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THE RELATION BETWEEN FIXATION OF PENICILLIN SULPHUR AND PENICILLINASE ADAPTATION IN *B. CEREUS*.

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IT was recently found (Pollock, 1950) that cells of *Bacillus cereus* reacted to treatment with penicillin for 1 hr. at 0°, by rapidly forming penicillinase when subsequently incubated in a penicillin-free casein hydrolysate medium. During incubation the rate of penicillinase production was approximately constant, and depended upon the penicillin concentration used for pre-treatment up to 1·0 unit/ml., at which level maximal effect occurred. These facts suggested that the effect of the penicillin might be due to its interaction with some specific receptor within the cell to form a complex or compound functioning as some sort of specific catalyst whose concentration determined the rate of penicillin formation.

Pasynskii and Kastorskaya (1947) reported that very small quantities of penicillin (amounting to about 1000 molecules/cell) were absorbed by *Staphylococcus aureus* growing in penicillin broth. Later, Maass and Johnson (1949*a*) and Rowley, Cooper, Roberts and Lester Smith (1950) showed that minute quantities of S (corresponding to a penicillin fixation per cell of the same order as that reported by Pasynskii and Kastorskaya were irreversibly fixed by *Staph. aureus* cells when treated with a very low concentration ($<$ 0·1 unit/ml.) of S^{35}-labelled penicillin.

The work here reported was therefore undertaken in order to see whether penicillin S was similarly fixed by *B. cereus* ; and if so, whether any correlation could be found between the extent of such fixation and the rate of penicillinase production—as might be expected were the two phenomena interrelated.

METHODS.

Organism : *B. cereus* NRRL 569.—Washed suspensions were grown in tryptic meat broth from spore inocula for 16 hr. at 35° on a bacteriological shaker.

Penicillin.—Ordinary sodium benzylpenicillin was obtained from Glaxo Ltd. Purified S^{35}-labelled penicillin containing 0·16 μc/unit and prepared by growing *Penicillium chrysogenum* in a synthetic medium containing radioactive sulphate was kindly given us by Dr. Lester Smith of Glaxo Laboratories. Benzylpenicillin

constituted over 85 per cent of the total penicillin in this radioactive product and non-penicillin radioactivity amounted to about 5 per cent of the total.

Bioassay of penicillin was carried out by measuring zones of growth inhibition of a sensitive strain of *Bacillus subtilis* around cylinders containing penicillin solutions on a 0·3 per cent Lab Lemco, 1 per cent peptone, 2 per cent agar base (containing about 10^5 *B. subtilis* spores/ml.) poured into large square plates (10 in. × 10 in.). Two concentrations of standard Penicillin G and of the unknown were tested in octuplicate on the same plate. The solutions were allowed to diffuse out into the medium at + 2° for 4 hr. before incubation (16 hr. at 35°) in order to increase the sensitivity of the assay. The mean values for inhibition zone diameters and thus the potency ratio, with limits of probable error, of the unknown to the standard were then calculated.

Penicillinase activities were assayed by the manometric method of Henry and Housewright (1947), and refer to the total enzyme (combined extra- and intracellular fractions) present in the culture.

Penicillinase production was followed in 0·8 per cent casein hydrolysate medium (CH) with M/600 $MgSO_4$ and M/50 phosphate buffer, pH 7·0, aerobically, at 35°, samples being removed at intervals and M/1200 oxine added to stop further enzyme production (as described by Pollock, 1950). The only modification was the addition of 1 per cent gelatin to the medium, since it had been shown that this stabilized the enzyme against possible inactivation before assay.

Radioactivity measurements were done with an end-window (1·9 mg./sq.cm.) Geiger-Müller Counter. Thick washed suspensions of cells for counting were suspended in water to about 50 mg./ml. About 0·2 ml. was added to 2 mm. deep, 15 mm. diameter rimmed polythene discs (previously weighed) so that the fluid was just level with the top of the rim, thus avoiding uneven deposition of cells. The samples were then frozen as rapidly as possible on dry ice, dried *in vacuo*, pressed down evenly on the discs with a metal plunger, re-weighed and counted. The average weight of dried cell samples was 10 mg. Corrections for self-absorption were made by means of a curve prepared by mixing 0·02 unit of radioactive penicillin with different weights of cells (ranging from 4·3 mg. to 13·6 mg.), freeze-drying and counting in a similar manner.

Penicilloic acid, from radioactive penicillin, was prepared by bringing a solution of 50 units/ml. to pH 12, heating at 100° for 30 min., leaving at room temperature overnight and then neutralizing to pH 7·0. Bioassay showed that no detectable biological activity (i.e., less than 0·1 per cent penicillin) remained. Concentrations are expressed in terms of the penicillin solution from which it was derived, as units-equivalent/ml.

S fixation by cells was followed under conditions previously used for pre-treatment of cells for penicillinase adaptation experiments (Pollock, 1950)—viz., in M/100 buffer at 0°, usually for 1 hr., followed by 3 washings. The only variation from the previously standardized technique was that cells were treated at a concentration of 2 mg./ml. instead of 1 mg./ml., in order to economise in penicillin.

RESULTS.

Effect of penicillin concentration on S fixation.

Fig. 1 shows the amount of S fixed by the cells after treatment for 1 hr. at 0° with different concentrations of radioactive penicillin followed by 3 washings.

The general form of the curve is not dissimilar to that found for penicillin-sensitive *Staph. aureus* by Rowley *et al.* (1950). There is a similar disproportionately high uptake at low concentration of penicillin, and the total amount of S bound/mg. cells is of the same order. There is a fairly definite point of inflection in the curve at about 1 unit/ml., above which the amount of S fixed is directly proportional to the concentration of penicillin used for treatment. The shape of the curve suggests that there are two types of S-fixation processes occurring: (*a*) a reaction with some receptor which becomes saturated at about 1·0 unit/ml. and will therefore take up no more S from penicillin above that concentration, and (*b*)

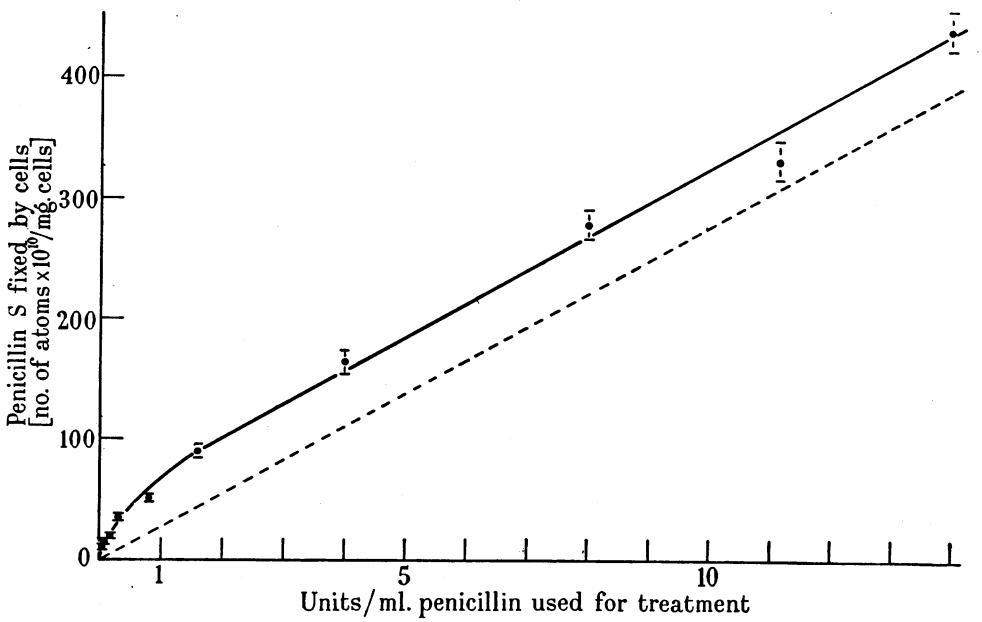

FIG. 1.—Fixation of S by *B. cereus* cells treated with S^{35}-labelled penicillin for 1 hr. at 0° followed by 3 cell washings. Limits of counting error are shown for P = 0·05. An interrupted line has been drawn through the origin parallel to the linear portion of the curve, to indicate the amount of " non-specific " S-fixation (see text).

fixation on to some other cell material which shows no sign—up to a level of 14 units/ml., at least—of being limited with respect to the amount absorbable.

That such an interpretation is reasonable is supported by results shown in Fig. 2 where the S-uptake curve from penicillin is compared with that obtained, under similar conditions, from equivalent concentrations of penicilloic acid. S-fixation from penicilloic acid is directly proportional to the concentration with which the cells are treated over the whole range tested, and is of the same order as the " non-specific " fraction of S-fixation from penicillin. The remaining S bound from penicillin is presumably specific for that compound and is of particular interest, since the fixation reaction appears to be complete at a level of about 1·0 unit/ml.—the concentration at which maximal effect on penicillinase production rate has already been shown to occur.

Errors inevitably associated with such fixation experiments do not allow a

very accurate estimation of the penicillin concentration at which maximal specific S-fixation occurs. But the curves illustrated in Fig. 1 and 2 have now been repeated 8 times with consistent results; and it is reasonable to put the probable level at between 0·5 and 2·0 units/ml.

Specific fixation of Penicillin S.

Fig. 1 and 2 show that 2·5 $\times$ 10^{11} atoms of S/mg./unit/ml. are fixed "non-specifically" from penicillin. If this quantity is subtracted from the curve in

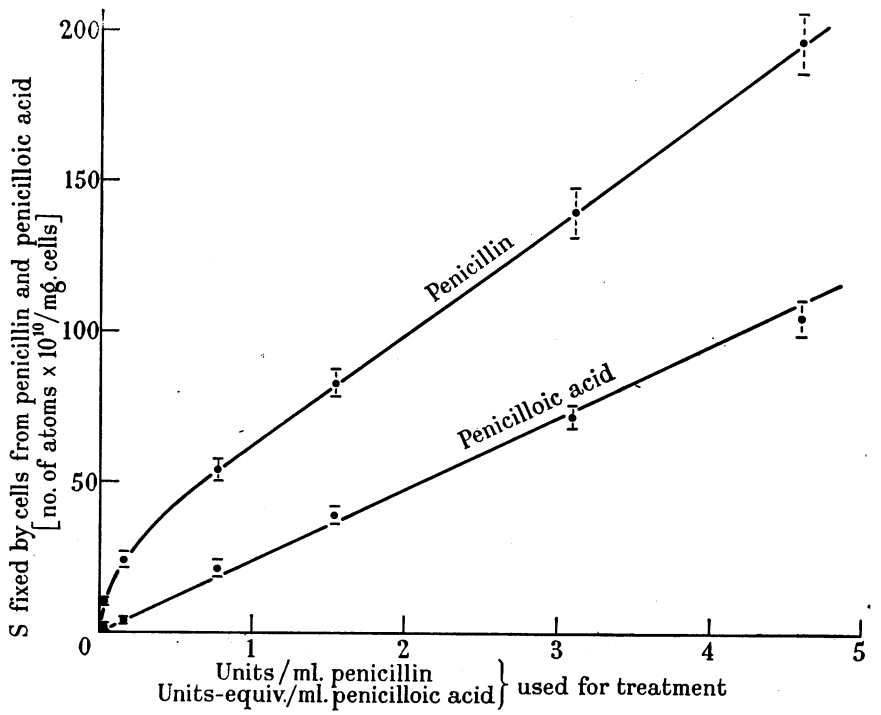

FIG. 2.—Fixation of S from S^{35}-labelled penicillin and penicilloic acid by *B. cereus* cells, showing limits of counting error for P = 0·05.

Fig. 1, it is possible to derive a curve for the specifically bound penicillin S—as shown in Fig. 3. In the same figure values are also plotted for penicillinase production rates obtained in the same experiment after treatment at 5 different penicillin concentrations (0, 0·08, 0·8, 8·0 and 14·0 units/ml.). It is, unfortunately, impossible to carry out the enzyme adaptation experiments at the same time at a greater number of different penicillin levels, and this figure must therefore be compared with the composite graph obtained from a number of different experiments published previously (Pollock, 1950). It does, however, clearly show maximal specific S-fixation occurring at a penicillin pre-treatment concentration of about 1·0 unit/ml.—the same value as for maximal stimulation of penicillinase production.

Rate of S-fixation from Penicillin.

Two lots of 30 ml. of cells at 2 mg./ml. were treated at 0° with 0·1 and 1·0 unit/ml. radioactive penicillin respectively and 10 ml. samples removed at 10, 60 and 180 minutes, spun down as rapidly as possible (6 minutes) and washed 3 times with water as usual. The amount of S fixed was then measured and the results plotted against time as shown in Fig. 4. At 1·0 unit/ml., after 10 minutes, the amount of S fixed was 48·5 per cent of that fixed after 3 hours, and after 60 minutes the percentage had risen to 73. It is clear that penicillin reacts with some cellular component very rapidly. The curve should be compared with that for penicillinase formation rates following identical penicillin treatment (Pollock, 1950) where the corresponding figures were 41 per cent and 67 per cent respectively. The correspondence between the two is striking.

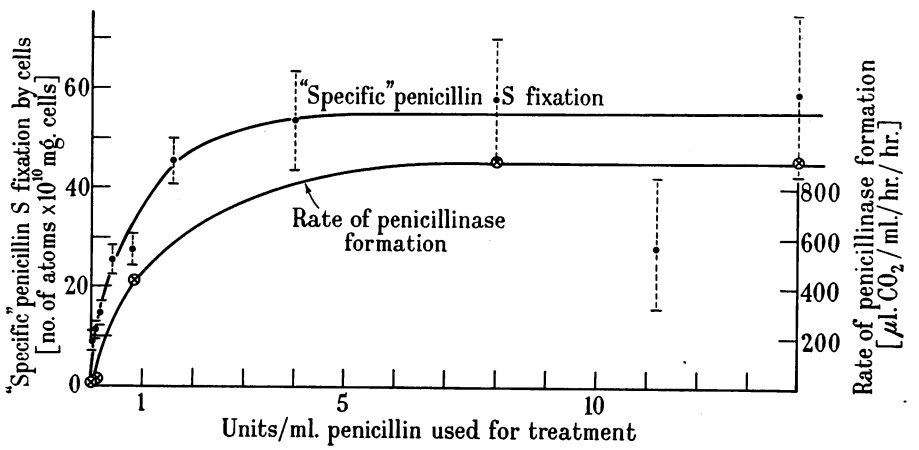

FIG. 3.—" Specific " fixation of S on to cells and rates of penicillinase formation following treatment of *B. cereus* with S^{35}-labelled penicillin. Limits of counting error are shown for P = 0·05.

Retention of fixed S during subsequent cell growth.

After standard treatment of cells with 0·5 unit/ml. S^{35}-labelled penicillin, they were inoculated at 1 mg./ml. into 0·8 per cent casein hydrolysate + M/50 glucose medium and incubated at 35° aerobically on a shaker. Samples were removed at 0, ½, 1, 1½, 2 and 2½ hours, the cells spun down, washed once and fixed radioactivity determined. The results are shown in Fig. 5 as total S fixed on cellular matter, derived (originally) from 1 mg. dry weight of cells, against time. Since during the 2½ hour period cell mass increased about 3-fold, the fixed S would be diluted in the growing cell protoplasm and results expressed per mg. of cells (as previously) would be confusing. The curve suggests that only about 30 per cent of S is probably lost during this period, and that therefore the greater part must be very firmly bound to cell material. Similar retention of penicillin S by *Staph. aureus* during subsequent growth in a penicillin-free medium has been reported by Maass and Johnson (1949*b*).

Proportion of penicillin S fixed on cells.

The penicillin concentration/S-fixation curve in Fig. 1 shows that about 2·5 $\times$ 10^{11} atoms of S per mg. of cells are fixed non-specifically from 1 unit/ml. and that the amount fixed specifically varies from 9 $\times$ 10^{10} atoms/mg. at 0·04 unit/ml. to about 50 $\times$ 10^{10} atoms/mg. at 1·5 units/ml. and above. The total penicillin S fixed on to cells treated at 2 mg./ml. varies from 0·50 per cent of that present in the medium at 0·04 unit/ml. to only 0·063 per cent at 14 units/ml. The proportion is thus exceedingly small. Assuming the average cell weight of *B. cereus* to be about 2 $\times$ 10^{-13} g., it appears that there are of the order of only 25 atoms of S specifically fixed per cell after treatment with 0·1 unit/ml. and 80 atoms/

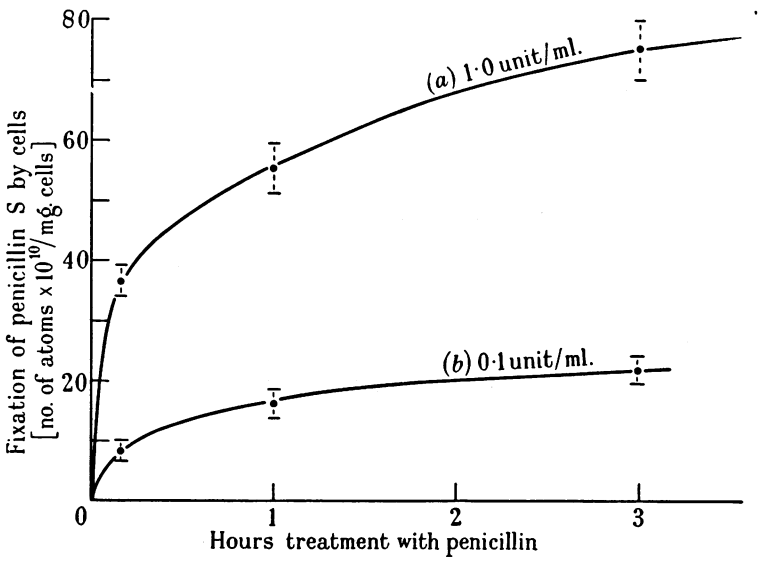

FIG. 4.—Rates of S fixation by *B. cereus* cells treated with S^{35}-labelled penicillin (*a*) at 1·0 unit/ml., (*b*) at 0·1 unit/ml. Limits of counting error are shown for P = 0·05. Penicillin assayed at 77 per cent nominal value.

cell after treatment with 1·0 unit/ml. (level at which maximal specific fixation and maximal penicillinase adaptation stimulation occurs).

Since measurement of S-fixation gives no information about the fate of the remaining penicillin S, attempts were made to estimate the actual amount of intact penicillin left in the medium after treatment of cells for 1 hour at 0° with 0·5 unit/ml. This level was chosen as being high enough to obtain reasonably accurate results by bio-assay, and yet still within the range known to be limiting both for specific S-fixation and penicillinase adaptation.

After treatment at 0·5 unit/ml. the cells were spun off as rapidly as possible in the cold room and the supernatant fluid divided into 2 parts. One part was heated for 30 minutes at 60° and then assayed on plates as described in " Methods." The preliminary heating was found necessary in order to avoid substantial loss of activity after cell treatment and during assay. This protective effect was probably due to destruction of traces of penicillinase already present, and to delay of

growth of the few living cells inevitably left behind in the supernatant after centrifugation. Both of these factors might be expected to contribute to the inactivation of penicillin during incubation of the assay plate. The other part of the supernatant fluid was used to treat a second lot of cells under the same conditions as the first. The two lots of cells thus treated for 1 hour at 0° [viz., (*a*) with penicillin at 0·5 unit/ml. and (*b*) with supernatant fluid from (*a*) after removal of the first lot of cells] were spun, washed 3 times, and their rates of penicillinase formation measured in CH at 35° as described in " Methods." The results are given in the Table. In two experiments there was a mean loss of 20

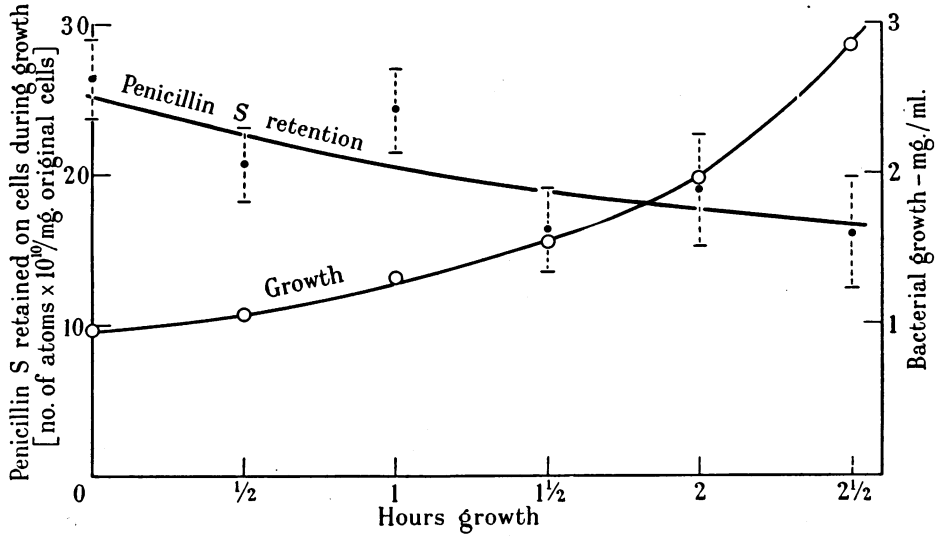

FIG. 5.—Loss of penicillin S from *B. cereus* cells during growth in casein hydrolysate following treatment for 1 hr. at 0° with 0·5 unit/ml. S^{35}-labelled penicillin. Limits of counting error are shown for P = 0·05.

per cent and 18 per cent respectively in antibacterial activity, and a falling off of only 9 per cent in ability to stimulate penicillinase adaptation (done in Expt. IV only). The limited accuracy of the technique is such that these results are consistent with the loss being, in both cases, little more than the fraction of penicillin S known to be actually fixed on the cells during pre-treatment.

DISCUSSION.

The general characters of S-fixation from penicillin by *B. cereus* are not dissimilar from those reported by Rowley *et al.* (1950) for other bacteria, although the total amount bound at very low penicillin concentration (0·04 unit/ml.) is 1/10 to 1/20 of what these workers found with penicillin-sensitive *Staph. aureus*. The shape of the curve of relationship between S fixed and concentration of penicillin used for treatment justifies its resolution into two components: (1) " specific " fixation with a maximum binding at about 1·0 unit/ml., and (2) " non-specific " fixation directly proportional to penicillin concentration over the

TABLE.—*Loss of Penicillin During Treatment of* B. cereus *with* 0·5 *unit/ml. for* 1 *hr. at* 0°.

Test used.			Cell concentration during treatment (mg./ml.).
A. Proportion of penicillin sulphur fixed on cells:			
Expt. I	0·16%	—	2·0
Expt. II	0·45%	—	5·0
B. Loss of antibacterial activity:			
	Mean loss from medium after treatment.	Limits of loss for P = 0·05.	
Expt. III	20%	10%–29%	1·0
Expt. IV.	18%	10%–25%	1·0
C. Loss of ability to stimulate penicillinase production:			
	Solution used for pre-treating cells.	Subsequent rate of enzyme formation in casein hydrolysate (μl. CO_2 ml./hr./hr.).	
	(*a*) Penicillin 0·5 unit/ml.	920	1·0
	(*b*) Supernatant from (*a*) after removal of treated cells	840	
	(*c*) Water (control)	10	
Loss of activity after treatment: 9%.			

whole range of values (from 0·04 to 14 units/ml.) tested. The fact that there is no corresponding "specific" S-fixation from penicilloic acid supports the conclusion that the "specifically-bound" fraction of penicillin S is due to an independent interaction between penicillin and some cell receptor specific for penicillin alone.

Comparing the two specific effects of penicillin treatment of *B. cereus*, (*a*) fixation of penicillin S by the cells and (*b*) stimulation of subsequent rate of penicillinase formation, it can be seen that—

(1) Both effects are maximal at a penicillin concentration of about 1·0 unit/ml. (Fig. 3).

(2) Both effects proceed at about the same rate (Fig. 4 and see Pollock, 1950).

(3) Both effects are more or less irreversible in the sense that bound S is retained (with little loss), and penicillinase continues to be formed at approximately constant rate, during subsequent growth in penicillin-free CH medium (Fig. 5 and see Pollock, 1950).

The significance of close correlation in (2) is somewhat less than might at first be expected, since it is possible that both penicillin effects might, initially, be limited by the rate of penetration of the penicillin into the cell. If this is so, all that is being measured in (2) is the speed of permeation of penicillin through the cell wall and membrane, and the only justifiable conclusion is that both effects take place very rapidly.

Considering the possible range of differences between the two effects in general the correlation is striking, and strongly suggests that they are not independent phenomena. Attempts, however, to show that all variations in the adaptive response of cells to penicillin treatment were due merely to differences in the amount of S specifically fixed were not successful. Different batches of cells and identical batches treated in different ways (e.g., with 1 hr. pre-incubation in CH medium + M/50 glucose) may differ considerably (5-fold or more) in their absolute rates of penicillinase formation following treatment with the same penicillin concentration, although they all show maximum response at about 1·0 unit/ml. Such variations were not associated with proportional variations in the quantity of specifically bound S. The latter, in fact, appeared to be fairly constant from one experiment to another, maximum variations not exceeding 30 per cent.

Nevertheless, the conditions affecting specific S-fixation are very similar to those pre-determining rate of penicillinase formation. The parellelism is too close to be merely coincidental, and indicates that the substance with which the penicillin reacts, when it exerts its effect, is qualitatively identical in the two instances. It seems reasonable, therefore, to conclude that the interaction between penicillin and some specific cell receptor which results in fixation of penicillin S is a necessary preliminary to the specific stimulating effect on subsequent penicillinase formation in a penicillin-free medium. The fact that there is not always a close quantitative relationship between the two effects only shows, as might be expected, that there are additional factors influencing penicillinase adaptation about which little is yet known. Moreover, the hypothetical S-fixing receptor with which the penicillin reacts exists in many species of bacteria—e.g., penicillin-sensitive *Staph. aureus* (Rowley *et al.*, (1950)—which do not produce any penicillinase whatever, adaptive or otherwise. It cannot therefore be the sole hereditary factor determining penicillinase adaptability.

It is not perhaps too early to speculate on whether some similar interaction between substrate and cell receptor might be the first essential preliminary to other enzyme adaptations in micro-organisms. If so, it would be neccesary to assume, on existing evidence, that the reaction is reversible, and in that case would be difficult to demonstrate. For instance, *Bact. coli* " 1433," if treated with nitrate, is not thereby endowed with any increased ability subsequently to form the adaptive nitratase in a nitrate-free medium (Wainwright and Pollock, 1949). The case of penicillinase may be a special one, and it is essential to make the qualification that the reaction between penicillin and receptor, which results in specific S-fixation, may not be necessary for penicillinase formation in the presence of excess free penicillin, though this possibility appears unlikely. Nothing is yet known about the extent to which the penicillin molecule is modified by its reaction with the cell. It is thus possible that the function of the specific receptor in penicillinase adaptation is simply protective—by retaining a small quantity of penicillin in a state in which it can constantly exert its catalyst-like activity in stimulating penicillinase formation after the removal of free penicillin. What is of particular interest, however, is the clear evidence that the ability of *B. cereus* cells to produce greatly increased amounts of an enzyme under certain defined conditions depends in the first place upon a definite chemical or physico-chemical reaction between the specific substrate and some substance already present within the cell. This focuses attention upon the nature and significance of the receptor substance, and the type of reaction it undergoes with penicillin.

Further information on this reaction should greatly assist in the study of the other factors which appear also to be necessary before the cell is able to produce penicillinase at maximal rate on subsequent incubation.

SUMMARY.

1. The uptake of S^{35} by *B. cereus* cells has been measured after treatment at 0° with varying concentrations of S^{35}-labelled benzyl penicillin and benzylpenicilloic acid and subsequent washing.

2. The proportion of S fixed from penicillin is very small, and decreases as the penicillin concentration increases, varying from 0·50 per cent of the total at 0·04 unit/ml. to 0·063 per cent at 14 units/ml., using a bacterial suspension of 2 mg./ml.

3. S-fixation from penicillin is made up of two superimposed fractions: (*a*) non-specific fixation—directly proportional to penicillin concentration in the medium and of the same order as the total S-fixation from penicilloic acid; and (*b*) specific fixation with maximum absorption at 1·0 unit/ml.

4. There is a correlation between the specific fixation of S by cells from penicillin and the effect of penicillin in specifically stimulating the subsequent rate of penicillinase formation, in that both processes (*a*) have a maximum at a penicillin concentration of 1·0 unit/ml., (*b*) proceed at the same rate, and (*c*) are more or less irreversible during subsequent incubation and cell growth for 2½ hours in casein hydrolysate.

5. It is concluded that the process entailing specific fixation of S by the cells from penicillin is essential to its action as a specific stimulator of penicillinase production in the absence of free penicillin.

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