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INTERACTION BETWEEN THE PARAMETERS OF HYDROSTATIC PRESSURE AND TEMPERATURE ON ASPARTASE OF *ESCHERICHIA COLI*[1]

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ABSTRACT

HAIGHT, ROGER D. (University of Nebraska, Lincoln) AND RICHARD Y. MORITA. Interaction between the parameters of hydrostatic pressure and temperature on aspartase of *Escherichia coli*. J. Bacteriol. **83**:112–120. 1962.—The data obtained from studies of an aspartase preparation and aspartase in cells of *Escherichia coli* indicate that there is an interaction between the parameters of hydrostatic pressure and temperature. Pressure was found to decrease aspartase activity at 45 C and lower in vitro and below 53 C in vivo, thereby indicating that when the enzyme-substrate complex is formed there is an increase in molecular volume which is counteracted by pressure. Above 53 C in vivo and above 45 C in vitro, temperature probably starts the unfolding process of the enzyme to expose more reactive sites, while pressure then pushes the enzyme and substrate into closer proximity with each other. Thus, pressure stimulated activity and also prevents further unfolding of the enzyme. Since the enzyme preparation retains about the same level of activity after being subjected first to 1000 atm at 56 C, the aspartase probably refolds into its original configuration or one similar to it, when subjected to 1 atm at 37 C.

In all cases, the presence of the substrate was found necessary to protect aspartase from thermal inactivation or denaturation.

In biochemical reactions where large molecules are concerned, the effect of moderate hydrostatic pressure (1 to 1000 atm) may be quite pronounced. In dealing with the influence of temperature on the response of biological processes to hydrostatic pressure, Brown, Johnson, and Marsland (1942) noted that, above normal optimal temperature for luminescent microorganisms, the intensity of light emitted increased with increased pressure; at lower temperatures, elevated pressures decreased the intensity; and at near optimal temperature, increased pressure had little effect. Morita and ZoBell (1956) demonstrated that the inactivating effect of hydrostatic pressure on succinic dehydrogenase of *Escherichia coli* was more pronounced at temperatures above or below the growth optimum for multiplication. A temperature-pressure relationship concerned with muscle contraction was noted by Brown (1934). Muscle contraction could be increased, decreased, or unaffected by pressure, according to the temperature of the experiment and source of the muscle.

Berger (1958), working with pressure effects on phenylglycosidase activity, also demonstrated a temperature-pressure relationship. His data also illustrate that, in the absence of substrate, enzyme inactivation was accelerated by increased pressure.

This paper deals with the pressure-temperature effects on aspartase in washed cells and in a cell-free preparation.

MATERIALS AND METHODS

Washed cell preparations. E. coli (University of Nebraska strain U58) was grown in a medium which contained K_2HPO_4, 5 g; KH_2PO_4, 2 g; peptone (Difco), 5 g; yeast extract (Difco), 1 g; glucose, 2 g; and 1000 ml of distilled water. After autoclaving, the pH was 7.0. *E. coli* was inoculated into 100 ml of the medium in 250-ml Erlenmeyer flasks and incubated at 37 C for 18 hr with continuous shaking. The cells were harvested by centrifugation at 23,000 × *g* for 10 min, washed twice with 0.07 M phosphate buffer (pH 7.2), resuspended in buffer, and aerated for 30 min to reduce the endogenous substrate level. After aeration, the cells were centrifuged and resuspended in buffer, so that a 1:1 dilution of the cells gave a transmittance reading of 45% at 600 mμ in a Bausch and Lomb

[1] This paper was taken from a dissertation submitted in partial fulfillment of the requirements for the Master of Science degree at the University of Nebraska.

Spectronic 20 colorimeter. The cells were then used immediately for washed-cell experiments.

Preparation of aspartase. E. coli was grown in 20-liter carboys, containing 15 liters of medium, with vigorous aeration for 18 hr at 37 C. This medium was autoclaved for 2 hr at 15 psi. The cells were harvested with a Sharples Super centrifuge and frozen immediately.

The cells were thawed, taken up with 0.07 M phosphate buffer (pH 7.2), and treated for 30 min in a Raytheon 10 kc sonic oscillator. The preparation was then clarified by two consecutive centrifugations at 144,000 × *g* in a Spinco Model L preparative ultracentrifuge, first for 60 min and then for 90 min. Microscopic examination showed the extract to be cell-free. The clear straw-colored supernatant was then frozen and used as the enzyme preparation, hereafter referred to as the aspartase preparation. It was diluted 1:500 with phosphate buffer for use in cell-free aspartase experiments; the nitrogen content at this dilution was 11.2 μg per ml.

Preliminary studies by Haight (1961) on this enzyme preparation showed that it was free of proteinase and fumarase. The presence of proteinase might destroy the enzyme during the incubation period, and thereby invalidate the results obtained. Fumarase activity could cause a shift in the aspartase equilibrium. A test for fumarase was developed for this investigation.

Assay of the aspartase activity. L-Aspartic acid (Nutritional Biochemical Corp.) was used as the substrate. The amount of ammonia produced, determined by Nesslerization, was used as the index of aspartase activity. The Nessler reagent was dissolved in gum ghatti (Haight, 1961). Three volumes of Nessler reagent followed by four volumes of 5 N NaOH were added in all ammonia determinations of the reaction mixtures. Good mixing and rapid addition of the base were found to be essential for good reproducibility. Color intensity was read in a Bausch and Lomb Spectronic 20 colorimeter at 450 mμ. The resultant optical densities were converted to micromoles of ammonia by comparison with a standard curve prepared with reagent grade ammonium sulfate. The ammonia determinations were always determined in the desired linear range.

In all cases where the aspartase preparation was used, the reaction was stopped by pipetting a sample of the reaction mixture into a known volume of 2 N H_2SO_4. In washed-cell studies, 2-ml samples of the reaction mixture were transferred into conical polyethylene centrifuge tubes containing 2 ml of 10% chilled trichloroacetic acid. Cells were removed by centrifugation at 23,000 × *g* for 8 min in a Servall SS-1 centrifuge. The cell-free supernatant was analyzed for ammonia. All experiments were run in triplicate.

Pressurization technique. The pressure equipment used in this investigation was essentially the same as that described by ZoBell and Oppenheimer (1950). Each pressure cylinder used in any particular study was filled with distilled water and brought to thermal equilibrium in a water bath prior to use. The reaction mixtures were placed in Pyrex test tubes of appropriate size and carefully stoppered to exclude air, thus maintaining only dissolved air in the reaction mixture. Preliminary studies demonstrated that the dissolved air did not affect the aspartase reaction at elevated pressures. The reaction mixtures were then pressurized (where desired) and incubated at various temperatures and incubation periods. After incubation the material was treated immediately to stop the enzymatic action.

A biological ammonia production from the substrate and nonbiological substrate resynthesis did not occur under the experimental conditions (Haight, 1961).

RESULTS

Effect of hydrogen ion concentration. An optimal pH of 6.9 was observed for the aspartase reaction at 37 and 45 C (Fig. 1). It was tacitly assumed that the pH optimum would not shift appreciably at temperatures of 50, 53, and 56 C and that pH 6.9 would be satisfactory for studies at various hydrostatic pressures and temperatures. It was realized that increased hydrostatic pressure would decrease the pH (see Discussion) and, therefore, that the amount of ammonia produced by the enzyme might be lowered when pressures above 1 atm were used. A broad pH range was also noted for this system.

For washed-cell studies a pH of 7.2 was selected; Figure 2 shows that the optimum for the aspartase reaction was 7.1 at 37 C and 1000 atm, and the optimum was close to pH 6.9 at 37 C and 1 atm.

Substrate saturation studies. When aspartic

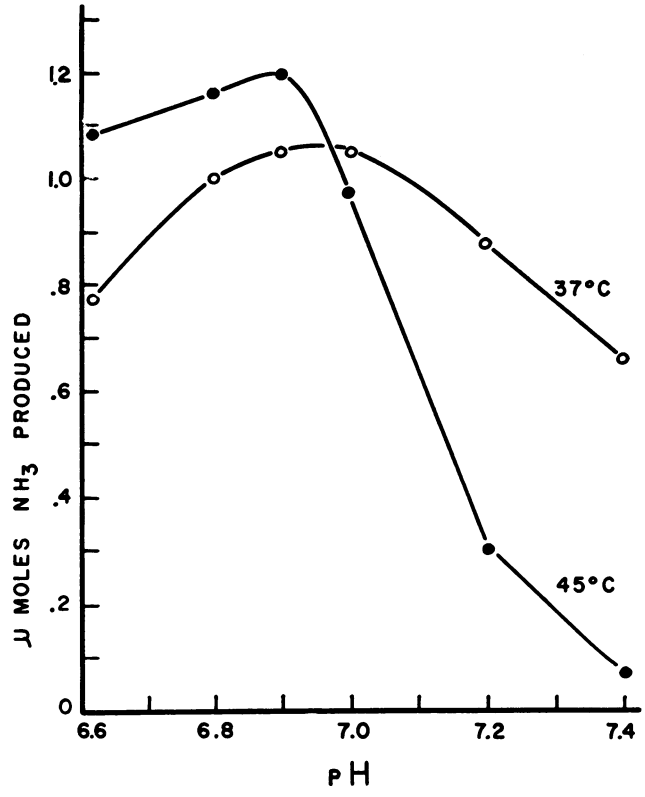

FIG. 1. *Effect of pH on aspartase activity. The reaction mixture contained equal volumes of the aspartase preparation (11.2 μg N/ml) and* L*-aspartic acid (800 μmoles/ml) in 0.2* M *phosphate buffer of the desired pH, and was incubated at 37 and 45 C for 35 min. The values were corrected for residual ammonia in the enzyme and substrate preparations.*

acid was used in a concentration of 400 μmoles/ml of reaction mixture, the aspartase preparation was saturated at 45 C (Fig. 3). At lower substrate concentrations, a lag was noted, which at present cannot be explained. Since a maximal catalytic rate was observed at 45 C, with a substrate concentration of 400 μmoles/ml, this level of substrate was used in all subsequent investigations with the aspartase preparation.

Rate studies on the aspartase preparation. As in all enzyme studies, it was necessary to know whether the enzyme preparation attained equilibrium during the incubation period or whether the rate of catalysis remained linear with time within the period of incubation. The catalytic rate remained linear during the 35-min incubation period at the various temperatures used in this investigation (Fig. 4). Since the substrate level was high, the aspartic acid does not become a limiting factor.

Between 37 and 53 C, no enzyme activity was lost by the enzyme preparation in the presence of the substrate during the 35-min incubation period at 1 atm (Fig. 4). This is not very surprising, since heat protection has been shown to occur with the addition of substrates and cofactors to the enzyme (Grisolia and Joyce, 1959; Berger, 1958). A slight activity loss occurred at 56 C.

Effect of temperature and pressure on aspartase activity. By use of the background data obtained at 1 atm, investigations of hydrostatic pressure as a parameter of temperature were initiated. The results obtained are shown in Fig. 5. At 37 C, aspartase activity decreased linearly with increased pressure, whereas at 45 C the pressure effect is less noticeable. A 31% increase in the 50 C and 1000 atm activity over the 50 C and 1 atm activity was noted. The 56-C curve behaves similarly, except that at 1000 atm some loss in activity occurred. The pressure effect at 53 C again shows a similar loss pattern.

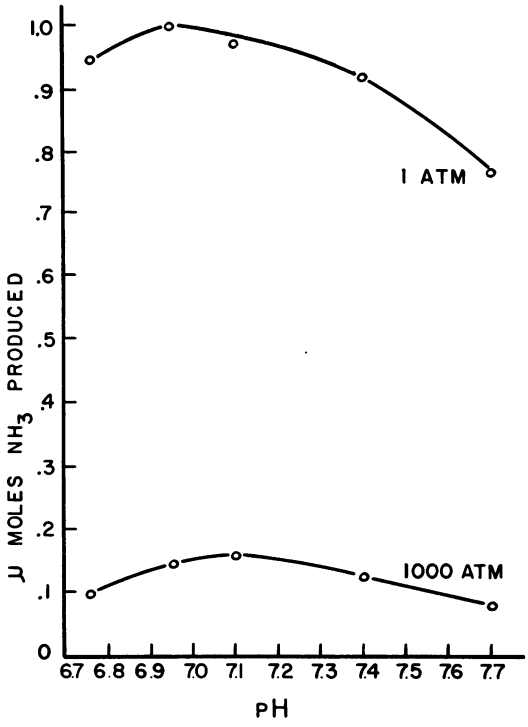

FIG. 2. *Effect of pH on the deamination of* L-*aspartic acid by washed cells of Escherichia coli. The reaction mixture contained equal volumes of undiluted fresh washed cells and 0.07* M *phosphate buffer at the desired pH, and was incubated at 37 C at 1 and 1000 atm for 35 min. Washed cells were made up in buffer so that a 1:1 (v/v) dilution with buffer gave a 45% transmittance reading at 600 mμ. The values were corrected for endogenous ammonia production.*

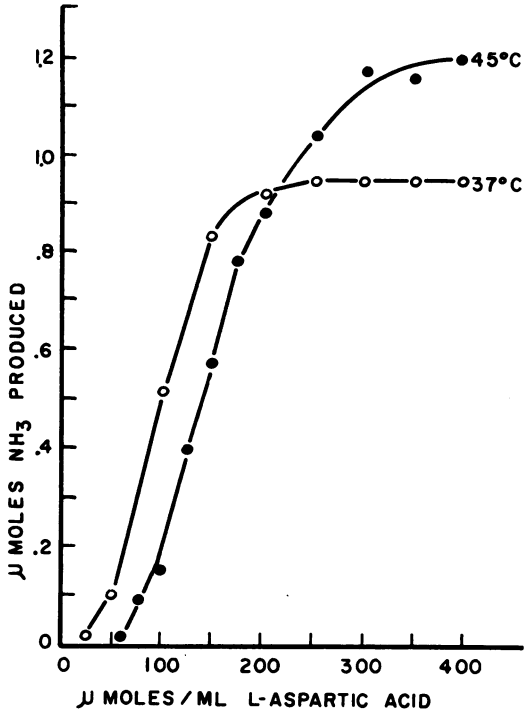

FIG. 3. *Effect of substrate concentration on aspartase activity. Equal volumes of the aspartase preparation (11.2 μg N/ml) and* L-*aspartic acid (various concentrations) in 0.2* M *phosphate buffer (pH 6.9) were used as the reaction mixture. Incubation period was 35 min at 37 and 45 C. The values were corrected for residual ammonia in the enzyme and substrate preparations.*

By plotting the data in Fig. 5 against temperature, the trends shown in Fig. 6 are obtained. There is a parallel nature of the activity curves between 37 and 45 C, where pressure increases in this temperature range show a consistent decrease in activity of the enzyme. This increased activity between 37 and 45 C follows van't Hoff's Law. Between 45 and 50 C, however, there is a switch-over where increased pressure increases the aspartase activity. The temperature increment between 45 and 50 C represents a critical temperature range where the action of pressure is reversed. Since there is an increase in activity at all pressures above 1 atm and at all temperatures above 45 C, there is some pressure-temperature interaction on the enzyme that yields the above results.

Role of substrate, temperature, and pressure on aspartase activity. The amount of ammonia produced at 37 C and 1 atm in 35 min of incubation is represented by bar A of Fig. 7. Incubation of the same reaction mixture under identical conditions for an additional 35 min produces approximately the same level of ammonia (bar A'). All bars indicated with a prime in Fig. 7 represent the amount of ammonia produced when a sample of the original reaction mixture, indicated by letters, is further incubated for 35 min at 37 C and 1 atm. When the reaction mixture is incubated for 35 min at 1000 atm and 37 C (bar B), there is a decrease in ammonia production. A further increase of ammonia is noted when the condition of incubation is changed to 1 atm and 56 C with the same time element (bar C). A still further increase in ammonia production occurs at 56 C and 700 atm (bar D).

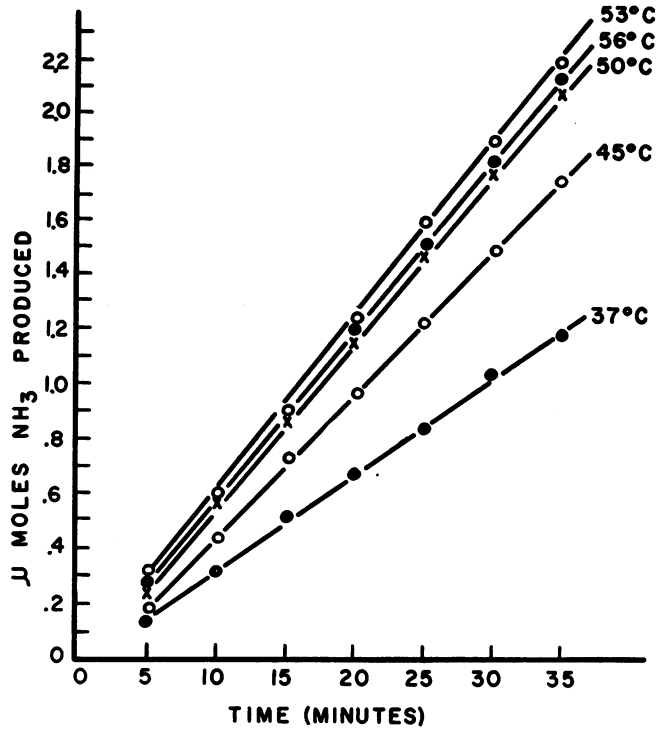

FIG. 4. *Rate of aspartase activity as a function of temperature. The reaction mixture consisted of equal volumes of the aspartase preparation (11.2 μg N/ml) and* L*-aspartic acid (800 μmoles/ml) in 0.2* M *phosphate buffer (pH 6.9) and was incubated at various temperatures. Ammonia was determined at 5-min intervals for each temperature. The values were corrected for residual ammonia in the enzyme and substrate preparations.*

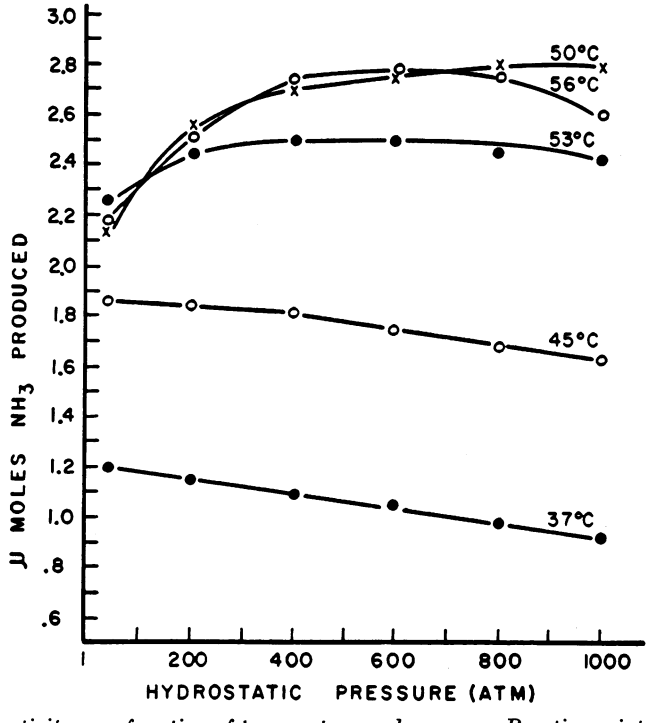

FIG. 5. *Aspartase activity as a function of temperature and pressure. Reaction mixtures were identical to those in Fig. 4.*

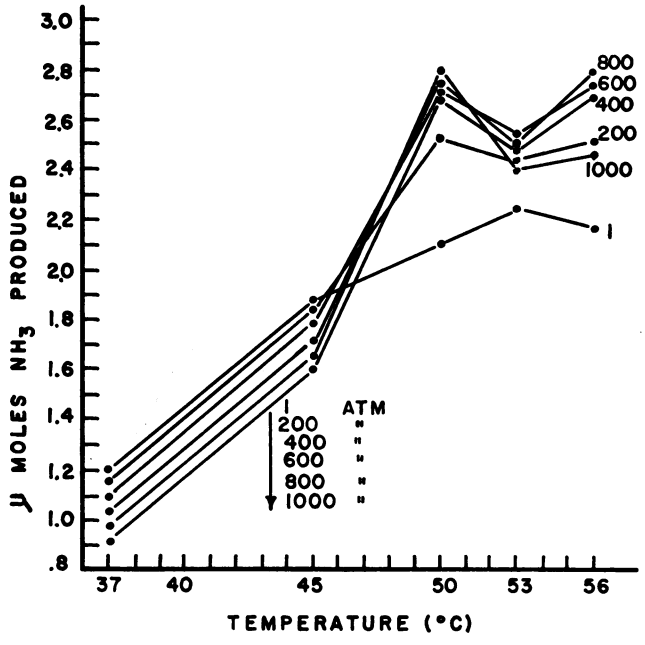

FIG. 6. *Temperature-pressure effects on aspartase activity. Replot of Fig. 5, using temperature as the abscissa instead of pressure.*

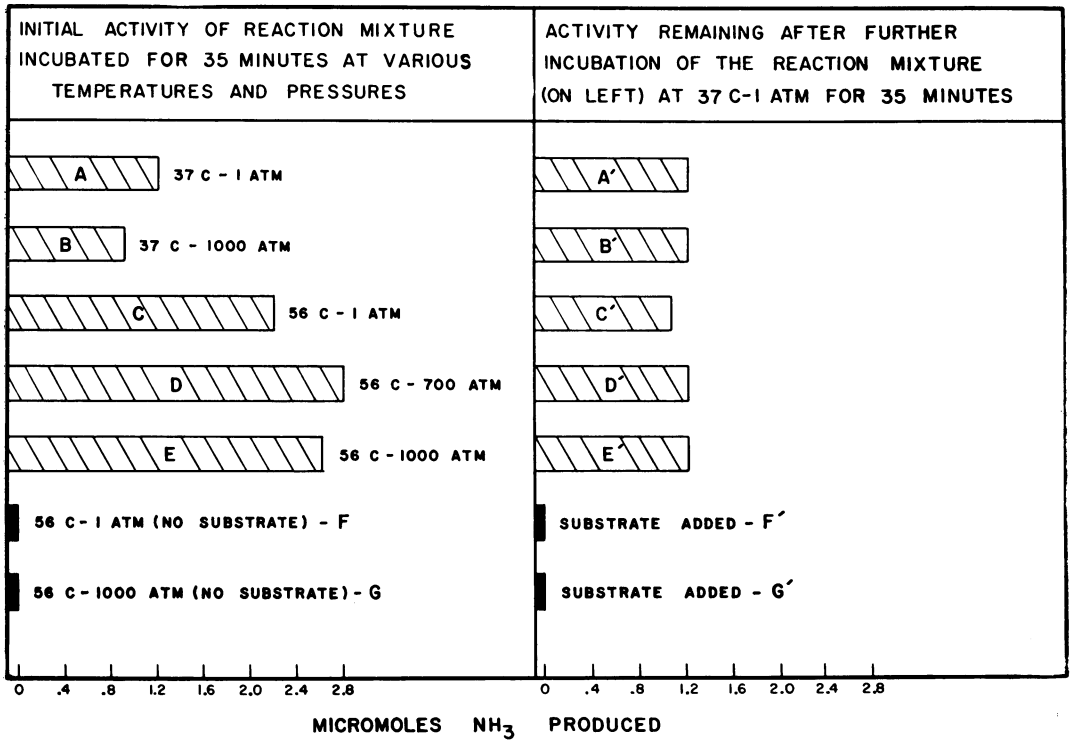

FIG. 7. *Activity of aspartase remaining after teratment at various hydrostatic pressures and temperatures in the presence and absence of* L*-aspartic acid. The reaction mixtures were identical to those in Fig. 4, except where no substrate was used initially.*

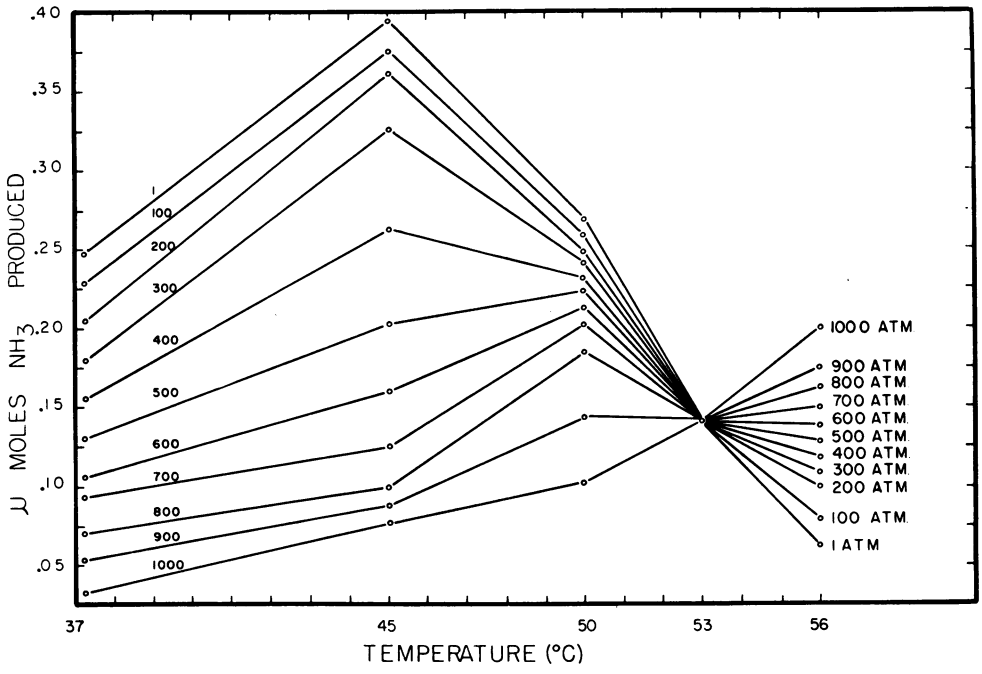

FIG. 8. *Effect of temperature and pressure on the deamination of* L*-aspartic acid by washed cells of Escherichia coli. The reaction mixtures were identical to those in Fig. 2, except that the reaction mixtures were made with 0.07* M*, pH 7.2 phosphate buffer in all cases. The values were corrected for endogenous ammonia production.*

At 56 C and 1000 atm (bar E), there is a slight drop in activity compared to the activity at 56 C and 700 atm (but the level remains higher than at 56 C and 1 atm). It appears that there was a stimulation of enzyme activity when the pressure was elevated at 56 C, with maximal activity at 700 atm.

Bar B′ represents the amount of ammonia produced by the reaction mixture at 37 C and 1 atm after two 35-min incubation periods. This bar shows approximately the same activity as bar A′ and indicates that a pressure of 1000 atm at 37 C does not permanently damage the enzyme.

The activity of the enzyme after treatment at 56 C and 1 atm is represented by bar C′, which shows a slight decrease in activity. This slight decrease in activity may be due to the inactivation of the enzyme at 56 C (Fig. 4). However, bars D′ and E′ show the same activity level as bars A′ and B′, which suggests that the increase in enzyme activity (bars D and E) is not permanent and that pressure protects the enzyme against permanent heat inactivation when the substrate is present.

When the enzyme is treated at 56 C and either 1 or 1000 atm without substrate, no ammonia can be detected (bars F and G). When these two reaction mixtures are incubated further (37 C and 1 atm for 35 min) with substrate added, no enzyme activity is detected. The results demonstrate that substrate must be present for the protection of this enzyme against heat inactivation and that pressure alone cannot protect the enzyme.

Effect of temperature on aspartase activity by washed cells at various pressures. The effects of temperature on the system at various pressures are shown in Fig. 8. Figure 9 shows the effect of pressure on the system at various temperatures. The 1-atm curve in Fig. 8 illustrates the effect of temperature on the whole cell system. An increase of temperature (45 C over 37 C) results in an increase of activity, as one would expect according to van't Hoff's Law. This increased activity due to temperature occurs at all pressures, but the maximal level of activity decreases with increases in pressure. At 1 atm, an increase in temperature (above 45 C) decreased the activity of the cells, which is to be

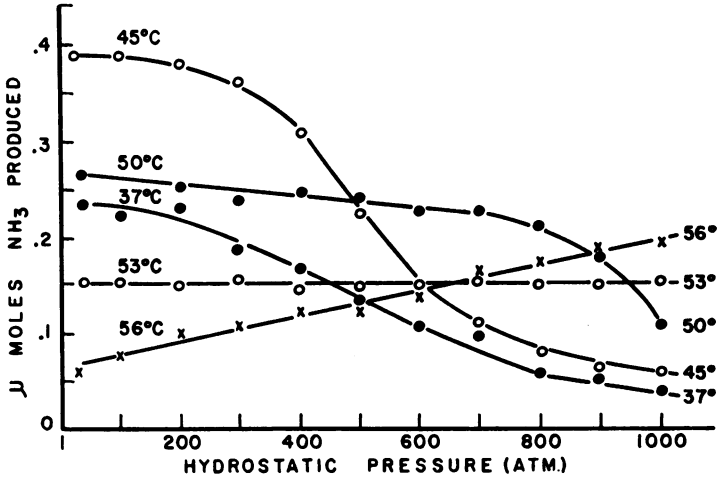

FIG. 9. *Temperature-pressure effects on the deamination of* L*-aspartic acid by washed cells of Escherichia coli. Replot of Fig. 8, using pressure as the abscissa instead of temperature.*

expected when thermal inactivation or denaturation of the cells takes place. In the 45 to 50 C temperature increment, the 100, 200, 300, 400, and 500 atm curves display the same phenomenon, but the degree decreases with increased pressure. However, the 600, 700, 800, 900, and 1000 atm curves do not show a similar effect.

In the 50 to 53 C temperature increment of Fig. 8, the 1, 100, 200, 300, 400, 500, 600, 700, and 800 atm curves all show a decrease in activity, whereas the 900 curve shows no change. The 1000-atm plot shows a continuing activity increase.

At 53 C, there appears to be a switch-over, that is, at 56 C the greatest activity is shown at 1000 atm and the lowest at 1 atm rather than the highest at 1 atm and the lowest at 1000 atm, as occurs at 37 C.

When the same data is replotted (Fig. 9), it can be seen that pressure adversely affects the activity at 37 C, that is, where temperature is ruled out as the cause of inactivation, pressure causes the system to decrease the rate of deamination.

The 45-C curve of Fig. 9 again illustrates this point, but a rapid decline occurs between 300 and 700 atm. The 53-C curve remains rather constant with increased pressure.

DISCUSSION

The aspartase preparation was studied under optimal pH and substrate conditions at 37 and 45 C at 1 atm. All the data obtained in this investigation were within the linear portion of the enzyme rate curves. We recognize the difficulties, both theoretical and mechanical, involved in trying to measure the pH change with increased hydrostatic pressure. Since an optimum of 6.9 was observed at two different temperatures (Fig. 1), this pH was selected for the aspartase preparation studies; it was tacitly assumed that the change in pH with temperatures above 45 C would not appreciably change the enzyme activity. At a pressure of 10,000 psi, the pH shift is toward the acid side, approximately 0.4 pH units for phosphate buffer at neutrality, according to Kauzmann (see Johnson, Eyring, and Polissar, 1954). Therefore, for all studies with the aspartase preparation, the results expressed in terms of μmoles of ammonia produced are actually biased toward a lower value if the pH change due to pressure is taken into consideration. The same can be said for the washed cells at elevated pressures.

According to Johnson et al. (1954), there are four possible mechanisms involving volume changes when high pressure is applied to the protein molecule. These mechanisms are (i) the amount of dipolar ion formation, (ii) the process of unfolding, which follows the weakening of the hydrogen bonds, (iii) the structure of the solvent, and (iv) the degree of ionization of the buffer system. We recognize the difficulties of analyzing the data when so many facets enter into the total picture. As a result we have chosen only the

parameters of pressure and temperature, because we feel that these are the two main factors involved in a rational analysis of our data. Furthermore, pressure and temperature were the two main conditions varied purposely in our investigations.

When the aspartase preparation was used (Fig. 5) at 45 C or lower, the activity of the enzyme decreased with increased pressure, thereby indicating an increase in the molecular volume. This increase is counteracted by pressure and, therefore, a slower enzyme catalytic rate occurs. At temperatures above 45 C, the rate of reaction is increased. At 53 and 56 C, the optimal activity is at 700 atm; a drop in activity occurs at higher pressures. Above 700 atm at 53 and 56 C, the effect of pressure on the molecular volume change is apparently the dominant force acting on the system. In other words, pressure causes an apparent stimulation in activity above 45 C. The curves for this pressure-temperature relationship are more clearly represented in the washed-cell data (Fig. 8 and 9), which show a critical temperature of 53 C instead of 45 C. This apparent stimulation in activity occurs even though the pH shifts slightly away from the optimum.

The key to the interpretation of the data is the function of temperature. It is recognized that when heat is applied to proteins (in this case, enzymes) the proteins become denatured. This denaturation process is due to the unfolding of the molecule, which is accompanied by a change in molecular volume. Therefore, the control of the molecular volume by the temperature-pressure interaction seems to be the most likely interpretation. Heat was shown to inactivate the enzyme in the absence of the substrate. Conversely, heat does not inactivate the enzyme in the presence of substrate. If inactivation by heat is due to unfolding of the enzyme molecule, the presence of the substrate may decelerate this process by virtue of the enzyme-substrate complex. This would then account for thermal protection.

However, the stimulation of activity above 45 C for the aspartase preparation and 53 C for the washed-cell preparation might be explained by a partial unfolding of the enzyme to expose more active catalytic sites, this process being controlled by temperature. The application of pressure may then increase the incidence of substrate-active site collision and prevent further unfolding of the enzyme. The former would increase the specific activity of the enzyme, and the latter would prevent partial or total inactivation from occurring. When pressure is reduced and temperature lowered, the enzyme may refold into its original configuration or one similar to it, through a process analagous to that shown by Pauling and Campbell (1942) and Doty et al. (1960). Thus, no inactivation of the enzyme occurs. This entire process of unfolding must be accompanied by an increase in molecular volume.

ACKNOWLEDGMENTS

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