# Adenosine Deaminase and Ribosidase in Spores of *Bacillus cereus*

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Inosine is a more effective germination stimulant than adenosine for spores of *Bacillus cereus* and *B. anthracis* (Powell & Hunter, 1955). A crude method of assessment, based on the paper-ionophoresis technique of Wade & Morgan (1954), indicated that extracts from disintegrated resting spores of *B. cereus* converted adenosine into inosine. This suggested the possibility that inosine might be, in fact, the germination stimulant, and that germination of a given spore suspension in adenosine might depend, to some extent, on its adenosine-deaminase activity. We have now studied quantitatively the deamination of adenosine by resting and germinated spores of *B. cereus*, and also its further breakdown to the purine and free ribose. The possible relationships between these reactions and spore germination in *B. cereus* are discussed. Part of this work has already been briefly reported (Powell, 1955).

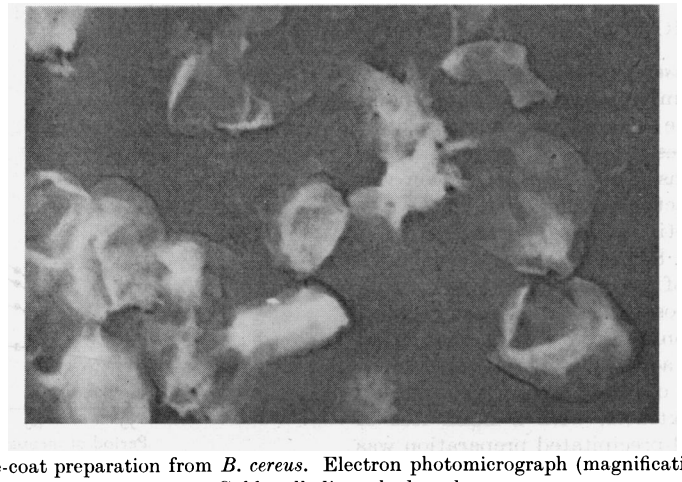

Fig. 1. Spore-coat preparation from *B. cereus*. Electron photomicrograph (magnification × 10 000). Gold–palladium shadowed.

## ORGANISMS AND METHODS

Resting-spore suspensions of a laboratory strain of *B. cereus* were obtained by growth in liquid-shaken culture as previously described (Powell & Hunter, 1955). Germinated forms were prepared by incubating resting spore suspensions containing about $2 \times 10^9$ spores/ml. for 30 min. with 2 mM inosine in 40 mM sodium potassium phosphate buffer pH 7·3. Resting and germinated spores were disintegrated in suspensions containing $2 \times 10^{10}$ spores/ml., using the Mickle (1948) tissue disintegrator with Ballotini beads size 12. The suspensions were ice-cooled at 10 min. intervals during disintegration, which usually took 1 hr. Homogenates were separated by centrifuging for 30 min. at 6000 *g* to give extract and 'debris' preparations. For some experiments (see below) the deaminase was precipitated from extracts with 30% (v/v) ethanol at 0°. It redissolved readily in water and buffer solutions. A spore-coat preparation was obtained by disintegrating a resting-spore suspension for approximately 20 min., i.e. until the appearance of stained films indicated that disruption had occurred, but the suspension still consisted mainly of recognizable spore-coat fragments. Smaller particles of doubtful origin were removed by repeatedly suspending in water, centrifuging for 15 min. at 900 *g*, and discarding the supernatant fluid. Five such treatments gave the suspension shown in Fig. 1.

Homogenates, extracts and debris fractions representing $0{\cdot}5$–$1{\cdot}0 \times 10^{10}$ spores/ml. were incubated with 2 mM adenosine and 40 mM sodium potassium phosphate pH 7·3 for various periods. The deamination reaction was stopped by adding

0·1 vol. of 25% (w/w) $HClO_4$. The ammonia produced was determined by the Conway (1947) technique, followed by nesslerization, allowance being made for endogenous ammonia production in the absence of adenosine. With extracts, the deamination reaction was also followed in a few experiments by measurements of light absorption at 265 mμ. (Kalckar, 1947), after appropriate dilution and correction for 'blank' absorption in the absence of adenosine. This method was more suitable for deaminase assay in ethanol-precipitated enzyme preparations than in crude extracts: crude extracts contained calcium dipicolinate (Powell, 1953) and gave a very high 'blank' absorption. The paper-ionophoresis method of Wade & Morgan (1954) was sometimes used as a further check on results obtained by the other methods. The ribosidase reaction was studied by determination of free ribose by the reductimetric method of Hagedorn & Jensen (1923) after protein precipitation with 0·25 vol. of 0·8% (w/v) NaOH and 0·75 vol. of 1·5% (w/v) $ZnSO_4,7H_2O$.

An ethanol-precipitated deaminase preparation from $10^{10}$ spores in 1 ml. of reaction mixture was tested for transaminase activity, the test system of Gunsalus & Tonzitich (1952) being used with adenosine as the amino-donor. After protein precipitation with 2·5% $HClO_4$, paper chromatograms were run in collidine–lutidine.

## RESULTS

*Adenosine deaminase* (*adenosine* → *inosine* + $NH_3$). Crude extracts from resting *B. cereus* spores deaminated adenosine more rapidly and completely than did homogenates (Fig. 2). This result is discussed below. The insoluble 'debris' fraction was without deaminase activity. Fig. 3 shows the rate of ammonia production during incubation of a mixture containing 1·8 mg. dry wt. of extract/ml., i.e. the equivalent of $5 \times 10^9$ spores/ml., and concentrations of adenosine between 1 and 8 mM. Under these conditions, the enzyme was saturated at concentrations of adenosine greater than 8 mM.

Addition of $\frac{1}{3}$ vol. of ethanol at 0° precipitated the enzyme from extracts, with a small loss of activity. The ethanol-precipitated preparation was freeze-dried and stored without further loss. Solutions of the ethanol-precipitated enzyme in phosphate buffer at pH 7·3 were relatively unstable, losing 50% of their activity during 18 hr. storage at 4°. The crude extract gradually lost about 50% of its activity during 3–4 days' storage at 4°. The change in ultraviolet absorption spectrum brought about by incubating adenosine with an ethanol-precipitated enzyme preparation is shown in Fig. 4. In Fig. 5 a comparison is made between the Conway and ultraviolet absorption methods of assaying adenosine deaminase, with an ethanol-precipitated preparation of rather low activity. The effect of pH on the deaminase activity of an ethanol-precipitated preparation dissolved in 25 mM acetate–barbiturate buffers (Michaelis, 1931) is shown in Fig. 6. The reaction attained a maximum rate at pH 8·7. Similar results were obtained in phosphate buffers.

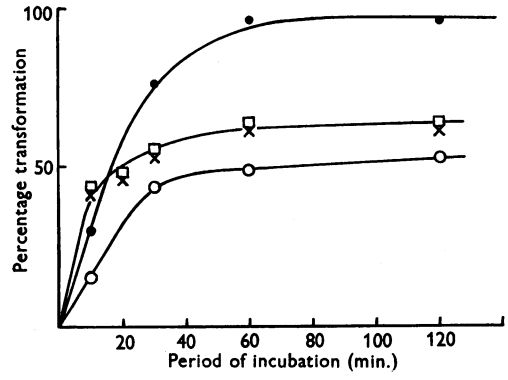


Fig. 2. Deaminase and ribosidase activity of disintegrated *B. cereus* spores. The equivalent of $10^{10}$ spores/ml. (7·2 mg. dry wt./ml.) incubated with 2 mM adenosine in 40 mM sodium potassium phosphate pH 7·3. ○, Deaminase activity of homogenate (7·2 mg. dry wt./ml.); ●, deaminase activity of extract (3·2 mg. dry wt./ml.); ×, ribosidase activity of homogenate (7·2 mg. dry wt./ml.); □, ribosidase activity of debris (4·0 mg. dry wt./ml.).

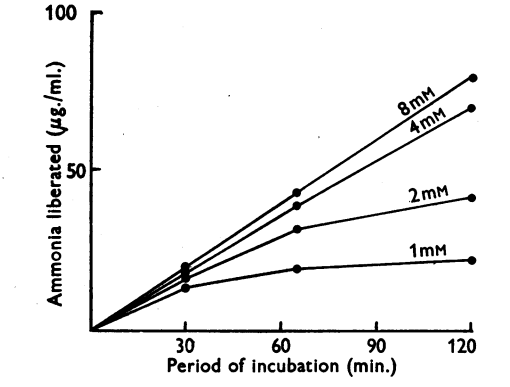


Fig. 3. Ammonia liberated during incubation of extract (1·8 mg. dry wt./ml.) from *B. cereus* spores with 1, 2, 4 and 8 mM adenosine in 40 mM sodium potassium phosphate (pH 7·3).

The reaction appeared to be a simple deamination. No oxygen uptake could be detected during incubation of adenosine with extracts or homogenates, nor any stimulation of ammonia production on the addition of crude boiled yeast extract (1·7 mg./ml.) or pyridoxal phosphate (10 μg./ml.).

There was no detectable conversion of oxoglutarate into glutamic acid in the presence of the ethanol-precipitated enzyme, adenosine and pyridoxal phosphate. Under the experimental conditions, a 10% conversion would have been readily observed.

Spore extracts were also tested for their deaminase activity on other naturally occurring purines and pyrimidines. Cytidine was deaminated at almost the same rate as adenosine (Fig. 7). Adenine, guanine, guanosine and cytosine were not attacked by the deaminase.

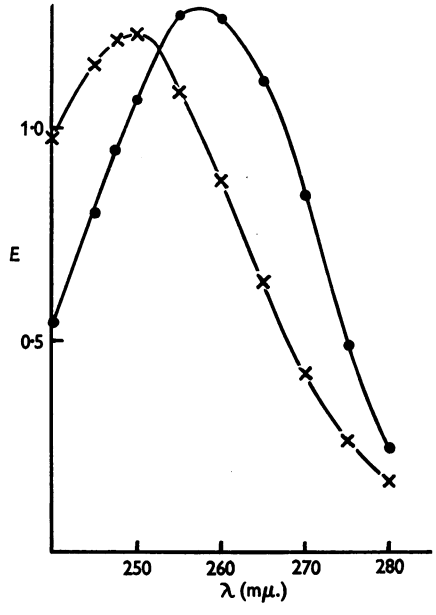


Fig. 4. Change in ultraviolet absorption spectrum during incubation of ethanol-precipitated deaminase with 2 mM adenosine in 40 mM sodium potassium phosphate (pH 7·3). Ethanol precipitate from spore extract ($2 \times 10^{10}$ spores/ml.) reconstituted in 40 mM sodium potassium phosphate to original volume of extract. Reaction mixture diluted 1:20 in 0·1 N-HCl. ●, $t=0$ min.; ×, $t=120$ min. Light path 1 cm.

Perhaps the most interesting property of the enzyme already reported (Powell & Hunter, 1955) was its heat stability in intact resting spores and its instability in extracts. We have confirmed this observation and found that deaminase activity both in homogenates and extracts was destroyed by heating for 15 min. at 60°. The deaminase survived heating at 60° for several hours in intact resting spores. As was suggested by previous semiquantitative experiments (Powell & Hunter, 1955), there appeared to be no correlation between the rate of adenosine deamination by homogenates or extracts from a given spore suspension and its germination rate in adenosine. Thus preheating a resting spore suspension for 1–2 hr. at 60° had no significant effect on the deaminase activity of homogenates prepared from it, nor did the addition of 1 mM tyrosine + 1 mM L-alanine to homogenates increase their adenosine-deaminase activity (see below).

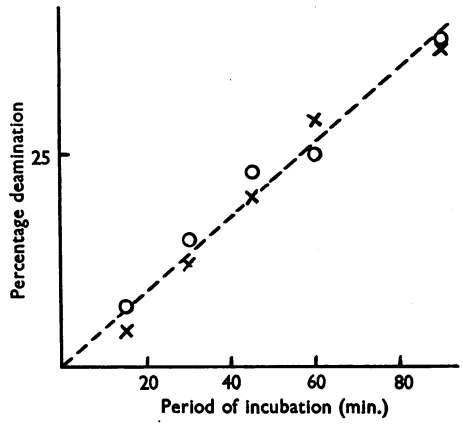


Fig. 5. Deamination of 2 mM adenosine in 40 mM sodium potassium phosphate (pH 7·3) by ethanol-precipitated deaminase. Ethanol precipitate from spore extract ($10^{10}$ spores/ml.) reconstituted in 40 mM sodium potassium phosphate to original volume of extract. O, Ammonia estimated by Conway technique; ×, deamination assessed from decrease in light absorption at 265 mμ. (60% decrease = 100% deamination).

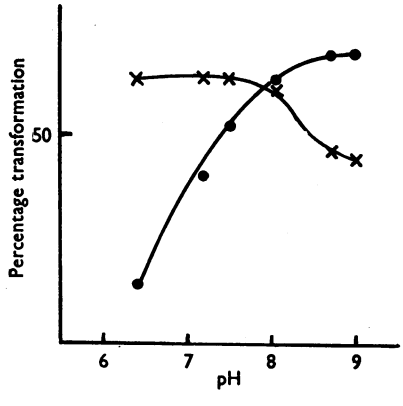


Fig. 6. Effect of pH on ethanol-precipitated deaminase (●) and debris ribosidase (×) activity. Ethanol-precipitated deaminase and resuspended debris incubated for 50 min. with 2 mM adenosine.

A few experiments were made with germinated spores of *B. cereus*. Both intact and disintegrated suspensions converted adenosine into inosine and all the deaminase activity appeared in the extracts.

In contrast with resting spores, the adenosine deaminase of intact germinated spores was heat-labile, being completely destroyed by heating for 15 min. at 60°. Homogenates and extracts also lost their deaminase activity during this treatment.

*Adenosine ribosidase (adenosine→adenine + ribose).* The adenosine-ribosidase activity of homogenates was associated with the insoluble 'debris' fraction, the extract being completely inactive. The reaction appeared to be independent of phosphate (cf. Kalckar, 1945). Paper chromatography (Partridge, 1949) of the products of reaction in phosphate buffer indicated the presence of free ribose with no evidence of ribose phosphate. The debris was dialysed for 24 hr. against distilled water with no loss of activity, whether tested in the presence or absence of phosphate. A spore-coat preparation (Fig. 1) showed ribosidase activity roughly equivalent to that of the 'crude' debris fraction on a dry-weight basis.

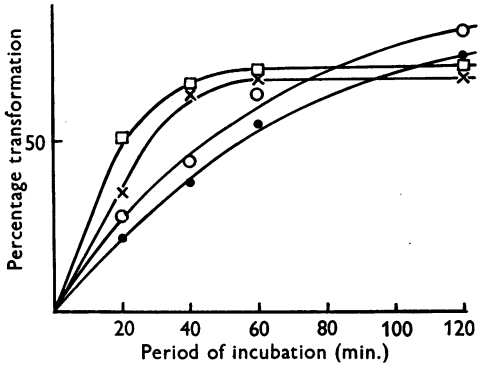


Fig. 7. Deamination of 2 mM adenosine (○) and 2 mM cytidine (●) in 40 mM sodium potassium phosphate (pH 7·3) by extract from *B. cereus* spores. Ribosidase activity of spore debris in 2 mM adenosine (□) and 2 mM inosine (×).

The ribosidase reaction proceeded at approximately the same rate in homogenates as in resuspended debris fractions (Fig. 2). When debris preparations were tested for their ribosidase activity on other naturally occurring nucleosides, it was found that inosine was hydrolysed at approximately the same rate as adenosine (Fig. 7). Guanosine was feebly attacked, but not xanthosine or cytidine.

The observation that deamination of adenosine was less rapid and complete in homogenates than in extracts (Fig. 2), whereas the ribosidase reaction proceeded at roughly the same rate in homogenates and in resuspended debris, can now be explained. Both deaminase and ribosidase were present in homogenates. The deaminase attacked adenosine, but not the product of the ribosidase reaction (adenine), whereas the ribosidase attacked both adenosine and the product of its deamination (inosine). Thus in homogenates the amount of substrate available to the deaminase was reduced by the ribosidase, whereas the ribosidase reaction was unaffected by the deamination of the substrate.

The relationship between ribosidase activity and pH is shown in Fig. 6. In contrast with the deaminase, there was a marked decrease in activity in solutions more alkaline than pH 7·5. The ribosidase was remarkably heat-stable, both in intact resting spores and in homogenates: there was no appreciable loss in activity when debris preparations, suspended in water, were heated at 100° for 1 hr. The heat stability of these preparations suspended in 40 mM sodium potassium phosphate buffer at pH 6, 7, 8 and in 40 mM borate pH 9·2 was also tested. At pH 6, there was no loss of activity during 1 hr. at 100°, and only a 10% loss at pH 7, 8 and 9·2.

Germinated spores of *B. cereus* also showed ribosidase activity of remarkable heat stability. This was again associated with the debris fraction after disintegration. When intact germinated spores were incubated with adenosine, a very low value for ribose production was obtained, although paper ionophoresis of the mixture indicated a disappearance of adenosine and formation of adenine and hypoxanthine. When these intact spores were heated at 60° for 15 min. before incubation with adenosine, the expected amounts of ribose appeared. The disappearance of ribose in the presence of intact germinated spores can therefore be ascribed to their ability to metabolize this substance. Homogenates of germinated spores were inactive in this respect and gave the expected value for ribose production without preliminary heating.

*Spore germination in* Bacillus cereus. In Fig. 8 the germination rate of preheated *B. cereus* spores in adenosine is compared with that of an unheated suspension in adenosine, inosine and an adenosine–tyrosine–alanine mixture. Different spore crops of *B. cereus* showed different degrees of sensitivity to adenosine alone (Powell & Hunter, 1955). The suspension referred to in Fig. 8 was extreme in its resistance to stimulation by adenosine, although germination proceeded rapidly in inosine and in the adenosine–tyrosine–alanine mixture. After heating at 60° for 1 hr. before incubation with adenosine, germination was rapid and complete.

As we have stated (see above), deaminase assays on homogenates from this and similar spore suspensions before and after heat stimulation did not support the suggestion that the adenosine deaminase system was activated during heat treatment. Results obtained from experiments with

spore homogenates may, however, be of doubtful value in attempting to correlate adenosine deaminase activity in *intact* spores with their rate of germination in the presence of adenosine (Powell & Hunter, 1955). On the other hand, it was not possible to assess the adenosine-deaminase activity of intact resting spores suspended in adenosine solution; in such experiments the deaminase activity measured was that of a mixture of resting and germinated forms. An interesting result was obtained, however, when the spore suspension referred to in Fig. 8 was incubated for 1 hr. with adenosine. There was no liberation of ammonia and no germination occurred, but the adenosine was converted entirely into adenine and free ribose, neither of which substances stimulates spore germination in *B. cereus*. This result provides some support for the suggestion that failure to deaminate adenosine may lead to failure to germinate, and also explains why this spore suspension remained 'dormant' on prolonged incubation with adenosine.

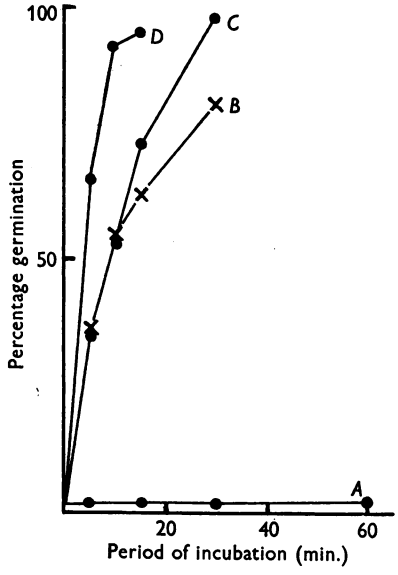


Fig. 8. Germination of *B. cereus* spores in *A*, 1 mM adenosine; *B*, 1 mM adenosine + 1 mM L-tyrosine + 1 mM L-alanine; *C*, 1 mM inosine. *D*, germination in 1 mM adenosine after pre-heating 1 hr. at 60°.

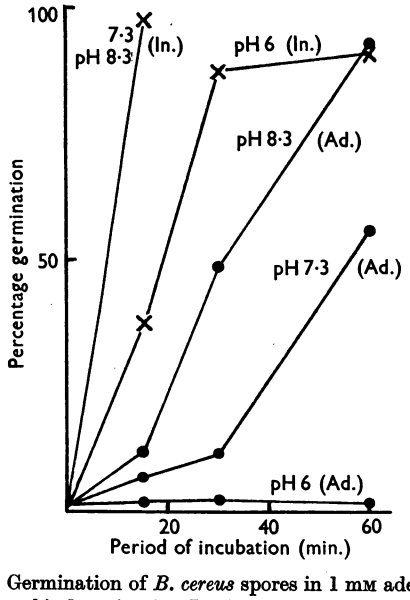


Fig. 9. Germination of *B. cereus* spores in 1 mM adenosine (Ad.) and in 1 mM inosine (In.) in 40 mM sodium potassium phosphate (pH 6, 7·3 and 8·3).

Since the activity of the deaminase increases and that of the ribosidase decreases as the pH becomes alkaline, it might be expected that germination of *B. cereus* spores would be more rapid and complete in alkaline solutions of adenosine. This was found to be the case. A typical set of results is shown in Fig. 9. On the other hand, the germination rate in inosine was much less dependent on pH (Fig. 9). This again suggests a correlation between adenosine deaminase activity and rate of germination in adenosine.

## DISCUSSION

Perhaps the chief point of interest in this investigation was the heat stability of adenosine deaminase in intact resting spores of *B. cereus* and its loss of heat stability during disintegration. In these properties, the enzyme resembled catalase and pyrophosphatase systems in extracts from resting *B. subtilis* spores (Murrell, 1952) and a glucose-dehydrogenase system in extracts from spores of *B. terminalis*, recently investigated by Church (1955). It is also interesting in this connexion that Keppie (1951) demonstrated heat-stable toxin in intact spores of *Clostridium botulinum* and heat-labile toxin in extracts from these spores after disintegration. We found that the adenosine deaminase in *B. cereus* spores became heat-labile during germination. The effect of germination on the other systems mentioned above does not appear to have been investigated.

The simplest way of accounting for these observations and for the high density (McIntosh & Selbie, 1937) and extremely low metabolism (Spencer & Powell, 1952; Crook, 1952) of bacterial spores is to postulate that their protoplasm is anhydrous. This view was first clearly stated by Lewith (1890), and has never been definitely disproved. Henry & Friedman (1937) attempted to compare the water content of spores with that of vegetative cells, and their conclusion—that spores and vegetative cells contained equal amounts of

water—appears to have been accepted until recently (Powell & Strange, 1953; Waldham & Halvorson, 1954) with less reservation than the authors themselves intended. Waldham & Halvorson (1954) found that dried spores of *B. terminalis* showed less affinity for water than did dried vegetative cells, and suggested that 'in spores, the polar groups necessary to attract water molecules are in some way masked'. We incline to the view that the spore protoplasm is a highly condensed 'waterproofed' system, possibly stabilized by calcium dipicolinate and by a certain peptide which appears to be associated with the spore coat (Powell & Strange, 1953; Strange & Powell, 1954; Strange & Dark, 1956). The general structure of the spore coat itself may also contribute to this stability (see below). During germination, calcium dipicolinate and the peptide are excreted (Powell & Strange, 1953), heat resistance is lost, and the spore becomes capable of active metabolism. It seems possible that at this stage the spore protoplasm becomes hydrated (Lewith, 1890), some process akin to depolymerization occurs, and the number of reactive enzyme groups is greatly increased. On this basis, it might be expected that disruption of the spore in water would produce changes similar to, but less orderly than those occurring during germination, and that enzyme systems which were heat-resisting in the intact spore would become heat-labile and possibly more reactive during disintegration. In our experiments with the adenosine deaminase system of *B. cereus* spores, we suggest that the possible stimulatory effect of pre-heating on this system in intact spores may have been masked by the changes which occurred during disintegration.

The heat stability of the adenosine ribosidase system which may be associated with the spore coat of *B. cereus* presents a rather different problem. It is possible that this heat stability is associated with certain other properties of the spore-coat preparations, e.g. their resistance to proteolytic enzymes (Strange & Dark, 1956). We found that ribosidase activity proceeded in intact spore suspensions unaccompanied by germination, and that the ribosidase system was heat-stable in resting and germinated spores both before and after disintegration. In these respects, the ribosidase resembled the alanine racemase of *B. terminalis* spores described by Stewart & Halvorson (1954). By ultrasonic disintegration these authors obtained an extract which contained 10–15 % of the original activity of the spores. This activity was associated with particles of indefinite size which the authors suggested may well have represented fragments of the spore surface. Further ultrasonic treatment of these particles rendered their alanine-racemase activity sensitive to heat and to attack by pepsin. It is possible that examination of spore-coat preparations of *B. terminalis* for alanine-racemase activity, and a study of the effect of ultrasonic treatment on the ribosidase activity of *B. cereus* spores might reveal further similarities between the two systems. Other enzyme systems, heat-resistant both in intact and distingegrated spores, and associated with the centrifuged deposit after disintegration, have been described. Thus, Murrell (1952) found that in addition to the heat-labile catalase present in extracts from disintegrated *B. subtilis* spores (see above) there was a less active heat-stable catalase activity associated with intact spores and with the centrifuged deposit after disintegration. This deposit also catalysed the oxidation of *p*-phenylenediamine, ascorbic acid and cysteine. Similar activity was shown by intact resting spores (Murrell, 1952; Spencer & Powell, 1952).

It therefore seems possible that the spore enzymes may be divided into two types, one of which loses heat stability during disintegration and germination, whereas the other retains it. The latter type may be closely associated with the spore coat. An investigation of the chemical structure of spore coats is now in progress in this Laboratory, and may throw some light on the properties of the ribosidase, and possibly other heat-stable systems, which we cannot at the moment attempt to explain.

## SUMMARY

1. Disintegrated resting-spore suspensions of *Bacillus cereus* deaminated adenosine and cytidine, but not adenine, guanosine, guanine or cytosine. These homogenates also showed ribosidase activity towards adenosine and inosine, but not towards guanosine, xanthosine, cytidine or uridine.

2. The deaminase activity was associated with the spore extract and the ribosidase with the spore debris.

3. Adenosine deaminase was heat-stable in intact resting spores, but heat-labile in homogenates and in germinated spores. The ribosidase was heat-stable both in intact and distintegrated spores. Germinated spores showed heat-stable ribosidase activity.

4. The deamination of adenosine was non-oxidative and appeared not to require a cofactor. The ribosidase reaction produced free ribose and no ribose phosphate.

5. The possible relationships between these reactions and the germination of *B. cereus* spores in adenosine are discussed.

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REFERENCES

Church, B. D. (1955). The role of L-alanine and glucose on dormancy in spores of aerobic bacteria. D.Phil. Thesis: University of Michigan.
Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*. London: Lockwood.
Crook, P. G. (1952). *J. Bact.* **63**, 193.
Gunsalus, C. F. & Tonzitich, J. (1952). *Nature, Lond.*, **170**, 162.
Hagedorn, J. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
Henry, B. S. & Friedman, C. A. (1937). *J. Bact.* **33**, 323.
Kalckar, H. M. (1945). *J. biol. Chem.* **158**, 723.
Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 445.
Keppie, J. (1951). *J. Hyg., Camb.*, **49**, 36.
Lewith, S. (1890). *Arch. exp. Path.* **26**, 341.
McIntosh, J. & Selbie, F. R. (1937). *Brit. J. exp. Path.* **18**, 162.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Mickle, H. (1948). *J. R. micr. Soc.* **68**, 10.
Murrell, W. G. (1952). Metabolism of bacterial spores and thermophilic bacteria. D.Phil. Thesis: University of Oxford.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Powell, J. F. (1953). *Biochem. J.* **54**, 210.
Powell, J. F. (1955). *Abstr. 3rd Int. Congr. Biochem., Brussels*, p. 93.
Powell, J. F. & Hunter, J. R. (1955). *J. gen. Microbiol.* **13**, 59.
Powell, J. F. & Strange, R. E. (1953). *Biochem. J.* **54**, 205.
Spencer, R. E. J. & Powell, J. F. (1952). *Biochem. J.* **51**, 239.
Stewart, B. T. & Halvorson, H. O. (1954). *Arch. Biochem. Biophys.* **49**, 168.
Strange, R. E. & Dark, F. A. (1956). *Biochem. J.* **62**, 459.
Strange, R. E. & Powell, J. F. (1954). *Biochem. J.* **58**, 80.
Wade, H. E. & Morgan, D. M. (1954). *Biochem. J.* **56**, 41.
Waldham, D. G. & Halvorson, H. O. (1954). *Appl. Microbiol.* **6**, 333.

# Crystalline Bacterial Penicillinase

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Bacterial penicillinase, which catalyses the hydrolysis of penicillin to the antibiotically inactive penicilloic acid, was first discovered by Abraham & Chain (1940) in a strain of *Escherichia coli* and has since been found in a wide variety of micro-organisms (Abraham, 1951). Several attempts have been made to isolate and purify the enzyme, amongst which the most successful appear to have been those reported by Wainwright (1950), Morgan & Campbell (1947), McQuarrie & Liebmann (1944) and Murao (1950). Although preparations of high activity were obtained by these workers and a crystalline product was claimed by Murao (1950) and by Baudet & Hagemann (1954), no criteria of purity, such as electrophoretic or ultracentrifugal analyses, were applied, and it is difficult to compare the specific activities achieved by different workers because of the varying and often unsatisfactory methods of assay employed. Strains of bacteria vary enormously in the amount of penicillinase they produce and in the extent to which its formation is stimulated by treatment of cells with penicillin. The organism most commonly used so far for isolation attempts has been *Bacillus cereus* strain NRRL 569, which produces and liberates into the medium large quantities of the enzyme after induction with penicillin (see Pollock, 1950). Pollock & Torriani (1953) succeeded in isolating the induced enzyme from the culture supernatant fluid of this strain, grown in a peptone medium, to a point which subsequent work (Kogut, Pollock & Tridgell, 1956) showed to have been nearly 50% pure. Experience with peptone showed that the difficulty of eliminating high molecular-weight substances present in the medium outweighed any of its possible advantages, such as increased yield of enzyme, and it was decided in future to use casein hydrolysate.

Late in 1953 a constitutive penicillinase-producing *B. cereus* strain, capable of forming large amounts of the enzyme without penicillin treatment, was isolated by Dr P. H. A. Sneath. This constitutive strain (*B. cereus* 5/B) appeared as a spontaneous mutant (Sneath, 1955) from a penicillin-sensitive, parent strain (*B. cereus* 5), physiologically distinct from *B. cereus* 569.

*B. cereus* 5/B was found to produce, in the absence of penicillin, penicillinase activities two to three times those of penicillin-induced *B. cereus* 569. Because of the high yield of enzyme, and the fact that penicillin additions to the medium during growth were not necessary, it was decided to use strain 5/B in an attempt to prepare a large batch by growth of cells in casein hydrolysate.