EFFECT OF HYDROSTATIC PRESSURE ON SUCCINIC, FORMIC, AND MALIC DEHYDROGENASES IN ESCHERICHIA COLI

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ZoBell and Morita (1957) demonstrated the existence of barophilic bacteria in various deeps and trenches of the Pacific and Indian Oceans where hydrostatic pressures up to 1,100 atm exist. However, the relationship between hydrostatic pressure and the activity of life in the deep sea is still unknown.

Johnson et al. (1954), for convenience of discussion, divided hydrostatic pressure into moderate or high pressures (ca. 1 to 1,000 atm) and very high pressures (ca. 1,000 atm and above). The effect of very high pressures has been known to inactive certain enzymes (Macheboeuf et al., 1933; Vignais et al., 1951; Matthews et al., 1940; Curl and Jansen, 1950a, 1950b). Using moderate pressures, Borrowman (1950) reported that hydrostatic pressures up to 700 atm at 30 C had no effect on the rate of formic dehydrogenase activity of Escherichia coli. In the latter work a pressure cylinder with optical windows was employed as well as cells of E. coli which were heated to 65 C for 5 min in order to inactivate as many interfering enzymes as possible. Since the cells were subjected to heat the “biological whole” was not represented.

Morita and ZoBell (1956) studied the effect of hydrostatic pressure on the succinic dehydrogenase system in cells of E. coli, which had been previously subjected to pressure. These measurements were carried out by the Thunberg technique after the pressure was released to 1 atm. This paper deals with the effect of pressure on the rate of formic, succinic, and malic dehydrogenase by cells of E. coli during the time of compression.

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MATERIAL AND METHODS

Preparation of cells. Cells of E. coli were grown in a Fernbach flask containing 200 ml of nutrient medium: glucose, 2 g; KH2PO4, 2 g; K2HPO4, 5 g; peptone (Difco), 5 g; distilled water, 1,000 ml. The pH of the medium after autoclaving was 7.0. This medium was inoculated with 20 ml of a similar medium in which E. coli had developed for 24 hr. The inoculated medium was incubated at 30 C for 16 hr. Cells were grown for each experiment.

The cells were harvested in a Servall type XL centrifuge and washed twice with equal volumes of m/100 phosphate buffer (pH 7.0) and resuspended in m/15 phosphate buffer (pH 7.0). The suspended cells were aerated with filtered air for 2 hr at room temperature (27 C). After aeration the cells were centrifuged and the final suspension adjusted to a turbidity of 250 on the Klett-Summerson apparatus using a red filter (640 to 700 mp). The zero setting was made with distilled water in a Klett tube. The final suspension was stored overnight in the refrigerator (5 C) and used the next morning.

Optical high pressure cylinder. Stainless steel pressure cylinders fitted with white sapphire windows were constructed (figure 1). A neutral piston prevented the mixing of the hydraulic fluid and the reaction mixture. This neutral piston was fitted with a 8/32 in. self-seal screw which had one side cut away to allow for the escape of gas and excess liquid in the optical high pressure cylinder. After filling the cylinder with the reaction mixture, the excess gas or liquid was replaced by the neutral piston and the self-seal screw was secured. The valve was attached and the optical high pressure cylinder attached to the pressure apparatus and pressurized to the desired hydrostatic pressure. The pressure apparatus was described by ZoBell and Oppenheimer (1950).

These optical high pressure cylinders were constructed to fit in a modified 10 cm cell compartment of a Beckman DU spectrophotometer. The
The total liquid volume of the optical high pressure cylinder was 7.5 ml.

Deoxygenating unit. A deoxygenating unit (figure 2) was constructed from a connecting tube (with a suction tube, standard taper 24/40) and a pair of ground joints (full length, standard taper 24/40, ends sealed). An 18 gauge syringe needle was inserted near the contact between the rubber tubing and the connecting tube so that the point of the syringe needle was at the base of the suction tube of the connecting tube. This syringe needle was plugged when the unit was evacuated.

Vacuum pump assembly. The lower arm of a three-way T-shaped stopcock was attached by a rubber tubing to a Cenco-Megavac vacuum pump while the upper arm was attached by rubber tubing to a nitrogen reservoir. Rubber tubing from the horizontal arm of the stopcock was attached to the deoxygenating unit. Vacuum or a slight nitrogen pressure could be applied by moving the stopcock to the appropriate position.

Methylene blue reduction measurements. Methylene blue was employed as the hydrogen acceptor. No change in methylene blue could be detected by use of the Beckman DU spectrophotometer after subjecting the methylene blue at 1,000 atm for 30 min.

The following constituents were added into the male ground joint of the deoxygenating unit (figure 2): 0.5 ml of 1:1,000 (0.000267 M) methylene blue, 2 ml of M/50 substrate, 11.5 ml of M/15 phosphate buffer (pH 7.0). One ml of cell suspension was added to the female ground joint. This deoxygenating unit was assembled and attached to the horizontal arm of the stopcock (see vacuum pump assembly) by rubber tubing and the stopcock turned so that vacuum could be applied. After evacuation, the deoxygenating unit was flushed with gaseous nitrogen (all nitrogen used was first passed through a mixture of pyrogallic acid and NaOH solution) and re-evacuated. After the second evacuation the cells were mixed with the methylene blue-substrate-buffer mixture and nitrogen allowed to pass back into the deoxygenating unit. This unit was under slight nitrogen pressure to prevent the entrance of atmospheric oxygen. The syringe needle plug was removed and a syringe attached to the syringe needle. The re-
action mixture was removed with the syringe and placed in the optical high pressure cylinder. The syringe and the optical high pressure cylinder were flushed with nitrogen before use. The optical high pressure cylinder was always kept under a nitrogen phase until the neutral piston was secured. The high pressure valve was attached to the optical high pressure cylinder and the entire unit connected to the hydrostatic pressure apparatus (ZoBell and Oppenheimer, 1950).

All measurements of methylene blue reduction on the Beckman DU spectrophotometer were started exactly 5 min after the cells were mixed with the methylene blue-substrate-buffer mixture. The first reading (5 min after mixing) was considered time "zero" in table 1 and figures 3 and 4. The amount of methylene blue reduced at 3 min intervals was followed. By subtracting the optical density reading at various time intervals from the initial optical density reading, the amount of methylene blue reduced could be determined.

The cell check (optical density of 0) for the Beckman DU spectrophotometer was made by employing the second optical high pressure cylinder filled with 7.5 ml of a solution: 1 ml cell suspension and 14 ml of phosphate buffer (pH 7.0).

RESULTS

Resting cells of E. coli show loss of succinic dehydrogenase activity during the period of applied hydrostatic pressure (figure 3). The amount of methylene blue reduced in 15 min, as well as the rate, decreased with increased hydrostatic pressure. At 1,000 atm the amount of methylene blue reduced in 15 min is just slightly more than

### Table 1

<table>
<thead>
<tr>
<th>Reaction Time (min)</th>
<th>1 Atm</th>
<th>200 Atm</th>
<th>600 Atm</th>
<th>1,000 Atm</th>
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<tbody>
<tr>
<td></td>
<td>µg</td>
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<td>0.7</td>
<td>8.7</td>
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<td>6.7</td>
</tr>
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<td>9</td>
<td>1.8</td>
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<td>12</td>
<td>1.9</td>
<td>16.0</td>
<td>2.7</td>
<td>16.5</td>
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<td>2.2</td>
<td>20.5</td>
<td>2.9</td>
<td>20.1</td>
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</table>

Figure 4. Micrograms of methylene blue reduced in the presence of succinate by resting cells of *Escherichia coli* (0.5 ml, Klett turbidimetric reading of 250 employing a red filter) at various hydrostatic pressures.

Figure 3. Micrograms of methylene blue reduced in the presence of succinate by resting cells of *Escherichia coli* (0.5 ml, Klett turbidimetric reading of 250 employing red filter) at various hydrostatic pressures.
the 1,000 atm endogenous methylene blue reduction, which indicates very little succinic dehydrogenase activity. After correcting for the endogenous methylene blue reduction, the cells at 200 atm reduced about 0.75 times the amount of methylene blue in 15 min than cells at 1 atm. Cells at 600 atm reduced about 0.4 times the amount of methylene blue reduced by cells at 1 atm.

Room temperature was employed throughout these experiments. The observed temperatures of the optical high pressure optical cylinder were found to be between 27.2 C and 27.7 C for the data recorded in figure 3. This represents a temperature difference of 0.5 C. This temperature difference was not considered significant.

In determining the action of hydrostatic pressure on resting cells of _E. coli_ when formate was employed as the substrate, it was noted that the amount of methylene blue reduced in 15 min decreased with increased pressure (figure 4). After correcting for the endogenous methylene blue reduction the cells at 200, 600, and 1,000 atm reduced about 0.83, 0.75, and 0.06 times the amount of methylene blue respectively as cells at 1 atm.

When malate was used as the substrate the amount of malate oxidized, as indicated by the methylene blue reduction, at 1, 200, and 600 atm is approximately equal (table 1). Again a large drop in the amount of methylene blue reduction is observed at 1,000 atm. The endogenous methylene blue reduction also increases with increasing pressure as noted in figures 3 and 4.

When the slopes of the line of the reaction rates at 1, 200, 600, and 1,000 atm on the oxidation of succinate, formate, and malate (as indicated by methylene blue reduction) were compared (table 2), it was noted that pressure applied does not affect each substrate in the same way. The differences in the slopes of the line at 1, 200, and 600 atm were small when malate was used as the substrate, increasing with formate and succinate respectively. In each case the slope of the line dropped drastically at 1,000 atm when compared to the increments of drop between other pressures.

The endogenous methylene blue reduction increased regularly with increasing pressure; at 1,000 atm a rate of 5 to 8 times the 1 atm value was observed.

**DISCUSSION**

The experimental data presented demonstrates the inactivation of formic, succinic, and malic dehydrogenase associated with the whole cells of _E. coli_ during the period of compression. It is not known whether the effects of moderate hydrostatic pressure are direct or indirect. This problem still remains for further investigation.

The application of hydrostatic pressure does not appear to inhibit the action of the three dehydrogenases to the same degree (table 2). The slopes of the line for formic and malic dehydrogenase action at 1, 200, and 600 atm did not differ appreciably. On the other hand, the slope of the line for succinic dehydrogenase action decreased considerably between 1 and 200 atm, between 200 and 600 atm, and between 600 and 1,000 atm.

Borrowman (1950) observed that pressures up to 700 atm did not change the formic dehydrogenase activity of cells of _E. coli_ which had been heated previously to 60 C for 5 min. Since heat was applied, the "biological whole" was not represented. Whether or not formic dehydrogenase associated with the "biological whole" is affected by hydrostatic pressure differently than crude or pure enzymes is not known. However, using cells of _E. coli_ at 1,000 atm a great drop in the methylene blue reduction is observed. Borrowman's (1950) maximum pressure was 700 atm. Thus, a pressure of 1,000 atm nearly inactivated formic dehydrogenase as well as succinic and malic dehydrogenase (table 1 and figures 3 and 4). Since the "biological whole" was used in these experiments, it is not known how other proto-

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**TABLE 2**

_Slope of the line of the methylene blue reduction for figures 3 and 4 and table 1. Corrected for endogenous methylene blue reduction_

<table>
<thead>
<tr>
<th>Atm</th>
<th>Malate*</th>
<th>Succinate†</th>
<th>Formate‡</th>
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<tr>
<td>1</td>
<td>1.27</td>
<td>1.93</td>
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</tr>
<tr>
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</table>

* Slope calculated from data in table 1 for the 3 to 15 min period.
† Slope calculated from data in figure 3 for the 3 to 15 min period.
‡ Slope calculated from data in figure 4 for the 6 to 15 min period.

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plasmic factors (other enzymes, cell permeability, etc.) affect the action of the enzymes tested.

Endogenous methylene blue reduction increased with increased hydrostatic pressure. Since methylene blue did not appear to break down under a pressure of 1,000 atm, the donation of hydrogen to methylene blue probably comes about by the dehydrogenation of some unknown component(s) of the cell. It is too early to speculate what this component(s) might be.

The harmful effects of oxygen at high pressures are due to the oxidation of the —SH group (glutathione, coenzyme A, cysteine, —SH enzymes) (Barron, 1955). In these experiments precautions were taken to exclude atmospheric oxygen at all stages of the experiment.

ACKNOWLEDGMENTS

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SUMMARY

A method and apparatus for the measurement of dehydrogenase activity under various hydrostatic pressures has been described.

Employing resting cells of *Escherichia coli*, the rate of succinic, formic, and malic dehydrogenase activity decreased with increased hydrostatic pressure during a period of compression. Under the conditions of the experiment, the effect of hydrostatic pressure on the rate of succinic, formic, and malic dehydrogenase varied according to the applied pressure. The endogenous methylene blue reduction by the resting cells was found to increase with increased hydrostatic pressure.

REFERENCES


