The Association of the Penicillin-binding Component of *Staphylococcus aureus* with a Lipid Fraction

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SUMMARY: Radiopenicillin is strongly bound by ultra-microscopic lipid-containing particles liberated on mechanical rupture of *Staphylococcus aureus* cells. The binding resembles that of intact cells in that it is irreversible and only occurs to a limited extent, but differs in that 7–12 times as much penicillin is bound per unit dry weight of material. The supernatant after centrifuging down the lipid particles decreases the titre of added penicillin as indicated by diffusion assay, possibly by a small irreversible inactivation superimposed upon a 'reversible' type of binding.

There is a correlation between the distribution of (a) the penicillin-binding component (P.B.c.), (b) $^{35}$S from radiopenicillin pretreated cells, and (c) lipid phosphorus, in the three fractions produced on rupture either in distilled water or in a formaldehyde solution. Rupture in formalin appears to allow the cells walls to retain most of the lipid particles and P.B.c., but only a little extra of the dry weight of the cells. Thus penicillin reacts with a lipid-containing fraction close to the cell wall in intact organisms. At least as much more P.B.c. is liberated on rupture of the cells as was available to the penicillin in the intact cell, but P.B.c. is somewhat unstable after rupture. These data are discussed in the light of evidence in the literature that penicillin may react initially with the osmotic barrier of bacteria.

It was noted several years ago in this laboratory that staphylococcal cell walls prepared by mechanical rupture of cells in distilled water (Cooper, Rowley & Dawson, 1949) were quite white, and that most of the yellow pigment present in the supernatant fraction was sedimented by high-speed centrifugation as a thin orange waxy layer coating the surface of the cell-wall layer. The lipid character of this fraction was suspected from its yellow colour, as nearly all the pigments of staphylococci when extracted by 90% phenol in water were soluble in chloroform. These pigments have been reported to be carotenoid in character (Sobin & Stahly, 1942). Mitchell & Moyle (1951b) confirmed the high proportion of phospholipid in the coloured fraction which sediments more slowly than the cell walls when a mechanically ruptured cell suspension is centrifuged.

It has been reported elsewhere (Rowley, Cooper, Roberts & Lester Smith, 1950) that penicillin-sensitive cells contain a penicillin-binding component (P.B.c.) which appears to be related to the mode of action of penicillin. Cell walls prepared in a formalin mixture were able to bind penicillin (Few, Cooper & Rowley, 1952), whereas cell walls prepared in distilled water were not (Cooper et al. 1949). It was noticed that cell walls which could bind penicillin were yellow and those which could not were quite white and that this colour difference always followed the penicillin-binding capacity. It was, therefore, considered possible that P.B.c. was associated with a lipid fraction of the cells, and the results presented below show that this is the case.
**METHODS**

**Measurement of penicillin uptake** by intact cells and cell walls using radiopenicillin has been previously described (Rowley et al. 1950; Few et al. 1952). Uptake by 'lipid particles' (see below) was measured as described in Table 1. The purity of the radiopenicillin was checked by the methods described by Cooper, Clowes & Rowley (1954).

**Cell preparation.** A penicillin-sensitive *Staphylococcus aureus* was grown on tryptic digest agar in Roux bottles for 16–40 hr., harvested in neutral 0·05M-phosphate solution, filtered through a no. 1 porosity sintered glass funnel to remove agar particles and washed 3 times with distilled water. The organisms were used at once or were freeze dried.

**Disruption of cells.** Cells were disrupted in distilled water or in 'medium A', which consisted of 1 ml. formalin solution, 0·1 ml. mercaptoacetic acid, 0·5 g. citric acid and 50 ml. distilled water saturated with octyl alcohol, made up to 100 ml. with water and adjusted to pH 6·5. Ten ml. of a cell suspension were agitated with 4 g. of glass ballotini beads, no. 12, with a Mickle shaker (Mickle, 1948; Mitchell & Moyle, 1951b). The concentration of cells was up to 25 mg. dry wt./ml. for distilled water, or up to 15 mg./ml. for medium A since the latter did not allow complete formation of typical penicillin-binding cell walls at concentrations higher than this. The rate of rupture was followed by a turbidimetric method (Cooper, 1953). After shaking for 1 hr. 10–15% of the organisms remained intact with distilled water as shaking mixture and 20% with medium A. Little further rupture occurred on continued shaking. In all quantitative analytical work unruptured cells present in the cell-wall preparation were allowed for.

**Separation of fractions after cell rupture.** The suspension of ruptured cells was removed by a pipette, and when quantitative recovery was required the ballotini were washed 5 times with 2 ml. portions of distilled water. The suspension was spun for 15 min. at 2500 g to remove cell walls and intact cells, and the supernatant was re-centrifuged twice at this speed to sediment cell wall debris which was added to the cell wall fraction. The supernatant, still quite turbid, was centrifuged for 30 min. at 20,000 g to sediment the 'lipid particle' fraction, which appeared as a homogeneous orange translucent pellet free from opaque white cell wall debris. No lipid particles were freed in medium A at concentrations below 15 mg. dry wt. bacteria/ml., but the yellow cell walls formed in this case had to be separated from a white heavier layer which appeared to be denatured protein and which contained no P.B.c., or 35S from radiopenicillin pretreated cells. The separation was easily effected with a Pasteur pipette followed by differential centrifuging. The white layer was added to the supernatant fraction for assay purposes.

**Biological penicillin assays** used an agar plate diffusion method with *Sarcina lutea* as test organism, porcelain cylinders of 8 mm. diameter, and an incubation time of 16 hr. at 27° (Welch, 1948). Penicillin solutions containing 0·02 and 0·05 u./ml. gave inhibition zones of 20 and 30 mm. diameter respectively so that the decrease in titre of about 0·01 u./ml. observed in presence of the
supernatant fraction was well outside experimental error. Tenfold replicates were used.

Assay of lipid phosphorus. The procedure followed was similar to that described by Mitchell & Moyle (1951b). The weighed freeze-dried samples (c. 20 mg.) were refluxed for 1 hr. with 2 ml. methanol in 20 ml. tubes with B14 sockets and condensers and the cooled extracts were transferred to fresh tubes. This procedure was repeated twice and the pooled methanolic extracts were evaporated to dryness and kept overnight in vacuo over P₄O₁₀. The dried extracts were then re-extracted overnight at room temperature with 3 ml. Na-dried ether, and the ether extracts transferred to fresh tubes; this step was also repeated twice. The pooled extracts were evaporated to dryness and the fatty residues digested with 0.5 ml. conc. HNO₃ (AR) + 1 drop of 100 vol. H₂O₂ until colourless. The total phosphate in these solutions was estimated by the colorimetric method of Fiske & SubbaRow (1925).

RESULTS

Penicillin binding by lipid particles

The lipid particle fraction obtained by high-speed centrifuging of the cell wall-free 'cytoplasmic contents' of the cells after rupture in distilled water was found to bind penicillin strongly (Table 1), the uptake being 10–15 u./g.,

Table 1. Uptake of radiopenicillin on lipid particles before and after addition of 0·2 u. ordinary penicillin/ml.

<table>
<thead>
<tr>
<th>Concentration (u./ml.)</th>
<th>35S in once-washed suspension (counts/ml/min.)</th>
<th>35S in supernatant after second centrifuging (counts/ml/min.)</th>
<th>Radio-penicillin uptake (u./g.)</th>
<th>35S in once-washed suspension (counts/ml/min.)</th>
<th>35S in supernatant after second centrifuging (counts/ml/min.)</th>
<th>Radio-penicillin uptake (u./g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0·1</td>
<td>1410</td>
<td>390</td>
<td>9·0</td>
<td>100</td>
<td>130</td>
<td>&lt;0·3</td>
</tr>
<tr>
<td>0·2</td>
<td>1650</td>
<td>420</td>
<td>10·6</td>
<td>190</td>
<td>220</td>
<td>&lt;0·3</td>
</tr>
<tr>
<td>0·3</td>
<td>1730</td>
<td>610</td>
<td>9·7</td>
<td>320</td>
<td>320</td>
<td>&lt;0·3</td>
</tr>
<tr>
<td>0·4</td>
<td>1960</td>
<td>800</td>
<td>10·0</td>
<td>330</td>
<td>340</td>
<td>&lt;0·3</td>
</tr>
</tbody>
</table>

i.e. 7–12 times greater than the original cells. It can be seen that this uptake is independent of penicillin concentration and is completely prevented by pre-treatment of the cells with a small amount of non-radioactive penicillin. The uptake by the lipid particles is thus similar to that by intact cells in that it appears to be an irreversible reaction with a component present in limited amount.

The significantly high value of the supernatant radioactivity of the untreated preparation (col. 3, Table 1), compared with that of the penicillin
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pre-treated controls (col. 6), suggests that about 20% of the P.B.C.-radio-
penicillin complex present in the lipid particles may be soluble, and indeed it
has been found that less material is precipitated after the second centrifugation
than after the first; thus 5–6% by weight of the bacteria appeared as lipid
particles (Table 2), but only 3–4% was left after one wash. Resuspension in
a larger volume of water (50 ml.) was sufficient to dissolve the lipid particles
completely, leaving no deposit on centrifuging.

The decrease in penicillin titre caused by lipid particles when resuspended
in distilled water, as determined by biological assay, agreed well with the
uptake of radiopenicillin. When the concentration of lipid particles was about
1 mg. dry wt./ml., about 25% of this biologically assayed P.B.C. was still
present in the supernatant after centrifuging for 1 hr. at 20,000 g. A similar
proportion of P.B.C. (about 30% of the total recovered) was present in the
supernatant fraction after rupture (see below). These data indicate that the
amount of P.B.C. present in the lipid particles which will dissolve in 1 ml. will
bind about 0.01 units of penicillin.

Decrease in penicillin titre caused by the supernatant fraction

In order to measure the amount of P.B.C. in the clear supernatant from
high-speed centrifugation after rupture in distilled water, standard penicillin
solutions were equally diluted in the supernatant and in distilled water,
placed at once on the assay plates and incubated. It was found that this
supernatant fraction decreased the titre of the penicillin solutions to an extent
which was dependent on penicillin concentration (Fig. 1). The straight-line
portion could be extrapolated to cut the vertical axis at 0.75 u. penicillin/g. dry
wt. of cytoplasmic contents (c. 0.01 u. penicillin/ml. supernatant). At low
penicillin concentrations only a small proportion of the penicillin which had
been added was still detectable, whereas this proportion was much larger at
higher penicillin concentrations. Fig. 1 suggested that the irreversible and
limited binding by the small amounts of soluble P.B.C. which were expected
to be present was superimposed on a different type of penicillin inactivation.
To see whether this was enzymic, in which case further incubation should
decrease the titre even more, the supernatant fraction was incubated with
penicillin for 6 hr. before adding to the assay cups. As the very small further
decrease in titre then observed was only of the same order as that of a control
in distilled water, it appeared more likely to be due to binding, perhaps by
protein, for example. If such were the case, the binding was reversible, as
estimation of bound radiopenicillin by removing excess radiopenicillin from
the supernatants by an electro-dialysis method (Few, Cooper & Rowley, 1953,
and unpublished results) gave a straight line of very much lower slope. As this
line passed through the origin no P.B.C. was detectable, and the P.B.C. must
therefore be considered to be dialysable. The rate of dialysis of the radio-
penicillin was also much slower in the presence of supernatant material than
in its absence, suggesting that the removal of the penicillin ions was being
hindered by a reversible binding.

It is interesting that the reversible binding by supernatant material is very
large compared with the irreversible binding by intact cells (Table 2). No reversible binding of a similar order by the intact cell was found in this present work or by Maass & Johnson (1949a, b) or by Rowley et al. (1950), using

Fig. 1. Decrease in penicillin titre caused by the supernatant fraction obtained by centrifuging ruptured suspensions of Staph. aureus (20 mg. dry wt./ml.) at 20,000 g for 30 min. The suspension was removed from the ballotini beads without dilution. 'Total penicillin available' represents the amount of penicillin inactivated if the penicillin titre was decreased to zero.

Table 2. Distribution of lipid P, P.B.C. and $^{35}$S (from radiopenicillin pre-treated staphylococci) in the three fractions obtained on mechanical rupture

P.B.C. and $^{35}$S are expressed as equivalent units of penicillin/g. dry wt. of fraction, lipid P as $\mu$ mole PO$_4^-$/g. Two values in a space indicate the range observed in several experiments. All percentages are in terms of intact cells.

<table>
<thead>
<tr>
<th></th>
<th>% dry wt</th>
<th>P.B.C.</th>
<th>35S</th>
<th>Lipid PO$_4^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells</td>
<td>100</td>
<td>1-2-1-7</td>
<td>1-2-1-7</td>
<td>32-2</td>
</tr>
<tr>
<td>Cell walls</td>
<td>20-28</td>
<td>0</td>
<td>0-2</td>
<td>&lt;0-01</td>
</tr>
<tr>
<td>'Lipid particle'</td>
<td>5-6</td>
<td>60-75</td>
<td>19-5</td>
<td>17-2</td>
</tr>
<tr>
<td>fraction</td>
<td></td>
<td>10-13-6</td>
<td>5-3</td>
<td></td>
</tr>
<tr>
<td>Supernatants</td>
<td>60-66</td>
<td>Probably</td>
<td>69</td>
<td>70</td>
</tr>
<tr>
<td>Ruptured in distilled water</td>
<td></td>
<td>30</td>
<td>1-6</td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>85-90</td>
<td>90-105</td>
<td></td>
<td>88</td>
</tr>
</tbody>
</table>

Ruptured in 'medium A'

<table>
<thead>
<tr>
<th></th>
<th>% dry wt</th>
<th>P.B.C.</th>
<th>35S</th>
<th>Lipid PO$_4^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact cells</td>
<td>100</td>
<td>2-2</td>
<td>1-5</td>
<td>32-2</td>
</tr>
<tr>
<td>Cell walls</td>
<td>33-40</td>
<td>60-75</td>
<td>75</td>
<td>63</td>
</tr>
<tr>
<td>'Lipid particle'</td>
<td>1-2</td>
<td>*</td>
<td>*</td>
<td>&lt;3</td>
</tr>
<tr>
<td>fraction</td>
<td></td>
<td>4-5-6</td>
<td>2-9</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Supernatants</td>
<td>60-70</td>
<td>*</td>
<td>12-5</td>
<td>0-3</td>
</tr>
<tr>
<td>Totals</td>
<td>95</td>
<td>60-75</td>
<td>88</td>
<td>87</td>
</tr>
</tbody>
</table>

* No suitable assay
biological and radioactive assay methods. Thus the reversible binding centres appear to be liberated on rupture. Reversible binding of penicillin G (Klotz, Urquhart & Weber, 1950) by bovine serum albumin, when expressed as u. penicillin/g. dry wt., is of a similar order to that of the supernatant fraction in the present case.

Correlation between distribution of penicillin-binding component from untreated cells, $^{35}$S from radiopenicillin pre-treated cells, and lipid phosphorus

Table 2 shows that P.B.C., $^{35}$S and lipid P are practically absent from cell walls prepared in distilled water, present in the lipid particles to a high concentration and in the supernatant fraction to less than or the same amount as in intact cells. The proportions of $^{35}$S closely followed the proportions of lipid P, and the proportions of P.B.C. are similar, although greater differences may be expected here since some extra P.B.C. is produced on rupture (see below) and the moderate stability of P.B.C. when in solution may differ from that of the lipid particles. A similar correlation was found in medium A, where the bulk of the P.B.C., $^{35}$S and lipid P were retained in the cell wall while only a small extra proportion of the dry weight was retained.

Differences in rupture products obtained from distilled water and medium A

In medium A the 'lipid particle fraction' was 1–2% as compared with 5–6% in distilled water (Table 2). In fact, the small sediment observed was not at all similar to the lipid particles from distilled water, being neither orange nor waxy, but white and opaque and resembling finely divided cell-wall material. The supernatants from medium A were practically colourless, whereas those from distilled water were yellow. On the other hand, the cell walls from medium A were orange, whereas those from distilled water were white.

Effect of lipid solvents

Refluxing for 1 hr. with methanol or pyridine, or standing in 90% (w/v) phenol in water removed the bulk of the $^{35}$S from radiopenicillin pre-treated cells. Refluxing dried organisms 3 times with 3 ml. MeOH for 5 min. (under $N_2$) destroyed or extracted all the P.B.C. from the cells, together with a considerable amount of lipid material. Unfortunately, whether destruction or extraction had occurred could not be decided as the extraction products interfered with the biological assay.

High recovery of penicillin-binding component after rupture in distilled water

Table 2 shows that, while the recoveries of material measured by dry weight, $^{35}$S and lipid P were 85–90%, the recovery of P.B.C. was always at least 90%. This was surprising since cell-free P.B.C. was found to be rather unstable, and 2–2½ hr. had elapsed between the start of rupture and the time of assay. Also, the uptake by lipid particles represented only that amount of penicillin remaining bound after one wash in distilled water, and as shown above P.B.C. is slightly soluble. This suggested that more P.B.C. was liberated on rupture than was available to the penicillin in the intact cells, an idea which was supported by the finding that the penicillin $^{35}$S bound by lipid particles
was 2–3 times as much per unit dry weight as the amount of $^{35}$S recovered in the lipid particle fraction from cells saturated with radiopenicillin before rupture (Table 2, cols. 3 and 5). When 0·3 u. radiopenicillin/ml. was present during cell rupture in distilled water instead of being added afterwards, twice as much radiopenicillin was bound by the lipid particles (i.e. 19–27 u./g. compared with 10–13 u./g.). Presumably the penicillin reacted with the P.B.C. immediately it was formed, thus avoiding any inactivation of the P.B.C.

The high recovery of P.B.C. was confirmed by pre-treating the cells with non-radioactive penicillin and removing the excess by thorough washing. The uptake of radiopenicillin by a sample of the intact cells was then less than 0·08 u./g., yet on rupturing the remainder the uptake by the lipid particles was 4–5 u./g., i.e. about the same as when radiopenicillin was added, and the excess removed, before rupture. The recovery of P.B.C. in the lipid particle fraction alone in this particular experiment, by radioactive assay, was then greater than 400% of that available to the penicillin in the starting material. These results indicate that considerably more P.B.C. is liberated on rupture of the cells than could react with the penicillin before rupture, and that P.B.C. is somewhat unstable during rupture.

**DISCUSSION**

The results presented above show that the component in staphylococci which binds penicillin is associated with a lipid fraction situated close to the cell wall of intact organisms, and suggest that this penicillin-binding component may itself be a lipid. However, this may not be the case, as, calculated on the basis of the lipid phosphorus, the lipid particles would only contain about 10% of material of the composition of lecithin. The lipid particles ('small particle fraction') prepared from a *Staphylococcus aureus* strain by Mitchell & Moyle (1951b) contained components other than lipids, some of which, as these authors pointed out, were very similar in composition to the cell-wall material which did not bind penicillin. They suggested that the small particle fraction was the lipid layer observed cytologically under the cell wall. Their analyses for this fraction were, in fact, consistent with 20–30% fat-soluble material, 30–70% of adhering cell-wall material and a few per cent of substances (e.g. nucleic acids) which were perhaps included from the soluble supernatant fraction, as the particles could not be washed.

Nevertheless, the association of P.B.C. with cell-wall lipid is very interesting in view of the fact that the component responsible for the osmotic properties of the cell, the 'osmotic barrier' (Mitchell, 1949a), must necessarily lie close to the cell surface, and is widely believed to be lipid in character (e.g. see Work & Work, 1948). The fraction associated with P.B.C. has both of these properties. If, therefore, in its initial rapid reaction with the cell the penicillin molecule inactivates some component of the osmotic barrier, then the metabolic defects whose appearance coincides with the addition of penicillin should be closely related to osmotic barrier malfunction. Many such relations appear to exist in the literature, so that it is worth while to consider those surface changes which begin immediately the penicillin is added.
(1) Effects of penicillin on the functions of an osmotic barrier. Gale & Taylor (1947) showed that viability and active amino-acid transport across the osmotic barrier diminishes in parallel fashion in cells growing in presence of penicillin. Hotchkiss (1950), with different bacteria and growth conditions, found that only a little glutamic acid assimilation was prevented by penicillin but that polypeptides were liberated into the medium. This effect was confirmed by Gale & Paine (1951). However, as penicillin inhibits Bacillus subtilis in a medium containing only inorganic N (Hunter & Baker, 1949), the cell’s disorganization is not limited to amino-acid uptake. A contrasting phenomenon, the effect of osmotic barrier destruction on penicillin uptake, is that loss of penicillin-binding capacity by intact cells and lipid particles proceeds simultaneously with cytolysis by a lipid-soluble substance such as phenol, whereas an anionic and a cationic detergent had no effect over the cytolytic range (Cooper, 1954).

(2) Effect of penicillin on surface properties of the cell. The cell surface is apparently synthesized in situ and is, therefore, either concerned in its own manufacture or is synthesized by the layer immediately underlying it, i.e. the lipid ‘cytoplasmic membrane’. (There is some cytological evidence of cell wall synthesis by the cytoplasmic membrane, e.g. Bisset, 1950.) Thus it is relevant to this discussion that penicillin added to growing cultures immediately slows the formation of a polyhydroxy phosphoric ester (XSP) which comprises a large proportion of the cell wall of most penicillin-sensitive, but not resistant, organisms (Mitchell & Moyle, 1950). Since the cell surface increases, because the cell also swells, it follows that either the cell wall becomes very much more elastic, perhaps due to loss of XSP, or that a very considerable amount of other cell wall components are produced to make good the deficiency of XSP. It may be a reflexion of this change that the suspension simultaneously loses its stability (Mitchell & Moyle, 1951a) and that there occurs a progressive drop in surface charge which starts very soon after addition of penicillin (McQuillen, 1950–1). The very small number of penicillin molecules attached (Rowley et al. 1950) would themselves contribute a negligible amount to the charge on the bacterial surface. Streptomycin causes an opposite type of effect (McQuillen, 1951) in that there is an immediate change in surface charge on addition of this compound to resting cells, corresponding to a reversible adsorption of it, but there is no further effect on growing cells. The effect of penicillin in this case is, therefore, not common to all antibiotics, as is, for instance, the formation of ‘giant forms’.

Another surface change reported is that a penicillin-resistant brucella organism lost the power to synthesize its surface capsule only when in presence of penicillin (Braun, Kraft, Mead & Goodlow, 1952). It is interesting that these surface changes presumably do not apply to the whole surface, as growth in penicillin does not affect the adsorption of phage on a sensitive staphylococcus (Elford, 1948).

(3) Changes in properties of the strain on adaptation or selection to penicillin resistance or sensitivity. When penicillinase production is not involved, changes in penicillin sensitivity are simultaneously accompanied by changes in many
properties. Among these are changes which appear to be related to osmotic or surface properties of the cells. For instance, highly resistant staphylococci lose ability to grow in 6.5% NaCl (Bellamy & Klimek, 1948; Klimek, Cavallito & Bailey, 1948), and resistant brucella mutants lose their smooth character (Braun et al. 1952). Gale (1947) found that an increase of, say, tenfold in penicillin resistance resulted in the glutamic acid assimilation system requiring tenfold the amount of penicillin to inhibit it, and the more resistant organisms were less able to assimilate glutamate (Gale, 1947). When relating changes in antibiotic sensitivities to the osmotic barrier, however, it must be remembered that any general change in cell permeability is likely to alter drug sensitivity.

In conclusion, the very small number of penicillin molecules bound by sensitive bacteria makes it seem probable that a very limited number of reactions, perhaps only one, may be governed by P.B.C. If this is so, the very diverse nature of the effects produced by penicillin in different organisms suggests that this reaction which is initially disorganized by addition of penicillin itself controls many diverse systems. The osmotic barrier in bacteria plays a vital part in the economy of the cell and it is shown above that there are many suggestions that the initial lesion caused by penicillin lies in the osmotic barrier. It is worth consideration, therefore, that the effects of penicillin in disorganizing, for example, nucleic acid metabolism (Mitchell, 1949b; Gale & Rodwell, 1948; Park & Johnson, 1949) may be due to the inability of the cell to assimilate, synthesize or retain some substance essential for such metabolism because of faulty functioning of the osmotic barrier.

I wish to thank Sir Alexander Fleming, F.R.S., for his continued interest in this work, and Dr D. Rowley for the help afforded by many useful discussions. Acknowledgements are due to the Central Research Fund, London University, for a grant for apparatus, and to Dr E. Lester Smith, of Glaxo Laboratories, for a generous gift of radio-penicillin G.

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REFERENCES


Penicillin binding and lipids


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