Influence of Organic Anions on the Liberation of Penicillinase from *Staphylococcus aureus*

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1. A further fraction of 'cell-bound exopenicillinase', in addition to that bound ionically to the surface of the cell wall, has been identified in *Staphylococcus aureus*.
2. Treatment of cells with low concentrations (0.01 M) of organic anions liberates part of this exopenicillinase, such liberation being dependent on both time and temperature, but independent of an external supply of energy.
3. Tricarboxylic acids tend to be more effective than dicarboxylic or monocarboxylic, although there are large differences between ionic species even when tested at the same normalities.

In the preceding paper (Coles & Gross, 1967) it was shown that *Staphylococcus aureus* cells grown under certain conditions retained all of their exopenicillinase and that part of this was bound ionically to the surface of the cell wall. In addition it was stated that occasionally there was a time-dependent increase in the amount of penicillinase liberated by phosphate. Investigations were therefore undertaken to determine whether there existed a portion of cell-bound penicillinase that required some metabolic process for its liberation. It is conceivable that this latter fraction of penicillinase might be actively transported across the plasma membrane.

MATERIALS AND METHODS

The ^{14}C -labelled protein hydrolysate from *Chlorella vulgaris*, of specific activity $600\ \mu\text{C}/\text{mg.}$, was obtained from The Radiochemical Centre, Amersham, Bucks. Samples for the measurement of ^{14}C incorporation were precipitated with 5% (w/v) trichloroacetic acid, heated at 90° for 30 min., collected and washed several times with 5% (w/v) trichloroacetic acid on DA Millipore filters. The filters were attached to planchets with rubber cement, dried and counted on a thin-window Nuclear-Chicago gas-flow counter.

Viable counts were performed in duplicate by plating 0.1 ml. portions of suitably diluted cell suspensions on nutrient agar and incubating overnight at 37° . The organisms and other materials and methods were as described previously (Coles & Gross, 1967), except that tris-CY medium was the growth medium used throughout.

RESULTS

The time-dependent increase in the amount of penicillinase liberated with phosphate (Coles &

Gross, 1967) is usually but not always considerably enhanced by the addition of glucose (Fig. 1). The rate of this reaction is also strongly dependent on temperature. Almost no increase with glucose occurs at 0° , whereas the rate at 37° is approximately double that at 22° . It should be emphasized that the addition of glucose alone to aqueous suspensions of *S. aureus* does not cause liberation of penicillinase, although a marked increase in respiration occurs.

The above results suggest that the observed time- and temperature-dependent liberation of penicillinase is linked with some metabolic event. There is now considerable evidence that ATP plays an essential role in the process of active transport of small metabolites (Scholefield, 1964) and it is probable that a similar requirement for ATP would exist for the active transport of proteins across membranes. Studies were therefore undertaken to determine if production of ATP was a prerequisite for the time- and temperature-dependent liberation of penicillinase.

Liberation by organic compounds. Other organic metabolites that might be expected to yield energy in the form of ATP were tested for their effect on the liberation of penicillinase from cells of *S. aureus*. The effect of some of these substrates is shown in Table 1. Not shown in Table 1, but tested at the same concentrations under the same conditions, were glucose, galactose, mannose, fructose, arabinose, ribose and glycerol, none of which promoted the liberation of penicillinase. This information and the data in Table 1 show that to stimulate the liberation of penicillinase the compounds must be ionized, and that in general the more ionic groups per molecule the greater their effectiveness in this reaction. Therefore other organic anions, not

Fig. 1. Liberation of penicillinase from *S. aureus* by phosphate and glucose plus phosphate. Cell suspensions were incubated at 37°, samples (1.0 ml.) were withdrawn at the times indicated and treated as described in the Materials and Methods section. ▲, Glucose (0.01 M); ●, phosphate (0.15 M); ○, glucose with phosphate.

Table 1. Liberation of penicillinase by organic anions

Incubation was for 20 min. at 37° with each compound at two concentrations.

Compound tested at ...	Liberated penicillinase (units/ml.)	
	0.01 M	0.05 M
Galacturonate	0	0
Glucuronate	0	39
Lactate	0	66
Propionate	166	204
Pyruvate	110	894
Formate	252	915
Benzoate	266	1244
Acetate	327	1324
Tartrate	0	157
Fumarate	375	786
Malate	517	1209
Succinate	788	1294
Oxalate	855	1109
Malonate	1312	1716
Maleate	1383	1754
Aconitate	1050	1774
Citrate	2082	1878

expected to furnish an increase in ATP production, were also included in this survey for their ability to liberate penicillinase, and these results are also in Table 1. It is obvious that the tricarboxylic acids tend to be more effective than the dicarboxylic, which in turn liberate more penicillinase than the monocarboxylic acids, but there are striking differences between ionic species even when compared at the same normality, e.g. lactate-acetate and tartrate-maleate. The release of penicillinase by such compounds as malonate and maleate indicates that the organic acids cause liberation by a process other than by increasing production of ATP.

Effect of anaerobiosis. As a further indication that the organic acids were not causing liberation of penicillinase by furnishing energy from oxidative metabolism, release experiments were performed under anaerobic conditions. An anabolic reaction known to be strongly dependent on a supply of ATP, namely the incorporation of amino acids into hot-trichloroacetic acid-insoluble material, was measured as a control. A representative experiment (Table 2) confirmed that, although *S. aureus* can derive and utilize glycolytic energy anaerobically from glucose in the presence of phosphate, the formation of ATP does not appear to be essential for liberation of penicillinase. Of the organic acids the greatest amount of penicillinase liberated under these conditions was obtained with maleate and

Table 2. Liberation of penicillinase and incorporation of ^{14}C from ^{14}C -labelled protein hydrolysate by *S. aureus* under anaerobic conditions

Incubations were carried out in a solution containing 18 amino acids at a concentration of $10\text{ }\mu\text{g./ml.}$ (L-isomer) at 37° in Warburg manometer flasks in $\text{N}_2 + \text{CO}_2$ (95:5); the total volume was 3.0 ml. Cell concentration was approx. 2 mg./ml. Substrates and $0.075\text{ }\mu\text{C}$ of ^{14}C -labelled protein hydrolysate were tipped from the side arm after temperature equilibration. After incubation 1.0 ml. was removed and precipitated for measurement of ^{14}C incorporation as described in the Materials and Methods section. The remainder was centrifuged and penicillinase assayed in the supernatant. All substrates were 0.01 M except phosphate (0.15 M).

Incubation for 60 min. with	Liberated penicillinase (units/ml.)	^{14}C incorporation (counts/min./ml.)
Water	0	15
Glucose + phosphate	100	728
Glucose	0	42
Malate	10	29
Citrate	45	18
Pyruvate	4	528
Maleate	45	32

citrate, neither of which supported the incorporation of ^{14}C -labelled amino acids into hot-trichloroacetic acid-insoluble material. A further indication that the extent of liberation was not directly controlled by the supply of energy was obtained by incubating under aerobic conditions with glucose and phosphate the same cell suspension as reported in Table 2. Under the latter conditions, although the incorporation of ^{14}C was approximately quadrupled to 2900 counts/min./ml., the amount of penicillinase liberated was unchanged at 100 units/ml. In all experiments measuring ^{14}C incorporation, the cell concentration was lowered to favour incorporation and facilitate the filtration of precipitates.

Effect of inhibitors. The fact that malonate promotes rather than inhibits the liberation of penicillinase strongly suggests that energy derived from the oxidation of the tricarboxylic acid-cycle intermediates is not essential for this process. 2,4-Dinitrophenol (0.2 mM), which gave up to 90% inhibition of the incorporation of amino acids into hot-trichloroacetic acid-insoluble material by washed suspensions of *S. aureus*, failed to influence the liberation of penicillinase caused by succinate, malate or citrate. Nevertheless, iodoacetate, as shown in Table 3, completely abolished the liberation of that fraction of penicillinase that is stimulated by glucose in the presence of phosphate, indicating that a metabolite derived from glucose beyond triose phosphate may participate in the liberation reaction. This is further substantiated by the fact that, in the presence of glucose, arsenate may quantitatively replace phosphate in the stimulated liberation reaction, although in this case no net gain of energy would be expected. Iodoacetate did not influence release of penicillinase by low concentrations of citrate and therefore appears to be without direct action on the liberation process.

In none of the experiments described in this paper was liberation of penicillinase dependent on further

protein synthesis, as shown by insensitivity of the process to 60 μg . of chloramphenicol/ml.

Effect of organic anions. The fact that of the compounds tested only those containing ionic groups promoted liberation of penicillinase and that this process appeared independent of a concomitant supply of energy prompted us to compare the process of liberation mediated by the organic acids with the purely ionic liberation as exemplified by phosphate and dextran sulphate at 0 min. (Coles & Gross, 1967). Unlike the latter, liberation promoted by the organic acids (10 mM) was strongly dependent on time as shown in Fig. 2. In contrast with that brought about by 0.15 M-phosphate, only minimal liberation occurred spontaneously with pyruvate, succinate, malonate and citrate (however, cf. liberation by borate; Coles & Gross, 1967). For further detailed studies, citrate was chosen as an example of an organic acid with strong releasing ability.

Increasing concentrations of citrate (0.05–0.1 M) liberated increasing amounts of penicillinase at 0 min., as shown in Fig. 3, higher concentrations having no further effect. The largest amount of penicillinase liberated spontaneously by citrate was the same as that liberated by 0.15 M-phosphate under the same conditions and no additional amount was released by a subsequent wash with 0.15 M-phosphate. Liberation by 0.15 M-citrate at 0 min. was also largely independent of temperature (Fig. 4). However, incubation at 37° for 30 min. caused

Table 3. *Inhibition by iodoacetate of glucose-stimulated liberation of penicillinase from S. aureus in the presence of phosphate*

Incubation was at 37° for 60 min.

Additions	Liberated penicillinase (units/ml.)	
	Phosphate (0.15 M)	Phosphate (0.15 M) + glucose (0.01 M)
Nil	540	1060
Iodoacetate (5 mM)	530	430

Fig. 2. Time-dependent liberation of penicillinase by organic anions. Cell suspensions were treated as in Fig. 1. ○, Citrate (0.01 M); ●, malonate (0.01 M); △, succinate (0.01 M); ■, pyruvate (0.01 M); ▲, phosphate (0.15 M).

Fig. 3. Penicillinase liberation promoted by increasing concentrations of citrate. Conditions were as in Fig. 1. ●, 0 min. incubation; ○, 30 min. incubation.

further liberation of penicillinase; this increase was strongly dependent on temperature (Fig. 4) and increased with increasing concentration of citrate from 0.05M to 0.15M (Fig. 3). A curious phenomenon, consistently observed in these experiments, was the slight inhibition of liberation of penicillinase with increasing concentrations of citrate between 0.01M and 0.05M.

The above results indicate that a type of binding other than the ionic at the cell wall may be involved in fixation of potential exopenicillinase to the cell. Evidence supporting this conclusion has been obtained by incubating cell suspensions with low concentrations of citrate for 30 min. at 37° and then treating them with high concentrations of phosphate. Table 4 shows the results of two such experiments in which the time-dependent liberation of penicillinase caused by citrate was followed on addition of phosphate by a further liberation, independent of time. The total amount of penicillinase thus liberated was in excess of that spontaneously liberated by phosphate alone, indicating that at least part of the fraction liberated by low concentrations of citrate is distinct from the surface-located ionically bound portion of penicillinase.

Cell lysis. Many electron micrographs taken of cells after a variety of liberation treatments described in this paper showed no increase in the number of damaged cells over the few found in untreated cultures. Viable cell counts performed before and after anaerobic incubation for 2 hr. in 0.01M-citrate or a mixture of 0.01M-glucose and

Fig. 4. Effect of temperature on liberation of penicillinase by 0.15M-sodium citrate. Cell suspensions were mixed with 0.225M-sodium citrate as in the Materials and Methods section at 0°, 22° and 37°; samples were taken immediately and after 30 min. incubation at these temperatures were treated as described in the Materials and Methods section. ○, 0 min.; ●, 30 min.

Table 4. Liberation of ionically bound penicillinase after incubation of cells in the presence of low concentrations of citrate

Cell suspensions were incubated at 37°. Those receiving a further treatment with 0.15M-phosphate were incubated for the 30 min., a portion was removed and pipetted into sodium phosphate solution to give a final concentration of 0.15M-sodium phosphate and immediately centrifuged. The supernatant was assayed for penicillinase and the results are expressed per ml. of original incubation mixture.

Cells treated with	Liberated penicillinase (units/ml.)	
	Expt. I	Expt. II
Phosphate (0.15M), 0 min.	1350	860
Citrate (0.01M), 0 min.	430	90
Citrate (0.01M), 30 min.	1160	680
Citrate (0.01M), 30 min., followed by phosphate (0.15M), 0 min.	1525	1230
Water, 30 min.	0	0
Water, 30 min., followed by phosphate (0.15M), 0 min.	1320	850

0.15M-phosphate showed good agreement. Thus the treatment to which the cells were subjected did not cause any alteration to cell structure greater

than the maximum compatible with the cell's normal processes of growth and reproduction' (Pollock, 1962). These considerations in conjunction with those already mentioned (Coles & Gross, 1967) make it unlikely that cell lysis contributed to the liberation of penicillinase into the supernatant.

DISCUSSION

The liberation of penicillinase described in this paper does not appear to be by a process involving active transport across the plasma membrane. This statement is based on the generally accepted belief that ATP plays an essential role in processes of active transport (Scholefield, 1964), and there is no evidence from the work described here for the involvement of ATP in penicillinase liberation. It is convenient to refer to the portion of penicillinase liberated as 'cell-bound exopenicillinase'. A fraction of cell-bound exopenicillinase distinct from that bound ionically to the surface of the cell wall is strongly suggested by: (i) the kinetics of liberation—the dependence of its liberation by organic anions on both time and temperature contrast markedly with the characteristics of purely ionically released enzyme; (ii) the quantitative aspects of the amounts liberated (Fig. 3 and Table 4). The existence of two separate pools from which penicillinase may be selectively liberated is therefore indicated. Penicillinase bound ionically to the cell wall represents one of these pools. The fixation of the anatomical site of the second pool is much less certain. If it is possible for penicillinase to be passively transported across the plasma membrane to the medium, then we have no indication as to the location of this pool. However, if passage of penicillinase across the membrane is by processes involving active transport, then this process must have preceded our experiments and the second pool is fixed as being outside the permeability barrier of the cell membrane.

It is tempting to suggest that the mesosomal

membrane forms part of the site from which penicillinase is liberated by organic anions. The outside surface of this structure, being continuous with the plasma membrane, is in direct contact with the periplasm, and liberation of penicillinase from this site should not require the expenditure of large amounts of energy. This surface has been shown to be brought into even closer contact with the periplasm by the treatments described (Beaton, 1965). Mesosomal structures have been implicated in the formation and externalization of exoenzymes in *Bacillus* spp. (Lampen, 1965).

Although the compounds active in liberation from this second pool have all been shown to be ionic, the features of the release do not seem to be those of a purely ionic exchange e.g. time- and temperature-dependence, but rather those of an enzymic process. The anions may then play the role of an enzyme activator of such a process. This possibility is supported by work from Pollock's Laboratory, where it was shown that penicillinase attached to membranes of disrupted protoplasts of *Bacillus licheniformis* could be liberated by trypsin (Kushner & Pollock, 1961; Pollock, 1961). It was concluded that an enzymic process is involved in the normal liberation of penicillinase in this organism.

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