Thermodynamics of Protein Denaturation. Effect of Pressure on the Denaturation of Ribonuclease A*

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ABSTRACT: The effects of pressure on the denaturation of ribonuclease have been examined in an optical pressure cell at pressures up to 50,000 psi and over the temperature range from 0 to 65°. The denaturation is virtually 100% reversible over the wide range of conditions employed at the low protein concentrations which were used. The spectrophotometric results have been analyzed in terms of a two-state thermodynamic process. Over the acid pH range studied, the indicated volume changes are very small; varying from about -45 ml mole^{-1} (25°) at pH 2 to about -5 ml mole^{-1} (50°) at pH 4 for a pressure near 1 atm. The ΔV values are a strong function of pressure under all conditions studied, becoming more negative as the pressure is increased. These results suggest that denatured ribonuclease has a partial molal compressibility which is larger than that of the native protein by about 1.5×10^{-6} atm^{-1}. The results are discussed with respect to the various molecular processes which might have some importance in determining volume and compressibility changes. It is concluded that the hydrophobic contribution to the denaturation reaction, expected to be large on the basis of model compound estimates, is not at all apparent in these experimental data.

Earlier papers from this laboratory (Brandts, 1964a,-b; Brandts and Hunt, 1967) have attempted to provide experimental information on the thermodynamics of protein conformational transitions for small proteins such as ribonuclease and chymotrypsinogen. Prior work has dealt with temperature effects, effects of changes in solvent composition, and with the interrelationship of temperature and solvent effects. Perhaps the most important general findings from the early work relate: (1) to the very large heat capacity changes for denaturation reactions which have now been confirmed in a number of other laboratories both by indirect two-state methods (Pace and Tanford, 1968; Biltonen and Lumry, 1969; Hermans and Acamporo, 1967; Pohl, 1968a,b) and by direct calorimetric measurements (Danforth *et al.*, 1967; T. Schwarz, unpublished observations; I. Wadso, unpublished observations; W. Jackson and J. F. Brandts, unpublished observations); (2) to the very profound effect of small amounts of alcohol-like (Brandts, 1968) additives on the thermodynamic functions $\Delta S°$, $\Delta H°$, and ΔC_p; and (3) to the extremely strong coupling between temperature effects and composition effects in the case of alcohol-like additives. Large heat capacity changes and strong coupling between temperature and composition variables are known to be intrinsic characteristics of reactions which involve net exposure of hydrophobic groups to aqueous solution (Brandts, 1968).

The present study supplements the list of intensive variables available for analysis of the ribonuclease denaturation. This work is concerned primarily with pressure effects and, to some extent, the coupling of temperature and pressure effects. It is known that reactions involving hydrophobic exposure to aqueous solution give rise to large volume changes (Kauzmann, 1959; Friedman and Scheraga, 1965). With this in mind, it might be anticipated that the solvation of hydrophobic bonds during the denaturation process might also be of critical importance in determining the sign and magnitude of the pressure effects arising from volume changes and compressibility changes.

There has been a moderate amount of work done in the past on the effects of pressure on protein denaturation reactions (Johnson *et al.*, 1954; Suzuki, 1960, 1963; Tongur, 1952; Curl and Jansen, 1950; Miyagawa and Suzuki, 1963, 1964; Miyagawa *et al.*, 1963, 1964; Suzuki and Miyosawa, 1965). In most cases, the protein systems studied exhibited complete or partial nonreversibility upon release of pressure, so that no thermodynamic analysis of the data was possible. The usual mode of operation has been to subject the protein solution to high pressures in a bomb for a fixed period of time, release the pressure, remove the solution from the bomb, and analyze the resulting solution for native protein by activity assay or for denatured protein by measuring the extent of precipitation, sulfhydryl exposure, etc. Thus, the experimental data obtained depend upon the time of pressing, time lapse between pressure release and analysis, the method of analysis, and other variables so that it is difficult to extract any fundamental information.

The present studies were designed largely to overcome some of the above problems. It is known that the denaturation of ribonuclease is reversible in acid solutions at one atmosphere pressure if the total protein concentration is 0.05% or less (Brandts and Hunt, 1967). This also appears to be the case at other pressures below 50,000 psi, as will be shown. The use in the present work of a high-pressure optical cell, coupled into a high-resolution spectrophotometer, permits rapid sampling and a higher degree of precision than has usually been achieved in the past. The advantages offered by this method permit estimates not only of the two-state volume changes for ribonu-

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clease denaturation, but also allow reasonably good estimates of the change in compressibility.

Experimental Section

The pressurizing system and optical cell used in this study were commercial units (Superpressure Division, American Instrument Co., Silver Springs, Md.). The optical cell, with a maximum pressure rating of 50,000 psi, was constructed of stainless steel with a special Hastelloy C liner to prevent corrosion from exposure to acid solutions. The high-pressure windows were of sapphire and had excellent transmittance at the wavelengths used in this study. The optical path length of the cell was 2.0 cm. and the overall length of the cell was 5.2 in. The diameter of the cell body was 3.8 in.

A thermostatting jacket was constructed for the cell. This was made from brass and machined to fit snugly along the entire length of the cell. It included a baffle system to ensure uniform circulation of the thermostatting fluid throughout the length of the jacket. The cell, with its thermostatting jacket, was securely fastened into a metal supporting frame which positioned directly into the sample compartment of the Cary 14 spectrophotometer. The frame was designed so that the optical aperture of the cell automatically coincided with the light beam when the frame was in place.

The cell was connected by stainless steel tubing to a separator. The traveling Teflon piston in the separator served as a physical barrier to mixing of the protein solution with the pressurizing fluid (water). The separator was connected to a high-pressure pump through stainless steel tubing, and the high-pressure pump was driven by air from a small air compressor operating at 120 psi or below.

In spite of the large bulk of the pressure cell, temperature equilibration required less than 15 min. The temperatures of the protein solutions were continuously monitored on a strip-chart recorder connected to a high-pressure thermocouple located in the solution directly above the light path. Temperature fluctuations in the equilibrated cell were less than 0.05°. Each time the pressure was changed, the temperature would also change due to the work of compression, so that time would again be required for temperature equilibrium to be reestablished.

All absorbance measurements were made on the sensitive slide wire of the Cary 14 (0.0–0.2 optical density unit), using a suitable reference solution containing ribonuclease. There were initially problems obtaining reproducible data at this resolution. It was found that the buna-NO rings, supplied by the manufacturer, that seal the optical cell were leaching small amounts of absorbing species into solution as the pressure was changed. This problem was corrected by substituting silicone O rings.

Protein solutions were prepared from Worthington ribonuclease (RAF 8 CA) and concentrations determined using an extinction coefficient of 7.4 at 277 nm for a 1% solution in a 1-cm cell.

The method of operation was to equilibrate the sample initially at low temperature and low pressure. After data were obtained, the pressure was raised slightly and the system allowed to reequilibrate. After the highest pressure was attained the system was returned to the initial low pressure to check for reversibility. This procedure was repeated at each temperature, working from the lowest to the highest temperature, except as noted in the text.

Results

Reversibility. During the course of these studies, the reversibility of the ribonuclease solutions was constantly monitored. A reversible equilibrium can usually be detected in either of two ways: (1) In the transition region, the attainment of a time-independent value of the physical observable (optical density) implies the establishment of a reversible equilibrium; (2) the reversal of solution conditions (temperature or pressure) will lead to a reversal in the value of the physical observable if the transition under study is under thermodynamic control. Both of these tests were made continuously during the course of this investigation. All solutions passed the first test to a tolerance limited by the drift rate of the spectrophotometer (*ca.* 0.0002 optical density unit/hr). Above room temperature, the time required for equilibration of the spectrophotometric readings (upon changing temperature or pressure) was determined by the rate of thermal equilibrium, *i.e.*, about 15 min. Below 25°, equilibration in the transition region lagged considerably behind thermal equilibration, implying that the denaturation reaction was considerably slower than at high temperature. In the 0–15° temperature interval, complete equilibration required up to 3 hr and, to a good approximation, obeyed first-order kinetics.

With one exception, to be noted, all solutions displayed good reversibility according to the second criterion. After attaining the highest pressure at any given temperature, the pressure was lowered to the starting point and the extent of reversibility determined after reequilibration. Indicated reversibility was always in the range 98–103% (using the optical density change for complete denaturation as the 100% base). In nearly all cases, it was in the 100–102% range, implying a small systematic error in the measurements. The indicated reversibility under conditions where the protein passed through the transition region over the pressure range of interest was, on average, equivalent to the reversibility achieved under conditions where the native protein (or the denatured protein) was stable over the entire pressure range.

The single exception occurred with solutions of low pH (pH 2 and 2.6) when the temperature was very close to 0°. As the pressure was increased, these solutions passed into the transition region and displayed good "reversibility" as judged by the first criterion. This was true all the way up to the highest pressures. Upon release of the pressure, the system began to return slowly to the high optical densities characteristic of the native protein. However, these solutions continued to increase in optical density for very long time periods (at least 24 hr) and were not reversible as judged by the second criterion. Although we did not investigate this problem in detail, we feel that the slow increase in optical density was probably caused by light scattering resulting from irreversible aggregation. Since the data obtained from these solutions at 0° were internally consistent and what might be expected on the basis of data at higher temperatures which showed no such peculiarities, we feel that the system was actually under thermodynamic control up to the highest pressures attained and that irreversible effects set in only after release of the pressure. After discovering this low-temperature effect, the mode of operation was changed such that data at or near 0°

was obtained only after studies at all other temperatures had been completed for a particular solution.

Treatment of the Data. When protein solutions are subjected to changes in hydrostatic pressure, several factors arise which may lead to changes in the ultraviolet absorption properties of the solutions: (1) Under conditions where two or more conformational states have comparable free energies and differing partial molal volumes and/or compressibilities, the application of pressure will act *directly* to shift the conformational equilibrium toward those states of lowest volume and accordingly will be reflected in spectroscopic parameters. (2) In buffered solutions, the application of pressure may shift the acid–base equilibrium of the buffer. Since most protein conformational reactions are strongly dependent on the pH, this may act to *indirectly* shift the conformational equilibrium toward those states favored by the change in pH and these states will not necessarily be the protein states of lowest volume. (3) Even in the absence of any conformational changes, pressure may cause small changes in the absorption properties of native (or denatured) proteins. For example, it is well known that the energy gap for electronic transitions frequently depends on the polarizability of the suspending medium, which in turn is governed largely by the density for any given solvent. (4) In pressure experiments, a large trivial effect arises for any optical observable which depends on the concentration of solute per unit volume, due to the rather large compressibility of most solvents. In water, the concentration (grams per milliliter, moles per liter, etc.) of chromophores will increase by more than 10% in going from ambient pressure to 50,000 psi, resulting in a large increase in absorption. This effect can be eliminated from the data by expressing results in terms of extinction coefficients rather than optical density or absorbance units, but it still represents a source of experimental error since it means that a large proportion of the total change in signal upon pressurizing must in effect be subtracted off, leaving all of the error in the small signal which remains. Although the primary purpose of this paper is to obtain information on the direct effect of pressure on the conformational equilibrium of ribonuclease, all of the factors above must be properly dealt with in order to obtain a meaningful analysis of the data.

Extinction coefficients for ribonuclease were calculated at 287 nm from the relation

$$\epsilon_{(P,T)} = \frac{OD(P,T)}{LC(P,T)}$$

using optical densities corrected for a water blank in the pressure cell. It was assumed that the effective path length, L, is independent of pressure (and temperature as well) since Young's modulus for Hastelloy C steel (3×10^7 psi) and for sapphire (5×10^7 psi) is very large at pressures below the elastic limit. The protein concentration, C (g/100 ml), is quite dependent on temperature and pressure due to the expansibility and, more importantly, to the compressibility of water. The concentration was determined separately at each temperature and pressure from the known density of water over the range of conditions used in this study.

In the treatment of the data, the assumption will be made that the ribonuclease denaturation is a two-state process under our conditions of acid pH. This assumption has been used previously (Brandts and Hunt, 1967; Hermans and Scheraga, 1961) for the ribonuclease transition. Arguments against the two-state mechanism have been made by Poland and Scheraga (1965). Evidence in favor of the two-state assumption for the thermal transition at acid pH has also been presented (Lumry *et al.*, 1966; Brandts, 1968). Recent studies in our laboratory (unpublished data) indicate that calorimetrically determined ΔH° and ΔC_p values, obtained by a differential heat capacity method, for the ribonuclease denaturation at acid pH agree well with the values determined by a two-state analysis of spectrophotometric data, suggesting that this assumption will not lead us too far astray. However, all of the previous experimental evidence bearing on the two-state character of this denaturation was collected at a pressure of 1 atm. The data in this paper are consistent with a two-state transition at higher pressures but are not accurate enough nor extensive enough to provide a critical test. Therefore, although we will proceed under this assumption, present information does not permit us to completely rule out the possibility that the transition is more complex at higher pressures.

The two-state thermodynamic parameters may be obtained from the following equation, *i.e.*

$$K = e^{-\Delta F^\circ/RT} = \frac{\epsilon(P,T) - \epsilon_N(P,T)}{\epsilon_D(P,T) - \epsilon(P,T)} \qquad (1)$$

where K is the two-state equilibrium constant and ΔF° the free energy of denaturation. In the expression on the right-hand side of eq 1, ϵ is the experimentally determined extinction coefficient in the transition region and ϵ_N and ϵ_D are the extinction coefficients which the native and denatured protein would have under the *same conditions* of temperature and pressure. The latter two quantities are never directly measurable (Brandts, 1968) and must be obtained by an extrapolation procedure to be discussed.

The expressions for the volume change and compressibility change during denaturation may be readily obtained from standard thermodynamics. The change in partial molar volume can be determined from the pressure derivative of the free energy, *i.e.*

$$\left(\frac{\partial \Delta F^\circ}{\partial P}\right)_T = \Delta V = V_D - V_N \qquad (2)$$

where V_N and V_D are the partial molar volumes of the native and denatured forms. The compressibilities are defined as follows

$$\beta_D V_D = -\left(\frac{\partial V_D}{\partial P}\right)_T \qquad (3)$$

$$\beta_N V_N = -\left(\frac{\partial V_N}{\partial P}\right)_T \qquad (4)$$

It will be assumed that the pressure derivatives of volume on the right of eq 3 and 4 are independent of pressure, so that the terms on the left-hand side may be replaced by $\beta_D^0 V_D^0$ and $\beta_N^0 V_N^0$, respectively. The superscripts refer to the value of the corresponding parameters at "zero pressure," which will be virtually identical with the value at 1 atm. It is not known exactly how reliable this approximation might be, but the present experimental accuracy is such that it is pointless to

retain terms which involve the pressure dependency of compressibility effects.

With this approximation, it then follows that

$$V_D = V_D^0 - V_D^0\beta_D^0 P \quad (5)$$

$$V_N = V_N^0 - V_N^0\beta_N^0 P \quad (6)$$

The combination of eq 2, 5, and 6 followed by integration gives

$$\frac{\Delta F^\circ - \Delta F_0^\circ}{P} = V_D^0 - V_N^0 - \left(\frac{V_D^0\beta_D^0}{2} - \frac{V_N^0\beta_N^0}{2}\right)P$$

or

$$\frac{\Delta F^\circ - \Delta F_0^\circ}{P} = \Delta V^0 - \frac{\Delta(V^0\beta^0)}{2}P \quad (7)$$

where $\Delta F^\circ - \Delta F_0^\circ$ is the change in free energy in going from zero pressure to pressure P (other solution variables being held constant), ΔV^0 is the volume change at zero pressure, and $\Delta(V^0\beta^0)$ is the change in the product "volume times compressibility" during denaturation at zero pressure.

Equation 7 does not yield directly the change in compressibility ($\Delta\beta^0$) during denaturation, but only the change in the compressibility–volume product. In order to obtain $\Delta\beta^0$ exactly from this equation, it would be necessary to know the absolute partial molal compressibility of either the native or denatured protein, and these absolute values are not known. However, it can be deduced from the measurements of Fahey *et al.* (1969) of the specific volume of native ribonuclease (pH 9.1) up to pressures of 400 atm that the compressibility of this protein is *less than* 5×10^{-6} atm^{-1}. Considering this and the additional fact that ΔV^0 is always smaller in magnitude than 50 ml mole^{-1} for these studies, it will be true that $\Delta(V^0\beta^0)$ will be equal to $V_N^0\Delta\beta^0$ within a 3% tolerance and this is well within the experimental error. Using this approximation, eq 7 becomes

$$\frac{\Delta F^\circ - \Delta F_0^\circ}{P} = \Delta V^0 - \frac{V_N^0\Delta\beta^0}{2}P \quad (8)$$

A small portion of our data is shown in Figure 1, in the form of extinction coefficient versus pressure at several temperatures for a solution of pH 4.0 (0.04 M sodium acetate buffer). These data are generally typical of other data obtained at pH 4.0, 2.6, (HCl, no salt), and 2.0 (HCl, no salt). The transition region is of course shifted to lower temperatures at the lower pH values. Of the curves shown in Figure 1, only that curve at 51.7° significantly reflects the shift in the denaturation equilibrium brought about by increasing the pressure. The curve at 15° is at low enough temperature so that the native state is stable over the entire pressure range, while the curve at 65° reflects only the behavior of the denatured protein. A number of similar curves have been obtained for both native and denatured ribonuclease at other pH values and at other temperatures. The pressure dependence of ϵ_D has been examined at temperatures ranging from 38.7 (pH 2.0) to 65° (Figure 1). Although the absolute value of ϵ_D does depend on the temperature, as indicated in Figure 1, the pressure dependence of ϵ_D is, within our accuracy, independent of temperature and was always found to *parallel* the curve shown at 65°. Consequently, all that is required to establish the complete pressure behavior of ϵ_D in the transition region is an absolute value of ϵ_D at a single pressure. This can be obtained in either of two ways for the curve at 51.7°, for example. (1) From the shape of the sigmoid curve at 51.7°, the extrapolation to 100% denaturation can be made along the pressure axis. (2) Extrapolations can be made along a temperature axis as well. For example, data are available at pH 4.0 at 65, 60, and 55° (the latter two temperatures not shown in Figure 1). At all three of these temperatures, the denatured form is present to the extent of 100% at *ca.* 50,000 psi. The experimental points at these higher temperatures and 50,000 psi can be extrapolated back to 51.7° to obtain the absolute value of ϵ_D at that temperature and pressure.

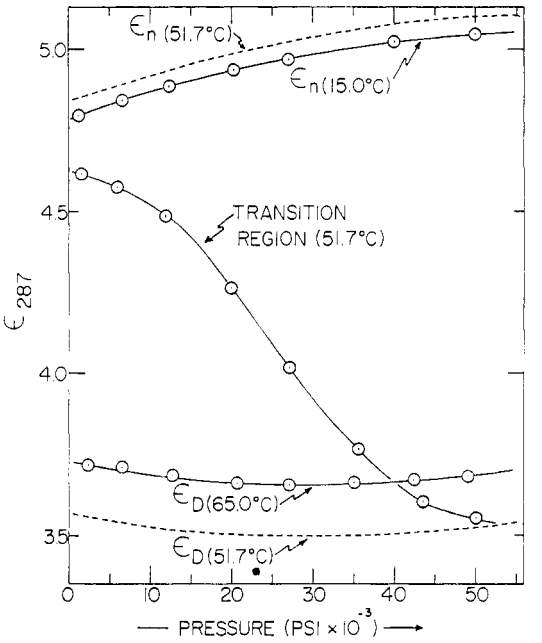

FIGURE 1: Experimental values of the extinction coefficient (287 nm) of ribonuclease A at pH 4.0, 0.04 M acetate buffer. Data points show the behavior of the native protein (15.0°), denatured protein (65.0°), and an equilibrium mixture (51.7°) in the transition region. The dashed curves show the extrapolated extinction coefficients of the native and denatured states in the transition region (51.7°), where they cannot be observed directly (see text).

These two methods show good agreement and the dashed line in Figure 1 shows the ϵ_D curve at 51.7° which is obtained. The values of $(\partial\epsilon_D/\partial T)_P$ obtained by this method are in very good agreement with those measured earlier using conventional cells at 1 atm (Brandts and Hunt, 1967) where experimental accuracy is better.

The determination of ϵ_N values under conditions in the transition region was accomplished in much the same way. In this case, the native protein was examined not only at the pH values indicated above but also at higher pH values where denaturation occurs to a very limited extent even at 30° and 50,000 psi. Over the temperature range 0–30° there was no detectable change in the pressure dependence of ϵ_N and the curves were parallel to that shown at 15° in Figure 1. There is a small temperature dependence of ϵ_N, observed

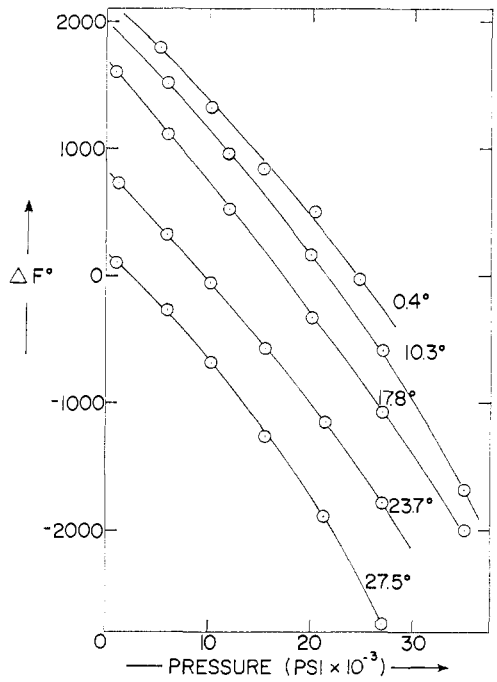

FIGURE 2: Calculated values of the free energy of denaturation (two state) at various temperatures and pressures and at pH 2.0 (HCl).

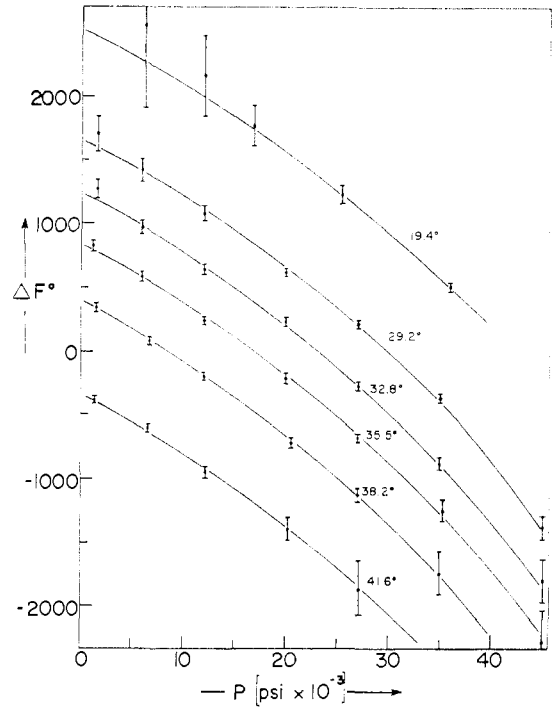

FIGURE 3: Calculated values of the free energy of denaturation (two state) at pH 2.6 (HCl). Approximate errors in free energies are indicated (see text).

previously at 1 atm (Brandts and Hunt, 1967), and this was taken into account in the same way as above. The resulting values of ϵ_N at 51.7° are shown as the dashed line in Figure 1.

With these approximations, equilibrium constants can be calculated from eq 1 at 51.7° and pH 4.0. The procedure was repeated for each temperature, pressure, and pH where thermodynamic parameters were calculated. The free-energy values, $\Delta F°$, which result from this analysis are shown in Figures 2, 3, and 4 for the three pH values where extensive measurements were made. As is to be expected, the scatter of the experimental points from a smooth curve increases as $\Delta F°$ values become larger in magnitude. Since we are interested only in the pressure dependence of free energy, the data are limited primarily by the relative errors in extinction coefficients and not by the absolute errors. If it is assumed that the errors in ϵ, ϵ_N, and ϵ_D are all of equivalent magnitude and equal to $\pm\sigma(\epsilon)$, then the approximate error in $\Delta F°$, $\sigma(\Delta F°)$, will be given by

$$(\Delta F°) = 2RT\frac{\epsilon_D - \epsilon_N}{(\epsilon - \epsilon_N)(\epsilon_D - \epsilon)}\sigma(\epsilon)$$

Using an estimated $\sigma(\epsilon)$ of 0.5×10^{-3}, which is a reasonable but approximate choice, the error bars which result are shown in Figure 3. Similar error estimates would hold for the data of Figures 2 and 4 at equivalent values of $\Delta F°$. The errors in pressure (*ca.* 100 psi) are small and need not be considered.

It should be noted that, in regions where the estimated errors are small, the two-state ΔV values (tangents to the curves in Figures 2, 3, and 4) become increasingly negative with increasing pressure, implying that denatured ribonuclease has a larger compressibility than does native ribonuclease. Within the limitations of the data, this situation prevails at all temperatures, pressures and pH values examined.

If the data are plotted according to eq 8, as in Figure 5, the compressibility effects can be seen more clearly. This type of plot tends to accentuate the errors in $\Delta F°$ so that all of the data are not suitable for this type of analysis. In particular, this method of plotting requires accurate $\Delta F_0°$ values, which can be obtained from extrapolation of the data in Figures 2, 3, and 4 to zero pressure. The curves of Figure 5 show data obtained at several temperatures where the $\Delta F_0°$ value are close to zero. At other temperatures where $\Delta F_0°$ becomes large in magnitude, such plots exhibit more scatter and become virtually incoherent at temperatures much further than about 5° from the transition temperature (1 atm).

The experimental estimates of ΔV^0 and $\Delta\beta^0$ can then be obtained directly from the intercepts and slopes, respectively, of plots similiar to those shown in Figure 5. There is still a problem in comparing directly the estimates of these parameters obtained in the buffered (pH 4.0) as opposed to the unbuffered (pH 2.0 and 2.6) solutions due to the fact that the denaturation of ribonuclease occurs with the uptake of protons. In the buffered solution the equilibrium that is being studied is

$$\nu HAc + N = DH_\nu + \nu Ac^-$$

where ν is the net difference in proton binding between the native (N) and denatured (D) states at the experimental pH. If the denaturation were studied under the same condi-

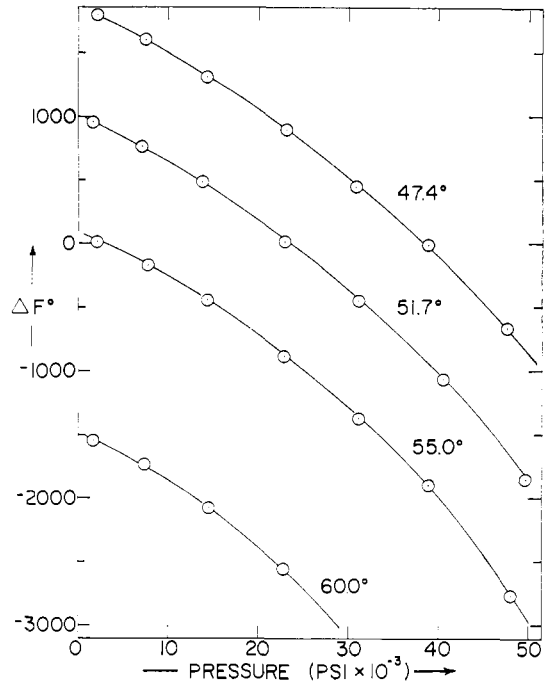

FIGURE 4: Calculated values of the free energy of denaturation (two state) at pH 4.0, 0.04 M acetate buffer.

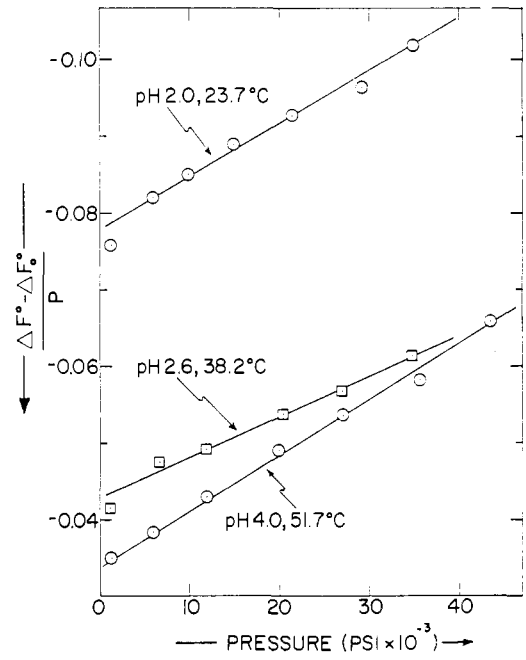

FIGURE 5: Two-state free-energy values for the ribonuclease denaturation plotted according to eq 8, under the indicated conditions of temperature, pressure, and pH.

tions but with no buffer participation, the experimental ΔV^0 values would correspond to a different process, *i.e.*

$$\nu H^+ + N = DH_\nu$$

The difference in ΔV^0 values between the two processes will be equal to ν times the volume change for ionization of the buffer. The volume of ionization of acetic acid is -12 ml/mole and very nearly independent of pressure (Hamann and Strauss, 1955). Assuming a two-state process, ν is given by the equation

$$\nu = \frac{1}{2.3RT}\left(\frac{\partial \Delta F^\circ}{\partial \mathrm{pH}}\right)_{T,P}$$

and the evaluation of ν from unpublished data on ribonuclease denaturation at one atmosphere leads to an estimate of 1.2 at pH 4.0 and 50°. Consequently, if the volume change associated with ionization of the buffer is subtracted out of the experimental ΔV^0 values obtained at pH 4.0, they will become more positive by 14.5 ml/mole of protein and the resulting values can then be compared directly with the experimental estimates obtained in the unbuffered solutions.

Although there is, in principle, also a buffer correction to be applied to the compressibility parameter obtained in the buffered solution, the correction in this case is very small and need not be made.

The final values of ΔV^0 and $\Delta\beta^0$ are shown in Table I, after the indicated corrections have been made at pH 4.0. These volume and compressibility estimates then refer, in all cases, to the changes which occur when the denaturation reaction proceeds under conditions of nearly constant pH since it is known that changes in the pH of HCl solutions (as pressure is applied) are very small (Distéche, 1959; Bates, 1964) and since the pH change in the acetate buffering system has, in effect, been corrected for by subtracting out the volume of ionization, as discussed above.

Discussion

Before proceeding to discuss the thermodynamic results obtained in this study, mention should be made of the behavior of native and denatured ribonuclease under pressure. Native ribonuclease (Figure 1) was found to have a much higher pressure coefficient of absorbance at 287 nm than that exhibited by denatured ribonuclease. This could conceivably be an indication that the conformation of the native protein is pressure dependent to some extent, but it seems possible that there is a simpler explanation. It is known that the π–π^* transitions of certain aromatic chromophores exhibit a rather

TABLE I: Two-State Estimates of Volume and Compressibility Parameters for the Denaturation of Ribonuclease A.

pH	Temp (°C)	ΔV^0 (ml/mole)	$\Delta\beta^0$ (atm^{-1})
2.0	23.7	−46.5	1.5×10^{-6}
2.0	27.5	−43.0	2.0×10^{-6}
2.6	35.5	−27.0	0.9×17^{-6}
2.6	38.2	−27.0	1.0×10^{-6}
2.6	41.6	−20.0	1.6×10^{-6}
4.0	47.4	−7.5	1.1×10^{-6}
4.0	51.7	−6.5	1.3×10^{-6}
4.0	55.1	−4.5	1.5×10^{-6}

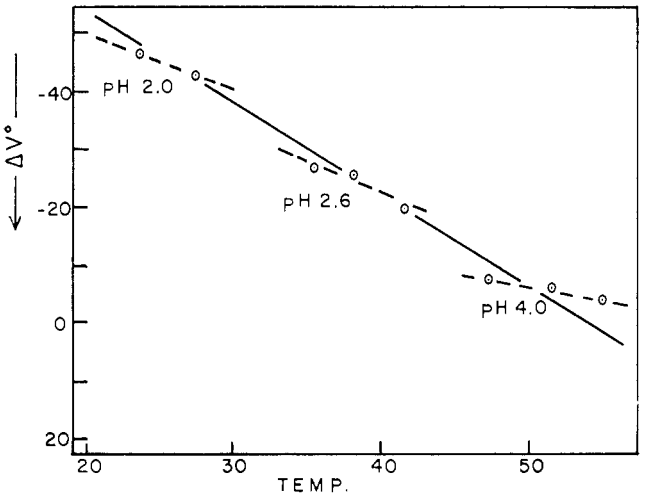

FIGURE 6: Values of ΔV^0 at different pH values, plotted *vs.* the temperatures at which they were determined. See text.

strong dependence on both temperature and pressure. In organic solvents, such chromophores undergo a red shift as the density of the solvent is increased by either lowering the temperature (Robertson, 1960; Tilley, 1967; J. F. Brandts and W. Cane, unpublished observations) or increasing the pressure (Weigang and Robertson, 1963; Robertson *et al.*, 1957; Robertson and King, 1961; Robertson, 1960). In aqueous solvents, similar chromophores undergo a blue shift when solvent density is decreased by lowering the temperature (Tilley, 1967; J. F. Brandts and W. Cane, unpublished observations). In our laboratory, we have found that acetyl-L-tyrosine ethyl ester in water exhibits a positive difference spectrum over the 270–300-nm range (after correction for concentration effects) as the pressure is increased. Thus this model compound behaves qualitatively the same as the tyrosine chromophores in native ribonuclease, so that the behavior observed for this protein may arise from intrinsic effects of pressure on the chromophores rather than from conformational changes as such, although more information is necessary before any conclusions can be made.

In spite of the relatively high accuracy of these experimental data it is still not possible to draw absolutely firm conclusions regarding the pH dependence and temperature dependence of the ΔV^0 and $\Delta\beta^0$ parameters. The ΔV^0 values are plotted in Figure 6, as a function of the temperature at which they were measured. These data suffer from a problem which is common to all studies of denaturation thermodynamics. Denaturation reactions are so cooperative with respect to most intensive variables that accurate thermodynamic parameters can only be obtained over a very limited range of the variable. For example, the ΔV^0 values cover a temperature range of only 5–8° *at any single pH.* In order to extend the temperature range, the pH must be varied and data of the type shown in Figure 6 are then obtained. In this case it is possible to say that, as the transition temperature is shifted to higher temperatures by raising the pH, the values of ΔV^0 become less negative. However, it is not possible to ascertain how much of the change in ΔV^0 is brought about by the higher pH and how much is brought about by the higher temperature at which the data were obtained.

In this study, it has consistently been found that ΔV^0 becomes more positive as the temperature is increased at any constant pH, implying that the coefficient of thermal expansion, α, of denatured ribonuclease is larger than that for native ribonuclease. Nevertheless, because the changes in ΔV^0 over the small temperature span available at constant pH are very small, as seen in Figure 6, experimental uncertainties become important. It was previously suggested by Kauzmann (1959) that denatured proteins may have larger α values than native proteins, an observation consistent with the early data collected by Johnson *et al.* (1954) on partially reversible protein systems which suggest that moderate pressure tends to stabilize most native proteins at high temperature while it has the opposite effect at low temperature. (These data also suffer from the fact that, in order to change the working temperature, a second variable (usually pH) was also changed. In addition, the earlier work was not corrected for the effect of pressure on the buffer systems employed.) Our data under reversible conditions might tend to support this idea, at least for a single protein.

At any rate, the indicated temperature dependence of ΔV^0 at constant pH (the dashed lines in Figure 6) does not appear to be large enough to account for the total change in ΔV^0 which results from simultaneously altering the temperature and pH (solid line in Figure 6). The data then suggest that at least part of the change in ΔV^0 arises from the pH change, so that the derivative $(\partial\Delta V^0/\partial\mathrm{pH})_{T,P}$ is probably positive for ribonuclease in the acid range.

The only conclusion which can be made regarding compressibility effects is that $\Delta\beta^0$ is positive for ribonuclease denaturation and of the order of magnitude $1\text{–}2 \times 10^{-6}$ atm^{-1}. The data in Table 1 appear to show systematic increases in $\Delta\beta^0$ with increasing temperature at all pH values, but the uncertainties in the $\Delta\beta^0$ parameter are even greater than those in ΔV^0 and we would not attempt to attach any significance to these small changes.

Mention should be made of the previous pressure work of Gill and Glogovsky (1965) on the ribonuclease denaturation. These workers estimated two-state volume changes over the pressure range 0–20,000 psi using specific rotation at 589 nm as the physical observable. Their results were not precise enough to observe compressibility effects so that their ΔV estimates correspond to an average pressure of *ca.* 10,000 psi. Their estimate at pH 2.80 is -30 ± 10 ml/mole, which is in satisfactory agreement with our estimates under comparable conditions. Under their conditions (5.5% protein, 0.01 M potassium acid pthalate), however, the buffering capacity of the solution is predominantly due to the protein itself. Since there is proton uptake by ribonuclease during denaturation there will be a tendency for the pH to increase as denaturation progresses by applying pressure. This effect will be partially counterbalanced by the increased acidity of all protein carboxyl groups (and the pthalate buffer) at high pressures. Since they did not "correct" for these effects, their ΔV estimates will contain contributions not contained in our estimates. In addition the two sets of results are not directly comparable because of the 100-fold difference in protein concentration. Although Gill and Glogovsky obtained good reversibility in optical rotation at these high concentrations, we have found less than 100% reversibility (1 atm) in the ribonuclease transition in difference spectra measurements, viscosity measurements, and measurements of calorimetric

heats and heat capacities of denaturation at concentrations in excess of those used in this study (unpublished observations). In view of the uncertainties as to how important these differences in solution conditions are, we would not attach much significance to the favorable comparison of ΔV estimates.

Also, the direct measurements of pycnometric density changes during ribonuclease denaturation (1 atm) by Holcomb and van Holde (1962) were made under conditions identical with those of Gill and Glogovsky. These measurements lead to estimates of ΔV and $\Delta\alpha$ which are much too large in magnitude to be consistent with our estimates from pressure perturbation of a two-state equilibrium. Whether these discrepancies are the result of the lack of validity of the two-state assumption, as suggested by Gill and Glogovsky (1965), or whether they result directly from the differences in solution conditions discussed above, we cannot say with certainty.

Fahey *et al.* (1969) recently attempted to measure directly the apparent specific volume of ribonuclease up to pressures of 400 atm using a magnetic balance. They conclude that, within their experimental errors, there is no change in the specific volume of the protein (pH 2.02, 20°) over the pressure range from 1 to 400 atm. From the scatter of their data at different pressures, it appears that their resolution is about 0.0005 ml g^{-1} or about 5 ml $mole^{-1}$. Using our data (Figure 2), we estimate that the extent of denaturation will change from 10 to 20% over their pressure range so that the change in molar volume which we would expect from our data is about 5 ml $mole^{-1}$ (*i.e.*, *ca.* 10% × ΔV^0). Thus, their data cannot be used to support or refute the quantitative validity of our two-state estimates, although the data of Fahey *et al.* would appear to be inconsistent with ΔV^0 values much larger in magnitude than those reported in this study.

It is virtually impossible to draw any conclusions regarding the more important factors which contribute to the volume and compressibility effects observed for the ribonuclease denaturation. In the case of the volume change, in particular, it is so small (less than 0.5% of the volume of the native protein) at 1 atm that one cannot ascribe it to any particular partial process (hydrogen bond breaking, hydrophobic exposure, electrostatics, etc.) occurring during denaturation. Rather, it seems likely that the volume changes for certain of the partial processes are larger in magnitude than the total ΔV^0 and that the important feature is the nearly complete cancellation of positive and negative contributions from all of the partial processes which occur.

The small volume change associated with the ribonuclease denaturation may be fairly typical of denaturation reactions in general. Densitometric and dilatometric volume changes have been measured for numerous denaturation reactions (see discussion in Kauzmann, 1959) and these show volume decreases of the order of 0.5% of the total molar volume, or less. Studies of the effects of pressure on denaturation reactions (Johnson *et al.*, 1954; Suzuki, 1960, 1963; Tongur, 1952; Curl and Jansen, 1950; Miyagawa and Suzuki, 1963, 1964; Miyagawa *et al.*, 1964; Suzuki and Miyosawa, 1965) indicate equally small volume changes (sometimes positive, sometimes negative, but always small) for many different proteins although, as mentioned, the reversibility in these experiments is frequently not good.

Attempts have been made to estimate the various volume contributions from the partial processes which occur during denaturation (Brandts, 1968; Kauzmann, 1959). Schellman (1955) originally estimated that the interchange of peptide–peptide hydrogen bonds for peptide–water hydrogen bonds should result in a volume *decrease* of *ca.* −2 ml/mole of peptide. Effects from packing efficiency might also tend to lead to volume *decreases* during denaturation. Assarsson and Eirich (1968) have pointed out that the void volume associated with packing large particles together will generally be much larger than for packing large particles in a dilute solution consisting of a solvent of small molecules. They estimate for example that pure dimethylacetamide, when diluted by a continuum solvent, will show a contraction in partial molar volume of 30% (in the limit of infinite dilution) for this effect alone. This process bears some relationship to protein denaturation since, in the native protein, the bulky organic groups must be packed together with little or no solvent penetration into voids while, in the denatured state, a portion of the polypeptide chain unfolds and will, presumably, be packed together with the smaller solvent molecules. These arguments assume of course that there is no change in solvent "structure" when the bulky groups are brought into contact with solvent, so that they would not apply strictly to any real systems.

In support of the qualitative arguments above, Nogochi and Yang (1963) have estimated from dilatometric and refractometric measurements that the helix–coil transition of poly-L-glutamic acid in water, corrected for titration effects, leads to a volume *decrease* of *ca.* −1 ml/mole of peptide groups. This observation could be construed as an indication that the combined effects of hydrogen-bond solvation and packing will give rise to a decrease in volume for unfolding reactions, since hydrophobic effects associated with poly-L-glutamic acid unfolding should not be too significant.

It is likely that there will be moderately large volume contributions to denaturation reactions from electrostrictive effects. Results from Kauzmann's (Rasper and Kauzmann, 1962; Kauzmann *et al.*, 1962) laboratory suggest that the carboxyl groups of several *native* proteins, including ribonuclease, show volume changes during ionization of about −11 ml $mole^{-1}$ of carboxyl groups; this value being in excellent agreement with those exhibited by small carboxylic acids in water. On the other hand, dilatometric titration of ribonuclease and other *native* proteins in the pH 6–10 region suggests that the average molar volume of charged basic side chains is larger, by about 8 ml/mole, than the corresponding model compounds in water. Since there are a total of 19 basic side chains in ribonuclease (all of these will be charged in both native and denatured forms at the acid pH values of interest here) there is a potential *decrease* in volume of *ca.* −150 ml/mole of ribonuclease if one makes the extreme assumption that all basic side chains are normalized during denaturation.

In addition, it is known (Hermans and Scheraga, 1961) that a maximum of one to two carboxyl groups is neutralized during denaturation in the acid range. This should contribute positively to ΔV^0, but the magnitude of the contribution should only be about 10–20 ml/mole of ribonuclease and will depend on pH.

The transfer of hydrophobic solutes from organic solvents to dilute aqueous solution gives rise to a large "contraction" in the volume of the hydrophobic solute (Kauzmann, 1959; Friedman and Scheraga, 1965; Masterton, 1954). A portion of

this contraction may be due to packing problems, discussed earlier with reference to the ideas of Assarsson and Eirich (1968), since these hydrophobic solutes are normally considerably larger than water molecules. It has also been suggested that interstitial solvation and an actual increase in the density of water molecules around the hydrophobic solute may contribute to the reduced volume in the aqueous phase. On the basis of model compound transfer data on pure hydrocarbons, Kauzmann (1959) has estimated that the solvation of hydrophobic bonds in water should lead to a volume *decrease* of about −20 ml/mole of aliphatic side chains (25°) with smaller negative contributions from aromatic side chains. The data of Friedman and Scheraga (1965) on the molar volumes of pure alcohols and alcohols in infinitely dilute aqueous solution indicate somewhat smaller volume effects of about −1 ml/mole of CH_2 groups for transfer into the aqueous phase. Although these quantitative estimates of volume changes for hydrophobic bond solvation do not agree, the qualitative picture is that the exposure of hydrophobic residues to solvent during the denaturation process should contribute negatively to ΔV^0 and the magnitude of the contribution could conceivably be several hundreds of milliliters for denaturation of a protein the size of ribonuclease if the model compound estimates are considered to give a reliable semiquantitative estimate.

This concludes the list of factors normally considered to be important sources of volume change during denaturation. It is apparent that *something is missing* from this list since all of the potentially large sources of volume change are negative if the above estimates are reasonable. Stating the problem somewhat differently, there appears to be no correspondingly large positive contribution to ΔV^0 which is capable of cancelling the expected negative contribution from hydrophobic solvation such that the total volume changes will be close to zero as observed for ribonuclease and other denaturations. This represents a deficiency in our present knowledge of the volumetric properties of proteins, and it is not apparent how to resolve the problem. The difficulty may lie in the estimate of the hydrophobic contribution. These estimates are based on the assumption that solvated hydrophobic residues in the denatured protein are effectively at infinite dilution. If the unfolding process in water is incomplete, as seems likely (Aune *et al.*, 1967), then there may be regions of the denatured protein where there exists high local concentrations of hydrophobic side chains interspersed with a few water molecules. The process of hydrophobic solvation would accordingly not strictly resemble the oil-to-water transfers described by the model compound reactions discussed above but would also contain some characteristics of a water-to-oil transfer process (Brandts and Lumry, 1963) since, in the partially unfolded denatured protein, some water molecules which are normal "bulk" molecules in the native state will find themselves surrounded predominantly by hydrophobic side chains and portions of the polypeptide backbone in the denatured state. Volume changes for the *transfer of water* from the pure state to dilute solution in organic solvents have been measured (Masterton and Seiler, 1968) and vary from + 2 ml/mole of water for transfer to dichlorethane, + 4 ml/mole for transfer to benzene or trichloroethane, to + 14 ml/mole for transfer to carbon tetrachloride. If this type of "solvation" does take place in denatured proteins it may tend to negate part of the contraction associated with the oil-to-water transfers, although there is absolutely no substantial evidence to support its occurrence.

The changes in compressibility upon denaturation are equally difficult to interpret. Resorting again to evidence from model compound studies, there is a clear suggestion that the partial molal compressibilities of hydrophobic solutes in dilute aqueous solution are smaller than their compressibilities in other solvent systems. Corroboration of this statement comes from several sources. Basset and Dole (1936) have examined the solubility of the hydrophobic N_2 molecule (liquid state) in water at various pressures up to about 4500 atm. They observed that the solubility passed through a maximum at about 3000 atm, implying that N_2 has a smaller volume (at low pressures) and a *smaller compressibility* when dissolved in water than it has in the pure liquid state. Phase diagrams for systems such as nicotine–water and 4-methylpiperidine–water also suggest that these hydrophobic solutes have a considerably smaller partial molal compressibility in dilute aqueous solution than in the pure liquid state (Schneider, 1963). Alexander and Hill (1965) have shown that there is a negative excess compressibility associated with aqueous propanol solutions below room temperature, although it apparently changes sign at higher temperatures. F. Franks and H. T. Smith (to be published) have suggested that the negative excess compressibility arises because the normally vulnerable structure of water is protected against the effect of pressure by the hydrophobic solute. Finally, the variation in critical micelle concentration with pressure for a number of long-chain aliphatics (Tuddenham and Alexander, 1962; Hamann, 1962) is consistent with the idea that the fully solvated monomers have a smaller compressibility than do the micellar species.

It seems then that the increase in compressibility observed for the ribonuclease denaturation is in the *opposite direction* to the expected contribution arising from exposure of hydrophobic side chains if one is willing to accept the usual analogy with model compound transfer reactions. Older data on other denaturation reactions seems to reinforce the generality of this conclusion, since it has been observed for several proteins (see Johnson *et al.*, 1954) that, at temperatures in excess of 50°, the application of moderate pressure retards both the rate and extent of denaturation while the application of higher pressures greatly accelerates the ease of denaturation; an observation consistent with an increase in compressibility upon denaturation.

The only conclusion of an interpretive nature which can be made from this study is a negative one. *The volume and compressibility changes during denaturation are not completely dominated by the "hydrophobic effects" which are described by the usual model compound transfer reactions.* This may mean that other partial processes contribute in a more important way than do the hydrophobic processes to the volume and compressibility changes. It could also mean that the model compound transfer reactions are, for one reason or another, poor systems from which to estimate the hydrophobic effects for denaturation reactions, at least with respect to volume and compressibility changes.

Under certain circumstances it appears that protein reactions involving hydrophobic bonding do give rise to large volume changes. The aggregation of poly-L-valyl ribonuclease (Kettman *et al.*, 1966) has the thermodynamic characteristics of a reaction controlled by hydrophobic interactions. The

rate of aggregation has been studied under pressure and it was found that the volume of activation for the aggregation process was very large (260 ml/mole at moderate ionic strength) and Kettman *et al.* have suggested that the major factor which causes the large volume change is the reduction in oily side-chain–water contacts during aggregation. If this is true, it is all the more puzzling why large volume contractions are not frequently observed for denaturation reactions.

Since preparation of this manuscript, the recent work of Kliman (1969), carried out in the laboratory of W. Kauzmann, has come to our attention. He has measured the solubility of the hydrophobic solute 4-octanone over a large pressure range up to 5000 atm. The comparison of data on the model solute with existing data on proteins has led him to conclusions similar to those stated in this manuscript. Kliman concludes that "We seem to be faced with two choices. Either the model compounds used are not indicative of the forces that control the overall structures of protein molecules or the portions of the protein molecule the models represent do behave as predicted, but other forces acting in different portions of the protein molecule determine the observed behavior. . . . There seems to be serious deficiencies in the models generally used for describing hydrophobic interactions in proteins or in the notion that these interactions are the major source of stability of the native forms of proteins."

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