

HYDRATION AND DENATURATION OF COLLAGEN AS OBSERVED BY INFRARED SPECTROSCOPY*

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ABSTRACT

A procedure is described to obtain very thin films (~ 0.01 mm.) of undenatured collagen which are suitable for obtaining infrared spectra without denaturation. An infrared cell is described which permits the measurement of infrared spectra of collagen at controlled temperature and humidity. The spectra obtained are discussed in relation to denaturation and hydration of collagen. Hydration of collagen is observed in the frequency range $2900\text{--}3500\text{ cm}^{-1}$ where OH, NH, and CH bonds absorb. Denaturation results in changes of the amide I (C=O stretching) band in the $1630\text{--}1660\text{ cm}^{-1}$ region. A detailed study of the amide II band ($\sim 1530\text{--}1560\text{ cm}^{-1}$) has been carried out in order to determine the range of relative humidity over which water molecules are attached to the triple helix backbone of undenatured collagen molecules.

INTRODUCTION

Polypeptides and proteins exhibit several characteristic absorption bands in the infrared region of the spectrum. These characteristic bands occur in the spectra of all secondary amides and have been commonly labeled amide A, amide B, amide I, II, III, IV, V, VI, and VII (1). Each one of these bands is associated with a particular type of vibration of the polypeptide repeat unit. Figure 1 shows these absorption bands as observed in the spectrum of the model compound *N*-methyl acetamide. In actual proteins all the characteristic bands are not so easily observed. The amide A, B, I, II, III, and V bands are usually recognized without difficulty and are, on the basis of theoretical calculations (2), assigned to specific molecular vibrations as indicated in Table I. As shown in Table I, the characteristic protein bands absorb in relatively narrow frequency ranges. Never-

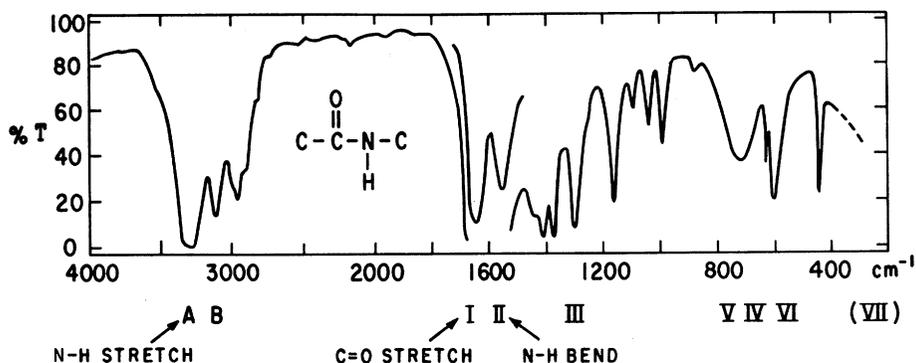


FIGURE 1.—Characteristic amide bands as exhibited by the model compound *N*-methyl acetamide.

theless, minor shifts within these ranges and splitting of the bands into components can be correlated with the molecular structure of the sample under investigation (3). For most proteins, a considerable amount of information can be deduced from the study of the precise position and splitting of the amide I and II bands. Thus, it is possible to distinguish between the α -helix, various pleated sheet structures, and random conformations of fibrous proteins such as silk and the keratins (3). In favorable instances, such studies are also possible on globular proteins in aqueous or D_2O solutions (4, 5).

TABLE I
CHARACTERISTIC PROTEIN BANDS

Designation	Approximate Frequency (cm^{-1})	Description
Amide A	~ 3300	NH stretching
Amide B	~ 3100	Overtone of NH bending
Amide I	1590–1680	C=O stretching
Amide II	1480–1580	NH bending and CN stretching
Amide III	1230–1300	CN stretching and NH bending
Amide V	640–800	Out-of-plane NH bending

Collagen presents a special case because of its unusual amino acid composition and the resulting triple helical structure. The structural repeat unit within one fibril contains nine peptide groups (6). This makes theoretical predictions of the location and fine structure of the characteristic amide bands almost impossible and renders even empirical interpretations very difficult. It has been suggested that two tightly bound water molecules per three peptide units form an integral part of the three-dimensional structure of native collagen (6). One of these

water molecules forms a hydrogen-bonded bridge between two carbonyl groups of adjacent strands; the other forms a bridge between a carbonyl group and an NH group of adjacent strands. Two adjacent chains with two hydrogen-bonded water molecules in between are shown in Figure 2. The three hydrogen-bonded links (two involving water molecules) are marked 1, 2, and 3 in Figure 2. The infrared spectrum of collagen has been reported and discussed by several investigators (7-10). These published data show inconsistencies in the frequen-

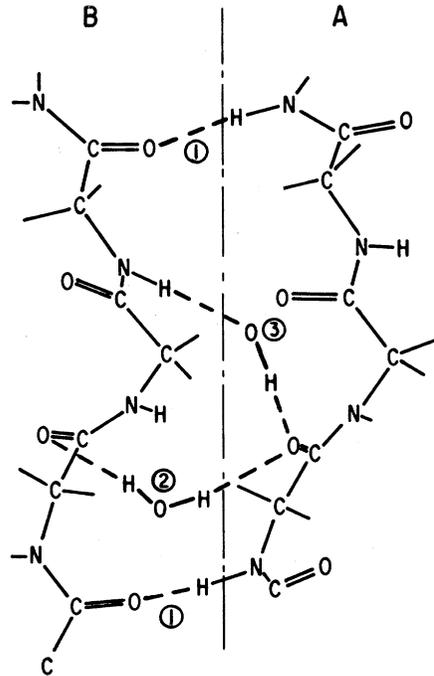


FIGURE 2.—Schematic presentation of two adjacent strands of native collagen. Hydrogen-bonded bridges are marked with encircled numbers. The precise location of the other H (not shown) of the water molecule at position 3 is not known.

cies of characteristic amide bands and the effects caused by humidity and denaturation. The purpose of this communication is to compare the infrared spectrum of native collagen with the spectrum of gelatin in order to pinpoint spectral changes caused by denaturation and to obtain additional information regarding the water binding of collagen.

EXPERIMENTAL

To obtain infrared absorption spectra of solid materials, films of approximately ten micron thickness are required. Gelatin films were cast from water on infrared-transparent silver chloride plates. Thin films of undenatured collagen were pre-

pared in the following way. Bovine Achilles tendon collagen, obtained from Calbiochem‡, Los Angeles, California, was cut into thin slices and the slices were suspended in a small amount of distilled water. The suspension was poured into a stainless steel cylinder, 16 mm. inner diameter, with an outer jacket and a bottom with a 1.4-mm. orifice. The outer jacket was filled with a dry ice and alcohol mixture. The suspension froze in a few seconds and was forced through the orifice by applying 40,000 p.s.i. pressure with the help of a stainless steel piston. This process was repeated eight times, until a very fine suspension was obtained. The final suspension was deposited on a silver chloride window and degassed to avoid bubbles, which prevent the formation of a smooth film. The water was evaporated under a stream of dry nitrogen, while the deposit was spread into a smooth film with the help of a Teflon spatula. The final film was inspected by electron microscopy in order to determine whether any collagen denaturation had taken place during the sample preparation.

An infrared absorption cell was constructed which permitted the measurement of spectra under controlled conditions of humidity and temperature. The cell consisted of a water-cooled outer brass jacket with silver chloride windows, an outer chamber filled with helium, which is a very good heat conductor, to provide heat conductance from the outer jacket to the sample, and an inner chamber with silver chloride windows, the sample being deposited on the inside of one of the inner windows. The infrared absorption cell is schematically pictured in Figure 3.

A constant, known humidity content of the air was achieved by recirculating a captured volume of air through sulfuric acid solutions of known concentration and the inner chamber of the absorption cell. Nitrogen gas from a liquid nitrogen container was employed for measurements at zero percent relative humidity.

After the infrared measurements had been completed, the collagen sample was again investigated by electron microscopy to ascertain that no denaturation had taken place by local heating caused by the beam of infrared radiation. Several spot checks over the entire area of the sample which had been exposed to infrared radiation showed no sign of denaturation. The electron micrographs were reproduced as negative images. A platinum preshadowed carbon replica 13 of the collagen sample was examined in an RCA EMU 3-G electron microscope at 50 kv. accelerating voltage.

The infrared spectra were obtained with a Beckman Model IR-7 double beam grating spectrophotometer. A gas cell of five cm. path length was inserted in the reference beam and filled with the proper amount of water vapor to compensate for the water absorption in the working beam. Equilibrium between the moist air and the collagen sample was attained within a few hours, as shown by the observed infrared spectra.

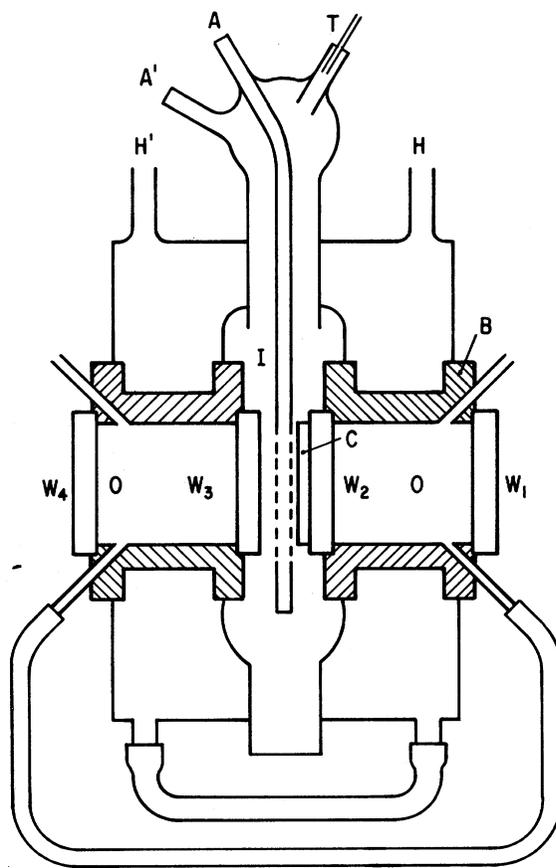


FIGURE 3.—Schematic cross section of the infrared absorption cell. A, inlet of constant humidity air. A', outlet of constant humidity air. B, brass jacket. C, sample film. H, inlet of cooling water. H', outlet of cooling water. I, inner chamber. O, outlet chamber. T, inlet of thermocouple wires. W₁–W₄, silver chloride windows.

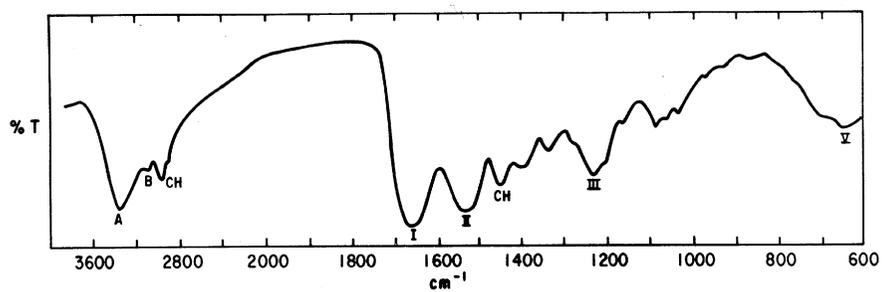


FIGURE 4.—Infrared spectrum of native collagen. Zero percent relative humidity.

RESULTS AND DISCUSSION

Figure 4 shows the infrared spectrum of native collagen as observed at zero percent relative humidity. The amide A, B, I, II, III, and V bands are easily observed. The bands close to 2940 cm^{-1} and 1450 cm^{-1} are most probably caused by CH stretching and bending motions, respectively (10, 11). The amide A, amide I, and amide II bands are discussed below in a little more detail in order to see what information can be obtained from a careful study.

Figure 5 shows the amide A band (NH stretching), the amide B band, and the CH stretching band of native collagen (solid line) and gelatin (dotted line) at zero relative humidity. Water is known to exhibit an OH stretching band at $\sim 3400\text{ cm}^{-1}$, considerably higher than the 3300 cm^{-1} NH stretching band. No specific OH absorption band at 3400 cm^{-1} is observed in either the spectrum of collagen or the spectrum of gelatin. It is noteworthy that the spectra of collagen and gelatin almost overlap in this frequency region. There are two ways to explain these data, in particular, the absence of an OH absorption band. Either the structural water has been lost in the process of exposing the very thin films to dry nitrogen, or some very tightly bound water molecules absorb at a very unusual frequency which coincides with the NH stretching band at 3330 cm^{-1} . Because

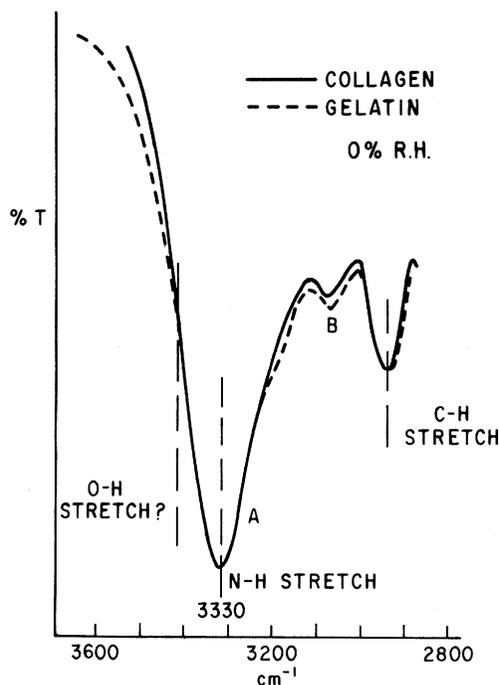


FIGURE 5.—Infrared spectra of collagen and gelatin in the $2800\text{--}3600\text{ cm}^{-1}$ region. Zero percent relative humidity.

no further change was observed in the spectra on exposing the sample films to dry nitrogen for a considerably longer time, the first alternative is favored, *i.e.*, the films at zero humidity appear to correspond to essentially anhydrous samples. The similarity of the absorption curves of collagen and gelatin, as shown in Figure 5, suggests that the state of hydration is similar for both substances under the described conditions and that the gelatin film has also been dehydrated. Figure 6 shows the same spectral region as exhibited by collagen at 90 percent relative

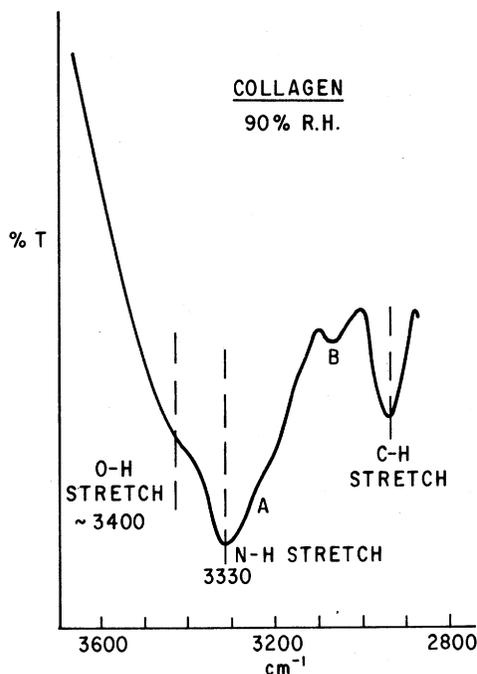


FIGURE 6.—Infrared spectrum of native collagen in the 2800 to 3600 cm^{-1} region. 90 percent relative humidity.

humidity. The absorption of the OH bonds of water is clearly discernible at $\sim 3400 \text{ cm}^{-1}$. The spectral region from 2800 cm^{-1} to 3600 cm^{-1} thus yields some information about the state of hydration of collagen and gelatin, but provides no way to differentiate between the two, *i.e.*, to obtain information about denaturation of collagen.

Figure 7 shows the amide I and amide II absorption bands of native collagen. The amide I band exhibits a low frequency shoulder and can be looked upon as a composite band with components at 1657 cm^{-1} and 1625 cm^{-1} , as indicated by dotted lines in Figure 7. The presence of at least two C=O stretching bands is in agreement with the molecular structure of collagen, as shown in Figure 2.

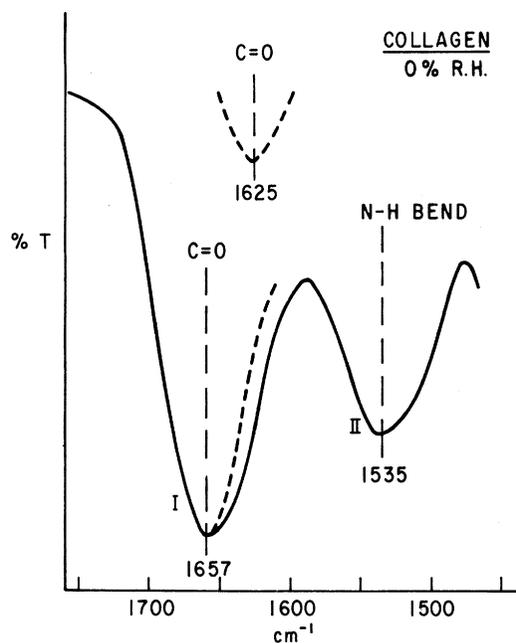


FIGURE 7.—Amide I and amide II bands of native collagen. Zero percent relative humidity.

Figure 8 shows the amide I and amide II bands as exhibited by gelatin at zero relative humidity. The amide I band is almost perfectly symmetrical, reflecting the breakdown of the well-ordered collagen structure. Disappearance of the low frequency shoulder of the amide I (C=O stretching) band is thus an indication of denaturation of collagen.

Figure 9 shows an expanded spectrum of native collagen in the amide II absorption region as a function of relative humidity. The curves are plotted as absorbance *vs.* wave number ($A = \log 1/T$). The amide II band is primarily caused by NH bending vibrations and CN stretching vibrations of the peptide repeat unit (2). Water itself does not absorb in this region of the spectrum. The gradual change of the band shape and the peak frequency of the amide II band as a function of relative humidity indicates that water molecules are gradually attached to the backbone peptide groups over the entire range of relative humidity from zero to 70 percent. These results are in agreement with work done by Mellon and coworkers on water absorption of various proteins and polypeptides (12). It seems, therefore, to be incorrect to assume that a small amount of water, which forms an integral part of the three-dimensional structure (6), is absorbed by the backbone at very low relative humidity values and that the remaining absorbed water (taken up at higher relative humidities) does not influence the basic skeleton of the three stranded molecules. The data indicate that the environment

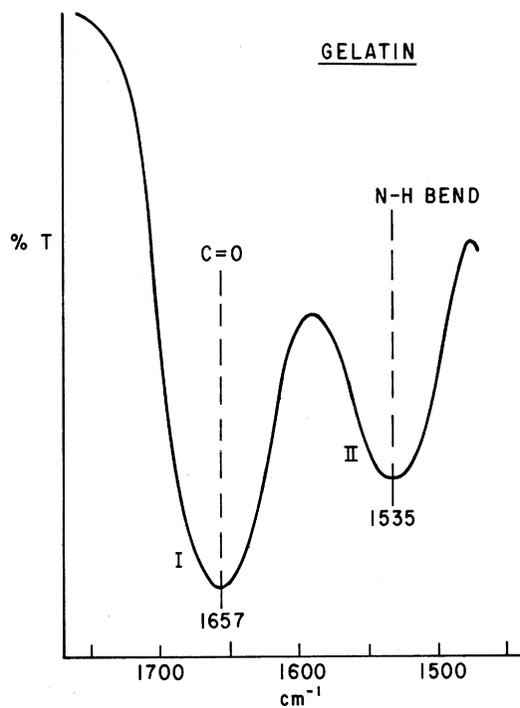


FIGURE 8.—Amide I and amide II bands of gelatin. Zero percent relative humidity.

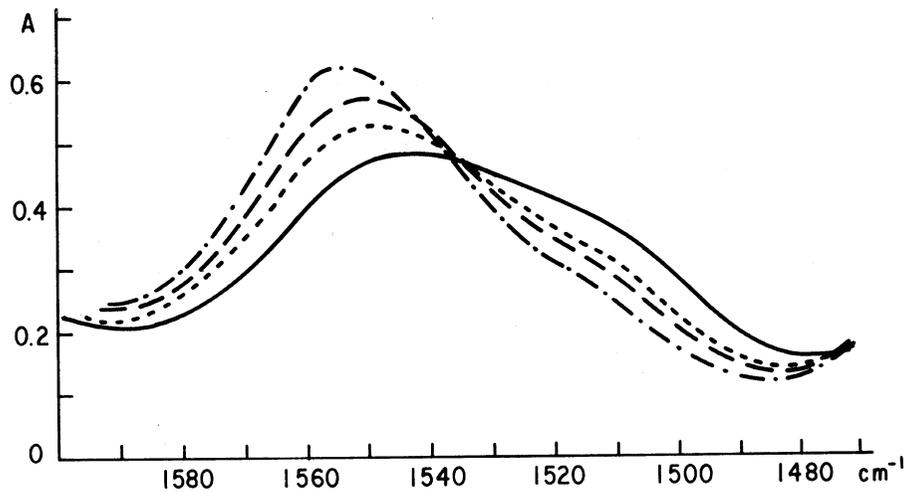


FIGURE 9.—Expanded presentation of amide II band of native collagen as a function of relative humidity.

——— 0 %. - - - - 10 %. - . - . 38 %. 70%.

of the polypeptide backbones changes over the entire relative humidity range studied. It could be argued that some very tightly bound water molecules, which form a part of the basic collagen structure, remain attached to collagen even at relative humidities approaching zero. In view of Figure 5, this would require a very unusual and unlikely absorption frequency for the OH bonds of these water molecules. The gradual changes observed in the amide II band over the entire relative humidity range from zero to 70 percent do in any case indicate that water is attached to backbone peptide groups over a very wide range of relative humidity.

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DISCUSSION

DR. TU: Dr. Mellon, will you please lead the discussion.

DR. MELLON (U. S. Department of Agriculture): We have just heard a very thorough presentation of the application of a sophisticated technique to the determination of the water relationships in the collagen molecule. Although this presentation was devoted chiefly to detecting the structural water molecules proposed by Dr. Ramachandran as crossbonds in the collagen molecule, the methodology can be applied to studies on water molecules bound to other groups in other ways. This will require considerably more research.

I have one question for Dr. Susi.

How does this type of water that you discussed today compare with the bound water that the tanner always talks about?

DR. SUSI: I think the combined water that the tanner talks about is water of the nature that Dr. Keller discussed here earlier in the morning, where he found a breaking point, or optimal point, of around 30 percent. It seems that, consider-

ing the water that is not actually within the backbone structure, there is still more tightly bound and less tightly bound water present, and about 30 percent seems to represent the amount of water which is tightly bound, which includes probably the backbone water as well as some water which is either between the protofibrils or between the fibrils, but which is tightly bound. This 30 percent really represents only about two water molecules per one polypeptide residue, so it isn't an awful lot. In most proteins you have about this type of hydration when you have a protein under normal conditions. Once you go beyond this 30 percent, then the tightly bound water in a general sense starts coming off, but the 30 percent is much more loosely bound than the last five or ten percent.

I think this answer is qualitative, but that's the best we can do right now.

DR. BUECHLER (K. J. Quinn and Company): There has been a lot of discussion in the past year about poly-water, which is formed by having glass capillaries that are completely dry, and letting water condense within those capillaries. The water has a very different infrared spectrum than normal water. It also has a much higher boiling point. These changed characteristics have been attributed to polymerization of water to a high polymer.

There are other people who feel that the orientation which is achieved at the silicate surface by a new type of hydrogen bond of the water with that silicate is the determining factor, which then orients other water molecules at some distance therefrom.

I was just wondering if you had compared the IR spectrum of this water at below ten percent humidity levels, that is, this very differently bound water that you speak of, with the IR spectrum and other characterizations which are being reported for poly-water.

DR. SUSI: No, I haven't really compared it with poly-water, but, from what I've seen and read, it would not be — the absorption spectrum is not really similar to the poly-water spectrum. The absorption is certainly stronger and the hydrogen bonds are probably stronger than you find in water-to-water molecules, in liquid water; but I don't think it resembles anything as drastic as the poly-water. It hasn't really been established whether the poly-water can be prepared or not, aside from conditions where you have thin films, capillary films, and surface films.

There'll be a presentation next July at the Gordon Conference on Infrared Spectroscopy. I think Professor Lippincott is the one who has done the most work on that, but there are others who claim that the whole thing is just a publicity stunt. I am not taking a position here.

DR. BUECHLER: I think it's been done in enough laboratories by now. There have been some five involved, so one would not say it's a publicity stunt, particularly since the original work was severely criticized by some of the people who are now agreeing with it.

I don't want to get into the politics of the situation. I wish to stress that when I asked my question before on Dr. Keller's paper, what I meant was that when water was picked up on completely anhydrous collagen, insofar as this is obtainable from acetone dehydration, there were changes occurring in the protein structure. The evidence is that this is in the region of this very tightly bound water you are talking about. The fact that one would get, at 33 percent water content, a similar picture to what went before, I don't think is really material to what I was trying to ask about.

I'm trying to ask what about this other area of low moisture content which you are studying now. With respect to that, you say you went to essentially a zero relative humidity. Collagen is itself a wonderful desiccant and has been shown to rob water from other desiccants, and I wonder which desiccant system you used.

DR. SUSI: We didn't use a desiccant system at all. What we have is a stream of extreme anhydrous nitrogen passing over and around a film which is, as I say, ten microns thick, and after 48 hours you can keep the sample in there for a week or more and you don't observe any more changes in the infrared spectrum. It's not a desiccant system; it's a system where you dry it, where you blow at a considerable rate very dry anhydrous nitrogen over a very thin film. And, of course, any kinetic phenomena will be fantastically accelerated by this technique, rather than having it in a desiccated condition or above a saturated salt solution.

Whether we do really have, in a true sense, anhydrous collagen, I just wouldn't know, and wouldn't claim. These are just experimental results. I do think we are touching the very tightly bound water, but I am not sure that we really have gotten rid of it.

MR. DONOVAN: You have been working with transmission on thin films; I wonder if you have any observations on multiple internal reflection with solid collagen.

DR. SUSI: No, I don't. We plan to do these experiments, but I haven't gotten to them yet. There was one paper published on that, about two years ago; and, from what I can tell, the spectrum doesn't look at all like what we consider to be native collagen, but we haven't worked with this.

I think it is very promising, but you have to be very careful. You have to work at very low angles and be careful that you really don't get distortion because of the dispersion mode which you superimpose on the absorption mode in these multiple reflections.

DR. MELLON: Are there any more questions?

I wish to thank Dr. Susi for a very interesting presentation.