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ABSTRACT

MORITA, RICHARD Y. (University of Nebraska, Lincoln) AND ROGER D. HAIGHT. Malic dehydrogenase activity at 101 C under hydrostatic pressure. J. Bacteriol. **83**:1341–1346. 1962.—No malic dehydrogenase activity was found to occur at 101 C at various hydrostatic pressures from 1 to 700 atm. However, activity was demonstrated with hydrostatic pressures above 700 atm, with optimal activity at 1,300 atm at the same temperature. Explanation of the data is based upon thermal denaturation of the enzyme, which involves a molecular volume increase of the enzyme. The molecular volume increase is counteracted by hydrostatic pressure. Pressures above 700 atm to 1,500 atm (highest employed) were sufficient to offset the denaturation by 101 C which probably resulted in an incomplete denaturation of malic dehydrogenase.

It is well known that high temperature will denature enzymes and that the denaturation process involves a molecular volume increase (Johnson, Eyring, and Polissar, 1954). Molecular volume increases are opposed by pressure. Brown, Johnson, and Marsland (1942) presented evidence that, above optimal temperature for luminescent bacteria, pressure increased the luminescence by reactivating the thermally inactive luciferase. At temperatures causing a progressive destruction of luminescence at favorable pH, pressure retarded the irreversible denaturation (Johnson et al., 1945). Johnson and Campbell (1945) demonstrated that hydrostatic pressure retarded the precipitation of highly purified human globulin and egg albumen at 65 C. Pressure also retarded the thermal denaturation of tobacco mosaic virus (Johnson, Baylor, and Frazer, 1948). Evidence was presented that substrate must be present to demonstrate the protective effect of pressure on the enzymes, phenylglycosidase (Berger, 1958) and aspartase (Haight and Morita, 1962), against thermal inactivation.

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MATERIALS AND METHODS

Source and preparation of enzyme. The malic dehydrogenase preparation was obtained from R. J. Downey of this laboratory. The enzyme was prepared by treating a thick cell suspension of *Bacillus stearothermophilus* NCA 2184 with a 10-kc Raytheon sonic oscillator for 30 min and centrifuging it for 60 min at 23,500 $\times$ *g* at 0 C in a Servall RC-2 refrigerated centrifuge. The supernatant liquid was decanted and centrifuged at 144,000 $\times$ *g* for 120 min in a Spinco Model L preparative ultracentrifuge. The clear straw-colored supernatant liquid, which contained the enzyme, malic dehydrogenase, was decanted and frozen. When needed, the enzyme preparation was thawed and diluted 1:10 (v/v) with tris (hydroxymethyl)aminomethane-HCl (pH 8.5, 0.2 M). This enzyme preparation contained 153 μg protein nitrogen per 0.5 ml.

Assay of malic dehydrogenase activity. Malic dehydrogenase activity was measured by the procedure of Marsh (1956), which involves the reduction of triphenyltetrazolium chloride (TPTZ) to formazan (reduced TPTZ) in the presence of NaCN, diphosphopyridine nucleotide (DPN), tris buffer, L-malic acid, and malic dehydrogenase.

The reaction mixture (4.0 ml total volume) contained: tris-HCl buffer, 1.3 ml (pH 8.5, 0.2 M); L-malic acid, 1.0 ml (pH 8.5, 400 μmoles/ml); TPTZ, 0.5 ml (0.02 M); NaCN, 0.2 ml (0.05 M); DPN, 0.5 ml (1 mg/ml); and the diluted malic dehydrogenase preparation (0.5 ml). When total volumes larger than 4.0 ml were needed, the respective reagent volumes were scaled accordingly.

The reaction mixture was placed in test tubes

(12 by 16.5 mm) and stoppered with no. 00 white rubber stoppers. No air phase remained in the stoppered test tubes. The test tubes were then placed in the pressure cylinder for treatment. Certain experiments called for the pretreatment of either the enzyme or DPN before addition to the rest of the reaction mixture.

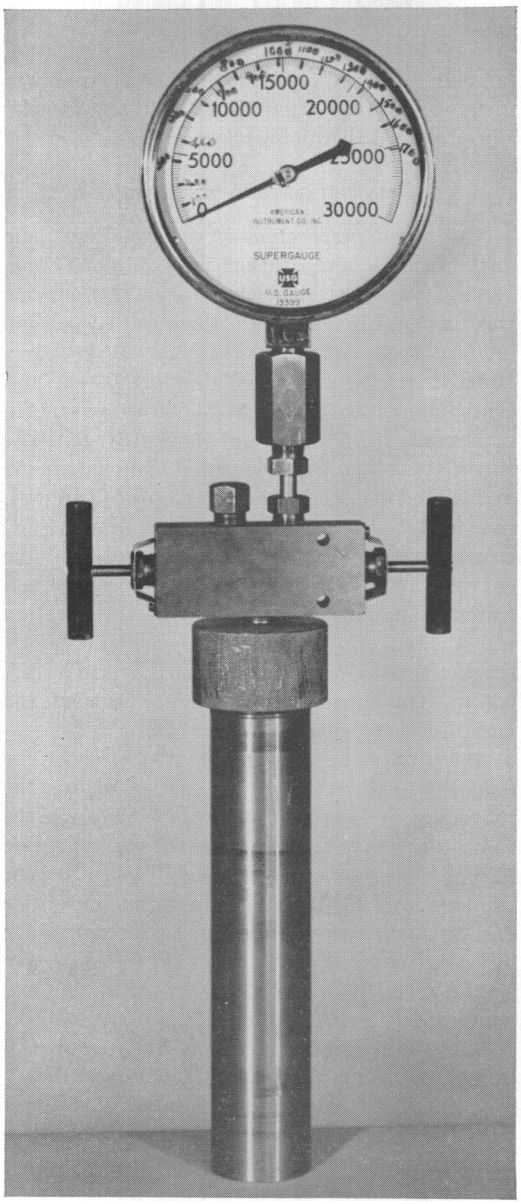

FIG. 1. *Pressure cylinder used for studies at 101 C at various hydrostatic pressures.*

After incubation at the desired temperature and pressure, 4 ml of the reaction mixture were pipetted immediately into screw-cap test tubes containing 1.0 ml of chilled trichloroacetic acid (50%) to stop the reaction. All tubes were kept cold in an ice bath and in the dark until analyzed. The formazan was extracted with 5.0 ml of ethyl acetate. A sharp interface between the ethyl acetate and the reaction mixture was brought about by centrifugation for 5 min at 1,230 × *g*. A known amount of ethyl acetate containing formazan was diluted with a known amount of ethyl acetate to obtain a reading within the linear range of the standard curve. The standard curve was obtained by diluting known amounts of formazan with ethyl acetate, and the optical densities of the solutions were read at 495 mμ on a spectrophotometer (Bausch and Lomb Spectronic 20). The activity of the malic dehydrogenase preparation was expressed as mg formazan produced per ml of reaction mixture. No formazan was produced under the conditions of our

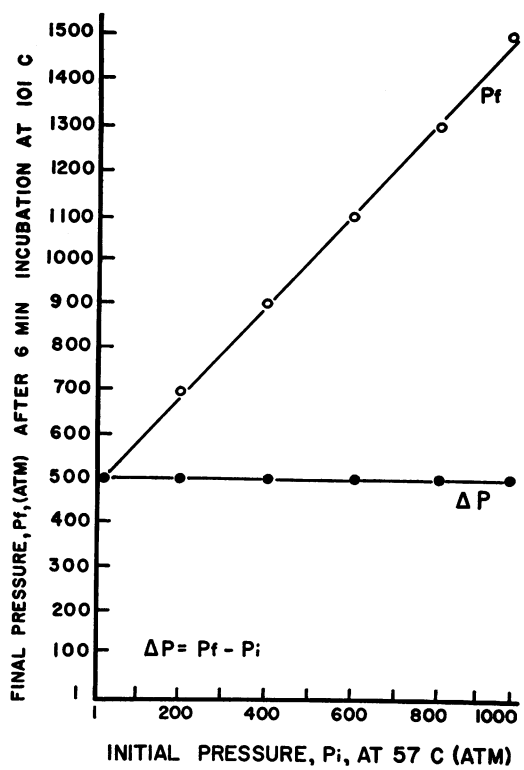

FIG. 2. *Initial pressure—final pressure relationship for the pressure cylinder illustrated in Fig. 1.*

experiments in the absence of the enzyme preparation.

Pressurization technique. The pressure apparatus and technique described by ZoBell and Oppenheimer (1950) was employed with one exception. The pressure-cylinder cap was fitted with a three-way valve so that a pressure gauge could be attached (Fig. 1). Rupturing of the neoprene "O" ring seals at 101 C under pressure caused difficulties. For this reason, constant observation of the pressure gauge was necessary to insure correct incubation times.

The pressure cylinder was filled with water and equilibrated to 57 C. After the stoppered test tubes filled with reaction mixture were placed in the pressure cylinder and the pressure-cylinder cap secured, the pressure was elevated to the desired initial pressure at 57 C (P_i in Fig. 2). If the pressure cylinder was equilibrated to 101 C before pressure was applied, the enzyme would undergo thermal denaturation before the hydrostatic pressure could be elevated. A total of 6 min was required for the initial pressure in the cylinder to reach the final pressure (Pf in Fig. 2). The data in Fig. 2 were obtained by use of the pressure cylinder shown in Fig. 1. In all cases where the temperature of 101 C was used, the pressure cylinder was cooled with cold water before the pressure was released and the pressure-cylinder cap removed to obtain the reaction mixture. This process took approximately 1 min.

For experiments at 1 atm and 101 C, the pressure-cylinder cap was removed and the cylinder filled with ethylene glycol and equilibrated at 57 C. The reaction mixture was added to a long test tube fitted with a vapor stop and was then placed in the pressure cylinder. The entire assembly was immersed in the 101 C ethylene glycol bath as shown in Fig. 3. This procedure was necessary since the increase in temperature from 57 to 101 C would increase the pressure if the pressure cylinder was capped. Also, approximately the same time element was required for it to obtain thermal equilibrium as the entire pressure cylinder.

An Aminco constant-temperature bath, type R, which can maintain a liquid temperature of up to 200 C (± 0.2 C), was utilized for this investigation. The temperature was checked with a National Bureau of Standards Certified thermometer.

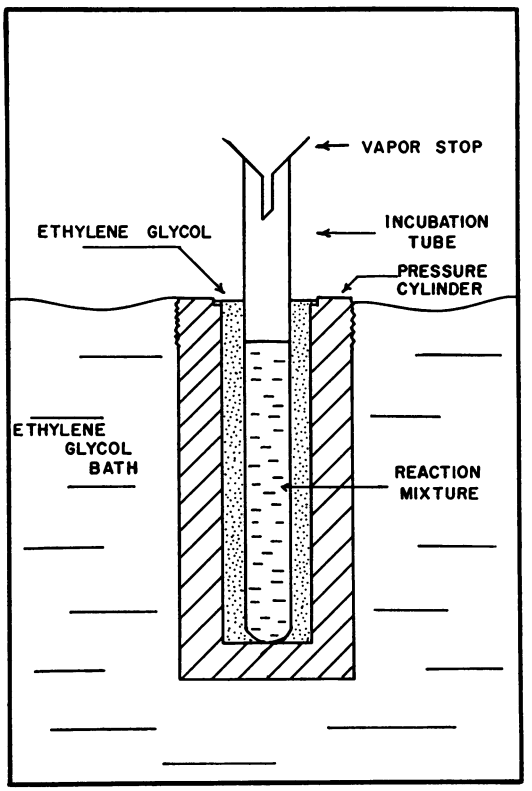

FIG. 3. *Apparatus used for 1 atm studies at 101 C*

RESULTS

Effect of pH. Preliminary studies on the malic dehydrogenase activity at 60 C demonstrated that substrate saturation level was 50 μmoles/ml of reaction mixture. In order that the substrate would not be a limiting factor, 100 μmoles of L-malic acid/ml of reaction mixture were used. This level showed no substrate inhibition. The rate of enzyme activity during a 6-min period did not decrease. By use of a substrate level of 100 μmoles/ml of reaction mixture, the reaction mixtures were made up with varying pH values by using 0.2 M tris(hydroxymethyl) a mino methane (tris) buffer and incubated for 35 min at 60 C. The data in Fig. 4 show an optimal pH of approximately 8.5 and also show that the activity curve in relation to pH is not sharp. For all subsequent studies, a pH of 8.5 was selected.

Stability of DPN. Treatment of DPN at various pressures and temperatures for a period of 44 min before addition to the rest of the reaction mixture revealed that activity was lost

at 100 C (Table 1). DPN, in a concentration of 1 mg/ml, was employed in the reaction mixture since 60% activity was lost when DPN was treated at 100 C and 1 atm for 44 min. Destruction of DPN occurred more when treated at 100 C and 1 atm for 44 min than when treated at 100 C and 1,500 atm for 44 min.

Activity of malic dehydrogenase at various temperatures at 1 atm. When the enzyme reaction mixture was subjected to various temperatures, an optimum of 55 C was noted (Fig. 5). No activity of the enzyme could be detected at 80 C, which indicated that thermal inactivation occurred just below 80 C.

Activity of malic dehydrogenase at 101 C at various pressures. When the malic dehydrogenase reaction mixtures were subjected to a temperature of 101 C at various pressures, it was noted that no activity could be demonstrated at 1 to 700 atm (Fig. 6). However, activity of the malic dehydrogenase was demonstrated when hydrostatic pressure was elevated above 700 atm. Since it took 6 min for the initial pressure to reach the final pressure in the system, the 6-min points served as the base line for these studies. At 1,300 atm and 101 C, the amount of formazan produced by the reaction mixture in 41 min was approximately 350% greater than the amount produced in 6 min. The net activity at 101 C at various pressures, represented in Fig. 6 as delta (Δ), is the difference between the amount of

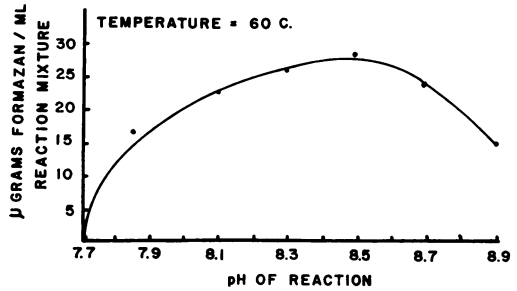

FIG. 4. *Effect of pH on malic dehydrogenase activity at 60 C. The reaction mixture contained tris-HCl buffer, 1.3 ml (various pH levels, 0.2 M); L-malic acid, 1.0 ml (various pH levels, 400 μmoles/ml); TPTZ, 2.5 ml (0.02 M); NaCN, 0.2 ml (0.05 M); DPN, 0.5 ml (1 mg/ml); and 0.5 ml of diluted malic dehydrogenase preparation (153 μg protein N/0.5 ml). Values are corrected for controls. Incubation time was 35 min.*

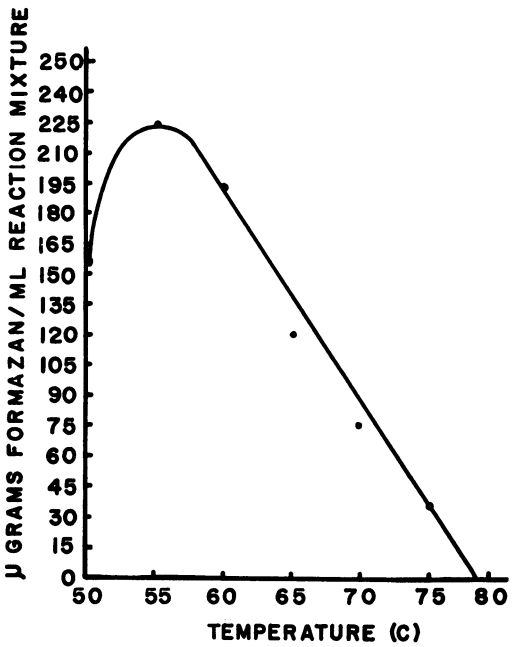

FIG. 5. *Effect of temperature on malic dehydrogenase at 1 atm. The reaction mixture was identical to that in Fig. 4 except that the pH level was 8.5. Incubation time was 35 min. Values are corrected for controls.*

TABLE 1. *Stability of DPN at various hydrostatic pressures and temperatures as measured by the amount of formazan produced*[a]

Treatment of DPN[b]	Formazan produced[c]	Activity lost[d]
	μg/ml	*%*
28 C, 1 atm, 44 min.....	190	
60 C, 1 atm, 44 min.....	190	0
100 C, 1 atm, 44 min.....	79	60
100 C, 1,500 atm, 44 min.....	108	40
No DPN.....	0	

[a] The reaction mixture contained tris-HCl buffer, 1.3 ml (pH 8.5, 0.2 M); L-malic acid, 1.0 ml (pH 8.5, 400 μmoles/ml); TPTZ, 0.5 ml (0.02 M); NaCN, 0.2 ml (0.05 M); 0.5 ml DPN (1 mg/ml); and 0.5 ml of the diluted malic dehydrogenase preparation (153 μg per protein N per 0.5 ml).

[b] Before addition to the malic dehydrogenase reaction mixture.

[c] At 60 C for 35 min; tests were run in duplicate and corrected for controls.

[d] Relative to treatment at 28 C and 1 atm for 44 min.

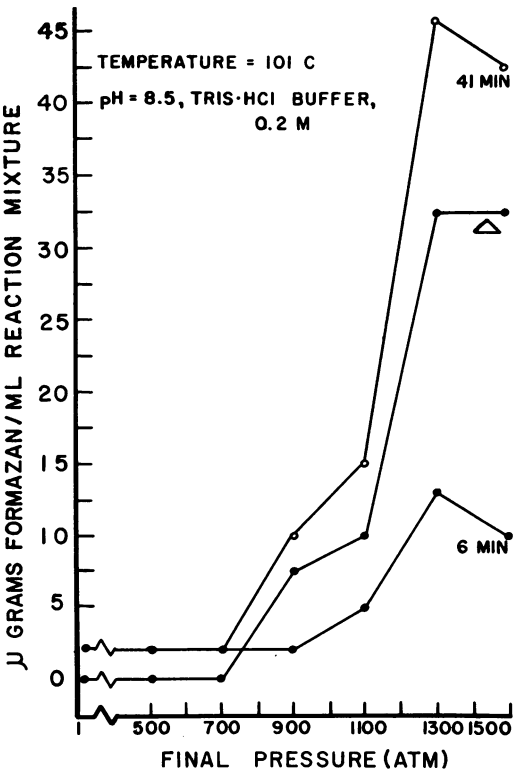

FIG. 6. *Activity of malic dehydrogenase at 101 C under various hydrostatic pressures. The reaction mixture was identical to that in Fig. 4 except that the pH level was 8.5. Delta (Δ) represents the difference between the 6 min and 41 min incubation times. Values are corrected for controls.*

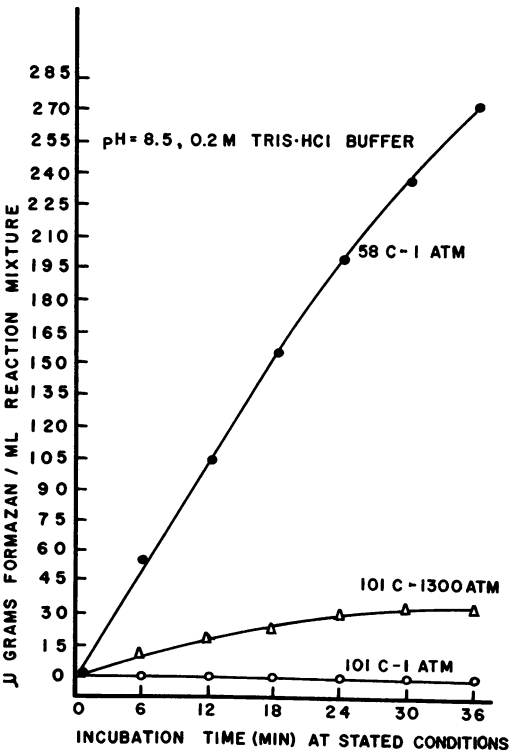

FIG. 7. *Rate of malic dehydrogenase activity. The reaction mixture was identical to that in Fig. 4 except that the pH level was 8.5. The curve of 101 C and 1,300 atm is corrected for the 6-min period required for the initial pressure to reach the final pressure. Values are corrected for controls.*

formazan produced in 41 min minus the amount formed in 6 min.

Malic dehydrogenase displayed activity with time at 101 C and 1,300 atm, whereas the curve at 101 C and 1 atm curve showed no activity (Fig. 7). The curve at 101 C and 1,300 atm is definitely not as pronounced as the curve at 58 C and 1 atm.

DISCUSSION

Since it is extremely difficult to measure pH shifts with increased hydrostatic pressure and temperature, tris-HCl buffer was employed. According to Kauzmann (*personal communication*), the organic amine types of buffers possess a small Δ volume (v) (Δv = +2 to +4 ml at 25 C and 1 atm at infinite dilution) as compared to phosphate buffer (Δv = −29 ml at 25 C and 1 atm at infinite dilution). The pH shifts with pressure are minimized with systems that possess small Δv values. Since the pH curve at 60 C and 1 atm (Fig. 4) does not display a sharp peak, a slight shift in pH would not alter the main point of this investigation, which is the activity of malic dehydrogenase at 101 C under hydrostatic pressure.

Malic dehydrogenase activity at 1 atm was destroyed at approximately 78 C (Fig. 5), but, when the pressure was increased beyond 700 atm (Fig. 6), the molecular volume increase brought about by the increase in temperature was counteracted by the applied hydrostatic pressure. As a result of the applied pressure, the enzyme does not undergo complete thermal denaturation. The extent of denaturation of the enzyme under the pressure applied at 101 C awaits further investigation. Since a pressure cylinder has not

been developed to compensate for the increase in pressure brought about by increased temperature, there is undoubtedly some denaturation of the enzyme during the time the pressure cylinder goes from its initial pressure to the final pressure (Fig. 2). Denaturation would probably take place more at the lower pressures applied in our studies.

We also realize that the rate of activity at 101 C and 1,300 atm is quite low compared to the rate at 58 C and 1 atm. However, it should be remembered that we are probably not operating under the optimal temperature for the malic dehydrogenase system. The data obtained, however, show clearly that malic dehydrogenase can operate at 101 C when hydrostatic pressures of 700 to 1,500 atm are applied.

ACKNOWLEDGMENTS

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