Comparison of Protein Structure in Crystals, in Lyophilized State, and in Solution by Laser Raman Scattering. III.[1] α-Lactalbumin

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Abstract: Possible conformational changes of α-lactalbumin associated with lyophilization and crystallization were investigated in detail by Raman scattering technique It was found that lyophilization altered the protein three-dimensional structure considerably, but crystallization produced *no* detectable effect on backbone conformation. This conclusion is the same as that reached earlier for lysozyme. In the amide III region (both crystals and solution spectra) three well-defined peaks were observed at 1274, 1260, and 1238 cm^{-1} very similar to those of lysozyme at 1272, 1258, and 1238 cm^{-1}, respectively, indicating that α-lactalbumin may have a conformation similar to that of hen's egg-white lysozyme. This is consistent with the "analogy" molecular model proposed by Browne, *et al.* To demonstrate the sensitivity of the amide III contour with respect to change in α-lactalbumin backbone conformation, Raman spectra of aqueous solution at pH 6.6 and 2.1 were compared. Spectral evidence was presented that the distinct feature of an extremely sharp peak at 1361 cm^{-1} in both α-lactalbumin and lysozyme was related to some specific interactions involving those "buried" indole rings.

One of the two protein components of lactose synthetase, α-lactalbumin, has a covalent structure very similar to that of egg-white lysozyme.[2] When the sequences of the two proteins are compared, at least 42 amino acid residues are identical at corresponding positions and an additional 27 residues are chemically similar. From these observations Brew, *et al.*,[2a] concluded that the genes for α-lactalbumin and egg-white lysozyme were derived from a common ancestor. Because of this similarity in sequence, Browne, *et al.*,[3] have constructed an "analogy" molecular model for α-lactalbumin on the basis of the main chain conformation established by X-ray diffraction for lysozyme. This predicted three-dimensional structure of α-lactalbumin has been tested against various studies of the physical and chemical properties of the protein in solution.[4-7] Comparisons of the circular dichroism (CD)[6] and optical rotatory dispersion (ORD)[4] curves of α-lactalbumin and lysozyme indicated that the two molecules have similar conformation. Solvent perturbation[6,7] and hydrogen ion titration studies[5] revealed that two of the four tryptophan residues were "buried" and two were "exposed" at 25° and that the four tyrosyl residues are located near the surfaces. These are all consistent with the model of Browne, *et al.*[3]

Despite the similarities in sequence and conformation, an apparent distinction between the two molecules is the large difference in the overall charge of the molecules. α-Lactalbumin has an isoionic point of about pH 5 and lysozyme one of pH 10.5.[2a] Moreover, in contrast with α-lactalbumin, lysozyme shows a tendency to resist unfolding.[8,9] The conformation of lysozyme has been shown to be unaffected by a reduction in pH at room temperature. The pH value can be reduced to below pH 2.0 without any sign of conformation change.[9] These facts are consistent with the studies of lysozyme solution at pH 2.0 by Raman effect,[10] which showed that no significant spectral changes in the amide III backbone region (1220–1300 cm^{-1}) had resulted in going from pH 5.2 to 2.0.

In this paper, we wish to show that the amide III frequencies and contour in the Raman spectra of α-lactalbumin undergo drastic changes upon reduction in pH value. This should provide some indication of how sensitive this spectral region is with respect to change in backbone conformation. We will then proceed to compare the Raman spectra of the protein in crystals, in the lyophilized state, and in solution to determine whether there exist some conformational differences (both main chain and side chain) between different physical states. This is a continuation of our recent Raman studies on protein crystals and solutions. Previous work on lysozyme[11] has shown that the main chain conformation is the same in the crystal and in solution. This is not surprising and should be expected in view of the fact that lysozyme has a high resistance to unfolding by pH[9,10] and by heat.[12] In the case of carboxypeptidase A, a protein twice as large as lysozyme in molecular weight and containing only one disulfide bond, the amide III contour was somewhat altered upon crystallization, indicating conformational change.[13] More recently, we investigated insulin single crystals by Raman scattering[14] and found that the Raman amide III contour in the spectra of crystals was different from that of solutions at acidic pH and pH 8. Before any general-

(1) Papers I and II in this series are ref 10 and 13.
(2) (a) K. Brew, J. T. Canaman, and R. L. Hill, *J. Biol. Chem.*, **242**, 3747 (1967); (b) F. J. Castellino and R. L. Hill, *ibid.*, **245**, 417 (1970).
(3) W. J. Browne, A. C. T. North, D. C. Phillips, K. Brew, T. C. Vanaman, and R. L. Hill, *J. Mol. Biol.*, **42**, 65 (1969).
(4) A. Aune, Ph.D. Thesis, Duke University, Durham, N. C., 1968.
(5) F. M. Robbins, R. E. Andreotti, L. G. Holmes, and M. J. Kronman, *Biochim. Biophys. Acta*, **133**, 33 (1967).
(6) M. J. Kronman, *Biochem. Biophys. Res. Commun.*, **33**, 535 (1968).
(7) M. J. Kronman, W. B. Hoffman, J. Jeroszko, and G. W. Sage, *Biochim. Biophys. Acta.*, **285**, 124 (1972).
(8) M. Z. Atassi, A. F. S. A. Habeeb, and L. Rydstedt, *Biochim. Biophys. Acta*, 200, 184 (1970).
(9) C. Tanfold, *Advan. Protein Chem.*, **23**, 121–282 (1968).
(10) N. T. Yu, unpublished results; see also Ph.D. Thesis, Massachusetts Institute of Technology, 1969.
(11) N. T. Yu and B. H. Jo, *Arch. Biochem. Biophys.*, **156**, 469 (1973).
(12) M. C. Chen, R. C. Lord, and R. Mendelsohn, *Biochim. Biophys. Acta*, **328**, 252 (1973).
(13) N. T. Yu and B. H. Jo, *J. Amer. Chem. Soc.*, **95**, 5033 (1973).
(14) N. Y. Yu, B. H. Jo, Robert C. C. Chang, and J. D. Huber, *Arch. Biochem. Biophys.*, **160**, 614 (1974).

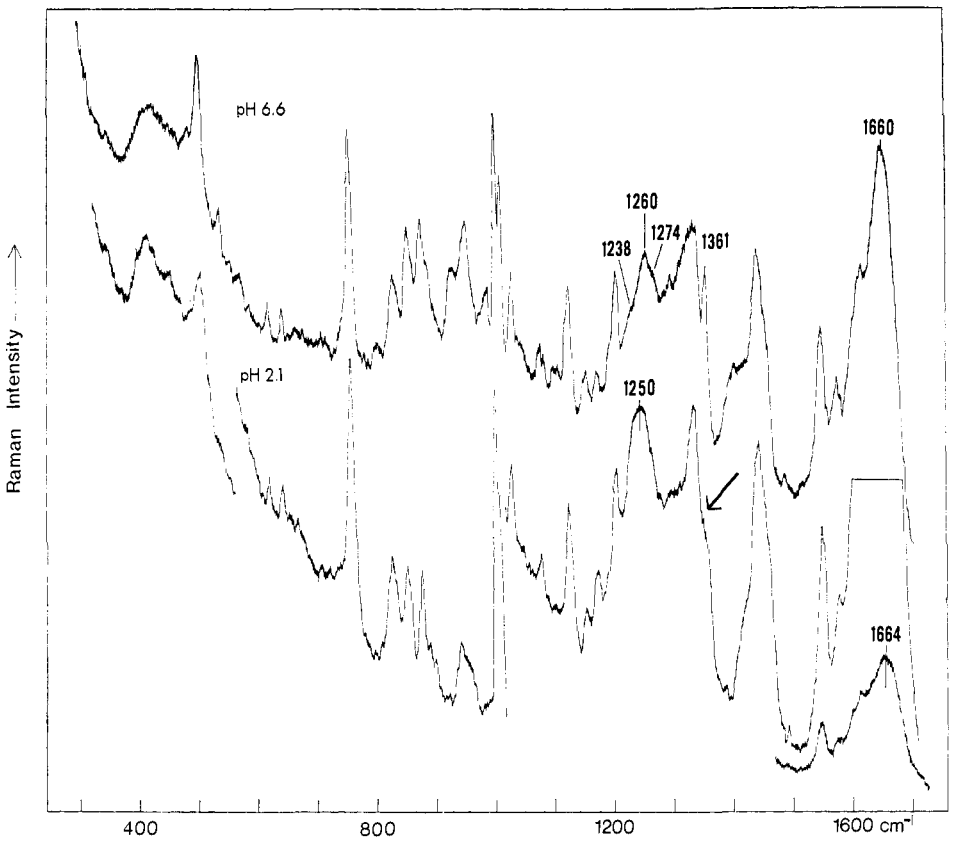

Figure 1. Raman spectra of α-lactalbumin solution (10%, salt free) at pH 6.6 and 2.1. Conditions for spectra: spectral slit width ($\Delta\sigma$), 3 cm^{-1}; rate of scan (γ), 25 cm^{-1}/min; standard deviation (Ds), 1%; sensitivity (s), 2500 cps full scale; laser power (p) at the sample, 150 mW.

ization can be made with certitude concerning protein structure in crystals and in solution, we feel that Raman studies on this subject should be extended to include those proteins susceptible to conformational changes by pH and heat.

Experimental Section

Materials. Bovine α-lactalbumin (twice crystallized, salt-free lyophilized powder) was a gift from Dr. M. P. Thompson of Eastern Marketing and Nutrition Research Divison of U. S. Department of Agriculture. An additional sample which has been purified from the crystalline product by means of gel filtration[15] was kindly provided to us by Professor D. Puett of the Department of Biochemistry at Vanderbilt University. Raman spectra obtained from both types of samples were identical.

Crystals of α-lactalbumin for Raman measurements were grown from ammonium sulfate solution at pH 6.6 according to the manner recommended by Aschaffenburg and Drewry.[16] The small crystals (about 20–65 μ) obtained by this method were of nonuniform shapes and sizes. Before Raman experiments, they were kept in 70% saturated $(NH_4)_2SO_4$ solution. Hen's egg-white lysozyme crystals in bromide form (the crystals used in the previous studies[11] were in chloride form) were grown from 5% sodium bromide solution at pH 6.0 by the method of Alderton and Fevold.[17]

Methods. Raman measurements on crystals were made with the crystals soaked in 70% saturated $(NH_4)_2SO_4$ solution. The technique of obtaining Raman spectra under mother liquor conditions has been described previously.[13] Solution measurements were made under solvent conditions very similar to those of crystals. The solutions were prepared by adding a maximum amount of saturated ammonium sulfate solution (pH 6.6) to a 10% salt-free solution of α-lactalbumin at pH 6.6 without developing turbidity. Spectra of salt-free solutions at pH 6.6 and 2.1 were also obtained. The pH value was adjusted by 1.0 *N* NaOH or HCl and determined by a Radiometer Model 26 pH meter.

Several Raman systems have been employed in this investigation. This work was started at Massachusetts Institute of Technology in 1968 and continued at Georgia Institute of Technology (1970–present). During the earlier investigation at Georgia Tech (1970–1972), we used a laboratory-assembled laser Raman system[18] in the School of Physics, equipped with a Spex 1401 double monochromator, ITT FW 130 phototube, Coherent Radiation Model 52B argon ion laser, and standard nuclear counting electronics. The spectra presented in Figure 1 were two of the satisfactory spectra directly recorded on this equipment. High quality Raman spectra of α-lactalbumin crystals and lyophilized powder were recently obtained on a new Spex Ramalog system with triple monochromator (1401 double monochromator plus third monochromator) in the School of Chemistry, equipped with a RCA Model C-31034 phototube and a modified Spex pc-1 photon-counting system.

All the spectra reported here were obtained with the 514.5-nm line of an argon ion laser and at 3-cm^{-1} resolution. The sample handling and spectroscopic methods used were similar to those described earlier.[11,13,18]

Results and Discussion

(a) Conformation Change of α-Lactalbumin in Solution Induced by pH. A Demonstration of the Sensitivity of the Amide III Contour and a Tryptophan Line at 1361 cm^{-1} to Protein Conformation. The top curve of Figure 1 shows the laser Raman spectrum of salt-free α-lactalbumin in solution at pH 6.6 and 25°. The spectrum obtained under identical conditions except at pH 2.1 is also presented in Figure 1 (bottom curve) for comparison. At pH 6.6, the amide III contour (centered at 1260 cm^{-1} with two shoulders at 1238 and 1274 cm^{-1}) is very similar to that of native lysozyme solution,[11] indicating similar backbone con-

(15) J. Hermans, Jr., and D. Puett, *Biopolymers*, **10**, 895 (1971).
(16) R. Aschaffenburg and J. Drewry, *Biochem. J.*, **65**, 273 (1957).
(17) G. Alderton and J. Fevold, *J. Biol. Chem.*, **164**, 1 (1946).
(18) N. T. Yu, C. S. Liu, and D. C. O'Shea, *J. Mol. Biol.*, **70**, 117 (1972).

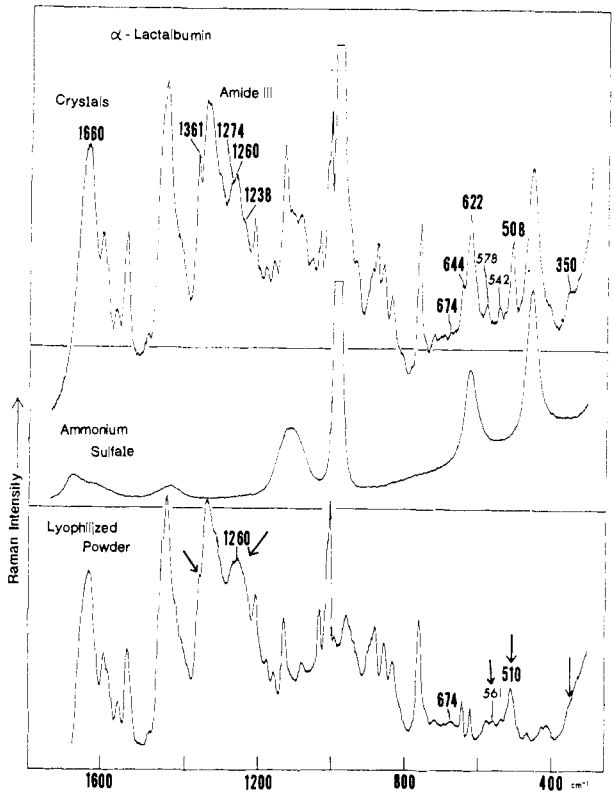

Figure 2. Comparison of the Raman spectrum (top) of α-lactalbumin crystals to that (bottom) of lyophilized powder. The middle curve shows the contribution of ammonium sulfate solution to the top spectrum. All the spectra were taken with the same spectral slit width of 3 cm^{-1} and a scanning speed of 30 cm^{-1}/min. Laser power (p) at the samples is about 70 mW.

formation between the two proteins. Upon reduction in pH value from 6.6 to 2.1, the amide III contour has undergone drastic alteration into a stronger, structureless broad band centered at 1250 cm^{-1}. At the same time, the amide I band shifts from 1660 to 1664 cm^{-1}. Since the spectral features at 1664 and 1250 cm^{-1} closely resemble those of random-coiled glucagon[19] and poly-L-glutamate,[20] it is concluded that the polypeptide backbone of α-lactalbumin has been unfolded and solvated by water on going from pH 6.6 to pH 2.1.

The sharp tryptophan line at 1361 cm^{-1} becomes barely detectable at pH 2.1. However, in view of the data of Kronman, *et al.*,[7] it is not possible to say that the decrease in intensity of this line means that tryptophan residues become exposed to solvent. It may be interpreted as due to changes in the environments of the two "buried" Trp residues. The indole rings accessible to water are not expected to contribute to the distinct "sharp" feature at 1361 cm^{-1} on the basis of the Raman spectra of random-coiled glucagon[19] and amino acid tryptophan in solution.[12,21] The effect of conformational change on this tryptophan line has been discussed in some detail by Lord and coworkers.[12]

(b) Spectral Evidence for Conformational Difference between Crystals and Lyophilized Powder. The top and bottom curves of Figure 2 are the Raman spectra of α-lactalbumin crystals and lyophilized powder. Since the spectrum of crystals was taken in the presence of mother liquor, it contains several intense lines due to ammonium sulfate (see middle curve of Figure 2). However, all the Raman lines displayed in the bottom spectrum originated from the salt-free protein. The spectral regions carrying the information of the C–S–S–C stretching modes, indole ring vibrations, and the amide bands (I and III) are relatively clear and thus useful for our present purpose.

The differences between the two spectra are pointed out by arrows. Since the spectra were taken with identical spectral slit width (*i.e.*, 3 cm^{-1}) and reproduced at least five times, the line-broadening and the disappearance of sharp peaks (*e.g.*, at 1361 cm^{-1}) are a true reflection of structural changes and not due to artifact.

Backbone conformational difference between crystals and lyophilized powder may be seen in the amide III region (1220–1300 cm^{-1}). As we have discussed in part a, the frequencies and complex line shape in this region are related to the protein backbone conformation. In the spectrum of the crystals, the amide III contour resembles that of a salt-free solution at pH 6.6 (Figure 1). Upon lyophilization, three resolved peaks at 1274, 1260, and 1238 cm^{-1} have coalesced into a broad band centered at 1260 cm^{-1}. The exact nature of the structural change related to this line broadening is not completely clear, but line broadening in the amide III region would be expected if the hydrogen bonding in the backbone were less uniform. It should be noted that the amide III line broadening on dehydration has also been observed in insulin[14] and ribonuclease A.[13]

The spectral features near 508 (S–S) and 674 cm^{-1} (C–S) are of particular interest because of the specific correlation with the structural parameters of the disulfide links. The S–S stretching frequency is considered to depend on the torsional angles about the C–S bonds,[22] but independent of the CS–SC dihedral angle.[22,23] It should be pointed out that to the contrary of these results, Scheraga and coworkers[24] found that the S–S mode varied linearly with the CS–SC dihedral angle for compounds whose CC–SS dihedral angles were not very different. Despite this disagreement, the broadening of the S–S line at 508 cm^{-1} upon lyophilization can be interpreted as the result of conformation induced changes of the bond angles (either C–S torsional or CS–SC dihedral angles) into a less uniform state. This line broadening was also observed when α-lactalbumin was thermally denatured (unpublished results) and when lysozyme was denatured by LiBr and by sodium dodecyl sulfate.[25]

The intensity ratio of the C–S and S–S lines varies widely among model compounds[21,23,24] and proteins,[11,13,14,18,19] and is definitely related to the structure of the disulfide links. Although its correlation with specific parameters[21,23,24] is not well established, it is still useful in monitoring the structural changes associated with the disulfide environments. Recently, it has been shown[18] that this ratio undergoes quite a dramatic change in the conversion of globular to

(19) N. T. Yu and C. S. Liu, *J. Amer. Chem. Soc.*, **94**, 5127 (1972).
(20) R. C. Lord and N. T. Yu, *J. Mol. Biol.*, **51**, 203 (1970).
(21) R. C. Lord and N. T. Yu, *J. Mol. Biol.*, **50**, 509 (1970).
(22) H. Sugeta, A. Go, and T. Miyazawa, *Chem. Lett.*, 83 (1972).
(23) E. J. Bastian, Jr., and R. B. Martin, *J. Phys. Chem.*, **77**, 1129 (1973).
(24) H. E. Van Wart, A. Lewis, H. A. Scheraga, and F. D. Saeva, *Proc. Nat. Acad. Sci. U. S.*, **70**, 2619 (1973).
(25) M. C. Chen, R. C. Lord, and R. Mendelsohn, *J. Amer. Chem. Soc.*, **96**, 3038 (1974).

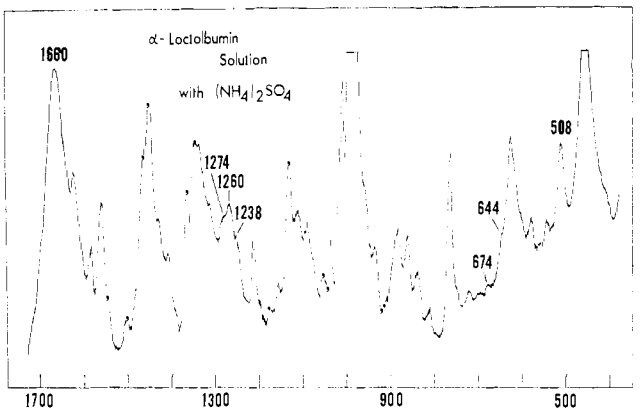

Figure 3. Raman spectrum of α-lactalbumin solution (10%) with saturated ammonium sulfate. $\Delta\sigma$, 3 cm^{-1}; γ, 30 cm^{-1}/min; s, 3000 cps; p, 200 mW.

fibrillar insulin. In Figure 2 the C–S intensity between crystals and lyophilized powder is nearly the same and the change in the intensity ratio upon freeze-drying is primarily due to the decrease of the 508-cm^{-1} peak.

The conformational alterations induced by lyophilization is also reflected in the intensity decrease of the tryptophan line at 1361 cm^{-1}. Consistent with the interpretation of part a, it means that the environments of those "buried" tryptophan residues (Trp-28 and -63) have been altered.

(c) Effect of Crystallization on the Conformation of α-Lactalbumin. In previous work on this subject,[11,13] Raman spectra of protein crystals were compared to that of solution at very low ionic strength. To examine whether the addition of salt (ammonium sulfate in this case) would induce conformational change before crystallization commenced, we have obtained the Raman spectrum of α-lactalbumin solution with saturated $(NH_4)_2SO_4$ (Figure 3). Comparison of this spectrum with that of the crystals (top curve of Figure 2) in the amide III region indicates that there is *no* effect of crystallization on the backbone conformation of α-lactalbumin. The spectra of the salt-free solution (top curve of Figure 1) and the solution with salt (Figure 3) also show similar features in the amide III region, suggesting *no* conformational difference. The results obtained so far from this laboratory indicate that small proteins with four disulfide bonds such as lysozyme, α-lactalbumin, and ribonuclease A do possess sufficiently large conformational energies to prevent distortion of backbone conformation by lattice forces during crystallization. At present, we are planning to investigate some proteins without disulfide bonds such as nuclease to see whether the backbone conformation is also the same in crystals as in solution.

Comparison of side-chain vibrations in crystals and in solution is not feasible in the regions where serious overlap exists with intense solvent lines. However, the S–S line at 508 cm^{-1}, the C–S line at 674 cm^{-1}, and the indole line at 1361 cm^{-1} closely resemble the corresponding lines in crystals (top curve of Figure 2). The tyrosyl line at 644 cm^{-1} is somewhat weaker in solution than in crystals (also lyophilized powder), which was also found in lysozyme.[11]

(d) Comparison of α-Lactalbumin and Lysozyme

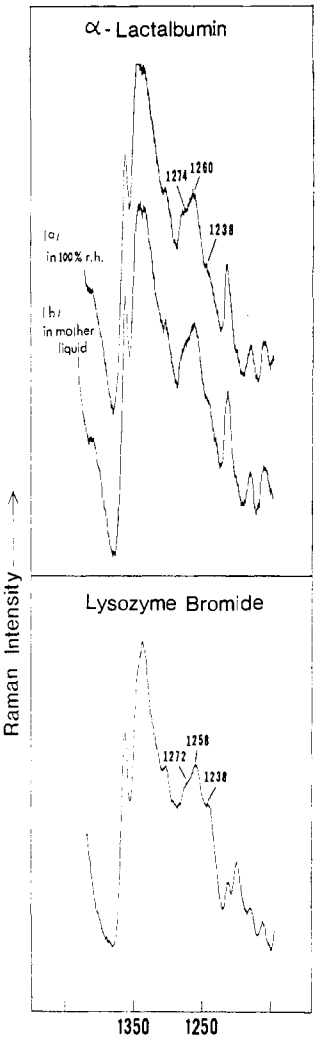

Figure 4. Comparison of Raman spectra of α-lactalbumin and lysozyme crystals in the 1100–1400-cm^{-1} region.

Conformation in Crystals. Circular dichroism[6] and optical rotatory dispersion[4] studies have shown that the three-dimensional structures of α-lactalbumin and lysozyme are similar. Comparison of Raman spectra in the amide III backbone region also reveals this similarity. In Figure 4, two spectra of α-lactalbumin are presented, one obtained in 100% relative humidity with unfocused laser powder of about 70 mW and the other taken from crystals immersed in mother liquid. These two spectra are identical, indicating the absence of laser dehydration effect[14] in curve a. The correlation of the three peaks in the amide III region with specific structural features has been discussed previously in the case of lysozyme.[11] The component at 1238 cm^{-1} was assigned to the antiparallel pleated sheet in the "hairpin turn" of the lysozyme backbone. Since this component was also found in the spectra of α-lactalbumin, it is concluded that such a "hairpin turn" may also exist in this protein. The component at 1274 cm^{-1} was quite definitely attributed to α helix,[11] while the one at 1260 cm^{-1} might be derived from random-coil structure with some contribution from α helix.

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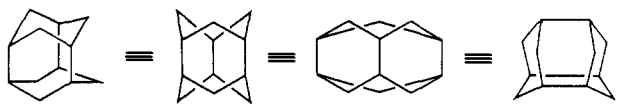
and Research Corporation. The author thanks the School of Chemistry and the Research Corporation for providing matching funds for the purchase of Raman equipment. The Raman spectra in Figure 1 were obtained by Mr. C. S. Liu in Professor D. C. O'Shea's light scattering laboratory. The author is indebted to Dr. M. P. Thompson and Professor D. Puett for the highly purified samples of α-lactalbumin.

Communications to the Editor

Iceane

Sir:

The synthesis of strained bridged polycyclic hydrocarbons of high symmetry and unusual structure continues to be a challenging objective to organic chemists.[1,2] Tetracyclo[5.3.1.1^{2,6}.0^{4,9}]dodecane (**1**)[2] is particularly interesting in this regard. This highly symmetrical (point group D_{3h}) rigid molecule has a carbon skeleton consisting of *two chair cyclohexanes* connected to each other by three axial bonds, and thus the periphery of this molecule is composed of *three boat cyclohexanes*. The trivial name "iceane" has been proposed[3] for **1** since this molecule is geometrically the hydrocarbon analog of crystalline water (Figure 1).[4] We wish to report the synthesis of iceane (**1**).



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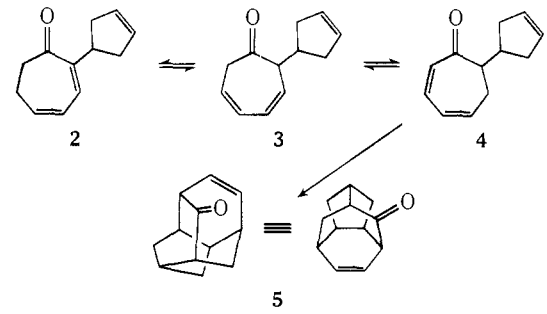
The facile construction of a C_{12} tetracyclic easily convertible to **1** was promised by the intramolecular $\pi_s^4 + \pi_s^2$ cyclization of the appropriate alkenyl dihydrotropone.[5] Reaction of Δ^3-cyclopentenylmagnesium bromide[6] with tropone[7] at 0° gave a mixture of 2-(Δ^3-cyclopentenyl)dihydrotropones, **2**–**4** (38%, bp 103–105° (1–3 mm)). Unlike our previously studied cases,[5] the symmetrical nature of the olefin and the geometric constraints imposed by the cyclopentene ring obviates the formation of tetracyclic cycloaddition products from dihydrotropones **2** and **3**. Pyrolysis of the dihydrotropones at 200–205° (heptane containing 5% *N*,*N*-dimethylaniline 24 hr) gave a single product, tetracyclo[5.3.2.0.^{2,4}.0^{4,9}]dodec-11-en-8-one (**5**) (mp 214.0–216.0°, $\nu_{max}^{CCl_4}$ 1719.3 cm^{-1}), in 21% yield.[8,9] Conversion of **5**



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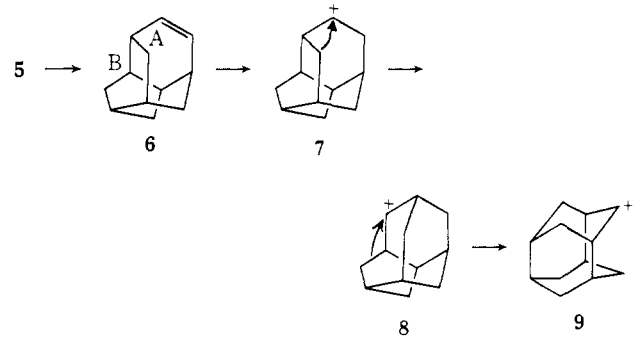
to its semicarbozone (mp 208.0–210.0°) followed by treatment with potassium hydroxide[10] gave olefin **6** (88%, mp 191.5–194.0°). The cmr spectrum of **6** (Figure 2A) exhibited seven lines which was consistent with its symmetrical structure.

Conceptually, two Wagner–Meerwein rearrangements separate the carbonium ion **7** from iceane. For example, migration of bond A results in the contraction of seven-member ring to produce **8** while a further 1,2-alkyl shift (bond B) produces the iceane skeleton **9**.[11]

Molecular mechanics calculations of these ring



6 7 8 9

(1) (a) G. Schroeder, *Angew. Chem., Int. Ed. Engl.*, **2**, 481 (1963); (b) P. E. Eaton and T. W. Cole, *J. Amer. Chem. Soc.*, **86**, 962, 3157 (1964); (c) P. v. R. Schleyer and M. M. Donaldson, *ibid.*, **82**, 4645 (1960); (d) C. A. Cupas, P. v. R. Schleyer, and D. J. Trecker, *ibid.*, **87**, 917 (1965).

(2) See also end paper, J. B. Hendrickson, D. J. Cram, and G. S. Hammond, "Organic Chemistry," 3rd ed, McGraw-Hill, New York, N. Y., 1970.

(3) L. F. Fieser, *J. Chem. Educ.*, **42**, 408 (1965).

(4) A hexagonal diamond having the ice structure has also been described. "Iceane" is the first member of this family: F. P. Bundy and J. S. Kaper, *J. Chem. Phys.*, **46**, 3437 (1967).

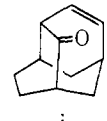
(5) C. A. Cupas, W. Schumann, and W. E. Heyd, *J. Amer. Chem. Soc.*, **92**, 3237 (1970); (b) C. A. Cupas, W. E. Heyd, and M. S. Kong, *ibid.*, **93**, 4623 (1971); (c) L. Hodakowski and C. A. Cupas, *Tetrahedron Lett.*, 1009 (1973).

(6) P. D. Bartlett and M. R. Rice, *J. Org. Chem.*, **28**, 3351 (1963).

(7) P. Radlick, *J. Org. Chem.*, **29**, 960 (1964).

(8) Satisfactory elemental analyses were obtained for all new compounds.

(9) The proton nmr spectrum of **5** showed the same features characteristic of that of 2-homoprotoadamantenone (i) exhibiting two symmetrical triplets centered at τ 3.72 and 4.03, while an unobscured triplet, assignable to the allylic bridgehead proton adjacent to the carbonyl, appeared at τ 7.12.



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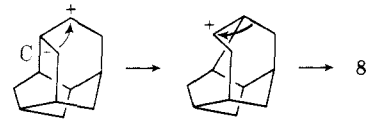
(10) D. Todd, *Org. React.*, **4**, 378 (1948).

(11) Alternately, migration of bond C would produce (ii). A further 1,2-alkyl shift, as indicated, would produce the identical ion **8**.



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