THE SITE OF ACTION OF PENICILLIN

III. EFFECT OF SURFACE-ACTIVE SUBSTANCES ON PENICILLIN UPTAKE BY *STAPHYLOCOCCUS AUREUS*

by

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During attempts to extract that component of penicillin sensitive staphylococci which is capable of binding the drug[1] it was observed that 30 mg/ml aqueous phenol solutions were capable of preventing penicillin uptake. As the penicillin binding component (PBC) is associated with a lipid fraction close to the cell wall in intact staphylococci[2], the effects of detergent substances on such a fraction is of interest in view of the disorganising effects of detergents on that fraction of the cell responsible for retaining cell solutes[3,4,5], which must certainly lie close to the cell surface and is widely believed to have lipoidal properties (see for example WORK AND WORK[6]).

METHODS

The method for determination of radiopenicillin uptake was described by ROWLEY *et al.*[1], and growth conditions, preparation of "lipid particles" by mechanical rupture of the cells and measurement of radiopenicillin uptake by these particles were described by COOPER[2]. The effect of detergents was determined by addition of detergent to the concentration indicated, after which cells were centrifuged once and resuspended in water. Samples were taken for viable counts and radiopenicillin was added to the suspensions. The supernatant after centrifuging was assayed for total inorganic phosphate by the method of FISKE AND SUBBAROW[7] and the amount of phosphate liberated was taken as an indication of the cytolysis which had occurred. To determine its effect on penicillin uptake by lipid particles, phenol was added directly to the suspension of lipid particles in the "cytoplasmic fluid" remaining after rupture of the cells in distilled water and removal of cell walls by centrifuging. Radiopenicillin was added, and the uptake of radiopenicillin was measured as described[2]. Viable counts were made by the method of MILES AND MISRA[8]. The purity of the radiopenicillin was ascertained by the method described by COOPER, CLOWES AND ROWLEY[9]. Phenol used was AR crystalline and dioctyl sodium sulphosuccinate (Aerosol OT), cetyl trimethylammonium bromide (CTAB) and Tween 80 were the commercial products.

RESULTS

Effect of phenol on penicillin uptake

In the experiments shown in Fig. 1 no cells were killed up to 3 mg phenol/ml but all cells were dead at 6 mg/ml. It can be seen that "cytolysis" (*i.e.* liberation of inorganic phosphate) appears to start only when all the cells are killed, although in fact the viable count is measured after a much longer time of contact with phenol than cytolysis or uptake, as there is presumably phenol still attached to the cells during the incubation period. Thus the process of death proceeds to completion while cytolysis is measured

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after only one hour. Cytolysis and prevention of penicillin uptake proceed at a measurable rate over concentration ranges which are identical (*i.e.* 5–20 mg/ml). As cytolysis increases, the uptake by intact cells and by lipid particles decreases (phenol added *after* rupture). Penicillin ^{35}S, once attached, is not removed by all concentrations up to 50 mg/ml phenol. Attempts to recover PBC from 30 mg/ml phenol extracts by freeze-drying were not successful. (PBC was estimated by addition of the extracts to penicillin solutions, which were then compared with known standards by a plate assay procedure. No inactivation of penicillin was found to occur.)

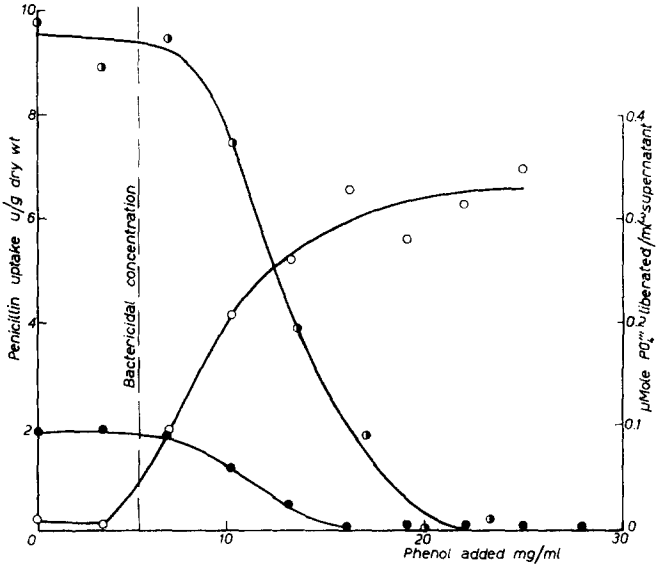

Fig. 1. Effect of different phenol concentrations on penicillin uptake by lipid particles (◑—◑) and by intact staphylococci (●—●), and on PO_4^{-3} liberation (○—○). Cells at 3 mg/ml were contacted with phenol at 18° for 1 h, centrifuged, the supernatant was removed for PO_4^{-3} assay, the cells were resuspended in distilled water for viable counts, and radiopenicillin was added. Cells were centrifuged after a further 30 min and assayed for radioactive uptake.

Phenol, in concentrations which completely prevented penicillin uptake, had no effect on the naked eye appearance of lipid particles when sedimented, *i.e.* they still appeared as an orange homogeneous translucent pellet of the same size. This suggests that, although phenol disorganises the osmotic barrier, this is not due to gross solubilisation of lipid material. Cells treated with phenol before rupture gave poor yields of whitish lipid particles, not well defined.

The inhibiting effect of 30 mg/ml phenol on penicillin uptake by intact cells appeared to be complete within the 10 minutes required for assay, and up to at least 45 mg dry wt. cells/ml. There was some indication that the reaction proceeded more slowly around 10 mg phenol/ml. MITCHELL AND MC QUILLEN[10] reported that cytolysis by phenol at 10 mg/ml required about one hour for completion.

Effect of dioctyl sodium sulphosuccinate (Aerosol OT) on penicillin uptake

The changes in Aerosol OT (Fig. 2) differ from those in phenol in that death and cytolysis start at lower concentrations and proceed simultaneously, although 90% death

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again occurs at much lower concentrations than 90% cytolysis. The logarithm of the viable counts falls off linearly with concentration. In contrast with phenol, even when 99.9% of the cells are killed and cytolysis is perhaps 90% complete, penicillin uptake is decreased only by 10–20%. No ^{35}S from radiopenicillin-pretreated cells is removed at 2 mg/ml Aerosol OT.

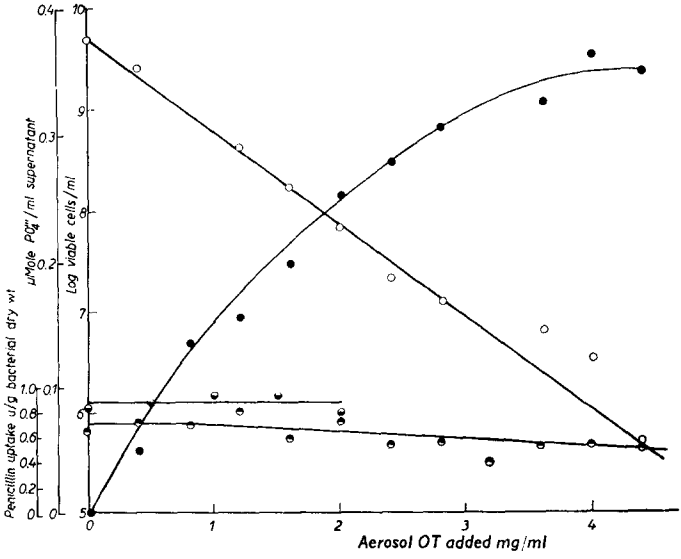

Fig. 2. Effect of different Aerosol OT concentrations on penicillin uptake by intact staphylococci (○—○), PO_4^{-3} liberation (●—●), viability (O—O) and ^{35}S remaining on radiopenicillin pretreated cells (◒—◒). Treated as for phenol, except that 5 mg dry wt. cells/ml were contacted with Aerosol for 2 h.

Effect of Tween 80 (polyoxyethylene sorbitan mono-oleate) on penicillin uptake

Penicillin uptake was not affected by Tween 80 up to 20 mg/ml but was reduced from about 2.2 u/g dry wt. at 0–20 mg Tween 80/ml to 1.75 u/g at 50 mg/ml. This slight drop in uptake at 50 mg/ml may be due to small quantities of oleic acid present as an impurity, functioning as an anionic detergent in a similar manner to Aerosol OT.

Effect of cetyl trimethylammonium bromide (CTAB) on penicillin uptake

In increasing CTAB concentrations penicillin uptake increases gradually until the cells agglutinate and are all killed, when very much more penicillin becomes attached (Fig. 3). Penicillin ^{35}S attached before contact with CTAB is little affected. Of the high radiopenicillin uptake at high CTAB concentrations (Fig. 4), some is prevented by addition of small amounts of non-radioactive penicillin after the CTAB but before the radiopenicillin, and is therefore irreversibly attached. This irreversible uptake is roughly independent of concentration. The remainder of the uptake is not prevented by ordinary penicillin and is proportional to penicillin concentration, and is presumably reversibly bound. This may well be due to radiopenicillin which has not been sufficiently washed out, as the agglutinated suspensions are very difficult to wash by centrifuging.

It can be calculated from SALTON's[5] data that the concentration of detergent added will be 2–3 × that of the true concentration of CTAB present in the supernatant at

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equilibrium due to the high binding of detergent by the cells. Bearing this in mind, there is a coincidence between the added CTAB concentrations where penicillin uptake increases sharply and the calculated supernatant CTAB concentration where (a) the surface properties of the cells change sharply (seen from agglutination, and found by electrophoresis by McQUILLEN[11]), (b) saturation of the cells with CTAB becomes

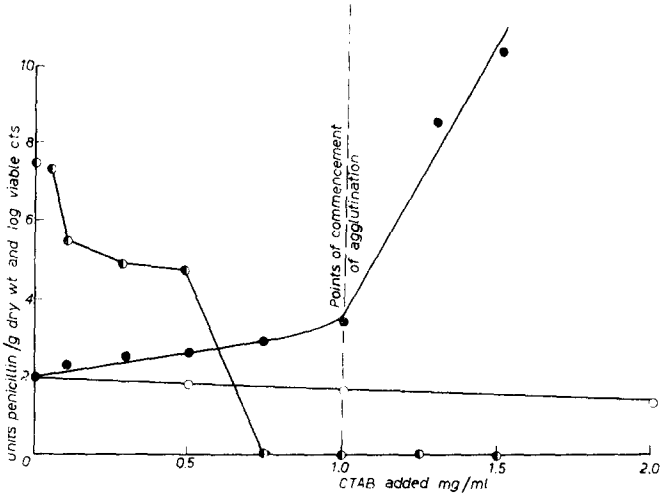

Fig. 3. Effect of different CTAB concentrations on penicillin uptake on intact staphylococci (●—●) viability (◐—◐), and ^{35}S on radiopenicillin pretreated cells (○—○). Treated as for phenol.

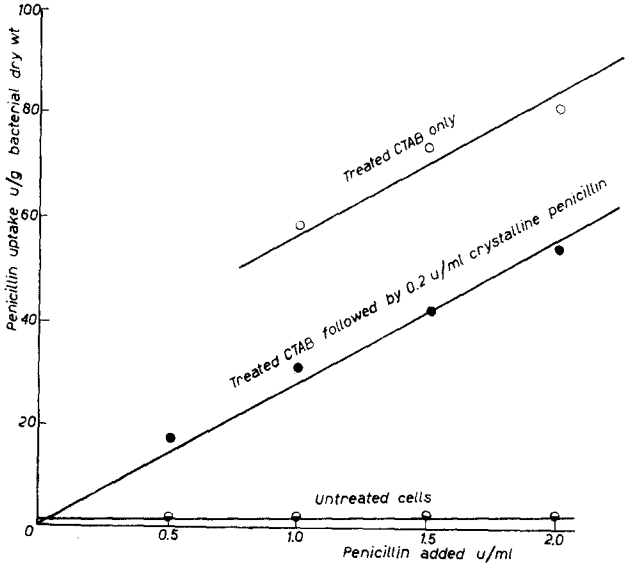

Fig. 4. Uptake of radiopenicillin on CTAB-treated staphylococci. Of 12 tubes containing cells at 3 mg/ml, 8 were treated with CTAB added to 2 mg/ml for 30 min, all 12 were centrifuged, and the cells resuspended in distilled water. Ordinary crystalline penicillin (0.2 u/ml) was added to 4 of the CTAB-treated tubes, all tubes were centrifuged again after 20 min and resuspended in distilled water. Radiopenicillin (0.5, 1.0, 1.5 and 2.0 u/ml respectively) was then added to each of the tubes in the three sets of four and the cells were re-centrifuged after 30 min for assay of ^{35}S uptake.

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maximal[5] and (c) micelles begin to form[12,13]. This coincidence suggests the probability that the high irreversible penicillin uptake in CTAB is due to a strongly adsorbed salt formation between penicillin and those CTAB molecules which may be secondarily adsorbed as micelles onto the primary monolayer of adsorbed CTAB.

DISCUSSION

The four types of detergent substances examined above each appear to cause different effects upon penicillin uptake by staphylococcal cells. Tween 80, electrically neutral and lipid insoluble, is not very cytolytic or bactericidal and so it is perhaps not surprising that penicillin uptake is not affected. Phenol, also a neutral detergent yet lipid soluble, causes a marked drop in penicillin uptake by intact cells and by lipid particles which exactly corresponds with cytolysis, while Aerosol OT, negatively charged and lipid insoluble in the usual sense, causes little effect on penicillin uptake over the cytolytic and bactericidal concentrations. CTAB, on the other hand, causes a marked increase in uptake of penicillin ^{35}S but not until concentrations have been reached where cytolysis may be presumed to be complete (SALTON[5]). Over the cytolytic range the uptake is increased less markedly, but no decrease in PBC similar to that with phenol was noticed.

As PBC was not recovered from phenol extracts, and no penicillin ^{35}S once attached was removed by any detergent, it appears likely that PBC is not removed from the cells by phenol but rather is blocked in some way. Thus although phenol and the ionic detergents all disorganise the osmotic barrier, the former prevents penicillin reacting with PBC during cytolysis of the cells while the latter do not. Some evidence has been presented[2] to suggest that PBC was actually associated with the osmotic barrier in that (a) it was in a lipid fraction close to the cell wall, and (b) some metabolic disturbances which occurred soon after contact with penicillin were cell wall phenomena. The coincidence of the prevention by phenol of penicillin uptake and of the destruction by phenol of the osmotic barrier affords further evidence in support of this idea. It also suggests a fundamental difference between the reactions of phenol and ionised detergents with the osmotic barrier, which may perhaps be expected from the ability of phenol to dissolve completely, and not just at the interface, in lipid bodies.

However, since it is not yet possible to isolate a moiety clearly recognisable as "the osmotic barrier", as is possible with the cell wall for instance, an association of penicillin mode of action with the osmotic barrier can only be assumed from indirect evidence. Several pieces of indirect evidence are available, and it remains to be seen whether sufficient further information on the properties of the osmotic barrier is forthcoming to make it seem more certain that such an association exists.

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SUMMARY

Phenol prevented penicillin uptake by *Staphylococcus aureus* whole cells and "lipid particles" at the same concentrations in which cytolysis and cell death occurred. Tween 80 had little effect on penicillin uptake. Aerosol OT caused only a small decrease in penicillin uptake over the concen-

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tration range in which cell death and cytolysis were maximal, but CTAB caused an increase in penicillin uptake which was especially marked above the agglutination level. No penicillin ^{35}S was removed from the cells by detergents once the penicillin had been attached. The relationship of these findings with the hypothesis that penicillin reacts initially with the osmotic barrier in *Staphylococcus aureus* is discussed.

RÉSUMÉ

Le phénol, à des concentrations qui provoquent la cytolyse et la mort des cellules, empêche l'absorption de la pénicilline sur les cellules entières de *Staph. aureus* et sur les "particules lipidiques". Le Tween 80 a peu d'influence sur l'absorption de la pénicilline. L'aérosol OT diminue peu l'absorption de la pénicilline dans les limites de concentration pour lesquelles la mort et la cytolyse des cellules est maximum, mais CTAB augmente l'absorption de la pénicilline surtout au-dessus du seuil d'agglutination. Quand de la pénicilline marquée par ^{35}S est fixée sur les cellules, des détergents ne peuvent la déplacer. Les auteurs rapprochent ces résultats de l'hypothèse selon laquelle la pénicilline réagirait d'abord avec la barrière osmotique de *Staph. aureus*.

ZUSAMMENFASSUNG

Phenol verhindert die Aufnahme von Penicillin der ganzen Zellen und "Lipoidteilchen" von *Staph. aureus* bei den gleichen Konzentrationen bei denen Cytolysis und Zelltod auftritt. Tween 80 hatte auf die Penicillinaufnahme geringe Wirkung. Aerosol OT verursachte nur geringe Verminderung der Penicillinaufnahme in dem Konzentrationsbereich in dem Zelltod und Cytolysis ihre Maximalwerte erreichten, CTAB jedoch verursachte einen Anstieg der Penicillinaufnahme, der besonders oberhalb des Agglutinationsniveaus zu bemerken war. Netzmittel entfernten kein Penicillin ^{35}S von den Zellen, wenn Penicillin erst einmal anhaftete. Die Beziehung dieser Entdeckungen zu der Hypothese, dass Penicillin anfangs mit der osmotischen Schranke in *Staphylococcus aureus* reagiert wird besprochen.

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