ENDOGENOUS RESPIRATION OF *BACILLUS CEREUS*

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ABSTRACT

CLIFTON, C. E. (Stanford University, Stanford, Calif.), AND J. M. SOBEK. Endogenous respiration of *Bacillus cereus*. J. Bacteriol. **82**:252–256. 1961.—The endogenous respiration of washed cells of *Bacillus cereus* varies with the nature of the growth medium and with time. The respiratory quotient of cells harvested from nutrient agar remained quite constant around 1.00 over a 2-hr period of respiration, whereas that of cells grown on glucose-nutrient agar decreased from 0.97 for the first hour to 0.87 for the second hour. Considerable amounts of ammonia were formed, the number of moles per mole of oxygen consumed decreasing with time for agar-grown cells and increasing for glucose-grown ones.

C^{14}-labeled, agar-grown cells utilized materials insoluble in cold or hot 5% trichloroacetic acid, ethanol, or chloroform as their endogenous substrate, the same behavior being noted with glucose-grown cells except that they utilized both hot trichloroacetic-soluble and -insoluble materials. These results indicate that the bulk of the endogenous substrates are chemically complex and, at least in part, are nitrogenous in character.

In a previous paper (Clifton and Sobek, 1961) it was reported that the endogenous respiration of *Bacillus cereus* appears to be reduced by 20 to 40% in the presence of glucose. It was thought desirable to study in greater detail the general nature of the endogenous respiration of this organism in an attempt to gain more insight regarding the assimilatory process and factors influencing it. In particular, attention was given to the possibility of poly-β-hydroxybutyrate serving as a substrate of endogenous respiration since the respiratory quotient (RQ) of *B. cereus* is around 0.92, and Williamson and Wilkinson (1958) have reported that the polymer is formed in quite appreciable amounts by this organism. Attention also was directed toward the possibility of nitrogenous compounds being involved in endogenous respiration since considerable production of ammonia (*see* Warren, Ells, and Campbell, 1960) has been noted in washed suspensions of various microorganisms.

MATERIALS AND METHODS

Cells of *B. cereus* (*Bacillus anthracis*, avirulent variant RS63 from C. F. Robinow's collection) were grown for 17 hr at 30 C on nutrient agar or nutrient agar containing 1% glucose in Roux bottles. The inoculum for a bottle consisted of the growth from an agar slant suspended in 2 ml of saline containing approximately 1 μc each of uniformly labeled glucose and glutamic acid. The test cells were harvested in water, removed by centrifugation, washed in water twice more, and finally suspended in 0.05 M phosphate buffer (pH 7.2) containing 5 ml of 0.1 M magnesium chloride per 100 ml of buffer. The suspensions were adjusted so that a 1 to 10 dilution read between 140 and 160 in a Klett-Summerson colorimeter (green filter, no. 54). All experiments were conducted at 30 C.

Oxygen consumption, carbon dioxide production, carbon determinations, and radioactivity measurements were carried out as previously described (Clifton and Sobek, 1961), 2.2 ml of cell suspensions being used in the Warburg vessels to remove two 1-ml portions readily for the analyses or fractionations. The determinations generally were conducted in duplicate. Ammonia was determined by nesslerization of steam distillates of the cell suspension, 3 ml of 20% sodium hydroxide being added to 1 ml of the suspension in a microstill.

Fractionation of the cells was carried out in a manner similar to that described by Park and Hancock (1960). In general, 2 ml of 10% cold trichloroacetic acid and 1 ml of water were added to 1 ml of the chilled suspension in the combustion tubes (Pyrex stoppers, Corning no. 7665) which were then placed in a refrigerator

(4 C) for 15 min. In preliminary experiments the cells were centrifuged (Servall SS4 centrifuge, 7,700 × *g* in all procedures) and 5% trichloroacetic acid added to the cells after removal of the supernatant. It was difficult to resuspend the cells in trichloroacetic acid to yield a uniform suspension without some loss of cells which stuck to the stirring rod or to the sides of the tube or pipette. Cold 10% trichloroacetic acid, therefore, was added in equal volume to the bacterial suspension rather than to the packed cells. Hence the extract commonly contained acid-soluble cell constituents as well as any material that leaked from the cells during the course of an experiment. In experiments where sulfuric acid was not added to the cell suspension it was observed that there was relatively little leakage from the cells.

The residue from the cold trichloroacetic acid extraction was suspended in 4 ml of 75% ethanol adjusted to pH 2.5 with dilute sulfuric acid and incubated at 45 C for 15 min. This extract contains ethanol-soluble protein and lipid. Little or no radioactivity was noted in ether extracts of the ethanol-extracted cells and hence ether extraction was omitted from most tests. The residue from the ethanol extraction, after centrifugation, was suspended in 4 ml of 5% trichloroacetic acid and heated in a boiling water bath for 15 min. This extract contains nucleic acids, cell wall acids, and miscellaneous materials, whereas the residue consists primarily of proteinaceous matter.

In some experiments, the ethanol residue was treated with 2 ml of hypochlorite (Williamson and Wilkinson, 1958); in others, the test suspensions were centrifuged directly and the cells were resuspended in the hypochlorite immediately since little or no change was noted in the radioactivity of the ethanol-soluble fraction. The hypochlorite suspension was incubated for 30 min at 37 C and 1 ml was then extracted for 2 hr with chloroform in a continuous liquid-liquid microextractor. The chloroform extract was transferred to a combustion tube and the chloroform removed by evaporation under a heat lamp before combustion in the combustion train (Nuclear-Chicago).

RESULTS AND DISCUSSION

The results of various studies (*see* Warren et al., 1960) have indicated that nitrogenous reserves may serve as the principal endogenous substrate for various microorganisms. It was observed in this study that ammonia was liberated in appreciable amounts during the course of endogenous respiration of *B. cereus* and, therefore, this behavior was studied in greater detail. Some of the ammonia was liberated into the suspension medium while a portion remained within the cells. The ammonia values reported herein are from determinations with the cell suspensions and hence represent both intra- and extracellular changes in ammonia over a period of time. It was observed that cells harvested from nutrient agar tended to form approximately 1 mole of ammonia per 6 moles of oxygen consumed and that the RQ was around 1.0. This RQ was higher than the one (0.92) reported (Clifton and Sobek, 1961) for cells grown on glucose agar. Comparative studies, therefore, were carried out on suspensions of cells grown on nutrient agar and on glucose-nutrient agar. The suspensions were made up to the same turbidity, a 1 to 10 dilution reading 160 on the Klett-Summerson colorimeter, filter no. 54. Typical results from a 2-hr period of respiration are presented in Table 1.

TABLE 1. *Influence of growth medium on the endogenous respiration of Bacillus cereus*

	Agar			Glucose agar		
	Time in hr					
	0–1	1–2	0–2	0–1	1–2	0–2
RQ	1.00	1.00	1.00	0.97	0.87	0.92
O_2 μmoles/ml	7.8	5.1	12.9	6.2	5.1	11.3
NH_3 μmoles/ml	1.4	0.6	2.0	0.3	0.4	0.7
O_2/NH_3	5.6	8.5	6.5	20.7	12.8	16.1

It is apparent from the results presented in Table 1 that although the rates of endogenous respiration of both suspensions fall to the same level by the second hour (and remain constant during this time) there is a marked decrease in the RQ of glucose-grown cells, whereas that of agar-grown cells remains constant. Also there is a decrease in the amount of oxygen consumed per mole of ammonia formed by glucose-grown cells, whereas an increase in this ratio is evident for agar-grown cells. These results suggest that there are changes in the nature of the endogenous respiration with time and differences between

TABLE 2. *Distribution of radioactivity in various fractions from 20 ml of a suspension of labeled cells of nutrient agar-grown Bacillus cereus before and after endogenous respiration for 150 min at 30 C*

Fraction	μcuries × 10^{-3} cellular-C^{14}	
	Original	After 150 min
Cold trichloroacetic acid soluble	17.3	17.5
Ethanol soluble	9.0	10.4
Hypochlorite soluble	141.0	113.0
Chloroform soluble	1.6	1.2
Hot trichloroacetic acid soluble	46.2	36.5
Residue	42.6	46.6
Total	257.7	225.2
Original cells	343.0	301.0

TABLE 3. *Distribution of C^{14} before and after a 2-hr period of endogenous respiration of labeled nutrient agar-grown cells of Bacillus cereus*

	μcuries × 10^{-3}				
	Cells	CO_2	Supernatant	Hot trichloroacetic acid soluble	Residue
Experiment A:					
Initial	25.8		2.8	7.8	18.0
Final	24.2		3.3	8.1	15.7
	−1.6		+0.5	+0.3	−2.3
*Experiment B:**					
Initial	31.7	0.1	1.3	11.7	22.1
Final	29.6	1.3	2.6	11.8	19.2
	−2.1	+1.2	+1.3	+0.1	−2.9
*Experiment C:**					
Initial	46.6				32.4
Final	44.1				29.8
	−2.5				−2.6
$CHCl_3$ extract, 0.52 and 0.55 μc × 10^{-3} at 0 and 2 hr					
*Experiment D:**					
Initial	20.7	0.2			14.7
Final	19.5	1.2			13.7
	−1.2	+1.0			−1.0

the endogenous substrates of cells grown on different media. Midwinter and Batt (1960) also have reported variation in RQ values from 0.89 to 1.00 for *Nocardia corallina* grown for different times on different substrates. It should be pointed out that heavier growth occurs on glucose agar and that this might influence cellular reserves, activities, or phase of growth. This study, however, was limited to cells grown for about 17 hr from inocula of approximately the same numbers of cells.

In an attempt to determine the general nature of the endogenous substrates the cells were subjected to various fractionation procedures followed by determinations of the C^{14} content of the various fractions. In preliminary experiments little change was noted in the distribution of radioactivity in cold trichloroacetic acid, ethanol (or ethanol followed by ether), and chloroform extracts (the latter of hypochlorite-treated cells) of agar-grown cells which had been shaken for 2 to 3 hr as compared with extracts of the original cells. These results indicate that compounds soluble in cold trichloroacetic acid, alcohol-soluble lipids, or chloroform-soluble poly-β-hydroxybutyrate are not the major endogenous substrates for agar-grown cells of *B. cereus*.

In one preliminary test the fractionation was carried out as indicated in Table 2. The results of the preliminary extractions reported in Table 2 are in general agreement with those summarized above. The most marked decrease in radioactivity was noted in the hypochlorite soluble fraction. Treatment with hypochlorite, however, may give rise to a residue for hot trichloroacetic acid extraction which is quite different from cells extracted only with cold trichloroacetic acid and ethanol. Also there may be some loss of radioactivity as a result of oxidation by chlorine and subsequent neutralization of the hypochlorite solution with 10% sulfuric acid. Hence the hypochlorite values are of little significance. The totals for the radioactivity recovered in the various fractions are low owing to loss of C^{14} in

In experiment B there was a decrease of 7.8% (15.7 μmoles) of cellular-C as contrasted with a 6.6% loss of C^{14}; corresponding figures for experiment C being 5.7 and 5.4; and for D, 6.6 and 5.8%, respectively. In experiment D the cellular-C decrease was 11.7 μmoles, the oxygen uptake 11.4 μmoles, the RQ 1.03, and 2.1 μmoles of ammonia were formed, representing a ratio of 5.4 moles of oxygen consumed per mole of ammonia formed.

respiratory carbon dioxide, in supernatant fluid, possibly during the hypochlorite treatment, and in the various manipulations involved in the procedures.

Portions of the cold trichloroacetic acid extracts obtained in the experiment summarized in Table 2 were tested for total radioactivity, other portions were tested for C^{14}-carbon dioxide liberated from amino acid carboxyl groups by ninhydrin at pH 4.5 (Van Slyke et al., 1941). The C^{14}-carboxyl carbon determinations were 2.2 and 2.4 $\times 10^{-3}$ μc for the original and final cells, respectively, thus indicating little or no change in the amino acid pool.

Since there was little change in radioactivity of the readily extractable components of labeled *B. cereus*, attention was directed to the hot trichloroacetic acid fractions. Results of four separate experiments with agar-grown cells are summarized in Table 3. Experiments A and B were carried out in Warburg flasks containing 2.2 ml of the cell suspension and 0.2 ml of 10% sulfuric acid in a side arm. The acid was tipped into the suspensions in the main part of some vessels at zero time, into others after a 2-hr period of respiration, for the determination of initial and final bound carbon dioxide and to stop respiration. Oxygen consumption was determined employing the same procedure, 0.2 ml of 20% potassium hydroxide being placed in the center well to absorb carbon dioxide. The hydroxide solution was transferred quantitatively to the combustion train where the carbon dioxide liberated on acidification and boiling was collected in the ionization chamber. Ammonia was determined in portions of the acidified suspension from the Warburg vessels. Acidification of the suspensions resulted in liberation from the cells of some radioactive materials which were not present in supernatants from nonacidified suspensions. Acidification also tended to cause some clumping of the cells and a greater tendency for them to cling to the sides of the vessels and pipettes. In experiments C and D the fractionations, centrifugation, and C^{14} determinations were carried out with separate 1-ml samples of the suspensions in the combustion tubes. These tubes were shaken in a rack placed in the Warburg bath. Since little change was noted in the C^{14}-content of the hot trichloracetic acid-soluble fraction in the earlier experiments this determination was omitted in these experiments. At zero time and after 2 hr, 10% trichloroacetic acid was added in equal volume to the suspensions and the tubes were then placed in a boiling water bath for 15 min. These procedures were simpler and reduced experimental errors. Carbon dioxide and ammonia production and oxygen consumption were determined at the same time with samples in or removed from Warburg flasks.

Radioactivity studies on the endogenous respiration of glucose-agar-grown cells also were made. It was observed that there was little or no change in the radioactivity of the cold trichloroacetic acid-soluble, the alcohol-soluble, or chloroform-soluble fractions over a 2-hr period of respiration. Likewise there was no change in activity noted for the C^{14}-carbon dioxide released by ninhydrin from the cold trichloroacetic acid-soluble material. In duplicate samples the residue after cold trichloroacetic acid and alcohol extractions contained 11.9 and 11.8 μc (all values $\times 10^{-3}$ per ml) of C^{14} and, after 2 hr, 10.0 and 10.1, an average decrease of 1.8 μc. The C^{14} determinations on the cells showed an average decrease of 1.85 μc (from 12.1 and 12.0 to 10.1 and 10.3). The hot trichloroacetic acid residue decreased from 10.7 and 10.9 to 9.4 and 9.4, an average decrease of 1.4 μc. Equipment failure prevented determination of the radioactivity of the hot trichloroacetic acid-soluble fractions but, by difference, the figures would indicate a loss of about 0.4 μc in this fraction.

Experiment D, Table 3, was repeated with cells grown on glucose agar, determinations of hot trichloroacetic acid-soluble radioactive materials being included. Since the results of the

TABLE 4. *Distribution of C^{14} before and after a 2-hr period of endogenous respiration of glucose agar-grown labeled cells of Bacillus cereus*

	μcuries $\times 10^{-3}$					
	Cells	Supernatant	CO_2	Chloroform soluble	Hot trichloroacetic acid soluble	Residue
Initial	15.3	1.1	0.2	1.7	3.4	13.0
Final	14.0	0.9	1.3	1.7	2.8	12.4
	−1.3	−0.2	+1.1	0	−0.6	−0.6

O_2, 15.5 μmoles; NH_3, 1.1 μmoles; O_2/NH_3, 14.1; RQ, 0.96.

experiments reported in Table 1 indicate a marked change with time in the nature of the endogenous reserves utilized by glucose-grown cells of *B. cereus* more detailed fractionations will be attempted later. Hence the hot trichloroacetic acid-soluble fraction includes any material that would be soluble in the cold acid. Again no decrease in radioactivity of chloroform-soluble material could be noted. The results of this experiment are recorded in Table 4. It is evident that the RQ is lower than that of cells grown on plain agar but has not fallen to the over-all value generally noted for a 2-hr experiment with glucose-grown cells. The O_2/NH_3 ratio is over twice that for agar-grown cells and the radioactivity of both the hot trichloroacetic acid-soluble and -insoluble fractions decreased by the same amount, a marked change only in the hot trichloroacetic acid-insoluble fraction being noted with agar-grown cells.

It is readily apparent from the above results that the endogenous substrates of glucose agar-grown cells differ, in part at least, from those of cells grown on agar. The RQ values for both types of cells, and in particular the agar-grown ones, are high for the oxidation of proteinaceous matter. In this study it was expected that poly-β-hydroxybutyrate might serve as a principal endogenous substrate. It was observed that this strain of *B. cereus* does form a chloroform-soluble substance, apparently the butyrate polymer, as previously reported by Macrae and Wilkinson (1958) for *B. cereus*. These workers also reported that *B. cereus* utilizes the polymer but did not give experimental data other than for *Bacillus megaterium*. A question could be raised regarding the specificity of their polybutyrate determinations since they are based on the decrease in turbidity of cellular suspensions on treatment with hypochlorite. Assuming that their measurements for poly-β-hydroxybutyrate are correct, in one experiment they noted with *B. megaterium* an oxygen consumption of 130 μl while only 57 μl would be required for the complete oxidation of the amount of the polymer reported lost by the cells. Hence, somewhat over half of the endogenous respiration of this organism would involve a substrate other than the polymer. If *B. cereus* were oxidizing the polymer to anywhere near the same extent as *B. megaterium* it would have been detected in changes in the radioactivity of the chloroform extracts. Much work remains to be done to determine the substrate(s) of endogenous respiration of even one strain of bacteria and the influence of various factors, including the growth medium, thereon.

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ADDENDUM

R. C. Urbá (1960. Protein breakdown in *Bacillus cereus*. Biochem. J. **71**:513–518) reports a 7% per hr protein breakdown in washed suspensions of *B. cereus*. This is in general agreement with the use of proteinaceous endogenous substrates reported in this paper.

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