μg.), 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 μmoles of substrate hydrolysed/hr./mg. of bacterial dry weight.

<table>
<thead>
<tr>
<th>Penicillin</th>
<th>Uninduced</th>
<th>Induced</th>
<th>Induced : uninduced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G</td>
<td>5.8</td>
<td>350</td>
<td>60</td>
</tr>
<tr>
<td>Penicillin V</td>
<td>6.3</td>
<td>370</td>
<td>63</td>
</tr>
<tr>
<td>DL-α-Phenoxyethylpenicillin</td>
<td>5.4</td>
<td>330</td>
<td>68</td>
</tr>
<tr>
<td>DL-α-Phenoxypropylpenicillin</td>
<td>5.0</td>
<td>300</td>
<td>60</td>
</tr>
<tr>
<td>6-Aminopenicillanic acid</td>
<td>1.2</td>
<td>70</td>
<td>62</td>
</tr>
<tr>
<td>2,6-Dimethoxyphenylpenicillin</td>
<td>—</td>
<td>1.8</td>
<td>—</td>
</tr>
</tbody>
</table>
penicillins G and V, and α-phenoxyethyl- and α-phenoxypropyl-penicillins were all hydrolysed at comparable rates; 6-aminopenicillic 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-phenoxyethylpenicillin 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-Phenoxyethylpenicillin and 6-aminopenicillic 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-aminopenicillic 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-phenoxyethyl-, α-phenoxypropyl- 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, $t_{1/2}$, 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.

Thus

$$\int_s^{tS} \frac{ds}{s} = \frac{K_m + s}{V_{max}} dt$$

and

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

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:

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

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 μmoles/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 $> \alpha$-phenoxypropylpenicillin $> \alpha$-phenoxyethylpenicillin $> \alpha$-phenoxyacetylpenicillin $> \alpha$-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 x 10⁶ 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 penicillinae 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).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (µmoles/ml/hr)</th>
<th>Minimal inhibitory conc. for sensitive staphylococci (µM)</th>
<th>Half-life at minimal inhibitory conc. (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free enzyme</td>
<td>Bound enzyme</td>
<td>Free enzyme</td>
<td>Bound enzyme</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>2-5</td>
<td>19</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Penicillin V</td>
<td>3-8</td>
<td>19</td>
<td>600</td>
<td>510</td>
</tr>
<tr>
<td>DL-α-Phenoxyethylenepenicillin</td>
<td>10</td>
<td>49</td>
<td>450</td>
<td>410</td>
</tr>
<tr>
<td>DL-α-Phenoxypropylenepenicillin</td>
<td>17</td>
<td>60</td>
<td>300</td>
<td>420</td>
</tr>
<tr>
<td>D-α-Phenoxypropylenepenicillin</td>
<td>13</td>
<td>—</td>
<td>325</td>
<td>—</td>
</tr>
<tr>
<td>L-α-Phenoxypropylenepenicillin</td>
<td>53</td>
<td>—</td>
<td>660</td>
<td>—</td>
</tr>
<tr>
<td>6-Aminopenicillanic acid</td>
<td>750</td>
<td>700</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>2,6-Dimethoxyphenylpenicillin</td>
<td>28 000</td>
<td>32 000</td>
<td>18-5</td>
<td>18-5</td>
</tr>
</tbody>
</table>

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 penicillinas (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 picramidopenicillanic acid is a good inducer, has no antimicrobial activity (at a concentration of
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.

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