Liberation of Surface-Located Penicillinase from *Staphylococcus aureus*

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1. Growth of *Staphylococcus aureus* (8325; α i⁻p⁺), constitutive for the production of penicillinase, in CY medium results in about 40% of the enzyme being free in the medium. By modifying the medium, 98% of the enzyme remains cell-bound.
2. Part of this is bound ionically to the surface of the cell wall and may be liberated instantaneously by certain inorganic anions. Maximum liberation was achieved with either phosphate or arsenate, both of which showed marked pH-dependence.
3. Polyanions that do not penetrate the cell wall, such as heparin, RNA and dextran sulphate, are also effective in liberating penicillinase. 4. Polyanions added to the growth medium prevent the appearance of ionically bound penicillinase owing to their strong affinity for the sites on the cell wall required for binding of the enzyme.

The process of the formation of extracellular enzymes is not clearly understood. Thus it is not yet known whether extracellular enzymes, and indeed other extracellular proteins, are formed on intracellular structures, such as polyribosomes located on the inside of the plasma membrane, through which they subsequently pass by some unknown mechanism to be liberated to the outside medium, or whether they are formed outside the permeability barrier of the cell membrane. In the latter case the only expected barrier to release from the cell might be the cell wall. Staphylococcal penicillinase is generally regarded as a truly extracellular enzyme according to the definition of Pollock (1962), although a high proportion of the total enzyme activity is intracellular. The production of penicillinase by *Staphylococcus aureus* is under the control of a plasmid-associated gene (Novick, 1963; Harmon & Baldwin, 1964), as is the degree of extracellularity of the formed enzyme (Novick & Richmond, 1965). With strain 8325 (α i⁻p⁺) grown in the CY medium of Novick (1963), approx. 40% of the total penicillinase is liberated into the growth medium. However, when this same strain is grown in a modified CY medium it has been found that more than 98% of the penicillinase formed is cell-bound.

As part of a programme of research into the formation and liberation of penicillinase by *S. aureus* (Coles & Gross, 1965*a,b*) the present investigations were started with the basic purpose of studying the pathway between the site of synthesis of exopenicillinase and its appearance free in the growth medium. It is therefore of considerable

importance to characterize the penicillinase that, although remaining cell-bound, may reside outside the plasma membrane. The present paper describes the properties of a fraction of penicillinase that is bound ionically to the surface of the cell wall.

MATERIALS AND METHODS

Organism. The strain of *S. aureus* (8325; α i⁻p⁺) (Novick, 1963; Novick & Richmond, 1965) used in this work was constitutive for the production of penicillinase and was kindly provided by Dr R. P. Novick. Cells were grown at 37° overnight with shaking in 11. Erlenmeyer flasks containing 240 ml. of Novick's CY medium or in the same medium modified by the omission of β -glycerophosphate and inclusion of 0.05 M-tris (pH 7.6). Cells, harvested by centrifugation, were washed once with distilled water and resuspended in water to give one-fifteenth of the original volume. For the release experiments described below, 1.0 ml. of this suspension containing approx. 45 mg. dry wt. of cells was rapidly mixed with 2.0 ml. of a solution of the ion to be tested (pH 7.6 unless otherwise stated), incubated for the prescribed time and centrifuged at 12000*g* for 10 min. at 0°. The supernatant was carefully pipetted off and assayed for penicillinase activity by the manometric method of Henry & Housewright (1947) at 37° in 0.017 M-NaHCO₃ in an atmosphere of O₂ + CO₂ (95:5). One unit of penicillinase is defined as the amount of enzyme capable of hydrolysing 1 μ mole of sodium benzylpenicillin in 1 hr. under the above conditions. This unit differs little in magnitude from that defined by Novick (1962), who measured activities at 30° and pH 5.8, the pH optimum for this enzyme. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Cell concentrations were determined at 700 m μ and dry weights estimated from a calibration curve.

Chemicals. Benzylpenicillin and heparin were commercial

products of these Laboratories, and chloramphenicol was obtained from Parke, Davis and Co., Sydney, N.S.W., Australia. The polyanions used were obtained from the following sources: sodium dextran sulphate 2000 (Pharmacia, Uppsala, Sweden); potassium polyvinylsulphate (K and K Laboratories Inc., Plainview, N.Y., U.S.A.); yeast RNA, sodium salt, and polygalacturonic acid (Koch-Light Laboratories Ltd., Colnbrook, Bucks.). The RNA and polygalacturonic acid (neutralized with NaOH) were extensively dialysed against distilled water and then freeze-dried before use. All other chemicals were of laboratory-reagent grade or better.

RESULTS

During growth of *S. aureus* for the production of exopenicillinase, it was observed that substitution of sodium β -glycerophosphate in CY medium by 0.05 M-tris (hereafter referred to as tris-CY medium) prevented almost completely the appearance of free penicillinase in the culture medium without affecting the total yield of cells. This was found not to inhibit the process of liberation, as addition of tris to normal CY medium caused no variation in the amount of penicillinase released, nor was the total yield of penicillinase changed. Sodium β -glycerophosphate, as such, was not essential for liberation of enzyme and could be replaced by the phosphate moiety of the molecule; glycerol had no effect.

The extent of liberation of penicillinase to the medium is a function of the phosphate concentration in the medium (Table 1), and the role played by phosphate in this release mechanism was therefore studied. Cells grown in tris-CY medium and washed with distilled water retained all their penicillinase and no loss occurred even during incubation in distilled water for long periods at 37°.

Table 1. Phosphate-dependent appearance of free penicillinase in overnight growth medium

S. aureus was grown for 16 hr. in 50 ml. Erlenmeyer flasks with shaking at 37° in 10 ml. amounts of tris-CY medium supplemented with Na₂HPO₄ (pH 7.6 with HCl) to the molarities indicated. Penicillinase determinations were performed on suitable samples of whole culture and supernatant.

Concn. of Na ₂ HPO ₄ (M)	Penicillinase (units/mg. dry wt. of cells)			Free penicillinase (%)
	Total	Free	Cell-bound (calc.)	
0.00	380	0	380	0
0.05	430	105	325	24.5
0.10	420	225	195	54
0.15	415	265	150	64
0.20	470	330	140	70
0.25	480	345	135	72

However, a portion of this cell-bound penicillinase was immediately liberated to the medium when such cells were incubated with various concentrations of sodium phosphate, as illustrated in Fig. 1. It must be pointed out that, in all experiments described here, penicillinase liberation was independent of further protein synthesis as shown by the insensitivity of this liberation process to 60 μ g. of chloramphenicol/ml., a concentration that completely inhibits penicillinase synthesis in *S. aureus* (Geronimus & Cohen, 1957). Fig. 1 shows that the lowest concentration of sodium phosphate causing maximal penicillinase release is approx. 0.15 M. At this concentration the amount of penicillinase released at 0 min. is independent of temperature between 0° and 37°, and release is usually, but not always, also independent of the time of incubation. The extent of penicillinase liberation achieved under these conditions compares very favourably (25–30%) with that obtained from the same quantity of cells grown in CY medium.

The above results strongly suggest that much of the penicillinase of *S. aureus* may be bound ionically to some cell structure. Table 2 shows that various anions will release bound penicillinase. No variation in the amount of penicillinase released under the above conditions was observed when K⁺ or Mg²⁺ was substituted for Na⁺. It therefore appeared that an ionic exchange between penicillinase and anions was taking place. Table 2 also shows that the anions at comparable ionic strength vary somewhat in their ability to liberate cell-bound penicillinase, so that the extent of ionic exchange that takes place is a property of the ionic species as well as of the

Fig. 1. Liberation of cell bound penicillinase from *S. aureus* grown in tris-CY medium by increasing concentrations of Na₂HPO₄ at 0 min. and 37°. Details were as described in the Materials and Methods section.

Table 2. Release of cell-bound penicillinase by anions

Cells were grown in tris-CY medium. Experiments were carried out at 0 min. and 25° as described in the Materials and Methods section. Higher bicarbonate concentrations could not be tested while maintaining the pH at 7.6.

	Anion	Final concn. (M)	Penicillinase liberated (units/ml.)
Expt. 1	Chloride	0.15	0
		0.3	130
	Nitrate	0.15	0
		0.3	540
	Sulphate	0.15	510
		0.3	2340
	Phosphate	0.15	2680
		0.3	2680
Expt. 2	Borate	0.15	1630
		0.3	1570
Expt. 3	Phosphate	0.15	3470
	Arsenate	0.015	420
		0.15	2810
	Bicarbonate-CO ₂	0.015	180
	Phosphate	0.015	490
		0.15	2880

Fig. 2. Effect of pH on the extent of liberation of penicillinase caused by phosphate, arsenate and borate. Cells were grown in tris-CY medium. Cell suspensions were mixed with 0.15M-phosphate, -arsenate or -borate at 25° then treated as described in the Materials and Methods section. The phosphate solutions were made by mixing appropriate quantities of equimolar Na₂HPO₄ and NaH₂PO₄; Na₂HAsO₄ solutions were adjusted to the desired pH with HCl; borate solutions of the appropriate pH were prepared from boric acid-borax mixtures. ○, Phosphate; ▲, arsenate; ●, borate.

concentration. With polybasic acids, the ionic species present in solution depend on the pH. Thus at pH 7.6, the pH at which the above studies were carried out, phosphate ions exist in the H₂PO₄⁻/HPO₄²⁻ ratio 13:87, whereas at pH 6.0 this ratio is 88:12. The liberation of penicillinase by phosphate, arsenate and borate as a function of pH is illustrated in Fig. 2. The activity curve for phosphate follows very closely that for the dissociation of the second H⁺, so that it would appear that the active phosphate ionic species is HPO₄²⁻. Both arsenate ions (H₂AsO₄⁻ and HAsO₄²⁻) show release ability, but free boric acid is without activity. However, there were several features of the process of liberation of penicillinase brought about by borate that distinguished it from that brought about by phosphate, as illustrated in Fig. 3. First, the process showed little dependence on the concentration of borate at the optimum pH, 8.0, between 0.01 and 0.7M. Liberation of enzyme by borate also increased with temperature between 0°

Fig. 3. Influence of time and temperature on liberation of penicillinase promoted by borate. Cell suspensions were incubated in 0.15M-borate, pH 8.0, at the following temperatures: ●, 0°; ○, 10°; △, 20°; ▲, 30°; ■, 37°. Samples removed at 10 min. intervals were treated as described in the Materials and Methods section.

and 37° and with time at temperatures between 10° and 37°. Suboptimum concentrations of phosphate (0.01M) also exhibited a time-dependent release of penicillinase, but at all concentrations of borate release of penicillinase depended on time and temperature.

Because of their small size the inorganic anions are likely to be freely diffusible, at least through the cell wall, and hence no definitive indication of the site of exchange with penicillinase can be obtained. A number of polyanions, of molecular weights ranging from 1.7×10^4 to 2×10^6 , have therefore been tested for their effect on cell-bound penicillinase. Table 3 shows that cells incubated in the presence of polyanions released a portion of their penicillinase to the medium. The amount of penicillinase released by the polyanions in each of several experiments was comparable with that released by the smaller anions previously tested, so that the site of penicillinase binding in *S. aureus* must have been accessible to these substances of high molecular weight.

At first glance the results obtained with the polyanions as represented by dextran sulphate appear to be at variance with those obtained by Rogers & Spensley (1955), who found that low concentrations of 'polyhydroquinone sulphonate' added to the medium almost completely inhibited the liberation of proteins, including coagulase and hyaluronidase from young cultures of *S. aureus*. We therefore undertook experiments along the lines of Rogers & Spensley (1955) with dextran sulphate, and also found that addition of this polyanion to tris-CY medium completely inhibited the appearance of ionically bound (liberated by 0.15M-

phosphate at 0min.) penicillinase on the cell wall, as shown in Table 4, with much less inhibition in the case of other proteins. The missing penicillinase was not in the culture supernatants. Similar results were obtained with polyvinyl sulphate as the inhibiting polyanion. The polyanions did not significantly affect the growth of the cells, making it unlikely that free passage of small metabolites into the cells was affected. Inhibition by dextran sulphate of the formation of penicillinase not liberated by 0.15M-phosphate does occur, but reaches a maximum at 15–20 $\mu\text{g./ml.}$, and concentrations as high as 5mg./ml. do not produce any further inhibition.

The preceding data must not be construed to indicate that only penicillinase is liberated by the conditions used. A variety of substances are known to be liberated by incubating *S. aureus* cells in distilled water (Salton, 1951). This liberation of material is greatly accentuated under the conditions used for penicillinase release. Prominent among high-molecular-weight substances found in cell-free supernatants were RNA and proteins (see Table 5), these latter far in excess of the small amount accounted for by penicillinase (calculated on a specific activity of 40 units/ $\mu\text{g.}$ for the purified enzyme; Richmond, 1963). The RNA released by

Table 3. *Liberation of penicillinase from S. aureus cells by polyanions*

Cells were grown in tris-CY medium. Cell suspensions were incubated at 25° for 0 to 5 min. with the polyanion, then treated as described in the Materials and Methods section.

	Anion	Concn. (mg./ml.)	Penicillinase liberated (units/ml.)
Expt. 1	Heparin	40	2840
	Polyvinyl sulphate	50	1740
	Phosphate (0.15M)	21	3185
Expt. 2	RNA	40	2140
	Phosphate	21	3050
Expt. 3	Dextran sulphate	40	1010
	Polygalacturonate	40	1480
	Phosphate	21	1440
Expt. 4	Polygalacturonate	30	2865
	Galacturonate (monomer)	30	90
	Phosphate	21	2880

Table 4. *Inhibition of formation of surface-located penicillinase and other proteins by dextran sulphate*

Cells were grown for 16 hr. in 50 ml. Erlenmeyer flasks with shaking at 37° in 10 ml. amounts of tris-CY medium containing dextran sulphate at the concentrations indicated. Penicillinase was determined on suitable samples of whole culture and supernatant. Cells were then centrifuged, washed once with 10 ml. of water, again centrifuged and the packed cells resuspended in 1.0 ml. of 0.15M-sodium phosphate. The cell suspension was immediately recentrifuged and the supernatant treated as in the Materials and Methods section. The amount of penicillinase in each culture supernatant was only 2–3 units/mg. dry wt. of cells. 'Intracellular' penicillinase amounts were calculated from: intracellular = total – released by phosphate – culture supernatant. All results are expressed per mg. dry wt. of cells. Protein was measured by the method of Lowry *et al.* (1951).

Concn. of dextran sulphate ($\mu\text{g./ml.}$)	Total penicil- linase (units)	Phosphate supernatant		Intra- cellular penicil- linase (units)
		Penicil- linase (units)	Protein ($\mu\text{g.}$)	
0	410	113	13.2	294
5	350	70	9.4	277
10	298	40	8.1	256
15	250	8	5.6	239
20	198	0	4.0	195
25	205	0	5.6	202

Table 5. *Analyses of washings of S. aureus cells*

Conditions were as for Table 2.

Wash solution	$E_{260}^{1\text{ cm.}}$ 960 m μ	$E_{260}^{1\text{ cm.}}$ 260 m μ	Protein ($\mu\text{g./ml.}$)	Penicillinase (units/ml.)
Expt. 1 Water	1.34	0.46	68	0
Phosphate (0.15M)	2.47	1.08	237	2025
Dextran sulphate (50 $\mu\text{g./ml.}$)	2.81	1.40	263	1210
Expt. 2 Water	1.01	0.43	77	0
Phosphate (0.15M)	1.49	0.64	213	1365
Dextran sulphate (50 $\mu\text{g./ml.}$)	2.46	1.30	250	1025

phosphate was identified by being made diffusible during treatment with ribonuclease. After purification by the method of Kirby (1956), and acid hydrolysis, it yielded the four main bases and ribose. Approx. 40% of the material with absorption maximum at 260 m μ was diffusible and more than 90% was not sedimented by centrifuging at 105 000g for 60 min., indicating that the RNA was not associated with ribosomes.

DISCUSSION

In any study of protein liberation from bacterial cells the possibility of cell lysis has to be considered. The conditions used in the present work are unlikely to cause lysis of staphylococcal cells because incubation times were short and conditions used for enzyme liberation mild. Moreover, prolonged incubation in distilled water with shaking failed to produce free enzyme. Salton (1951) found that much more severe conditions, such as incubation for up to 3 hr. in detergent, caused no appreciable lysis of staphylococcal cells.

Although the degree of extracellularity of penicillinase of *S. aureus* is believed to be under genetic control (Novick & Richmond, 1965) our results indicate that environmental conditions determine whether the exoenzyme is actually liberated from the cell. The fact that large amounts of penicillinase can readily be liberated by a variety of simple anions clearly indicates that under certain conditions staphylococcal exopenicillinase may remain ionically bound to the cell. That similar results to the above can be obtained with high-molecular-weight polyanions strongly suggests that this ionic binding takes place at, or very close to, the surface of the cell wall. The importance of the

cell wall for binding of the penicillinase of *Bacillus cereus* has been pointed out by Sheinin (1959). Mitchell & Moyle (1959) have stated that staphylococcal cell walls are impermeable to dextran of molecular weight 10⁴, so that even allowing for possible effects of the charge carried by these polyanions it is almost certain that those tested with molecular weights up to 2 $\times$ 10⁶ are unable to penetrate the cell wall.

The fact that polyanions are capable of displacing ionically bound penicillinase from the cell wall, and yet prevent the formation of this same enzyme fraction, can be readily explained on the basis of the strong affinity shown by these compounds for the staphylococcal cell wall (Rogers & Spensley, 1955) as follows. Exopenicillinase as it is formed in cells grown in tris-CY medium may become enmeshed in the cell wall at sites from which it can be displaced by anions. For the appearance of free exopenicillinase it is necessary that these sites be unoccupied by other substances. In contrast with the simple anions, polyanions permanently block these sites and prevent passage through the cell wall. It is not yet possible to say with certainty whether these sites at the cell wall are specific for penicillinase, but the results shown in Tables 4 and 5 indicate some degree of specificity. Although the appearance of ionically bound penicillinase was completely inhibited by dextran sulphate in the culture medium, that of other ionically bound protein was not. Though the polyanion-treated cell wall may be impermeable to penicillinase it is not so to all proteins. Similarly, phosphate is capable of liberating greater amounts of some proteins (at least penicillinase) than dextran sulphate, which, however, is the more effective anion for the liberation of total protein (Table 5).

It is apparent that potential exopenicillinase does not accumulate within the cells grown in the presence of dextran sulphate. Failure of exopenicillinase to find an unoccupied site in the cell wall may lead to inhibition, possibly by a mechanism of the feedback type, of synthesis of total penicillinase. Maximum inhibition is reached at the same dextran sulphate concentrations that cause complete cessation of the formation of surface-bound penicillinase. At this concentration, the depression of penicillinase synthesis is greater than would be observed if no surface-bound enzyme were formed, and the amount of intracellular penicillinase is therefore less than normal. Higher concentrations, however, do not decrease the intracellular fraction further. Thus the primary action of polyanions on the appearance of extracellular enzymes in *S. aureus* may be one of inhibition of liberation rather than of formation. There still remains the possibility that proteins such as exopenicillinase are formed outside the permeability barrier of the

plasma membrane (Pollock, 1962), in which case the polyanions might have a direct effect on formation. The presence of RNA, which is also liberated along with penicillinase, adds support to this possibility.

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