AN UNUSUAL PRESSURE DEPENDENCE FOR A REVERSIBLY ASSOCIATING PROTEIN SYSTEM; SEDIMENTATION STUDIES ON MYOSIN*

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The association of myosin molecules at low ionic strength to form long, filamentous macrostructures is well known and has been investigated in a number of laboratories.[1-8] In general, these particles have many structural features in common with those of the native thick filaments of muscle, but show a size distribution dependent on the ionic strength and other conditions prevailing during formation. Recent studies[7,8] have shown that in the pH range 8 to 8.5 (KCl = 0.10 to 0.20 *M*), the polymeric species formed has an unusually sharp size distribution. Only two sedimenting boundaries are observed in the ultracentrifuge: a hypersharp polymer peak with $s^0_{20,w}$ = 150*S* and a slower sedimenting monomer peak with $s^0_{20,w}$ = 6.5*S*. Detailed sedimentation studies reveal that the monomer is in rapid equilibrium with the high-molecular-weight polymeric species and that the equilibrium constant is critically dependent on the ionic strength and pH.[8] In the present communication we wish to present evidence that, in contrast to other rapidly associating protein systems which have been studied heretofore,[9] the equilibrium constant of the myosin monomer-polymer system shows a striking dependence on hydrostatic pressure.[10]

The effect of pressure on the sedimentation behavior of chemically reacting systems has been described recently by Kegeles, Rhodes, and Bethune[11] and by Ten-Eyck and Kauzmann.[12] The general effect of pressure on proteins has been reviewed by Johnson, Eyring, and Polissar[13] and by Kalckar.[14]

Results.—Figure 1*a* is a low-speed velocity sedimentation profile showing finite concentration gradients only at the monomer and polymer boundaries. At this low speed, the experimentally observed distribution of mass in the ultracentrifuge cell accords with that predicted by Gilbert for a rapidly reversible monomer→polymer equilibrium.[15,16] The unusual feature of a flat base line between the monomer and polymer boundaries is a consequence of the unusually large equilibrium constant which has been reported for this system ($K = 10^{30}$).[8,17] Figures 1*b*, *c*, and *d* present schlieren patterns of identical protein solutions run at three higher rotor velocities. The salient feature of these patterns is the marked elevation of the base line both between the monomer and polymer boundaries and in the region centrifugal to the polymer boundary. The elevation of the base line is seen to increase with the rotor velocity, suggesting that the monomer-polymer equilibrium is altered as a result of the increasing hydrostatic pressure gradient established throughout the liquid column. That this is indeed the case is demonstrated by the experiment summarized in Figure 2.

In this study increasing amounts of mineral oil (of density 0.85 gm/ml) were layered over myosin-polymer solutions (the total protein concentration in each case was 0.66%) of identical column height, and each of these preparations was centrifuged at 40,000 rpm for 75 minutes to resolve the monomer and polymer

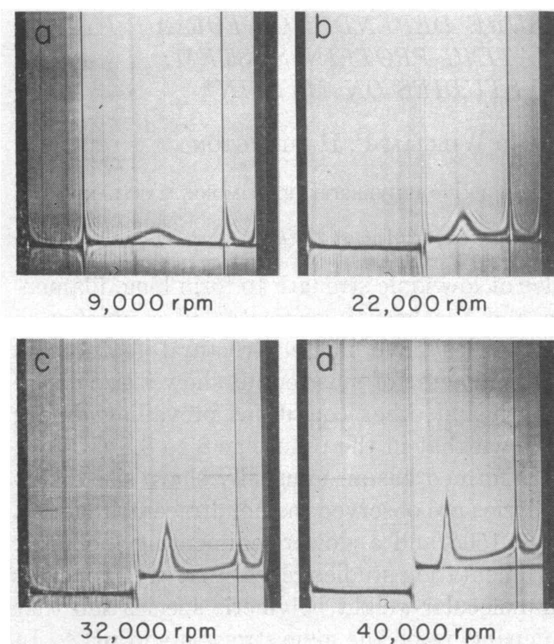

FIG. 1.—Effect of rotor velocity on myosin-polymer equilibrium.

(*a*) Total protein concentration, 0.4%. Time of centrifugation, 18 hr. The broadness of the monomer peak is due to diffusion. This experiment was carried out in a modified capillary-type double-sector synthetic boundary cell. The lower (centrifugal) capillary was 10.5 mm from the base of the cell instead of the usual 5.5 mm.

(*b*) Total protein concentration, 0.6%. Time of ultracentrifugation, 5 hr.

(*c*) Total protein concentration, 0.6%. Time of ultracentrifugation, 1.5 hr.

(*d*) Total concentration, 0.6%. Time of ultracentrifugation, 1 hr.

Aluminum double-sector cells coated with a thin (1/32 inch) layer of Kel-F were used. Each polymer solution was exhaustively dialyzed against a common buffered solvent of 0.18 *M* KCl, 2×10^{-3} veronal, pH 8.3, as described previously.[8] The rotor velocity is indicated in the figure. Temperature was 5°C.

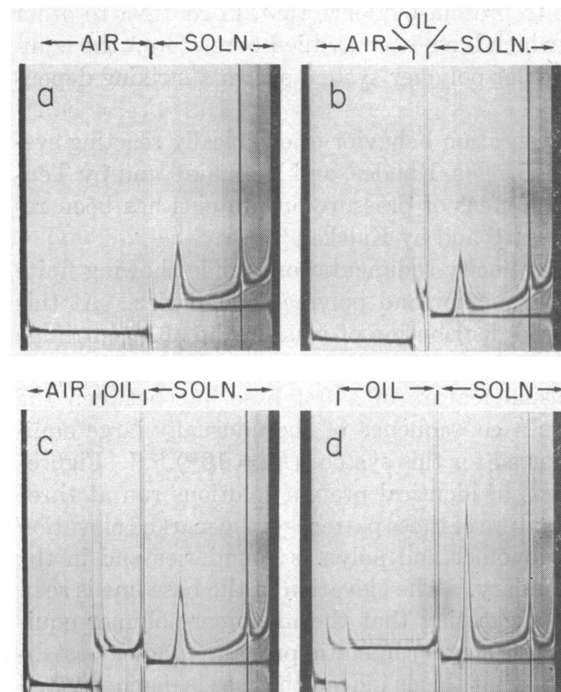

FIG. 2.—The effect of hydrostatic pressure on the monomer-polymer equilibrium at constant rotor velocity. Aluminum, double-sector cells coated with Kel-F (1/32 inch) were used. Rotor velocity, 40,000 rpm; temp, 5°C. Varying amounts of mineral oil (density = 0.85 gm/cc), previously equilibrated with dialysate, were added to aliquots of 0.66% myosin solution which had been dialyzed against 100 vol of 0.185 *M* KCl, 2×10^{-3} *M* veronal, pH 8.3, as previously described.[8] The lower (centrifugal) meniscus at the oil-solution interface is that of the protein solution, and the upper (centripetal) oil-air meniscus corresponds to the protein sector. The time of centrifugation for each frame was 75 min.

boundaries. Thus the effect of pressure on the myosin-polymer equilibrium is established before separation of the species occurs during centrifugation. The areas of the monomer schlieren peaks, extrapolated to the solvent base line, were measured by planimetry and converted to concentration units, taking into account

sectorial dilution by the usual procedure. As a result of the large equilibrium constant and the large value of n (the average number of myosin monomer units in the polymer), these areas give a very close approximation of the monomer concentration at the beginning of each experiment. The monomer concentrations thus derived from frames *a*, *b*, *c*, and *d* of Figure 2 were 0.11, 0.12, 0.15, and 0.26 gm/100 ml, respectively. It will also be noted (Fig. 2) that the elevation in base line between monomer and polymer boundaries increases with increasing pressure.

The two experiments presented above support the view that the monomer-polymer equilibrium is a function of hydrostatic pressure and that an increase in pressure shifts the equilibrium toward increased formation of monomer. In accordance with this thesis, concentration gradients (dc/dx) of both the polymer and monomer should exist throughout the liquid column as soon as the rotor is brought to speed and before any significant mass transport occurs. Thus, the monomer concentration should increase with increasing depth of the liquid column, while the polymer concentration should decrease.[18] At each level of the liquid column the concentrations of monomer and polymer adjust to satisfy the value of the equilibrium constant at that level. But, before any mass transport occurs (i.e., at "zero" time) the total protein concentration remains constant with respect to radial distance, and therefore this phenomenon cannot be detected by the schlieren or interference optical systems (assuming the refractive index increment (dn/dc) is identical for both monomer and polymer species). However, since the polymer (mol wt $= 50 \times 10^6$)[8] would be expected to scatter light far more strongly than the monomer, variations in the concentration of this species can be monitored by measuring changes in the optical density of the solution throughout the liquid column. Although this effect is hardly detectable at the normal wavelength of light used for the schlieren optical system (546 mμ), at lower wavelengths, as a result of the inverse fourth power dependence of light-scattering on wavelength, the effect is easily seen. Figure 3 shows the optical density at 365 mμ as a function of radial distance at four different rotor velocities obtained from schlieren photographs immediately after reaching speed. Thus it is clear from this experiment that the polymer concentration decreases continuously with increasing depth of the liquid column.

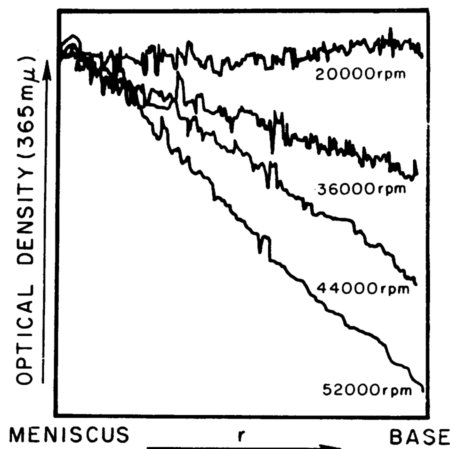

FIG. 3.—The effect of rotor velocity on the radial optical density profile. Protein concentration 1.0% in 0.178 M KCl, veronal 2×10^{-3} M, pH 8.3, 30 mm Kel-F single-sector cell. Rotor velocity as indicated in figure. Temp, 5°C. Identical results were obtained irrespective of whether the rotor velocity was raised from 20,000 to 52,000 rpm or first increased to 52,000 rpm and then decreased from 52,000 rpm to 20,000 rpm. Optical density is in arbitrary units and was obtained from a microdensitometer tracing of schlieren photographs taken with ultraviolet (365 mμ) light. At 52,000 rpm the extinction change across the photographic plate ($x_0 \rightarrow x_b$) is about 0.9 OD units.

In view of the dependence of the monomer-polymer equilibrium on the ionic strength and pH, a possible complication in the interpretation of Figures 2 and 3 is introduced if salt and pH gradients exist within the liquid column. In the case of the experiments summarized in Figure 2, separate sedimentation runs of the solvent against water demonstrated a concentration change (as measured by the interference optical system of the ultracentrifuge) of only 0.01 *M* between the meniscus and base of the liquid column over the short time period (75 min) used for this study. This gradient is far too small to account for the change in monomer concentration observed. Moreover, since the experimental conditions (rotor velocity, column height, temperature, time of centrifugation) were the same for each experiment (only the thickness of the oil layer changed), the salt gradient should be identical in each experiment. The pH gradient expected from the variation in hydrostatic pressure throughout the cell, 0.03 pH units,[19] is negligibly small. In Figure 3, the data were taken immediately after reaching the designated rotor velocity and long before any significant distribution of salt could occur.

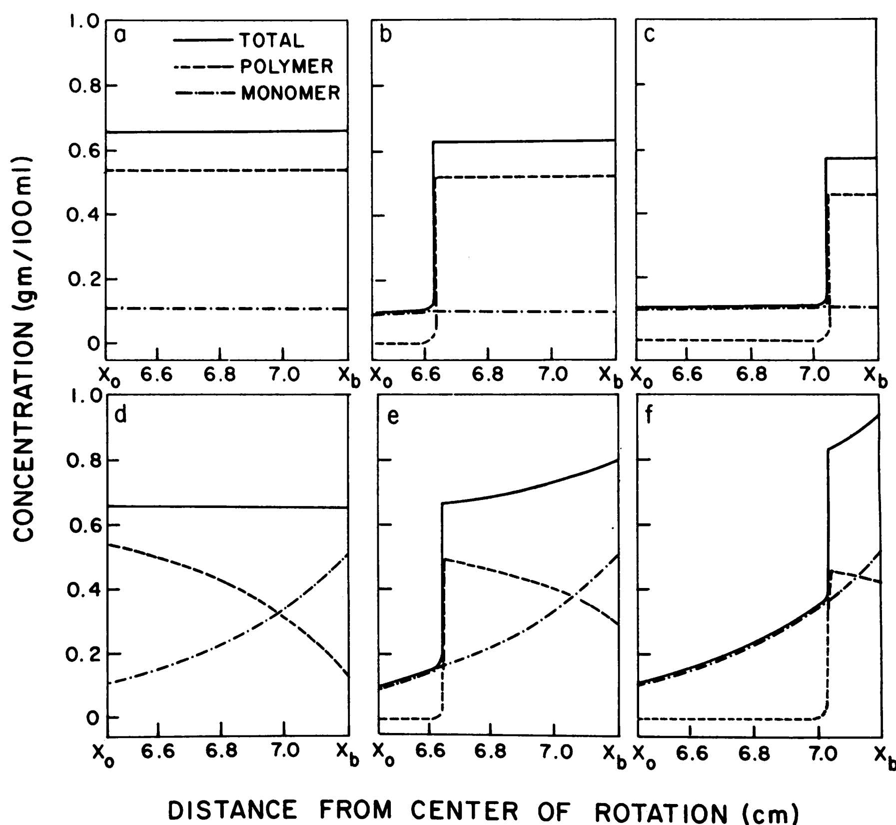

FIG. 4.—Concentration profiles (c vs. x) of the myosin polymer system. Curves were calculated for three radial positions of the polymer boundary (6.44, 6.64, and 7.04 cm) and incorporate radial dilution of the polymer. The monomer boundary was held fixed at the meniscus. Frames *a*, *b*, and *c* were calculated from Gilbert's equations.[15, 16] Frames *d*, *e*, and *f* describe the analogous concentration profiles (c vs. x) for the pressure-dependent polymerization equilibrium. The curves were plotted from data in Table 1.

Discussion.—The effect of pressure on the sedimentation patterns of the myosin-polymer system may be best understood by first considering the mass distribution in a centrifuge cell during a sedimentation velocity experiment in which the equilibrium between monomer and polymer is unaffected by pressure. Figure 4 (*a*, *b*, *c*) depicts plots of concentration versus distance expected for a monomer-polymer system at various positions of the polymer boundary as derived from Gilbert's equations.[15, 16] By virtue of the large difference between the sedimentation coefficient of the polymer and that of monomer, we can closely simulate the sedimentation experiment by holding the monomer boundary at the meniscus while sedimenting the polymer boundary through the cell. These plots show that neither the monomer concentration nor the polymer concentration changes significantly across the region between the two boundaries, in contrast to the usual situation observed for polymerizing systems. This feature is characteristic of equilibrium systems in which the polymer is formed by association of a large number of monomeric units (in the present case $n = 80$ to 90) and has been discussed previously.[8]

We now introduce a pressure dependence such that the monomeric species is favored by an increase in pressure. The explicit form of the pressure dependence of the equilibrium constant comes directly from a modified form of the second law which includes the potential energy of the centrifugal field and is simply

$$\ln K\,(x) = \ln K_0 - \frac{1}{RT}\int_{x=x_0}^{x=x} \Delta V \left(\frac{\partial P}{\partial x}\right) dx, \qquad (1)$$

where $K(x)$ is the equilibrium constant at any point x in the liquid column and K_0 is the equilibrium constant at the meniscus position x_0. The change in molar volume upon forming one mole of polymer from n moles of monomer is ΔV, and P, R, and T are the pressure, gas constant, and temperature, respectively.

This equation does not require that the molar volumes of the constituent species be independent of pressure. Due to the paucity of published data on the pressure dependence of interacting protein systems we cannot be certain that the molar volumes of proteins will not exhibit a small degree of pressure dependence.[20] Indirect evidence that the molar volumes of proteins are in fact pressure-dependent may be found in several known examples of pressure denaturation.[21–26] As a first approximation, however, we consider the volume for each species to be independent of pressure. Assuming solution incompressibility and making the substitution

$$\frac{\partial P}{\partial x} = \rho\omega^2 x \qquad (2)$$

where ρ is the solution density and ω the rotor velocity, equation (1) can be integrated to give

$$\ln K(x) = \ln K_0 - \left(\frac{\Delta V}{RT}\right)\left(\frac{\rho\omega^2}{2}\right)(x^2 - x_0^2). \qquad (3)$$

Using equation (3) the equilibrium constant has been estimated at different levels within the liquid column taking the change in partial specific volume upon polymerization, $\Delta\bar{v} = 6 \times 10^{-4}$ cc/gm (see below) and a rotor velocity of 40,000

TABLE 1

THE EFFECT OF PRESSURE ON THE EQUILIBRIUM CONSTANT FOR THE POLYMERIZATION OF MYOSIN

Radius (cm)	Pressure (atm)	$\log_{10} K$	Monomer (gm/100 ml)	Polymer (gm/100 ml)
6.44	0	78.0	0.11	0.55
6.54	11.2	71.2	0.14	0.52
6.64	22.6	64.2	0.17	0.49
6.74	34.2	57.2	0.20	0.46
6.84	45.9	50.1	0.25	0.41
6.94	57.9	42.8	0.30	0.36
7.04	70.0	35.5	0.37	0.29
7.14	82.2	28.0	0.46	0.20

Results give the distribution of reacting species at 40,000 rpm before mass transport begins. Pertinent parameters are $n = 83$, $x_0 = 6.44$ cm, $\Delta\bar{v} = 6.378 \times 10^{-4}$ cc/gm (corresponding to a vol change of 384 cc per monomer), $\log K_0 = 78.0$, total protein concentration = 0.66 gm/100 ml, temp = 5°C.

rpm. The concentrations of the constituent monomer and polymer species have thus been evaluated at each level in the liquid column and are presented in Table 1. As expected, the monomer concentration increases with increasing depth of the liquid column, while the polymer concentration decreases. This situation is depicted in Figure 4*d* and is confirmed experimentally in Figure 3.

Because n is very large (80 to 90) the monomer concentration is nearly independent of the polymer concentration. Consequently, sedimentation of the polymer boundary does not result in any significant alteration of the shape of the concentration profile (c vs. x) of either the monomer or polymer.

In Figure 4*e* and *f* the progress of the sedimentation velocity experiment is simulated for two different radial positions of the polymer boundary in a manner similar to that of frames *b* and *c*. As a result of the invarient shape of the monomer and polymer concentration profiles and the fixed position of the monomer boundary, the total protein concentration (monomer + polymer) will always be increasing radially as the polymer sediments into regions of ever-increasing monomer concentration. Positive concentration gradients should therefore be observed on both sides of the polymer boundary. This prediction has experimental confirmation in Figures 1 and 2.

Because of the positive gradients existing in the cell we do not expect this system to exhibit convection. We note, then, that the conditions leading to convection-free sedimentation in a pressure-dependent associating system are (1) that pressure favor formation of the more slowly sedimenting species, (2) that the polymer have a sedimentation coefficient much greater than that of the monomer, and (3) that the polymer be composed of a large number of monomeric units. If any of these conditions are not fulfilled, then convection may occur. For instance, calculations of Kegeles *et al.*[11] show that for a monomer-dimer equilibrium, convection will always take place irrespective of which species, dimer or monomer, is favored by increasing pressure. It is also clear for the case at hand that had the polymer rather than the monomer been favored by increased pressure, then convection would have occurred.

In the studies summarized in Figures 1–4, the rotor velocity was invariant throughout each experiment. However, a rapid increase in rotor velocity during the run may be expected to cause a rapid shift in the equilibrium and a correspondingly abrupt change in the sedimentation profile. That is, the rapid increase in

hydrostatic pressure within the liquid column will result in a readjustment of the concentrations of the two species. This process leads to a splitting of the hypersharp polymer boundary and results in the appearance of a slower sedimenting monomeric myosin boundary (a differential boundary) on the centripetal side of the polymeric peak. Schlieren patterns demonstrating this phenomenon have been presented in our earlier report (Fig. 12 of ref. 8).

Several laboratories have demonstrated that the contractile process in muscle fiber is markedly affected by the application of pressures comparable to those generated in high-speed ultracentrifugation.[27-31] The present findings demonstrating the striking instability of synthetic myosin filaments to such pressures may have relevance to these effects.

Conclusion.—From Rayleigh interference patterns of sedimentation velocity experiments similar to those present in Figures 1 and 2, we have evaluated the magnitude of the difference between the partial specific volumes of the monomer and polymer ($\Delta\bar{v}$) from plots of log K versus P. Details of this calculation will be presented in a later communication; however, we feel that the principal finding is of sufficient interest to warrant its presentation at the present time. From some 23 independent determinations, the difference between the partial specific volumes of the monomer and polymer is $6 \pm 1.2 \times 10^{-4}$ cc/gm, corresponding to a molar volume change (monomer $\rightarrow$ polymer) of 384 cc per monomer unit. Such small changes in the value of $\bar{v}$ lie below the range of routine experimental detection. At the present time the accuracy for routine partial specific volume determinations lies in the range of 0.01 cc/gm, which corresponds to a volume change of 1,000 cc per mole for a protein of molecular weight 100,000. Since the change in molar volume which can give rise to significant pressure dependence lies well below this figure, conclusions derived from ultracentrifuge studies of rapidly equilibrating interacting systems may require re-evaluation.

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[1] Jakus, M. A., and C. E. Hall, *J. Biol. Chem.*, **167**, 705 (1947).
[2] Noda, H., and S. Ebashi, *Biochem. Biophys. Acta*, **41**, 386 (1960).
[3] Zobel, C. R., and F. D. Carlson, *J. Mol. Biol.*, **7**, 78 (1963).
[4] Huxley, H. E., *J. Mol. Biol.*, **7**, 281 (1963).
[5] Josephs, R., Ph.D. dissertation, The Johns Hopkins University, Baltimore, Maryland (1966).
[6] Kaminer, B., and A. L. Bell, *Science*, **152**, 323 (1966).
[7] Kaminer, B., and A. L. Bell, *J. Mol. Biol.*, **20**, 391 (1966).
[8] Josephs, R., and W. F. Harrington, *Biochemistry*, **5**, 3474 (1966).
[9] Nichol, L. W., J. L. Bethune, G. Kegeles, and E. L. Hess, *Proteins*, **2**, 305 (1964).
[10] Josephs, R., and W. F. Harrington, *Federation Proc.*, **26**, 728 (1967).
[11] Kegeles, G., L. Rhodes, and J. L. Bethune, these PROCEEDINGS, **58**, 45 (1967).
[12] TenEyck, L. F., and W. Kauzmann, these PROCEEDINGS, **58**, 888 (1967).
[13] Johnson, F. H., H. Eyring, and M. J. Polissar, *The Kinetic Basis of Molecular Biology* (New York: Wiley, 1954), chaps. 9 and 10.
[14] Kalckar, H. M., in *Horizons in Biochemistry*, ed. M. Kasha and B. Pullman (New York: Academic Press, 1962), p. 513.
[15] Gilbert, G. A., *Discussions Faraday Soc.*, **20**, 68 (1955).

[16] Gilbert, G. A., *Proc. Roy. Soc.* (*London*), **A250,** 377 (1959).

[17] Previously reported values of K were in the range of 10^{480} to 10^{550}, and were expressed in terms of molar concentration units. In the present communication the more common convention of weight concentrations (gm/100 ml) was employed.

[18] Since the monomer is favored at higher pressures, its density must be greater than that of the polymer. The resulting small, but positive, density gradient acts to stabilize this system in the centrifugal field.

[19] Bates, R. G., *Determination of pH* (New York: Wiley, 1964), p. 117.

[20] According to the calculations of Kegeles *et al.*,[11] a protein system in which the interacting species has a mol wt 100,000 need undergo less than a 1% change in molar volume in order to effect a change of several orders of magnitude in the equilibrium constant.

[21] Ikkar, T., and T. Ooi, *Biochemistry,* **5,** 1551 (1966).

[22] Gill, S. J., and R. L. Glogovsky, *J. Phys. Chem.,* **69,** 1515 (1965).

[23] Kettman, M. S., A. H. Nishikawa, R. Y. Morita, and R. R. Becker, *Biochim. Biophys. Res. Commun.,* **22,** 262 (1966).

[24] Miyagawa, K., *Arch. Biochim. Biophys.,* **110,** 381 (1965).

[25] Hughes, F., and R. Steiner, *Biopolymers,* **4,** 1081 (1966). This article deals with pressure effects on nucleic acid denaturation.

[26] Cassel, J. M., and R. G. Christensen, *Biopolymers,* **5,** 403 (1967).

[27] Brown, D. E. S., *J. Cellular Comp. Physiol.,* **4,** 257 (1934).

[28] Cattell, M., *J. Cellular Comp. Physiol.,* **6,** 277 (1935).

[29] Brown, D. E. S., *J. Cellular Comp. Physiol.,* **8,** 141 (1936).

[30] Marsland, D. A., and D. E. S. Brown, *J. Cellular Comp. Physiol.,* **20,** 295 (1942).

[31] Hamann, S. D., *Australian J. Chem.,* **7,** 121 (1954).