The Site of Action of Penicillin: some Properties of the Penicillin-binding Component of *Staphylococcus aureus*

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SUMMARY: The penicillin-binding component (PBC) of *Staphylococcus aureus* is rapidly inactivated by acid but is more stable at neutral or alkaline pH values. At 2° various preparations lost 25-50 % activity overnight at the optimum pH. Cell-free preparations of PBC (penicillin-binding cell walls or 'lipid particles') showed an initial increase in the amount of PBC available to penicillin. PBC was heat-labile, being completely destroyed by 5 min. at 50°. The final amount of penicillin bound did not vary over the range pH 4.4-7.6, but the rate of binding was somewhat greater at the lower end of this range. No separation of PBC from lipid particles was achieved by several mild techniques. Organic solvents which did not remove lipid material from intact cells also failed to affect PBC; more drastic solvent procedures which were effective in removing lipid appeared to destroy PBC. Intact cells or lipid particles from a penicillin-resistant yeast did not bind penicillin.

The most likely interpretation of the experimental finding (Rowley, Cooper, Roberts & Lester Smith, 1950) that penicillin-sensitive cells were able to bind a small definite amount of penicillin in a specific and irreversible manner was that they contained a small amount of a chemical entity which was termed the penicillin-binding component (PBC). It was thought that PBC was likely to be the initial site of action of penicillin, although direct proof of this could not be afforded. PBC might exist in the cell as an easily removable co-factor or it might be an integral part of a large molecule, and its isolation would then involve the difficult task of separating a very small amount of material from a large amount of similarly constituted impurities. Alternatively, it is theoretically possible that PBC cannot exist separately, but is rather a steric effect produced by the conjunction of two large molecules between which the penicillin happens to fit. Any attempt to separate the two large molecules would then lead to the disappearance of PBC. Nevertheless, it seemed worth while to attempt the preparation of PBC in a soluble form from which chemical purification could begin. The results given below describe some properties of PBC and the lipid-containing particulate fraction in which it is concentrated (Cooper, 1954), and illustrate some of the difficulties encountered in obtaining PBC in soluble form.

METHODS

Uptake of radiopenicillin by intact cells was measured by the method described by Rowley *et al.* (1950), and the preparation of, and measurement of uptake by, penicillin-binding cell walls were as described by Few, Cooper & Rowley (1952). Methods of growing the staphylococcus used, preparation of 'lipid particles' and measurement of penicillin-binding by lipid particles by

use of radiopenicillin and by bioassay were described by Cooper (1954). The radiopenicillin purity was checked by methods described by Cooper, Clowes & Rowley (1954).

RESULTS

Stability of penicillin-binding component

It was important to know to which experimental conditions PBC could be submitted without undue loss. The results illustrated in Fig. 1 show that PBC could be kept at neutral or alkaline pH values for several hours at room temperature even in the cell-free state, but was rapidly and irreversibly inactivated by acid. About 25 % of the PBC was lost from intact cells when kept

Fig. 1

Fig. 2

Fig. 1. Effect of pH value on stability of PBC in 0.1 M-phosphate + 0.1 M-acetate. Assays of the initial activity were made separately in each case. ●—●, intact cells, left for 16 hr. at various pH values and 2°, washed 3 times and adjusted to pH 7 before addition of radiopenicillin. ●—●, cell walls prepared in formalin mixture left for 45 min. at various pH values and 24°, washed twice and adjusted to pH 7 before addition of radiopenicillin. ○—○, unwashed lipid particles, left for 90 min. at various pH values and 24°, neutralized by addition of penicillin in an equal vol. 2 M-phosphate buffer at pH 7.5; binding determined biologically by cup-plate assay of residual unbound penicillin.

Fig. 2. Effect of time of standing in distilled water at 18° on the penicillin-binding capacity of cell walls prepared in formalin mixture. Radiopenicillin was added at various times, and the suspensions then kept in the cold until the end of the experiment, when all were centrifuged and washed together for ³⁵S assay.

overnight at 2° at the optimum pH value (pH 6). In one sample of lipid particles stored overnight at 2° in the presence of cell cytoplasm about 50 % of the initial PBC was lost. The curious suggestion (in Fig. 1) that the penicillin-binding ability of cell walls prepared in formalin (Few *et al.* 1952) had increased slightly during the course of the experiment was confirmed in several experiments; it always occurred during the 1–2 hr. following the completion of the formalin cell-wall preparation and then fell fairly rapidly (Fig. 2). A similar

increase in available PBC but without subsequent rapid loss also occurred with lipid particles which had been separated from the cytoplasmic supernatant; it proceeded at about the same rate at 0° as at 37° (Tables 1 and 2). This, with the lack of inhibition of the effect by formalin pretreatment, suggested that the increase was not enzymic. Approximately twice as much PBC is liberated on cell rupture as is available to the penicillin in the intact cell (Cooper, 1954). The final uptake reached in Table 1 corresponds closely with the uptake by lipid particles when inactivation of PBC during rupture is minimized by shaking in the presence of radiopenicillin. The ability of the lipid particles to regain their penicillin-binding capacity on standing suggests that this inactivation may be reversible.

Table 1. *Stability of PBC in unwashed lipid particle suspensions in distilled water at 0° and 37°*

At the beginning of the experiment about 2 hr. had elapsed from start of cell rupture. Penicillin was added (0.07 unit/ml.) at various times and the suspensions then kept at 0° until the end of the experiment. The residual penicillin concentrations were determined by bioassay.

Time of observation (hr.)	Amount of penicillin bound by particle suspension (unit/ml.)		Penicillin bound by lipid particles (unit/g. dry wt. particles)	
	0°	37°	0°	37°
0	0.008	0.008	6.4	6.4
1½	0.031	0.041	25	33
2	0.036	0.028	29	22
3	0.040	0.032	32	25

Table 2. *Effect of temperature on stability of PBC*

Experimental method as in Table 1.

Initially	Amount of penicillin bound by lipid particle suspension (unit/ml.)							
	After 3¼ hr. at			After 5 min. at				
	0°	24°	37°	40°	50°	56°	60°	90°
0.031	0.021	0.018	0.014	—	—	< 0.003	—	< 0.003
0.015	—	—	—	0.011	< 0.004	—	< 0.004	—

The apparent difference in rates of loss of PBC from penicillin-binding cell walls and from lipid particles is probably due to the several washes of the former involved in the assay of bound penicillin. Thus PBC may become progressively more easily washed away from cell walls on standing.

PBC was unstable at higher temperatures, none being detectable after 5 min. at 50° (Table 2). Freeze-drying had no effect on the PBC content of intact cells, lipid particles or formalin-prepared cell walls. The PBC-radiopenicillin complex remained attached to intact cells in aqueous suspension very much longer than PBC itself was detectable.

Effect of pH value on penicillin uptake

Fig. 3 shows that the final amount of penicillin bound by resting cells (20 min. total contact) was only a little affected between pH 4.4 and 7.6. Very similar curves were obtained when the penicillin solutions were adjusted to the various pH values for the same period of time and neutralized before addition of cells, showing that the higher uptakes at pH 3 and 4.4 were entirely due to radio-active decomposition products of penicillin. The lowest pH value at which instability of penicillin is small over the course of the experiment is about pH 4.4 (Brodersen, 1947), increasing greatly at lower pH values. However, although the final amount of uptake of radiopenicillin is independent of pH, the rate at which this value is attained is decreased with increase in pH value (Fig. 4). The effect of pH value on the rate of penicillin uptake is of interest in view of reports (Abraham & Duthie, 1946; Eagle, Levy & Fleischman, 1952) which indicated that penicillin is considerably more active at pH 6 than at pH 7.5. Since the minimal inhibitory titre may be the result of a balance between the rate of inactivation of PBC by penicillin and the rate of PBC resynthesis (Maass & Johnson, 1949*b*), any increase in the rate of inactivation of PBC caused by pH changes should result in this balance being struck at lower titres, since the rates of penicillin-binding at pH 5.5 and 7.3 are roughly proportional to penicillin concentration (Fig. 5). It can be calculated from Figs. 4 and 5 that penicillin at 0.025 unit/ml. and pH 7.5 should inactivate PBC as fast as at 0.017 unit/ml. and pH 6.0. The difference between these theoretical minimal inhibition concentrations is rather less than the two- or fourfold differences observed by Abraham & Duthie and by Eagle and colleagues over this pH range, so that changes in penicillin binding-rate are probably not the only effects of pH value on the lethal process caused by penicillin.

Some properties of the lipid particles

The lipid particle suspension could not be fractionated by any of several mild methods. Electrophoresis at pH 9.0, 7.4 and 6.0 gave single ascending and descending boundaries which became less distinct with time and rapidly moved at different rates towards the anode, suggesting a polydisperse system bearing a negative charge. PBC remained in the fast-moving fraction. The pH value of maximum precipitation (iso-electric point?) was between pH 3 and 4, so that fractional precipitation at lower pH values would involve too much loss of PBC to be practicable. Differential centrifugation of lipid particles over the range 8000–16,000 *g* produced no changes in the PBC or lipid P assays. A fairly constant amount of lipid particle was soluble in each ml. of distilled water but the saturated solution was too dilute to produce a visible boundary in the electrophoresis apparatus. Both lipid particles and PBC were completely precipitated by calcium ions and by freezing and thawing, but were unaltered in appearance by boiling. A preparation of *Clostridium welchii* α -toxin (containing the lecithinase C) in 0.01 M-CaCl₂, or 1 mg. crystalline ribonuclease or trypsin per ml. at 37° for 30 min. had no effect on PBC or on the lipid particles.

Fig. 3. Effect of pH value on the uptake of radiopenicillin on intact cells suspended at 5 mg. dry wt./ml. in 0.1 M-phosphate + 0.1 M-acetate. Three samples of cells were adjusted to pH values of 3.0, 4.4, 7.6 respectively, each sample was divided into five portions and differing concentrations of radiopenicillin was added to each portion. After 15 min. at 18° the cells were rapidly centrifuged and washed 3 times in water for ^{35}S assay.

Fig. 4

Fig. 5

Fig. 4. Effect of pH on rate of penicillin uptake. Six samples of cells were adjusted to various pH values in 0.1 M-phosphate + 0.1 M-acetate and radiopenicillin was added to 0.02 u./ml. at zero time. Half of each sample was rapidly centrifuged and washed after 30 min. at 18° (○—○), and the remainder after 60 min. (●—●), for ^{35}S assay.

Fig. 5. Effect of penicillin concentration on rate of uptake at pH 5.53 (○—○) and pH 7.35 (●—●). Cells were suspended in 0.1 M-phosphate + 0.1 M-acetate, the suspensions were adjusted to pH 5.53 or pH 7.35 and added to the prepared radiopenicillin dilutions. After 30 min. at 18° the samples were centrifuged rapidly and washed for ^{35}S assay.

Attempts to extract PBC from intact cells

At room temperature addition of ethanol, 5 % ethanol, ethyl ether, chloroform, acetone, 5 % phosphate (pH 9.0), 9 % urea or 10 % NaCl solutions to freeze-dried cells did not remove PBC; nor did adjustment of a thick cell suspension to pH 9, 10, 11 or 12 followed by neutralization of the separated extracts. Under these conditions, the organic solvents mentioned were also ineffective in removing lipid. Trichloroacetic acid (10 %, w/v), anhydrous formic acid, anhydrous pyridine, *cyclohexylamine*, 90 % phenol, hot methanol and anhydrous diethylene glycol rendered the cells incapable of irreversibly binding radiopenicillin, but no PBC (by bioassay; or by electro dialysis assay method, Few, Cooper & Rowley, 1953) was detected in the residues from extracts made with trichloroacetic or formic acids, *cyclohexylamine*, pyridine or 90 % phenol (dialysed or freeze-dried to remove solvent), with diethylene glycol (dialysed or ethanol-precipitated) or with hot methanol (distilled off *in vacuo*).

Treatment with 90 % phenol, followed by cold acetone and ether, did not extract any ^{35}S from cells grown in radiopenicillin and washed thoroughly, although all the lipid and lipid P was extracted. This suggests that the penicillin did not react with the lipid fraction of the lipid particles, although any solvent procedures which succeeded in extracting cell lipid (90 % phenol, pyridine, hot methanol, *cyclohexylamine*) also succeeded in destroying PBC.

Uptake of penicillin by yeast

The findings of Maass & Johnson (1949*a*) that penicillin was not bound by yeast have been confirmed in this laboratory with a strain of baker's yeast. Daniel & Johnson (1954) showed that cell-free extracts of *Saccharomyces carlsbergensis* bound penicillin in an apparently specific manner, but radiopenicillin uptake experiments in the present work did not reveal any irreversible binding of penicillin by the lipid particles of the baker's yeast used (i.e. less than 0.1 unit penicillin at 0.4 unit/ml.). Perhaps yeast species contain PBC within the cell and sheltered from penicillin, rather than at or near the surface as in *Staphylococcus aureus*. It is interesting that briefly heating the supernatant from centrifuged ruptured staphylococcal cell suspensions appears to destroy the 'reversible' binding by cell contents (Daniel & Johnson, 1954; Cooper, 1954).

DISCUSSION

It can be seen that considerable difficulties are involved in the separation of PBC in a chemically definable form. All attempts to prepare a soluble extract were unsuccessful except for a simple saturated solution of lipid particles which was rather dilute to handle chemically or electrophoretically. Attempts to concentrate this solution produced coagulation on freeze-drying or inactivation by heat. PBC is very unstable to heat and acid, and cannot be kept long even at low temperature and at the optimum pH. Accordingly, extraction procedures must not be used which require prolonged standing in the presence of water, e.g. dialysis or solvent precipitation.

The so-called 'lipid particles' contain a large amount of protein (Mitchell & Moyle, 1951) as well as about 20% of lipid material and PBC, and their behaviour as outlined above indicates their chemical constitution to include a sparingly soluble lipo-protein complex in which the fatty material is strongly bound. PBC also appears to be strongly bound, but it may be more closely connected with the protein part. This is perhaps to be expected if penicillin specifically interferes with some enzymic activity, although binding is not affected by trypsin. Daniel & Johnson (1954) also found that their PBC was not affected by trypsin. The effect of trypsin may thus be a useful method of distinguishing the specific binding by PBC from the non-specific binding present in supernatants from ruptured cell suspensions (Cooper, 1954).

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