PENICILLINASE ADAPTATION IN *B. CEREUS*: ADAPTIVE ENZYME FORMATION IN THE ABSENCE OF FREE SUBSTRATE.

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THE apparently enzymic nature of various penicillin " inactivators " produced by a wide range of penicillin-resistant micro-organisms has been observed by a number of workers (see excellent review by Chain, Florey, Heatley and Jennings, 1949). The term " penicillinase " has, however, been used rather loosely to cover most cases where penicillin has been biologically inactivated by means of some sort of enzymic process, and few biochemical studies of the phenomenon have been undertaken. Foster (1945) observed that a cell-free preparation from *B. subtilis* inactivated penicillin with the formation of acid. The formation of an acidic group with pK 4·7 by the action of penicillinase from a strain of *B. cereus* on penicillin was confirmed by Benedict, Schmidt and Coghill (1945) ; and, later, Henry and Housewright (1947) showed conclusively, by measuring CO_2 evolution from a CO_2-bicarbonate buffer manometrically, that the penicillinase in a broth culture filtrate of *B. cereus* destroyed penicillin by quantitative hydrolysis to penicilloic acid, with the formation of one acid equivalent. It still, of course, remains to be shown that enzymic penicillin inactivators from other sources destroy penicillin in a similar manner.

The fact that penicillinase from some strains of bacteria behaves as an adaptive enzyme was first observed by Duthie (1944), who, using a strain of *B. subtilis*, found that addition of penicillin to the culture media—even after cessation of growth—produced up to 30-fold increase in yield of the enzyme. A similar adaptive effect has been reported by Lepage, Morgan and Campbell (1946) for strains of *B. megatherium* and *B. cereus*, and later again by Duthie (1947) for strains of *B. licheniformis*. Housewright and Henry (1947) confirmed the marked effect of penicillin on penicillinase production by *B. cereus*.

METHODS.

Organism.—*B. cereus* NRRL B-569 from Dr. R. D. Housewright.

Cell suspensions.—Prepared from 20-hr. growth at 35° in tryptic meat broth aerated by shaking, inoculated with one drop of a spore suspension prepared by heating 50 ml. of a 48-hr. culture in casein hydrolysate yeast extract medium (Gladstone and Fildes, 1940) for 1 hr. at 60°, washing the cells and resuspending

in 20 ml. water. Cells were spun down, washed once in water and made up to standard strength (10 mg./ml. or 20 mg./ml.) in water. Cell concentrations were measured by opacity, using a Spekker absorptiometer and a standard opacity/dry weight curve prepared as described for *Bact. coli* (Pollock and Wainwright, 1948).

Penicillinase activities were measured manometrically at 30° in a CO_2-bicarbonate buffer at pH 7·0, as described by Housewright and Henry (1947). One ml. or 2 ml. samples of the enzyme preparation were added to 0·5 ml. of 0·043 M sodium bicarbonate in the main compartment, and made up to 2·5 ml. with water. 0·1 ml. of penicillin solution 100,000 units/ml., 0·1 ml. of 0·043 M sodium bicarbonate and 0·3 ml. water were put in the side bulb. The manometers were gassed with 5 per cent CO_2 in N_2. After equilibration for 20 min. the contents of the side-bulb were tipped into the main flask. After 2 to 5 minutes, a constant rate of gas evolution was established. Penicillinase activities were assayed by measuring the rate of CO_2 production over the linear phase which persisted until about 75 per cent of the penicillin was destroyed. Even with enzyme preparations of very low activity, measurements were not continued for longer than 30 min.

All values were corrected for CO_2 retention caused by substances such as phosphate, amino-acids, etc., present in the sample. The CO_2 retention was calculated by adding a known amount of pure tartaric acid (dried in the side-bulbs) to samples of the various media, in presence of the CO_2-bicarbonate buffer, and measuring the per cent reduction in gas evolution compared to that from bicarbonate alone. It was confirmed that such substances causing CO_2 retention in no way affected the linearity of the reaction.

Penicillinase production was followed in M/50 glucose, M/600 $MgSO_4$, M/50 phosphate buffer pH 7·0, in the presence of varying quantities of penicillin up to 10,000 units/ml. and with or without 0·8 per cent " Vitamin-free " casein hydrolysate (Ashe), supplemented with M/12,500 tryptophan and M/5000 cystine. The cell concentration varied from 0·5 to 2·0 g./ml., and the suspensions were incubated in conical flasks (usually 20 ml. in 100 ml. flasks) at 35° on a shaker (100/min., amplitude 10 cm.). Samples of 3·0 ml. were removed for assay, and added to 0·6 ml. of M/200 oxine in order to stop further enzyme production. Except when specifically mentioned otherwise cells were not separated from medium, so that enzyme activities represent the combined extra- and endo-cellular fractions present in the whole sample. These were usually assayed within 3 to 4 hr., but had, occasionally, to be left overnight (18 hr.) at + 2° before testing. It was confirmed that there is no loss of activity of such preparations, in the presence of M/1200 oxine, for at least 3 hr. at 35° and 18 hr. at + 2°. Results are expressed as µl. CO_2 released per ml. per hr. ("Total penicillinase") or µl. CO_2 per hr. per mg. dry weight of cells (" Q penicillinase ").

Penicillin used was the pure sodium salt of penicillin G (benzylpenicillin) prepared by Glaxo Laboratories Ltd. Dilutions were made in M/100 phosphate buffer, pH 7·0.

Penicilloic acid was prepared by alkaline hydrolysis of sodium benzylpenicillin, using the method described in Clarke, Johnson and Robinson (1949). Attempts to obtain a crystalline product, however, failed, and a 30 per cent aqueous solution still showed antibiotic activity on *B. subtilis* layer plates several days after completion of hydrolysis. It was therefore reprecipitated with acid, freeze-dried

and twice dissolved in methanol on a water bath and evaporated down to dryness. The resulting product showed no antibiotic activity, and therefore contained less than 1 part per 10^6 of penicillin. Although it is possible that this amorphous product contained an appreciable amount of benzyl-penilloic acid, it can be regarded as consisting mainly (if not entirely) of benzylpenicilloic acid, and as being for all intents and purposes free from penicillin.

Growth was always measured by opacity estimation in a Spekker absorptiometer, and expressed as mg. dry bacterial wt./ml. after reference to the standard opacity/dry weight calibration curve.

RESULTS.

Preliminary.

The findings of Henry and Housewright (1947) regarding the pH/activity curve of penicillinase, the proportionality between rate of CO_2 evolution and enzyme concentration over a wide range, and the stimulatory effect of penicillin on penicillinase formation during growth in broth, were all confirmed. A small "basal" penicillinase activity was always detectable even if the cells were grown in the absence of added penicillin; while in the presence of 500 units/ml. enzyme yield was increased over 40-fold.

It was then shown that penicillinase adaptation would occur rapidly without growth in the presence of 10,000 units/ml. penicillin with M/50 glucose, M/10 phosphate buffer and M/600 $MgSO_4$ only, using washed cells at a concentration of 0·6 mg./ml. Apart from the penicillin itself no N source was added, and opacity measurements showed no change over the 2½ hr. of the experiment. The adaptation curve obtained (Fig. 1) is of the upward-sweeping, "logarithmic" type which occurs in other microbial enzyme adaptations.

Since penicillinase activities were usually measured using samples containing both intact cells and glucose, it would clearly have led to inaccurate results if there had been any marked gaseous change (e.g. due to anaërobic glycolysis) in the manometers before adding the pencillin, particularly if the penicillin had influenced the rate of any such change. In practice, however, it was found that gas changes before tipping were insignificantly small, and were therefore ignored from the point of view of estimating penicillinase activities. Moreover, penicillin at the concentration used for enzyme assay (3300 units/ml.) had no significant effect on O_2 uptake or on CO_2 output aërobically or anaërobically by cell suspensions using glucose as substrate.

Penicillin-sensitivity growth tests in tryptic meat broth showed the organism to be "intrinsically" fairly sensitive. An inoculum of 1 drop of a 10^{-7} dilution of an 18-hr. broth culture was completely inhibited by 1·5 units/ml. penicillin, but grew in 0·3 units/ml. Heavy inocula (e.g. up to 50 μg./ml.) would grow in 10,000 units/ml. within 24 hr. It is clear, therefore, that the organism's resistance to penicillin is of the "penicillinase" type (Spink and Ferris, 1947; Bondi and Dietz, 1948), and probably exists only in so far as it is able to destroy penicillin with the enzyme produced.

Extracellular production of penicillinase.

The penicillinase of B-569 has been described (Benedict *et al.*, 1945) as extracellular. By comparing activities of whole cultures or suspensions with those

of the supernatant fluid after centrifuging off the cells, it was indeed found that, with 48-hr. cultures, about 70 per cent of the enzyme activity was extracellular. During the early phases of enzyme production, however, the extracellular fraction was often much lower. For instance, during a 2½-hr. period of adaptation of washed cells in penicillin (as illustrated in Fig. 1) the extracellular fraction varied from 0 to only 38·5 per cent, and in some experiments never exceeded 6 per cent. The process appears to be some active form of enzyme secretion, since there was

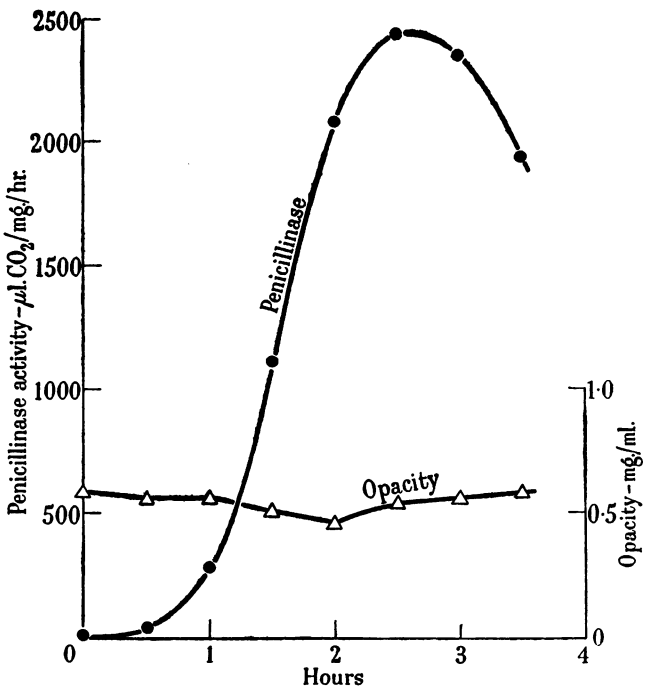

FIG. 1.—Penicillinase production by cells of *B. cereus* without added N source and in the absence of growth. Medium : M/50 glucose, M/50 phosphate buffer pH 7·0, M/600 $MgSO_4$. 35°, shaken in air.

no evidence at all of any cell lysis during this period, and addition of M/1200 oxine inhibited both further enzyme production and increase in the extracellular fraction.

Effect of oxine (*8-hydroxyquinoline*).

It has been found that suitable concentrations of oxine would inhibit nitratase adaptation in *Bact. coli*, without affecting the action of nitratase itself (Wainwright and Pollock, 1949). The same is true for pencillinase adaptation of *B. cereus* B 569 (Fig. 2) : M/1200 oxine completely inhibited further adaptation at 35°, while even double that concentration was found to have no significant effect whatever on the enzyme activity itself. Addition of M/1200 oxine to samples was therefore an ideal method for obtaining estimations of penicillinase activities at definite times during the period of enzyme production.

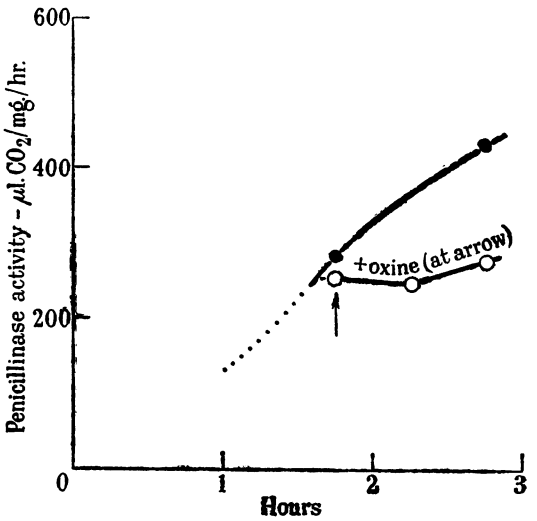

FIG. 2.—Inhibition of penicillinase production of *B. cereus* by M/1200 oxine. Medium : M/50 glucose, M/50 phosphate buffer pH 7·0, M/600 $MgSO_4$ + penicillin 10,000 units/ml. 1 mg./ml. cells. 35°, shaken in air. Open circles : M/1200 oxine added at 1¼ hr. and samples withdrawn at once, and after further ½ hr. and 1 hr. Filled circles : No oxine added. Samples withdrawn at 1¼ hr. and 2¼ hr. Cooled to 0° as rapidly as possible, and kept at 0° until assayed.

Effect of non-specific factors on penicillinase adaptation.

Oxygen.—Table I shows the effect of degrees of aëration on penicillinase adaptation with 10,000 units penicillin/ml. in the absence of added amino-acids. It is obvious that full aëration is necessary for maximal enzyme production, and it was repeatedly found that no significant amount was formed in the absence of O_2.

Mg.—Frequently no effect was found on the addition of M/600 $MgSO_4$, but since on some occasions stimulation of enzyme production occurred, Mg^{++} was added in all experiments.

Glucose was found to cause up to a 4-fold increase in the amount of enzyme produced in the absence of added N source and in the presence of 10,000 unit/ml. penicillin. The maximum effect occurred at a concentration of M/500. The action of glucose on penicillinase production is not, however, a simple one, and will be dealt with in more detail in a subsequent publication.

Temperature, pH.—No significant amount of enzyme was formed in 2 hr. at 18°, and about 4 times as much enzyme was produced at 35° as at 30°. Enzyme adaptation was maximal at pH 7·0, while below 6·5 it fell off rapidly.

Amino-acids, in the form of purified casein hydrolysate (Ashe) with added tryptophan and cystine, caused considerable stimulation of both basal penicillinase production (i.e. in the absence of penicillin) and penicillinase adaptation (with 10,000 units/ml. penicillin)—see Table II. No growth occurred in the presence of penicillin, even with added amino-acids, over the period of the experiment. Extent of stimulation by amino-acids was variable, and in some experiments amounted to a 6-fold increase in enzyme production.

TABLE I.—*Effect of Aëration on Penicillinase Adaptation of Washed Suspensions of B. cereus.*

Conditions.	Q penicillinase after 110 min. incubation at 35°.
In 6 in. × ¾ in. tube filled with argon	5
In 6 in. × ¾ in. tube open to air	9
In 50 ml. conical flask open to air	38
Ditto shaken at 100/min.	108
Ditto shaken at 350/min.	96

All vessels contained 5 ml. fluid with M/50 glucose, M/50 phosphate buffer pH 7·0, M/600 $MgSO_4$ and cells at 1 mg./ml.

TABLE II.—*Effect of Casein Hydrolysate on Pencillinase Production with and without Penicillin.*

Additions.	Q penicillinase after incubation for 100 min. at 35°.
Nil	6
10,000 units/ml. penicillin	1510
0·8 per cent casein hydrolysate	47
10,000 units/ml. penicillin + 0·8 per cent casein hydrolysate	2450

All flasks contained M50 glucose, M/50 phosphate buffer pH 7·0, M/600 $MgSO_4$ and initial cell concentration of 1 mg./ml. Casein hydrolysate was supplemented with tryptophan and cystine (see Methods).

TABLE III.—*Effect of Penicilloic Acid on Penicillinase Adaptation.*

Additions.	Q penicillinase after incubation for 2½ hr. at 35°.
Nil	10
2×10^{-7} M penicillin	790
2×10^{-2} M penicilloic acid	0
2×10^{-7} M penicilloic acid	10
2×10^{-7} M penicillin + 2×10^{-2} M penicilloic acid	905
2×10^{-7} M penicillin + 2×10^{-7} M penicilloic acid	640

All flasks contained 10 ml. of M/50 glucose, M/50 phosphate buffer, M/600 $MgSO_4$ and 1 mg./ml. washed cells of *B. cereus*.

TABLE IV.—*Effect of Penicillin Concentration on Penicillinase Adaptation.*

Penicillin conc. (units/ml.).	Q penicillinase after incubation for 2½ hr. at 35°.
0	46
0·0008	80
0·004	307
0·02	815
0·1	1190
10,000	133

All flasks contained M/50 glucose, M/50 phosphate buffer pH 7·0, M/600 $MgSO_4$ and cells (1 mg./ml.).

Specificity of stimulation of enzyme production by penicillin.

M/20 benzylalanylalanine (kindly supplied by Mr. H. Proom, of Burroughs Wellcome Laboratories, Beckenham) and M/25 glutathione, substances thought to be somewhat similar in structure to penicillin, were tested for ability to stimulate penicillinase production under the standard conditions (no added amino-acids) over a period of 2 hr. at 35°, but no stimulation occurred in either case. The first preparation of penicilloic acid (M/50) similarly tested was found to be nearly as active as penicillin in promoting adaptation. However, this had been prepared simply by alkaline hydrolysis of benzylpenicillin—without reprecipitation or methanol treatment—and biological assay showed it to contain about 1 : 100,000 active penicillin. This impurity was shown to be entirely responsible for the result (see below). A purified sample of penicilloic acid prepared as described in " Methods " was shown to be without significant activity on enzyme production (see Table III).

Effect of penicillin concentration.

Table IV shows the effect of different penicillin concentrations on penicillinase formation after 2½ hr. incubation in the standard glucose-Mg medium. It can be seen that a concentration of only ·004 units/ml. ($\triangleq 8 \times 10^{-9}$ M) caused a 7-fold increase in enzyme production over the control without penicillin, and even a concentration of ·0008 units/ml. produced significant stimulation. Moreover, the concentration previously employed (10^4 units/ml.) was actually inhibitory.

This result, though it adequately explains the effect first found with impure preparations of penicilloic acid, was unexpected. It was further found that samples of cells taken during adaptation in pencillin-glucose media, washed and resuspended in the same medium without penicillin, continued to form penicillinase. This was at first thought to be due to inadequate washing, but two further washings (five in all) only slightly reduced the rate of enzyme formation.

Enzyme formation by penicillin-treated cells after removal of penicillin.

It was eventually discovered that simple treatment of washed cells with penicillin for a short period at 0° conferred on them, even after thorough washing, the ability to form large amounts of penicillinase when subsequently incubated in the absence of penicillin (Fig. 3). This enzyme formation following penicillin-treatment was not qualitatively different from that occurring in the presence of penicillin. That is : it was stimulated by added amino-acids, it did not occur at all anaërobically, and it was inhibited by M/1200 oxine. The shape of the production curve, however, was different. Instead of being of the " logarithmic " type usually associated with enzyme adaptation, it had a short, but variable, lag phase (usually about 1 hr.) followed by a linear phase and, finally, a stationary phase which was, in some experiments, followed by a phase of decline —i.e. actual loss in enzyme activity.

Effect of added N source.—Although addition of casein-hydrolysate allowed cell growth to occur (up to a 3-fold increase in opacity during all except long-term experiments), this only had the effect of increasing the rate of enzyme formation and did not affect the linearity of the middle portion of the curve. In fact, the " tailing away " effect after the linear phase was shown conclusively to be due

to shortage of glucose and casein hydrolysate. Fig. 4 shows that the linear phase could be restored by replenishment of glucose and a source of amino-acids at the point where enzyme production was failing. In experiments where the medium was replenished earlier, no initial decrease in enzyme production occurred and

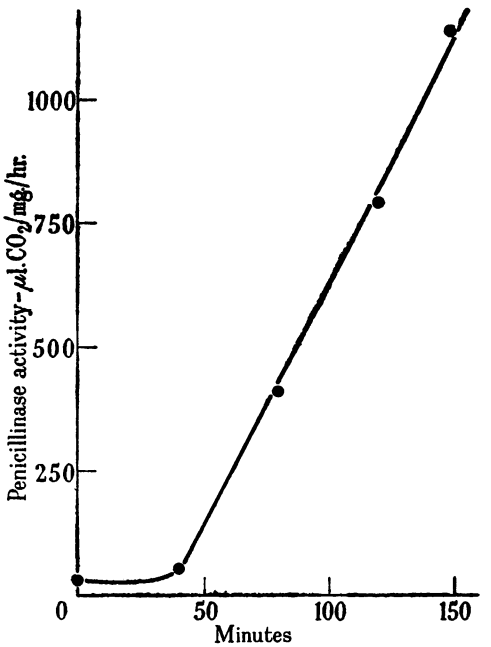

FIG. 3.—Linear production of penicillinase in M/50 glucose, M/50 phosphate buffer pH 7·0 and M/600 $MgSO_4$ by *B. cereus* cells following treatment with 1 unit/ml. penicillin. 35°, shaken in air. 1 mg./ml. cells.

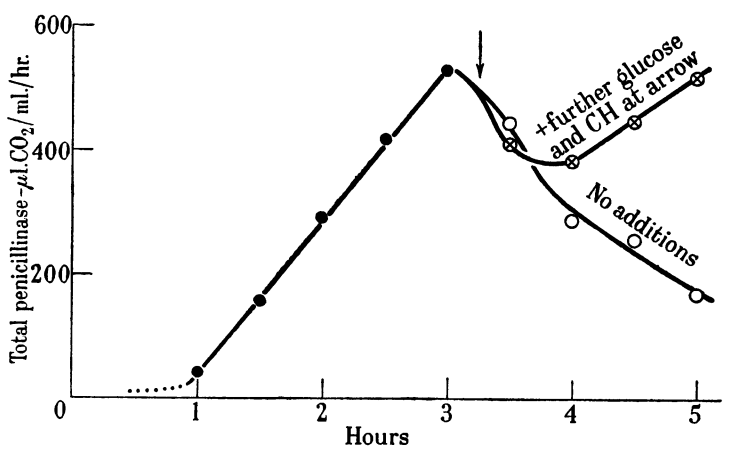

FIG. 4.—Effect of further addition of M/50 glucose and 0·8 per cent casein hydrolysate on penicillinase production by *B. cereus* cells previously treated with 1 unit/ml. penicillin for 1 hr. at 0°. Original medium : M/50 glucose, 0·8 per cent casein hydrolysate, M/600 $MgSO_4$ and M/50 phosphate buffer pH 7·0. 35°, shaken in air. Initial cell conc. 1 mg./ml.

the linear phase continued without interruption. This constant rate of penicillinase production could, in fact, be prolonged apparently indefinitely by suitable conditions (see below). The decrease in enzyme activity after 3 hr. shown in the culture without replenishment of glucose and casein hydrolysate has not been further studied. It is clearly not due to simple enzyme instability, since it has

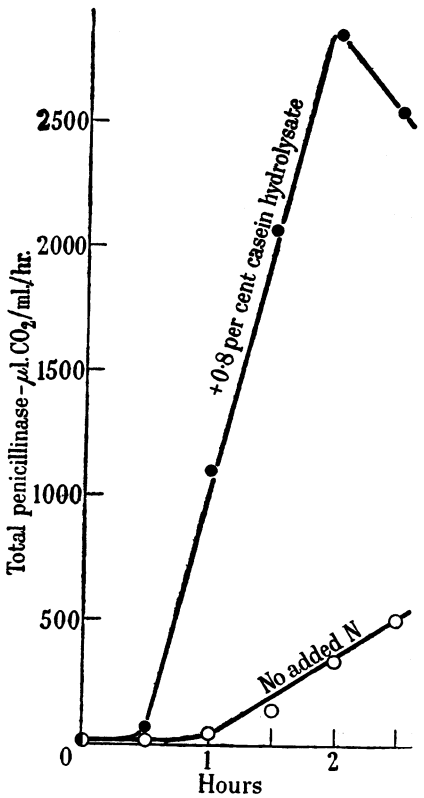

FIG. 5.

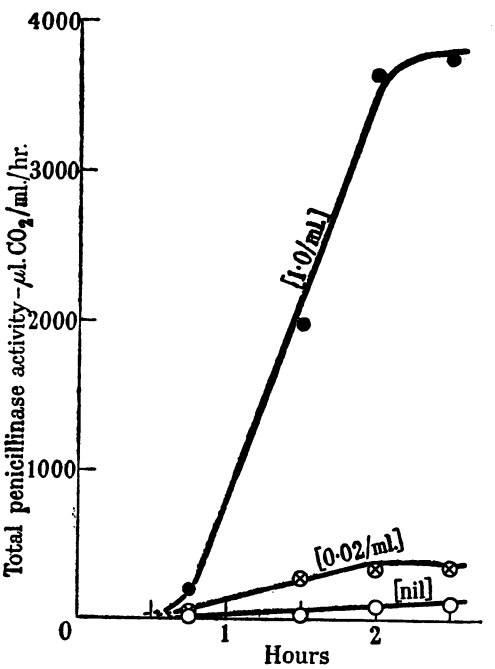

FIG. 6.

FIG. 5.—Effect of 0·8 per cent casein hydrolysate on rate of penicillinase production in M/50 glucose + M/600 $MgSO_4$ + M/50 phosphate buffer pH 7·0 by *B. cereus* cells previously treated with 1 unit/ml. penicillin. 35°, shaken in air. Initial cell conc. 1 mg./ml.

FIG. 6.—Rates of penicillinase production by *B. cereus* in glucose + casein hydrolysate after treatment of cells for 1 hr. at 0° with different concentrations of penicillin. Figures in brackets on the curves indicate concentrations of penicillin (units/ml.) used for pretreatment. 35°, shaken in air. Initial cell conc. 1 mg./ml.

been repeatedly shown that no loss of activity occurs at 35° in the presence of M/1200 oxine. It must therefore be connected with cell metabolism, and may be related to the phenomenon of enzyme "interaction" demonstrated by Spiegelman and Reiner (1947), which is said to occur more readily under conditions of N starvation.

Fig. 5 shows typical curves of enzyme formation, with and without added casein hydrolysate, by cells previously treated with 1 unit/ml. penicillin in M/100 phosphate buffer at 0° for 1 hr. followed by three thorough washings. This

stimulatory effect of amino-acids meant more accurate comparative results in the study of the pretreatment effect, and all subsequent experiments were done with added 0·8 per cent casein hydrolysate.

Effect of penicillin concentration.—The rate of penicillinase production after penicillin treatment was found to increase with increase in the concentration of penicillin used—up to a maximum of 1 unit/ml. A typical series of curves showing this effect is illustrated in Fig. 6. It was not practicable to test the effect of all the different concentrations of penicillin on the same suspension on the same day—and variability in adaptability between different suspensions and falling

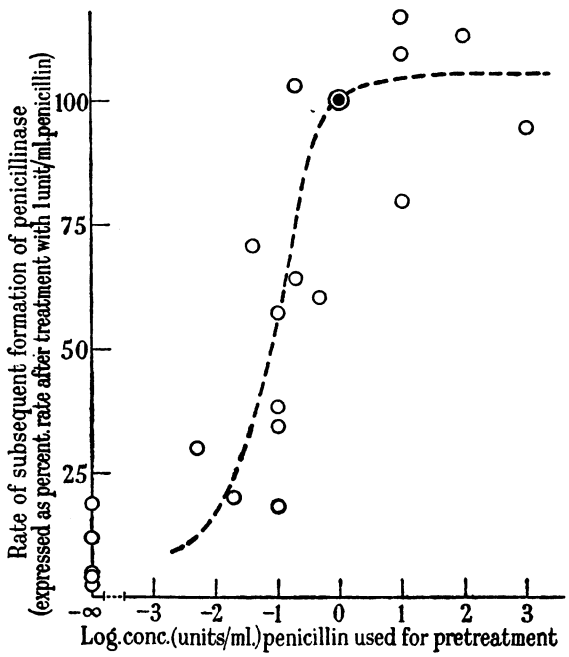

FIG. 7.—Relation between concentration of penicillin used for pretreatment of cells of *B. cereus* (for 1 hr. at 0°) and subsequent rate of penicillinase production in glucose + casein hydrolysate. 35°, shaken in air. Initial cell conc. 1 mg./ml.

off of adaptability of suspensions on storage precluded the comparison of absolute rates of enzyme formation between different experiments. If, however, rates of enzyme formation in all experiments are expressed as percentages of those obtained (always measured over the linear phase), using penicillin at 1 unit/ml., and plotted against penicillin concentration used, Fig. 7 is obtained. It can be seen that maximal rate of enzyme production occurs when the penicillin used for pretreatment was at a concentration of about 1 unit/ml., and half-maximal production at about 0·1 unit/ml. Maximal production rate is from 6 to 50 times that of the control without penicillin treatment.

Other factors.—It was found that this penicillin effect occurred very rapidly. Fig. 8 shows the comparative rates of penicillinase production measured over the linear phase after treatment with 1 unit/ml. penicillin at 0° for different lengths

of time. Washed cells, precooled to 0°, were added to M/100 phosphate buffer pH 7·0 containing 1 unit/ml. penicillin in an iced water-bath, to final cell concentration of 1 mg./ml. Samples were removed after 10, 60 and 180 minutes and the cells centrifuged down as rapidly as possible (10 min.), washed four times, resuspended to standard opacity (10 mg./ml.), and their rates of penicillinase formation at 35° in the glucose/casein hydrolysate medium compared to that of a similarly washed sample of cells treated for 10 min. in M/100 phosphate without penicillin. The rate of enzyme formation after only 10 min. treatment (to which perhaps should be added the time taken in the first centrifugation) was 66 per cent of that after 1 hr. treatment, and incubation for 3 hr. increased the rate only 35 per cent above that attained after 1 hr.

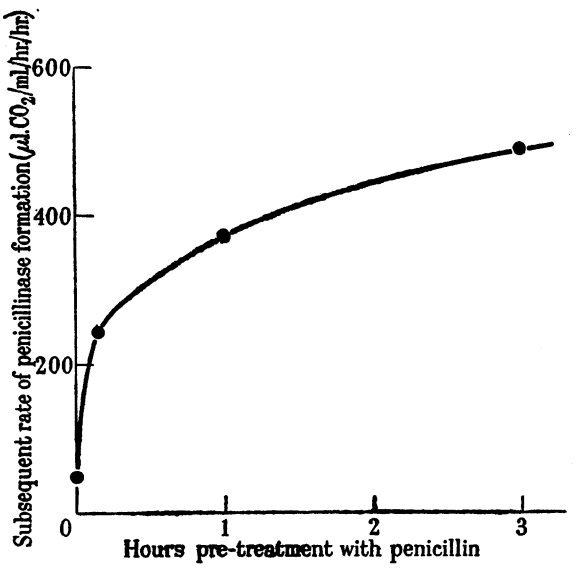

FIG. 8.—Relation between length of treatment of *B. cereus* cells at 0° with 1 unit/ml. penicillin and subsequent rate of penicillinase production in glucose + casein hydrolysate. 35°, shaken in air. Initial cell conc. 1 mg./ml.

The effect of penicillin treatment was more marked at 0° than at 35°. The rate of enzyme formation after treatment with penicillin for 1 hr. at 0° was 2½ times more rapid than after treatment at 35°. The effect of penicillin treatment was, however, not altered by the concentration of cells used during treatment—at least within the range of 1 to 5 mg./ml.

Effect of continued growth without penicillin.—By continually making 1 : 2 dilutions of a culture in fresh glucose-casein hydrolysate medium it was possible to follow penicillinase production and growth, using the heavy inoculum of 0·5 mg./ml. almost indefinitely. Fig. 9 illustrates an experiment where the cells were pretreated for 1 hr. at 0° with M/100 phosphate buffer containing 0 and 1 units/ml. penicillin respectively (followed by three washings), and were maintained in the logarithmic phase for about 3 hr. by half-hourly 1 : 2 dilutions in fresh medium. Growth opacity and penicillinase activities were measured every ½-hr. and the figures multiplied by the total dilution factor to give the results

in terms of 1 ml. of original medium. It is clear that: (*a*) Growth rate of the two lots of cells (untreated and treated with penicillin) is identical; (*b*) after the lag phase of 1 hr., penicillinase production by treated cells is approximately linear for the whole period of 5½ hr. of the experiment, and strictly linear up to 3½ hr. The relatively slight deviations from the straight line in the last 2 hr. are probably due to a combination of the high dilution correction (64-fold at 5½ hr.), and the increasing fraction of basal penicillinase that is being formed all the time in an amount roughly proportional to the total amount of growth; and (*c*) the Q penicillinase of the treated cells rises rapidly to a maximum at 2½ hr.

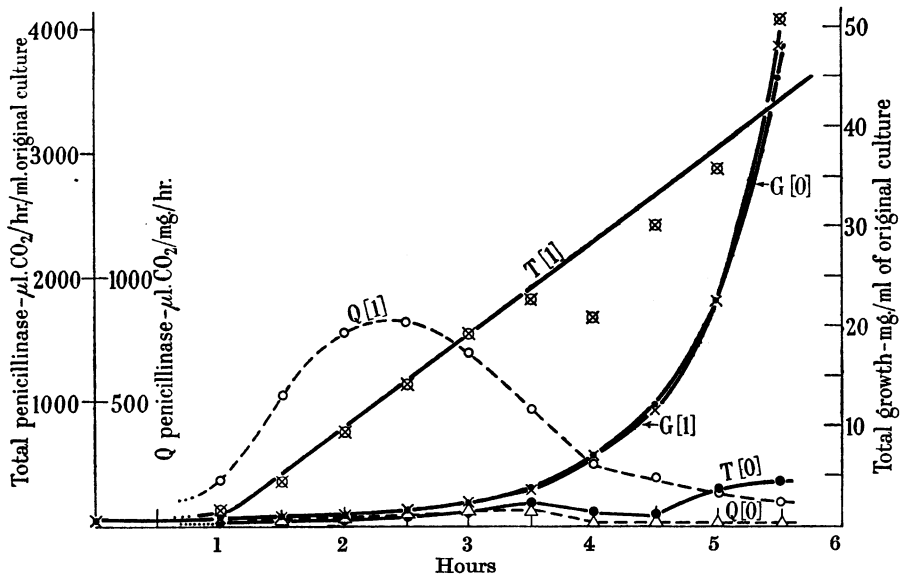

FIG. 9.—Continuous production of penicillinase during growth of *B. cereus* following treatment of cells with penicillin at 1 unit/ml., compared to control without penicillin pretreatment.

Q = Q penicillinase (μl. CO_2/mg. cells/hr.)
T = Total penicillinase (μl. CO_2/hr.)
G = Growth of cells (opacity measurements)
} Expressed as per ml. original medium.

[1] = Penicillin-treated cells (1 unit/ml.).
[0] = Untreated cells.

Medium: M/50 glucose, M/600 $MgSO_4$ + 0·8 per cent casein hydrolysate. 35°, shaken in air. Both cultures were diluted 1 : 2 in fresh medium after 2¾ hr. and every ½ hr. subsequently.

and then gradually falls towards that of the untreated cells, though even at 5½ hr., after nearly 7 cell generations, it is still about 7 times higher than in the control.

The constant rate of penicillinase production over a period when the cells are increasing their total mass over 100-fold, and are actually growing exponentially, is striking. The Q penicillinase of the treated cells does, however, eventually return to the normal basal level on continued incubation, as would be expected. An 18-hr. culture of penicillin-treated cells in broth, using a small inoculum, resulted in complete loss of all the adaptation originally developed, and no "genetically permanent" change has yet been found to follow the original penicillin treatment.

DISCUSSION.

The general characteristics of penicillinase production by *B. cereus* in the presence of 10,000 units/ml. penicillin are similar to those of other cases of microbial enzyme adaptation. This particular case of adaptation, however, presents two new features not hitherto reported. The first is the pronounced effect of minute concentrations of penicillin (less than 10^{-8} M) in stimulating adaptation; and the second is the acquired ability of cells to form penicillinase in a penicillin-free medium merely by treating them at 0° for a few minutes with small amounts of penicillin, followed by its removal. It is not improbable that the first phenomenon follows inevitably from the second.

It is, of course, uncertain how far the case of penicillinase adaptation may be unique—a possibility made, perhaps, more likely because of peculiarities associated with the marked antibacterial action of the substrate. It is certainly different from nitratase adaptation in *Bact. coli*, where it has already been shown conclusively that cells previously adapted to (i.e. treated with) nitrate do not form the enzyme, when incubated in a nitrate-free medium, any more rapidly than control untreated cells (Wainwright and Pollock, 1949). On the other hand, Wainwright (1950), in further studies on the effect of substrate concentration on nitratase adaptation, has found that a low concentration of nitrate (10^{-4} M) is sufficient to cause half-maximal adaptation when added directly to the medium. It is possible that similar results will be found in other systems. In any case, the fact that such small concentrations of substrate can promote rapid adaptation, even in one or two instances, should be a warning against ascribing activity in promoting adaptation to substrate analogues, etc., which are not of a very high degree of purity. In particular, it throws doubt on the significance of previous reports that the products of enzyme action are often as active in stimulating adaptation as the substrate itself (Yudkin, 1938). In the case of penicillinase, penicilloic acid, when eventually freed from contaminating traces of penicillin, was quite inactive; while as little as 0·001 per cent penicillin impurity was enough to give almost maximal adaptation.

Whether or no the case of penicillinase is unique, the continued production of the enzyme in the absence of penicillin by penicillin-treated cells is a striking phenomenon. Though not " heritable " in the strict genetic sense, the increased penicillinase content of adapted cells is a character acquired by contact with a specific factor in the environment, and has been shown to persist—in some degree—for at least 7 cell generations. That is genetically a very short time, but biochemically a long time, since it involves over a 100-fold increase in cell protoplasm.

The nature of the specific interaction between penicillin and cells which endows them with the new property of rapid penicillinase formation is being further studied, and will be discussed in greater detail in a subsequent publication. The two salient features of the phenomenon are: (*a*) linear formation of enzyme—even during logarithmic growth of cell mass—which continues apparently indefinitely as long as the cells are maintained in full activity by replenishment with glucose and amino-acids; and (*b*) the relationship between rate of enzyme formation and concentration of penicillin used for pretreatment, with maximal effect at 1 unit/ml. As previously pointed out, conclusions based on curves of enzyme formation must always be qualified by the reservation that the only method of measuring the amount of enzyme is by assaying its activity, and that

therefore it is at least theoretically possible to obtain misleading results due to the presence of enzyme inhibitors or activators. Nevertheless, apart from all the other factors which cannot be discussed here, it would be a most remarkable coincidence if the linear phase of penicillinase formation, as illustrated, for example, in Fig. 9, were simply an artefact. Assuming, therefore, that enzyme activity represents—i.e. is always directly proportional to—enzyme content, the two facts emphasized above suggest that the result of penicillin interaction with the cells may be the formation of a new compound or complex, stable enough to resist decomposition during subsequent cell metabolism and growth, which acts as some sort of specific catalyst stimulating the formation of penicillinase. The amount of this hypothetical catalyst formed would, of course, depend upon the concentration of penicillin present during pretreatment, and would determine the rate at which penicillinase was formed subsequently.

Three further points are worth mentioning :

(*a*) The relationship between penicillin concentration used in pretreatment and subsequent rate of enzyme formation, and in particular the low penicillin concentration needed for maximal effect, suggests that there may be some specific receptor substance within the cells with a high combining affinity for penicillin, which becomes " saturated " at a concentration of 1 unit/ml.

(*b*) Penicillin has, in fact, been shown to be specifically fixed by sensitive staphylococci, very rapidly and under conditions (i.e. at very low concentrations and at 0°) similar to those used in this work (Maass and Johnson, 1949 ; Rowley, Cooper, Roberts and Lester Smith, 1950). However, these penicillin-fixation experiments were done with radioactive (S_{35} marked) penicillin, and do not show to what extent absorbed penicillin remains unchanged within the cell.

(*c*) It seems likely that if absorbed penicillin is directly active in promoting adaptation by virtue of its specific molecular integrity, it must be fixed or protected in some way, such that it is not destroyed by the ever-increasing amounts of penicillinase present.

Whatever the mechanism of this " pretreatment effect," it must be regarded as established that penicillinase can be formed *adaptively* (that is, specifically in response to treatment with the substrate) in the absence of free penicillin. That is an important conclusion, since it disposes of any hypothesis, such as that advocated by Monod (1949) to explain enzyme adaptation, which ascribes to the substrate the role merely of stabilizer of enzyme formed as rapidly in its absence as in its presence. It shows also that it is not necessary (as first proposed by Dubos, 1940) for the substrate to be continuously metabolized in order for adaptation to occur.

SUMMARY.

(1) The adaptive production of penicillinase by washed suspensions of *B. cereus* has been followed by manometric assay of penicillin hydrolysis in a CO_2-bicarbonate buffer.

(2) In the presence of 10,000 units/ml. penicillin but without other source of N and in the absence of cell growth, penicillinase production at 35° follows the usual type of " logarithmic " curve characteristic of other enzyme adaptation processes, and may increase 40-fold over control cells, similarly treated but without penicillin.

(3) This adaptation is stimulated by glucose, O_2 and added amino-acids, and inhibited by oxine.

(4) Very small concentrations of penicillin, down to 0·004 units/ml. (8×10^{-9} M), are enough to cause marked adaptation.

(5) Cells treated at 0° with penicillin and then thoroughly washed will, on subsequent incubation in a penicillin-free medium, form penicillinase at a rate up to 30 times greater than untreated cells. This pretreatment effect occurs very rapidly—being about 50 per cent complete after only 10 minutes—and results in linear production of enzyme during subsequent logarithmic growth in a penicillin-free medium, the "Q penicillinase" value thus returning slowly to normal over a period covering several cell generations.

(6) The rate of penicillinase production increases with increase in the concentration of penicillin used for pretreatment up to 1 unit/ml., at which concentration maximal effect occurs.

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