PATHWAYS OF GLUCOSE CATABOLISM IN INTACT HEAT-ACTIVATED SPORES OF BACILLUS CEREUS

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When lyophilized dormant spores of Bacillus cereus strain terminalis are heated in phosphate buffer they become activated, gaining the ability to oxidize glucose, gluconate, 2-ketogluconate and pyruvate but not other hexoses, phosphorylated hexoses or acetate (Church and Halvorsnn, 1957). These results, along with the results of studies on the enzymes present in extracts of these heat-activated spores, were interpreted to mean that a functional Embden-Meyerhof (EM) system was absent and that glucose was utilized by means of a modified hexosemonophosphate (HMP) pathway coupled with an active system for acetate oxidation, presumably the tricarboxylic acid cycle (Halvorson and Church, 1957; Doi et al., 1959). On the other hand, when an isotopic technique was used to estimate the pathways of glucose catabolism in resting suspensions of vegetative cells of the same organism, harvested either in the early logarithmic or sporangial phase, the results indicated that about 98% of the glucose oxidized to CO_2 by the intact cells was catabolized via the EM pathway (Goldman and Blumenthal, 1960). In order to check the validity of the conclusion that spores of this organism do not possess an operative EM pathway, which is evident in the vegetative stage, we have now estimated the pathways of glucose catabolism in intact, heat-activated spores by using two different isotopic techniques with specifically C^{14}-labeled glucose. Our results indicate that the EM is a major pathway for glucose catabolism in these spores although the HMP pathway, or its equivalent, becomes quantitatively

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TABLE 1

DISTRIBUTION OF C^{14} IN PRODUCTS OF C^{14}-GLUCOSE CATABOLISM

Product Isolated	Glucose-U-C^{14} RSA	Glucose-U-C^{14} % of total C^{14}	Glucose-1-C^{14} RSA	Glucose-1-C^{14} % of total C^{14}	Glucose-3,4-C^{14} % of total C^{14}	Glucose-6-C^{14} RSA	Glucose-6-C^{14} % of total C^{14}	% glucose utilized, cumulative	% glucose via HMP pathway, cumulative	% spore germination, cumulative
Respiratory CO_2										(33)
0- 15 min.		0.1		0.1	0.3		0.01	7.9	1.3	48
15- 30 min.		0.5		1.2	2.5		0.02	24.3	5.5	62
30- 60 min.		3.8		3.9	8.8		0.04	54.4	9.5	73
60-120 min.		13.8		21.9	34.1		0.2	98.8	27.5	80
120-180 min.		8.4		11.5	23.0		0.2	100.0	38.0	80
Total		26.6		38.6	68.7		0.5	100.0	38.0	
Acetate	0.372	15.0	0.236	12.0		0.395	12.0			
COOH		46.3		6.9			0.8			
CH_3		53.7		93.1			99.2			
Lactate	0.345	4.0	0.209	2.7		0.398	3.7			

Clean, lyophilized spores of B. cereus strain terminalis, produced from cells grown in modified G medium (Hashimoto et al., 1960) were heated for 5 hr. at 65°C. in 0.05 M sodium phosphate buffer, pH 7.0 at a concentration of 100 mg. spores/ml. buffer. The spores were washed with, and resuspended in, the same volume of buffer. One ml. aliquots of the activated spores were added to duplicate flasks containing 650 μmoles sodium phosphate buffer, pH 7.0 and 50 μmoles of either glucose-U-, -1-, -3,4- or -6-C^{14} containing a total of 334,000 c.p.m. in a final volume of 15 ml. The flasks were incubated at 30°C. on a rotary shaker and the vials containing alkali, which were suspended from rubber stoppers on glass rods, were changed at the indicated time intervals. Aliquots of the medium were also removed at zero time and at various times thereafter for determination of the residual glucose by an anthrone procedure and for estimation of germinated spores. The number of viable spores were determined by plating aliquots on trypticase-soy agar (B.B.L.) before and after heating at 80°C. for 30 min. At zero time, 33% of the spores were already germinated. After 3 hr., 1 ml of 10 N H_2SO_4 was added to each flask and, after removal of the cells by centrifugation, the supernatant fluid was extracted continuously with ether for 18 hr. The ether-soluble acids were chromatographed on a silica gel column with a $CHCl_3$-n-butanol mobile phase. The acetic acid peak was further purified by steam distillation. All C^{14} was counted as $BaC^{14}O_3$. Total combustions, using persulfate, or degradations of acetate by the Schmidt-azide procedure, were performed in combustion-diffusion vessels.

important after the spores germinate. Furthermore, we observed an impairment in the terminal respiratory system so that acetate accumulates as a result of glucose oxidation.

The results of a typical experiment, in which both isotopic techniques were combined in a single experiment, are presented in Table 1. The rate and the extent of $C^{14}O_2$ production from glucose-3,4-C^{14} were about twice those from glucose-1-C^{14}, whereas only very small quantities of $C^{14}O_2$ were released from glucose-6-C^{14}. These data were used to estimate the percentage participation of the HMP and EM pathways by the method of Wang et al. (1958). Of the glucose oxidized to CO_2 during the first 15 min. interval, 99% proceeded via the EM pathway, while the cumulative figure gradually decreased to 62% at 180 min. The very low recovery of $C^{14}O_2$ from glucose-6-C^{14}, even after all of the glucose was utilized, suggested that there was an impairment in the further metabolism of C_3 and/or C_2 compounds derived from glucose. At the end of the experiment acetate recovered contained 15% of the C^{14} added initially as glucose-U-C^{14}, suggesting the possibility that the impairment was associated with the terminal respiratory system. Another 4% of the added C^{14} could be recovered as lactate from the ether soluble fraction and 15%, as an unidentified acidic compound. Furthermore, when pyruvate-2-C^{14} was incubated with activated spores for 8 hours, only 401 counts were recovered in the CO_2 while 231,000 counts were recovered as acetate.

Further evidence for the operation of the EM pathway was obtained when estimates were made of the pathways of glucose catabolism based on the relative specific activity (RSA) of the isolated acetate and lactate samples (Blumenthal et al., 1954). About 24 and 43% of the glucose-1-C^{14} yielding acetate and lactate, respectively, was calculated to be utilized by the EM pathway. Similar estimates of the percentage of glucose utilized by the EM pathway based on intermediates derived from glucose-6-C^{14} were always higher than those estimates based on glucose-1-C^{14} intermediates. Ordinarily, estimates based on intermediates derived from glucose-1-C^{14} are considered to be the more sensitive indicator of the HMP pathway (Dawes and Holms, 1958). In spite of

quantitative differences, however, all of the estimates indicated that the EM pathway was important for the utilization of glucose by the spores. The presence in the carboxyl group of less than 7% of the total C^{14} in the acetate derived from either glucose-1- or -6-C^{14} further supported this conclusion.

Germination, the end of the first phase in the complex development of a dormant spore into a vegetative cell, has been defined as the phase subsequent to the loss of heat resistance by the spore (Campbell, 1957), and is accompanied by increased metabolic activity and loss of refractility. Postgerminative development, or outgrowth, includes a series of morphological changes each of which is accompanied by changes in respiratory rate (Levinson and Hyatt, 1956). In the present experiment we found that 33% of the spores were already germinated at zero time and that this figure increased to 80% during glucose oxidation. We have been unable to prepare activated spores capable of oxidizing glucose without some germination, as reported (Church, 1955; Church and Halvorson, 1957). There was no postgerminative development of the spores in the present experiment. We have also observed that the degree of activation of glucose oxidation was dependent on the concentration of Na^+ present during the activation when the phosphate anion concentration remained constant (Goldman and Blumenthal, unpublished results).

The estimations of the pathways of glucose metabolism in the present report, then, were actually made on spore suspensions containing a constantly increasing percentage of germinated spores. During the first 15 min. interval 40% of the spores present were germinated and 60% ungerminated. If the ungerminated spores had used the HMP pathway exclusively, at least 50% of the glucose would be expected to be utilized via the HMP whereas the actual estimate for the interval indicated only 1% of the glucose traversed the HMP pathway. Furthermore, the extent of utilization of the EM pathway was greatest at the earliest portions of the experiment at a time when the per cent of germinated spores was lowest, and then gradually decreased as the per cent of germinated spores increased. These data suggest that the EM pathway is a major pathway for glucose oxidation by intact heat-activated spores and is quantitatively most

important during the early stages leading to germination. Sometime after germination, the HMP pathway also becomes a major pathway for glucose catabolism. Amaha et al. (1959) recently concluded that the EM pathway played an important role in the early stages of germination of heat-activated B. coagulans spores by glucose. This was based on the substrate specificity for germination and the EM enzymes present in spore extracts. Their results, together with the present results, indicate that the EM pathway must be considered an important route of glucose catabolism in spores of aerobic bacilli.

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