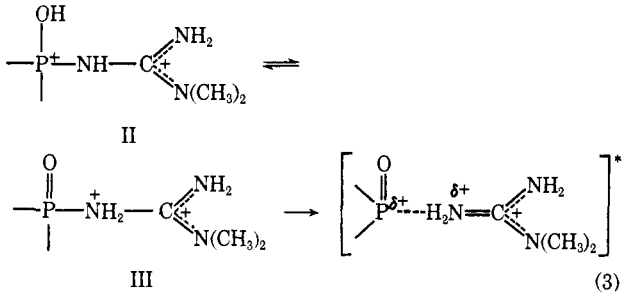
increases which would be expected to increase the concentration of the N-protonated tautomer III (eq 3) be-



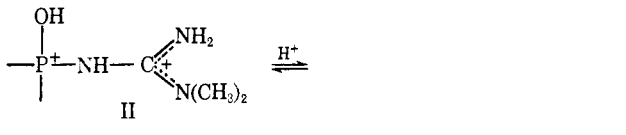
II

III (3)

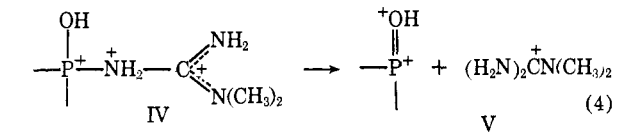
cause III has a higher charge density than II and its concentration should therefore increase as the ionizing power of the solvent increases.

This is a similar tautomerism to that observed for phosphinanilides[4] in which case the analog of III is the reactive species. Because of the excellent leaving group, III would be expected to be highly reactive and would undergo rapid cleavage of the P–N bond with dissociative character (A1-like) in the transition state.[10] Therefore, although the concentration of III may be small relative to II, reaction could proceed through this. This hypothesis is supported by the solvent isotope effect determined in 12 M H_2SO_4, $k_H/k_D = 0.5$, which is in agreement with an acid-catalyzed reaction with dissociative character.[9,11]

Although this is not the only possible mechanism for the hydrolysis of I in acidities above 10 M H_2SO_4, the only other pathways result in unlikely species such as the pathway involving intervention of a trication IV. This seems unlikely since: (1) a pK_a value of -11 for the *diprotonated* tetramethylguanidine has been determined,[12] and (2) reaction through IV would produce highly unstable intermediates (*e.g.*, V) of prohibitively high energy.



II



IV V (4)

Therefore, the reasons for the characteristic rate–acidity profile for the hydrolysis of I can be summarized at low acidity by protonation of an increasing fraction of substrate until decrease in water activity reduces the rate of the A2 process and at higher acidity by the fraction of the N-protonated dication which is the reactive species and which undergoes cleavage of the P–N bond by a mechanism with considerable dissociative character.

(10) P. Haake and P. S. Ossip, *J. Amer. Chem. Soc.*, **93**, 6924 (1971).

(11) From the rate of hydrolysis of I in 13.9 M H_2SO_4, we determined $\Delta S^*_{75.8} = -30$ eu. Although ΔS^* values are normally near zero in a reaction proceeding by an A1 mechanism, the high-solvation requirement of III would be expected to lead to a high negative entropy of formation of III so that the entropy change associated with the rate-determining step could be very small. These entropy considerations have been discussed briefly[4] and will be treated in more detail in a paper now in preparation (P. Haake and D. A. Tyssee).

(12) S. Limatibul and J. W. Watson, *J. Org. Chem.*, **36**, 3805 (1971).

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Laser Raman Spectra of Native and Denatured Insulin in the Solid State

Sir:

In order to demonstrate the sensitivity of Raman spectroscopy to protein conformation, we have selected a small protein, insulin, as a model and undertaken a systematic Raman spectroscopic study of it under various conditions. Recently we have reported a preliminary spectrum of native zinc-insulin crystal[1] in which a total of 45 lines were observed and tentative assignments were made. At present, we wish to report a higher resolution spectrum of the same sample together with a spectrum of denatured insulin for the purpose of comparison (Figure 1). These two spectra were obtained under comparable experimental conditions. Comparison of these two spectra (see Figure 2) reveals some striking and interesting spectral changes, indicating that the structure and conformation of insulin have undergone considerable changes during denaturation. It is obvious that a detailed analysis of these spectral changes will certainly provide detailed information about the nature of this structural transformation.

Biologically, insulin is a hormone concerned in the regulation of carbohydrate metabolism. It is well known that it undergoes a transformation from globular to fibrous form in dilute HCl solution by heating, with a concomitant loss in its activity and solubility.[2] However, both the activity and solubility can be recovered by treating the inactivated insulin with a 10% aqueous phenol solution.[3,4] The nature of this transformation has been extensively studied by various techniques.[2]

In 1951, Ambrose and Elliott[5] reported an infrared study of insulin in the amide I and amide II regions. It was found that the denaturation by heating at pH 2 causes a weakening of the C=O stretching band (amide I) at 1657 cm^{-1} and produces a new band at 1637 cm^{-1}. They also have shown that if the denatured insulin is dissolved in aqueous phenol and precipitated with acetone, the 1657-cm^{-1} band reappears. This observation is quite different from our present Raman results. We have observed that the amide I line at 1664 cm^{-1} (with a shoulder near 1680 cm^{-1}) is shifted to 1673 cm^{-1} and sharpened quite considerably upon denaturation. Obviously the infrared and Raman techniques are not detecting the same amide I vibrations in proteins. The coupling of adjacent peptide groups may be

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(3) T. D. Gerlough and R. W. Bates, *J. Pharmacol.*, **45**, 19 (1932).

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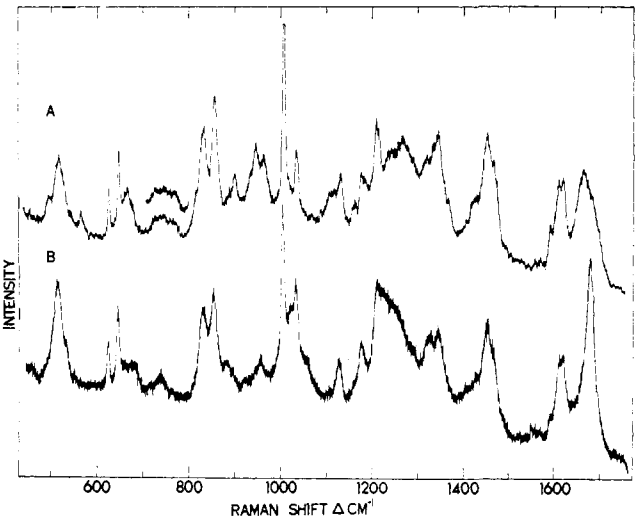

Figure 1. Raman spectra of beef insulin (actual tracings): (A) spectrum of native zinc-insulin crystalline powder; spectral slit width ($\Delta\sigma$), 4 cm^{-1}; sensitivity (s), 1000 counts per second (cps) full scale; rate of scan (r), 10 cm^{-1}/min; standard deviation (SD), 1%; (B) spectrum of denatured insulin (heat-precipitated); a solution of 10 mg/ml of insulin at pH 2.42 was heated at 100° for 45 min; the solid sample was obtained by air-drying the resulting fibrous gel; $\Delta\sigma$, 5 cm^{-1}; s, 5000 cps; r, 10 cm^{-1}/min; SD, 1%. The laser power at the samples is about 200 mW at 514.5 nm. The spectrometer was a laboratory-assembled Raman system using a Spex 1401 double monochromator, a thermoelectrically cooled detector (ITT FW130 photomultiplier, S-20 response), and a standard photon-counting read-out system.

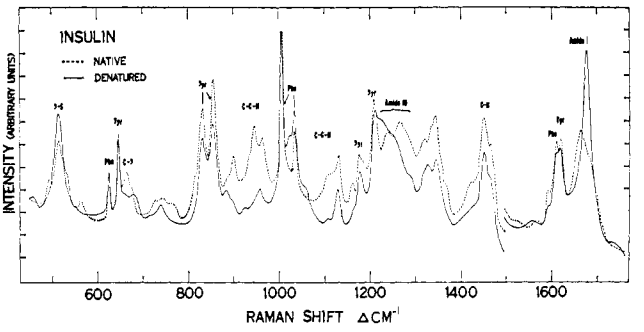

Figure 2. Raman spectra of beef insulin (redrawn from Figure 1 for better comparison). The line at 625 cm^{-1} due to phenylalanine residue is known to be comformation-independent[8] and is used as an internal reference. This line has equal intensity in both spectra.

responsible for this. A more detailed discussion on this point will appear in a later publication. It may be of interest to mention that Lord[6] and Mendelsohn[7] also have observed a Raman frequency shift from 1660 to 1674 cm^{-1} in the amide I region when lysozyme is chemically denatured (S-cyanoethylated).

Another strong Raman frequency characteristic of the peptide CONH group appears near 1260 cm^{-1} (amide III). Three resolved peaks at 1239, 1270, and 1288 cm^{-1} were observed in the spectrum of native insulin. Upon denaturation, the center of gravity was drastically shifted from 1260 to about 1230 cm^{-1}. This is a strong indication that the protein backbone has undergone an extensive unfolding and is in either β structure or random-coil form. Lord and Mendelsohn[6,7] have also observed a center of gravity shift from 1260 to 1247 cm^{-1} in the amide III region when lysozyme is denatured.

Raman spectroscopy is quite useful in providing direct evidence concerning the presence and number of disulfide linkages in proteins, and also useful in studying the local geometry of the CSSC group.[8] From the fact that the S–S stretching frequencies centered at 516 cm^{-1} do not weaken or disappear, one knows that the disulfide bonds of insulin still remain intact in the denatured state. The C–S stretching vibrations of the disulfide linkages in native insulin appear near 670 cm^{-1} (one at 668, the other at 680 cm^{-1} as a shoulder). Striking spectral changes in the 670-cm^{-1} region and changes in line shape and peak intensity of the line at 516 cm^{-1} strongly suggest that the dihedral angles and the CSS angles of the disulfide linkages have changed quite appreciably. It may be of interest to remark that this part of the Raman spectra does contain information, providing the kind of new insights which are not available by other techniques. A more complete interpretation will be given in a subsequent publication.

The speculation of extensive backbone unfolding in denatured insulin is supported by the spectral changes in the C–C–N skeletal vibrations region. This observation is in good agreement with the prediction made earlier by Lord and Yu.[8] Recently, we obtained a Raman spectrum of denatured insulin which was prepared from a tenfold more concentrated solution. It was found that the spectrum in this region is somewhat different from Figure 1B. This may be due to a difference in the extent of backbone unfolding.

Other interesting manifestations of the structural changes occur near 567, 748, and 840 cm^{-1}. The intensity drop of the lines at 828 and 853 cm^{-1} (assigned to the ring vibrations of tyrosine residues) may be due to the changes of the environments of these rings as a result of extensive unfolding. On the basis of present studies, we are in a position to conclude that Raman spectroscopy is a sensitive probe for the investigation of the structures and conformations of small proteins, and that it does provide the kind of new insights which are not obtainable by other research techniques.

In addition to the results presented here, we also have obtained high-resolution Raman spectra of amorphorus insulin and aqueous insulin (10% concentration, pH 2.8). These spectra agree closely with the spectra of native zinc-insulin crystals (Figure 1A). This means that, to the extent one can tell from laser-Raman spectroscopy, the conformation of insulin in amorphous form and in solution is the same as in the crystalline state.

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