EFFECT OF HYDROSTATIC PRESSURE ON THE SUCCINIC DEHYDROGENASE SYSTEM IN ESCHERICHIA COLI[1]

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Hydrostatic pressures of the order prevailing in the deep sea (up to about 1100 atm) have been found to affect the viability, reproduction, and metabolic reaction rates of microorganisms in different ways. Certain processes are accelerated whereas others are retarded by compression. Some of these differences appear to be inherent in the protoplasm or enzymes of organisms as a function of the pressure of their habitats. Certain bacteria from the deep sea, for example (ZoBell, 1952), tolerate or actually require much higher pressures than microorganisms whose normal habitat is near 1 atm (ZoBell and Johnson, 1949).

A rational explanation of how pressure may affect biological processes is complicated by the fact that compression appears to act differently upon diverse reactions catalyzed by the same cells. For example, we find the activity of aspartase of washed cells of *Escherichia coli* to be accelerated by compression up to 680 atm, but retarded by higher pressures and stopped at 1000 atm, ostensibly owing to the inactivation of the enzyme (ZoBell and Morita, 1954). On the other hand, the activity of succinic dehydrogenase from *E. coli* appears to be retarded by a pressure of only 200 atm. Observations described in this paper indicate that the succinic dehydrogenase system in *E. coli* is inactivated by moderate pressures.

MATERIALS AND METHODS

Preparation of cells. Cells of *E. coli* were grown in a 5-gallon carboy containing 14 L of nutrient medium: glucose, 2.0 g; KH_2PO_4 2 g; K_2HPO_4, 5 g; peptone (Difco), 3 g; yeast extract (Difco), 1 g; distilled water, 1,000 ml. Following sterilization in the autoclave the pH of the medium was 7.0. It was inoculated with 1.4 L of similar medium in which *E. coli* had developed for 24 hours. During the succeeding 18-hour incubation period at 22 C, the inoculated medium was agitated with filtered sterile air. Foaming was prevented by adding 0.2 g of Dow Corning AF Antifoam emulsion.

The cells were harvested in a Sharples supercentrifuge (ca 23,000 rpm), suspended in 15 L of ice cold (ca 4 C) water, and recentrifuged. The washed cells were lyophilized (−70 C at 300 μ Hg) and stored in a refrigerator over "Ascarite" *in vacuo* at 4 C.

Such lyophilized cells represent the "biological whole", a known quantity of which was used in each experiment.

Pressurization of material. Immediately prior to each series of experiments, sufficient quantities of lyophilized cells were weighed out to give 2 mg per ml in M/15 phosphate buffer (pH 7.0) solution in which they were evenly suspended. Equal aliquots of the cell suspension were pipetted into small (10 by 75 mm) test tubes. The latter were filled to capacity and closed with No. 000 neoprene stoppers, care being exercised to exclude air. The stoppered tubes were transferred at once to pressure vessels filled with hydraulic fluid equilibrated to the desired temperature in water baths. Pressure was applied for various periods of time by procedure described by ZoBell and Oppenheimer (1950).

Methylene blue reduction measurement. The Thunberg technique, described by Umbreit *et al.* (1949), was employed to measure dehydrogenase activity with methylene blue as the hydrogen acceptor. Elongated Thunberg tubes, 1.5 cm in diameter and 25 cm long, circumvented premature mixing of the bacterial suspension (in side arm) with the methylene blue succinate buffer mixture during evacuation of air, which resulted in some ebullience. In each tube was

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TABLE 1

Micrograms methylene blue reduced at 30 C in presence of succinate by 2 mg of lyophilized cells of E. coli stored at 5 C in vacuo

Reaction Time	7 Days' Storage	18 Days' Storage	33 Days' Storage
min	*μg*	*μg*	*μg*
0	0	0	0
3	21.2	10.3	2.9
6	34.9	16.3	6.6
9	54.3	23.2	8.6
12	76.4	32.2	8.9
15	92.9	44.6	10.3
18	108.4	52.6	12.0
21		62.9	15.2
24		74.6	17.2
27		85.2	18.0
30		99.2	19.0

placed 1 ml of 1:10,000 (0.000267 M) methylene blue, 2 ml of M/50 sodium succinate (freshly prepared for each experiment), and 2 ml of M/15 phosphate buffer (pH 7.0). In endogenous controls distilled water was substituted for the sodium succinate solution. After evacuating the air from each assembled Thunberg tube with a Cenco-Megavac pump, the tube was filled with CP nitrogen. The nitrogen was passed through a Deoxo catalytic unit to remove oxygen. Prior to adding the lyophilized cells, all solutions were boiled to exclude oxygen and cooled to 4 C. Spot tests on solutions and cell suspensions showed that in no stage of the experiments was the oxygen content as much as 0.1 ppm.

In the side arm of each tube was placed 1.0 ml of bacterial suspension (2 mg of lyophilized cells) that had been treated with different pressures at definite temperatures as indicated. The assembled Thunberg tubes were again evacuated, filled with oxygen-free nitrogen, and equilibrated in a water bath at 30 C before mixing the contents. In order to obtain comparable results in all experiments, the cells were mixed with the methylene blue-succinate buffer mixture exactly 30 minutes after they were depressurized. It would have been desirable to mix immediately after depressurization, because there may have been some regeneration or reversible recovery of dehydrogenase during this period, but it required about 30 minutes to make the necessary manipulations, including temperature-pressure equilibration of the system. As pointed out by Johnson *et al.* (1954), the effects of moderate pressures are frequently reversible, sometimes quantitatively so, as soon as pressure is released.

The amount of methylene blue reduced at intervals of 3 minutes following its mixing with the cells was determined with a Klett-Summerson photoelectric colorimeter connected to a Sola Type CVH constant voltage transformer (115 v, 1.04 amp). The zero setting was made with distilled water in a Klett tube employing a red filter (640–700 mμ). Since the Klett-Summerson potentiometer scale is logarithmic, the amount of methylene blue reduced is proportional to the Klett reading. By subtracting the Klett reading at various time intervals from the initial Klett reading, the amount of methylene blue reduced could be determined.

RESULTS

Lyophilized cells of *E. coli* undergo a gradual loss of succinic dehydrogenase activity during storage, even when refrigerated, as illustrated by the data in table 1. Consequently, it was necessary to compromise between performing all of the experiments with the same lot of lyophilized cells of increasing age and decreased activity or employing freshly prepared lots of lyophilized cells. It required about a week to prepare and standardize the cells for an experiment.

From 6 to 10 times as much methylene blue was reduced by lyophilized *E. coli* cells in the presence of succinate as by the endogenous controls without added succinate (table 2). The accelerating rate of methylene blue reduction by

TABLE 2

Micrograms methylene blue reduced by 2 mg lyophilized (9-day) cells of E. coli following 4 hours' pressurization at 30 C

Reaction Time	Treated at 1 atm		Treated at 600 atm	
	Endogenous control	With added succinate	Endogenous control	With added succinate
min	*μg*	*μg*	*μg*	*μg*
0	0	0	0	0
3	1.7	16.6	1.2	4.9
6	3.9	29.7	2.5	11.4
9	5.4	46.9	3.8	18.6
12	7.2	63.2	5.1	26.0
15	8.7	82.4	6.3	38.5
18	10.0	97.8	8.6	51.7

pressure treated cells in the presence of succinate may be due to the regeneration of succinic dehydrogenase that catalyzes the reaction:

$$HOOC \cdot CH_2 \cdot CH_2 \cdot COOH + MB \rightarrow$$

$$HOOC \cdot CH{=}CH \cdot COOH + MB{-}H_2$$

or the accelerating rate may be due to some other protoplasmic factor that is not understood. Since the "biological whole" was used in the experiments, the reactions were carried out anaerobically to render them independent of the action of cytochrome oxidase. The rate of acceleration with increasing reaction time was greater but the total amount of methylene blue reduced was less with lyophilized cells previously compressed at 600 atm for 4 hours.

The inactivation of succinic dehydrogenase was found to be progressively greater as the pressure was increased (figure 1). After 4 hours at 1000 atm virtually all of the succinic dehydrogenase appeared to be irreversibly inactivated. Little more methylene blue was reduced by such pressurized cells than by the endogenous controls. Pressures up to 1000 atm had little demonstrable effect on the endogenous activity at 30 C.

There is no indication that the loss of viability is responsible for the observed decreases in succinic dehydrogenase activity of cells that had been subjected to high pressure for short periods of time. The pressurized cells were found by phase microscope examination to be actively motile and morphologically normal in appearance. However, there was some diminution in the number of cells that could be demonstrated by plating procedures following compression of cell suspensions. This diminution in the number of colonies is believed to be due primarily to the clumping of cells caused by high pressure rather than due to the loss of viability. Few bacteria are killed by short exposure to moderate pressures, although a good many common forms gradually lose their viability when held for a few days at 400 to 1000 atm. ZoBell and Johnson (1949) have reported the survival of *E. coli* cultures for 2 days at 600 atm and 30 C; good growth at 600 atm and 40 C.

The effect of hydrostatic pressure on biological systems is a function of temperature. In general, the adverse effects of pressure on the viability of bacteria in nutrient media are more pronounced at low temperatures, say 0 to 20 C, than at the ordinary optimum, say 20 to 40 C, of the organisms. The optimum temperature or even the thermal death point for certain bacteria may be increased temporarily by a few

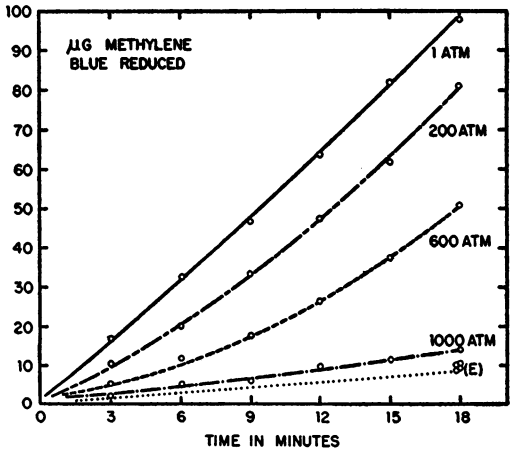

Figure 1. Micrograms of methylene blue reduced at 30 C after different periods of time by 2 mg of lyophilized (9-day) cells of *E. coli* which 30 minutes previously had been subjected to pressures of 1, 200, 600, or 1,000 atm for 4 hours at 30 C. The upper four curves represent activity with succinate as the hydrogen donor; the dotted line (*E*) at the bottom represents endogenous activity with no succinate added.

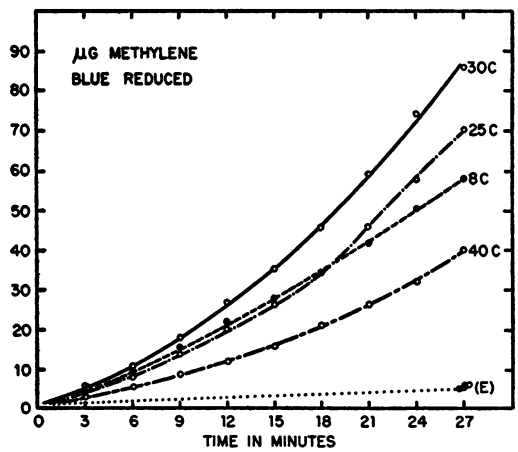

Figure 2. Micrograms of methylene blue reduced at 30 C after different periods of time by 2 mg of lyophilized (10-day) cells of *E. coli* which 30 minutes previously had been subjected to a pressure of 600 atm for 4 hours at 8, 25, 30, or 40 C. The upper four curves represent activity with succinate as the hydrogen donor; the dotted line (*E*) at the bottom represents endogenous activity with no succinate added.

TABLE 3

Micrograms methylene blue reduced at 30 C in presence of succinate by 2 mg of lyophilized (22-day) cells of E. coli previously pressurized for different periods of time at 600 atm and 30 C

Reaction Time	Period of Time Held at 600 atm		
	0 hours	1 hour	3 hours
min	*μg*	*μg*	*μg*
0	0	0	0
3	9.8	8.0	3.8
6	19.6	16.4	9.6
9	29.4	24.5	12.4
12	38.1	31.3	14.5
15	47.9	39.6	17.4

degrees during incubation at high hydrostatic pressure, but the adverse effects of pressure are aggravated by temperatures considerably in excess of the optimum of the organisms for reproduction. Similarly, the succinic dehydrogenase system of *E. coli* was found to be adversely affected by prolonged compression at temperatures that are either lower or higher than the optimum (30 C) of this strain for reproduction. During 4 hours' compression at 600 atm its succinic dehydrogenase system was inactivated more at 40 C or at 8 C than at 30 C (figure 2). Neither the rate nor the amount of endogenous activity, as indicated by methylene blue reduction in controls to which no succinate was added, was detectably affected by the temperature during 4 hours' compression at 600 atm.

The inactivation of succinic dehydrogenase increases with duration of compression. In a representative experiment (table 3) lyophilized *E. coli* cells held for 3 hours at 600 atm reduced only about half as much methylene blue as similar cells held for only 1 hour at this pressure.

DISCUSSION

Although many more observations must be made to ascertain whether the effects of hydrostatic pressure are direct or indirect, our experimental data demonstrate that moderate pressures inactivate the succinic dehydrogenase system of *E. coli*. The progressive inactivation of the succinic dehydrogenase system with time of compression makes extremely difficult any appraisal of the effects of pressure on the rate at which this enzyme system catalyzes the oxidation of succinic acid to fumaric acid in the presence of methylene blue.

According to Borrowman (1950), pressures up to 700 atm at 30 C had no effect on the rate of formic dehydrogenase activity of *E. coli* during an experimental period of 12 to 30 minutes in a pressure vessel equipped with optical windows through which methylene blue reduction could be observed. Since formic dehydrogenase, unlike succinic dehydrogenase, is a relatively stable enzyme, Borrowman heated the cells to 65 C for 5 minutes in order to denature as many interfering enzymes as possible. This is another example of different responses of enzyme systems to pressurization, perhaps attributable in part to intrinsic differences in the pressure tolerance of the enzyme system and possibly in part to differences in experimental conditions. Pressure tolerances and effects are dependent not only upon temperature and time; they are also influenced by gas tension, pH, osmotic pressure, chemical composition of the medium (Johnson *et al.*, 1954), and by other environmental factors.

Stadie and Haugaard (1945) reported the irreversible inactivation of succinic dehydrogenase system in rat tissues by 2 to 4 hours' exposure to 7 atm of oxygen. Haugaard (1946) attributed the inactivation to the oxidation of the SH group. Owing to the inactivation of succinic dehydrogenase by oxidation, particularly at high pressures, we endeavored to exclude free oxygen during the critical stages of our experiments.

SUMMARY

Employing methylene blue as the hydrogen acceptor, it has been demonstrated that the succinic dehydrogenase system associated with "biological whole" lyophilized cells of *Escherichia coli* is inactivated by moderate hydrostatic pressures.

There was some inactivation of the succinic dehydrogenase of *E. coli* at 200 atm at 30 C. The amount of inactivation increased progressively with time of compression. Approximately half of the enzyme system was inactivated after 4 hours at 600 atm and virtually all was irreversibly inactivated after 4 hours at 1000 atm.

The inactivating effects of pressure were most pronounced at temperatures either above (40 C) or below (8 C) the organism's optimum (30 C) for multiplication.

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