THE CLEAVAGE OF ADENOSINE BY SPORES OF *BACILLUS CEREUS*[1]

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Received for publication April 25, 1955

Recent investigations of the germination of bacterial spores of some species in systems where no subsequent vegetative development occurs have led to the elucidation of the rather simple requirements of the spores for germination. Some of these requirements have been summarized by Church *et al.* (1954). As a result of such investigations, Stewart and Halvorson (1953) found an active enzyme, alanine racemase, in certain aerobic spores. This enzyme was found to be unusually resistant to destruction by heat. Subsequent investigations, however (Church *et al.*, 1954), made it apparent that the activity of this enzyme is not directly related to the germination process, and consequently it was postulated that the L-alanine required by these spores for rapid germination was involved in another, as yet unspecified, reaction.

In addition to L-alanine, spores of certain species have been found to require adenosine for germination (Hills, 1949; Powell, 1951; Stewart and Halvorson, 1953, and others).

It has been shown (Lawrence, 1955) that the rapid germination of spores of *Bacillus cereus* var. *terminalis* in the presence of purine ribosides and alanine is always accompanied by the cleavage of the riboside.

Phosphorolytic cleavage of various nucleic acid derivatives has been demonstrated in animal and certain non-spore forming bacterial cells by a number of workers (Kalckar, 1945; Paege and Schlenk, 1950; Greenberg, 1951; Manson and Lampen, 1951*a*, 1951*b*; Klein, 1935; Friedkin and Kalckar, 1950, and others). Wajzer (1947) and Friedkin and Kalckar (1950) showed that a number of these systems are reversible. Nucleoside hydrolyzing enzymes have been found in yeast (Heppel and Hilmoe, 1952; Carter, 1951) and in lactobacilli (Lampen and Wang, 1952; Kalckar *et al.*, 1952.)

This report deals with the reaction between intact spores of *B. cereus* var. *terminalis* and adenosine. The products of the reaction were identified and the course of the reaction studied under various conditions.

MATERIALS AND METHODS

Washed and dried spores of *B. cereus* var. *terminalis* were obtained from Dr. H. O. Halvorson. They were stored at −20 C.

Paper chromatography, performed by the descending procedure in the 20 C constant temperature room, was used for identification of sugars and of purine derivatives. The solvent used for sugars was the ethanol-boric acid mixture of Cohen and Scott (1950). For locating the sugar spots, the paper strips were sprayed with either 1 per cent *p*-anisidine in *n*-butanol or with *m*-phenylene diamine according to the method of Chargaff, Levine and Green (1948). The method used to identify the purine derivatives was that described by Carter (1950).

The amount of reducing sugar was estimated by the procedure of Nelson (1944). Adenosine was determined by the spectrophotometric method of Kalckar (1947*b*), using as the source of adenosine deaminase a sample of intestinal phosphatase kindly supplied by Pentex, Incorporated, Kankakee, Illinois. One μg of this material as supplied will deaminate approximately 0.015 μg adenosine per minute and will not attack adenine.

RESULTS

Identification of adenine as a product of the action of spores on adenosine. It was considered that the adenosine molecule in the presence of spores might be deaminated to yield inosine, or might be split at the ribosidic bond to yield adenine.

In order to distinguish between these hypotheses, spores of *B. cereus* (5 mg per ml) were incubated at room temperature in a water solution of adenosine (3.75 μM ml). After 40 minutes, the mixture was added to an equal volume of cold 4 per cent perchloric acid to stop further reaction, and the spores were removed by centrifugation.

[1] Journal Paper No. 1001, New York State Agricultural Experiment Station, Cornell University, Geneva, New York.

TABLE 1

Identification of adenine as the product of the reaction between spores and adenosine

	Adenosine-Spore Supernate	Adenosine Control	Adenine Control	Adenosine-5-Phosphate Control	Inosine Control
Rf of spot absorbing UV light	0.41	0.23	0.41	0.0	0.14
Rf of spot stable to HNO_2 (method of Reguera and Asimov (1950))	0.41	no spot	0.41	no spot	no spot

The solvent used was *n*-butanol saturated with a 10 per cent aqueous solution of urea.

In each of several replicate experiments, the absorption spectrum of the cell-free supernatant fluid was found to have the peak at 259.5 to 260.5 mμ. This indicated that the product of the reaction was probably adenine or an adenine nucleotide. These compounds, and adenosine as well, have absorption peaks at 260 mμ and by ultraviolet absorption spectra are indistinguishable from one another, while the peak absorption of inosine occurs at 249–250 mμ (Kalckar, 1947*a*).

The identification of the reaction product as adenine was verified by paper chromatography using the butanol-urea solvent of Carter (1950) and by the color reaction of the ultraviolet absorbing spots as described by Reguera and Asimov (1950). It may be observed (table 1) that the supernatant fluid from spore-adenosine mixtures contained adenine but no adenosine, adenylic acid or inosine.

It was found that the presence of spores was necessary for the breakdown of adenosine in this system, as evidenced by the stability of adenosine in the absence of cells for at least 2 hours at 60 C and for at least 30 minutes at 100 C.

Identification of the reducing sugar produced from adenosine. The supernatant fluid resulting from the incubation of spores with adenosine gave a strong positive test for pentose by the methods of Tauber (1937) and of White and Green (1932). Ribose phosphates and adenosine which conceivably could have been present in the supernatant fluid gave negative tests by these procedures. Paper chromatography of the supernatant fluid by the procedure of Cohen and Scott (1950) indicated that the sugar was free ribose (Rf of both ribose and unknown was 0.65). Since ribose-l-phosphate, which is the probable product of a phosphorolytic cleavage, is rapidly hydrolyzed at the pH of 1 obtained by deproteinizing with perchloric acid (Lowry and Lopez, 1946), the identification of the sugar was repeated in supernatants which had not been subjected to acid deproteinization.

No evidence for the presence of a phosphate ester in the supernatant fluid was found by the use of the ammonium molybdate spray of Hanes and Isherwood (1949). In addition, attempts were made to precipitate by barium and ethanol any sugar phosphates present (Umbreit *et al.*, 1945). The slight precipitate which was obtained yielded no detectable sugar after 3.5 hours' hydrolysis with N HCl at 100 C. The material soluble in barium-ethanol contained all the reducing sugar. This sugar had an Rf of 0.65, corresponding to that of ribose.

Quantitative relation between adenosine and reducing sugar. An attempt was made to obtain a material balance between the adenosine and the reducing sugar during the course of the reaction. For the adenosine measurements, aliquots of the reaction mixture were removed, deproteinized as before, diluted and incubated at 20 C with a suspension of intestinal phosphatase which possessed adenosine deaminase activity. Kalckar (1947*b*) showed that the adenosine concentration could be measured by the decrease in optical density at 265 mμ, due to the deamination of adenosine to inosine. The initial absorption at 265 mμ was obtained from a control sample in which the deaminase preparation had been inactivated by boiling for 15 minutes. It was found that the deamination of 10 μg adenosine resulted in a decrease in optical density of 0.274. This value was used in estimating adenosine concentration.

The data (table 2) which is typical of three replicate experiments at various temperatures, show that when intact spores were used, the reducing sugar was found in smaller amounts than was necessary to account for the adenosine which had disappeared.

The relationship between the amounts of adenosine cleaved and sugar produced was also determined using extracts of spores rather than

TABLE 2

Material balance between adenosine disappearance and reducing sugar appearance during incubation of spores (5 mg per ml) with adenosine (5.62 μM per ml)

Time Incubated at 37 C Minutes	Adenosine Cleaved μM per ml	Reducing Sugar Produced. μM per ml. (assume MW = 150)
0	0.0	0.0
15	1.85	0.95
45	3.85	2.62
60	4.89	3.04

intact spores. In this case, the balance was even less satisfactory; only 0.18 μM reducing sugar was present even after all (5.62 μM) of the adenosine had disappeared.

The explanation for the lack of balance between the adenosine cleaved and the reducing sugar produced is not evident. In an attempt to demonstrate metabolism of the ribose by intact spores or spore extracts, these were incubated aerobically in a Warburg apparatus at 37 C with ribose, sodium pyruvate or L-alanine as substrates, each with and without adenosine. No gas exchange was noted with the spore extracts even after prolonged incubation (2 hours). With intact spores, no gas exchange occurred for the first 25 minutes. However, after 25 minutes in the presence of pyruvate or L-alanine with adenosine, both CO_2 evolution and oxygen uptake began. With ribose as a substrate for intact spores, no gas exchange was noted over a period of 2 hours' incubation, and all the reducing sugar was recovered at the end of that period. These results indicate that the spores before germination, as well as the spore extracts do not oxidize free ribose. The respiration after prolonged incubation of intact spores with adenosine in the presence of the other substrates was attributed to the metabolic activity of the newly germinated spores.

Studies on the rate of breakdown of adenosine. The reaction rate was followed by measuring the rate of production of the reducing sugar during incubation of 1 mg per ml spores with 1.5 mg (5.62 μM) adenosine per ml. Aliquots were removed at intervals, deproteinized with perchloric acid, the cells removed by centrifugation and the reducing sugar estimated by the method of Nelson (1944). Typical results of such determinations show (figure 1) that the concentration of reducing sugar increased linearly with time.

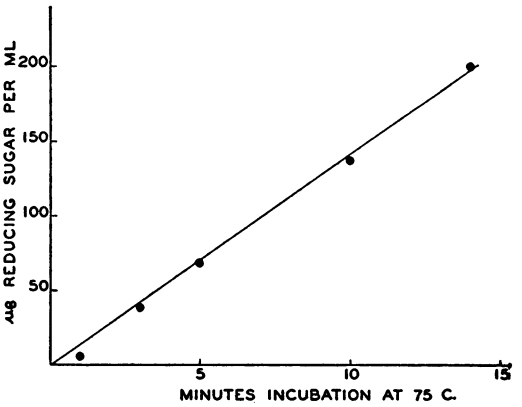

Figure 1. The increase in concentration of reducing sugar with time of incubation. Spores (1 mg per ml) incubated with 1500 μg (5.62 μM) adenosine per ml at 75 C.

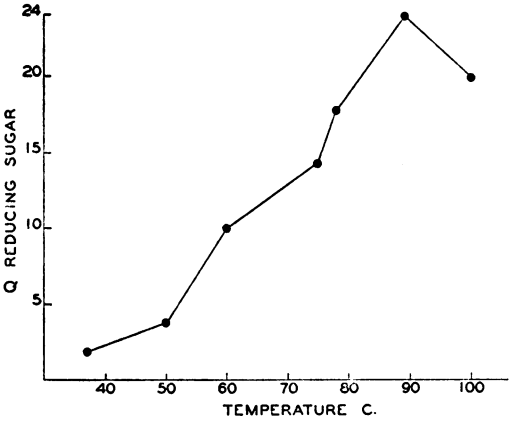

Figure 2. The effect of temperature of incubation on the rate of production of reducing sugar. Substrate: adenosine, 1500 μg (5.62 μM) per ml. A Q of 10 corresponds to 0.067 μM sugar as ribose per mg spores per minute.

In order to determine the effect of temperature on the rate of reaction, Q values, defined as μg reducing sugar produced per minute by 1 mg of spores from 5.62 μM adenosine were calculated from similar experiments at various temperatures. These Q values (figure 2) which were repeatedly obtained make it apparent that this reaction has a very high optimum temperature under the conditions described. The apparent energy of activation in the range from 37 to 89 C was calculated to be 11,500 calories, which is close to the value found for many other enzyme reactions (Lardy, 1949).

From data obtained on the rate of cleavage of

adenosine at 37 C, half maximum velocity was calculated to occur at an initial substrate concentration of 1.0×10^{-3} M. Heppel and Hilmoe (1952) found a value of 1.1×10^{-3} M for the phosphorolysis of inosine, and 6.5×10^{-4} M for the phosphorolysis of nicotinamide riboside by enzymes from yeast. This value of the Michaelis constant indicates a relatively low affinity of enzyme and substrate, in agreement with the finding of Stewart and Halvorson (1953) on the affinity for alanine of alanine racemase from these same spores.

Heat stability. The effect of prior heating of a spore suspension on its ability to catalyze the liberation of reducing sugar from adenosine was studied. A water suspension of spores (2 mg per ml) was immersed in a boiling water bath for various periods of time. Aliquots were removed, rapidly chilled, mixed with adenosine to a final concentration of 1 mg per ml spores and 5.62 μM per ml adenosine, and incubated at 60 C. During the incubation, the concentration of reducing sugar was estimated as described above and Q values were calculated.

The ability of these heated spores to germinate was also determined. It was found that heating for 60 minutes at 100 C prevented subsequent germination.

From the results of such determinations (figure 3) it can be seen that a heat treatment sufficient to prevent the germination of the spores has relatively little effect on the ability of the spores to cleave adenosine. In fact, the spores retain this ability to a remarkable degree after heating. Even after 4 hours at 100 C, the rate of sugar liberation was lowered by only 50 per cent. However, suspensions of spores (5 mg per ml) autoclaved for 15 minutes at 121 C were unable to liberate any measurable amount of reducing sugar from adenosine.

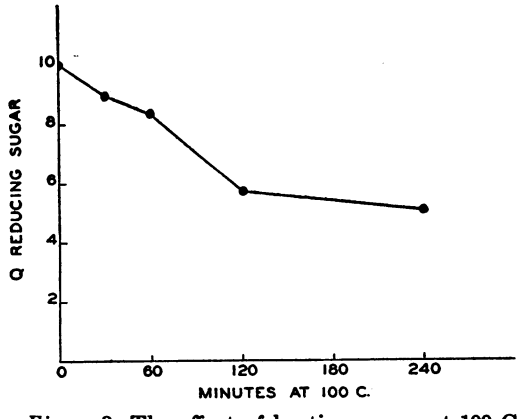

Figure 3. The effect of heating spores at 100 C for various periods of time on their ability to liberate reducing sugar when subsequently incubated with adenosine at 60 C.

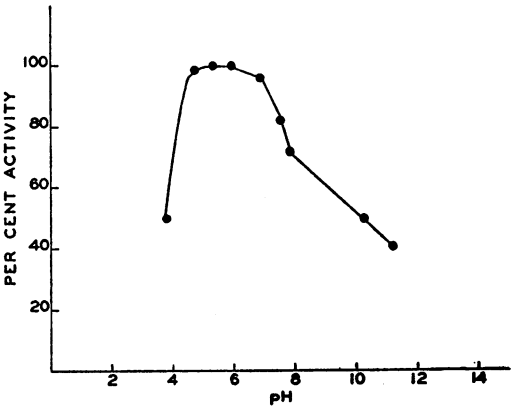

Figure 4. The effect of pH of the suspending medium on the rate of production of reducing sugar. Buffer: 0.0167 M acetate or phosphate. Substrate: adenosine 5.62 μM per ml.

Phosphate requirement. The results so far reported had been obtained using water alone as the suspending medium. Church *et al.* (1954) found that 0.067 M phosphate was required for optimum germination of these spores. Therefore, the effect of various concentrations of phosphate on the rate of sugar liberation was determined. The rates were measured as before, using 0.017, 0.034 and 0.067 M phosphate buffer, pH 6.9 as the suspending medium. Incubation was at 60 C. In each case, the rates were found to be identical with those obtained in distilled water, that is, 10 μg (0.067 μM) sugar per minute per mg spores. Similarly, the inclusion of 0.007 M disodium arsenate in place of the phosphate did not affect the rate.

pH optimum. The effect of the pH of the medium upon the rate of cleavage was studied using as substrate 5.62 μM per ml adenosine in 0.017 M buffers (phosphate and acetate). It appeared (figure 4) that the pH optimum was near pH 5.5.

DISCUSSION

The work reported here demonstrates that spores of a strain of *B. cereus* catalyze the cleavage of adenosine to yield the free base adenine and the free sugar ribose. The lack of effect of added phosphate or arsenate on the rate of cleavage, as

well as the absence of phosphorylated sugars among the reaction products, makes it likely that the cleavage occurs without involvement of phosphate.

It has not been possible to demonstrate synthesis of adenosine from adenine and ribose or ribose phosphate, using spores as a catalyst. No evidence was found for an exchange reaction upon incubation of adenosine and hypoxanthine with spores of *B. cereus*. These results are similar to those obtained with hydrolytic enzymes from yeast by Heppel and Hilmoe (1952) and are consistent with the finding (Lawrence, 1955) that inosine, the product of such an exchange, as well as adenosine is cleaved by spores of this organism.

In the experiments reported here, it was found that the sugar recovered was insufficient to account for all of the adenosine cleaved. This could be interpreted as indicating another pathway for attack on the adenosine, one in which ribose is not liberated. This explanation appears unlikely since adenine and ribose were the only products detected.

SUMMARY

Incubation of adenosine in the presence of spores of *Bacillus cereus* has been shown to result in the cleavage of the riboside into the free base adenine and the free sugar ribose. The amount of ribose recovered, however, was insufficient to account for all of the adenosine which was cleaved.

The optimum pH of the reaction was found to be between pH 5 and 6. Addition of phosphate or arsenate to the system had no effect on the rate or extent of cleavage.

The reaction was found to have a very high optimum temperature, near 90 C, with an energy of activation of 11,500 calories. The spore suspension retained activity to a remarkable degree after heating at 100 C for 4 hours, but the activity was destroyed by autoclaving the spores at 121 C for 15 minutes.

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