[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

# Structural Studies of Ribonuclease. IV. The Near Infrared Absorption of the Hydrogen-Bonded Peptide NH Group[1,2]

BY JAN HERMANS, JR., AND HAROLD A. SCHERAGA

RECEIVED FEBRUARY 29, 1960

Previous experiments had indicated that 20 of the hydrogens of ribonuclease do not exchange with deuterium below the transition temperature at *p*H 4.8, presumably because 20 groups are involved in hydrogen bonds which are inaccessible to the solvent. Near infrared absorption measurements (involving the first overtones of the fundamental frequencies) indicate that these hydrogens are bound to nitrogens, rather than oxygens, and therefore are probably part of the backbone peptide hydrogen-bond system. On the basis of near infrared studies of methanol, aniline and poly-$\gamma$-benzyl-L-glutamate it is possible to distinguish between hydrogen-bonded and non-hydrogen-bonded NH and OH groups. The 20 hydrogen-bonded NH groups of the "hard core" of ribonuclease can be detected in a near infrared difference spectrum when ribonuclease (containing these 20 NH groups) is measured against fully deuterated ribonuclease in $D_2O$. A fairly good quantitative estimate of the number of NH groups in ribonuclease in $D_2O$ (*i.e.* 20) was made with the aid of a calibration spectrum obtained with internally hydrogen-bonded poly-$\gamma$-benzyl-L-glutamate.

## Introduction

Previous deuterium–hydrogen exchange studies of ribonuclease[3] at *p*H 4.8 indicated that about 20 hydrogens do not exchange with deuterium unless the protein is above the transition temperature (60° at this *p*H). Presumably, the unfolding of the molecule at high temperature permits this "hard core" of 20 hydrogens to become accessible to the solvent. In previous interpretations[4,5] of the exchange measurements it was tentatively assumed that the 20 inaccessible hydrogens were part of the backbone peptide hydrogen-bond system rather than being involved in side-chain hydrogen bonds. Infrared evidence to be presented here indicates that these 20 groups at NH rather than OH donors, supporting the original hypothesis that they are involved in peptide NH. . .OC backbone hydrogen bonds.

The measurements reported here were made in the near infrared (0.7 to 3.5 $\mu$) where the absorption depends on the overtones and combinatory frequencies of the fundamental vibrational modes involving primarily hydrogenic stretching vibrations. There are several practical advantages in studying absorption in the overtone rather than in the fundamental frequency region. First of all, the resolving power of the available instruments (Beckman and Cary spectrophotometers) is higher in the overtone region. Secondly, the extinction coefficients are lower in the overtone region; this permits the use of moderately concentrated solutions with optical path lengths comparable to those usually used in the visible and ultraviolet regions (*i.e.* 1 to 10 cm.); furthermore ordinary silica cells are very suitable for the measurements. Therefore, near infrared measurements were carried out on solutions of ribonuclease in $D_2O$ and on several model systems in a variety of solvents. Details of near infrared spectroscopy may be found in the reviews of Kaye.[6]

The near infrared studies of this paper provide information on the hydrogen bonding of amide groups of a similar nature observed in the fundamental region by several workers.[7]

## Experimental

Materials.—The bovine ribonuclease was obtained from Armour Laboratories[8] (Lot Nos. 381-059 and 647-213). Poly-$\gamma$-benzyl-L-glutamate (PBG) of molecular weight of about $2 \times 10^5$, 1,2-dichloroethane (DCE) and $D_2O$ were the same materials used in a previous study.[10] Chloroform, carbon tetrachloride and methanol were A. R. grade Mallinckrodt products. Baker and Adamson purified grade aniline was distilled at atmospheric pressure to give a colorless product.

Deuteration of Ribonuclease.—Ribonuclease was deuterated by dissolving 1 g. in 10 ml. of $D_2O$ (alternatively, 4 g. in 25 ml. of $D_2O$), keeping the solution at 38° for 24 hr. and then lyophilizing the material. The process was then repeated on the lyophilized protein. Under these conditions, all but 20 of the exchangeable hydrogens were replaced by deuterium.[3] This material is designated RNase-$H_{20}$. Another sample was *fully* deuterated by performing the two stage deuteration at 65° for 15 minutes.[3,11] This material is referred to as RNase-all D.

Solutions of each of the samples were prepared by weighing equal amounts of both samples and dissolving them in equal amounts of $D_2O$. These solutions were then centrifuged at 10,000 *g* for 20 minutes to remove insoluble material. Concentrations were determined by measuring the optical density at 287 m$\mu$ of aliquots which were diluted with $H_2O$, using extinction coefficients which had been determined for each lot in separate dry-weight *vs.* optical density measurements.[12] By this method the concentrations of each pair of

(1) This investigation was supported by a research grant (E-1473) from the National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, U. S. Public Health Service, and by a research grant (G-6461) from the National Science Foundation.

(2) Presented before the Division of Biological Chemistry at the 138th meeting of the American Chemical Society, New York, New York, September, 1960.

(3) C. L. Schildkraut and H. A. Scheraga, THIS JOURNAL, **82**, 58 (1960).

(4) H. A. Scheraga, *ibid.*, **82**, 3847 (1960).

(5) H. A. Scheraga, C. Y. Cha, J. Hermans, Jr., and C. L. Schildkraut, "Amino Acids, Proteins, and Cancer Biochemistry," Academic Press, Inc., New York, N. Y., 1960, p. 31.

(6) W. Kaye, *Spectrochim. Acta*, **6**, 257 (1954); **7**, 181 (1955).

(7) See, for example, H. Lenormant and E. R. Blout, *Nature*, **172**, 770 (1953); G. H. Haggis, *Biochim. Biophys. Acta*, **19**, 545 (1956); **23**, 494 (1957); E. G. Bendit, M. Feughelman, R. D. H. Fraser and T. P. MacRae, *Textile Research Journal*, **29**, 284 (1959).

(8) Our previous exchange studies[3] were also carried out with Armour lot No. 381-059. In similar exchange experiments, Stracher[9] found that the protein from one bottle of lot 381-059 behaved similar to our samples (*i.e.*, there was a considerable number of non-exchangeable hydrogens below the transition temperature) but that the protein from a second bottle of lot 381-059 gave no indication of non-exchangeable hydrogens. We have used protein from three different bottles, the first (lot 381-059) for exchange studies,[3] the second (lot 381-059) and third (lot 647-213) for the infrared studies reported here. All three appeared to be identical as far as the presence of the non-exchangeable hydrogens was concerned.

(9) A. Stracher, *Compt. rend. trav. lab. Carlsberg*, in press. We are indebted to Dr. Stracher for sending us a copy of the manuscript.

(10) M. Calvin, J. Hermans, Jr., and H. A. Scheraga, THIS JOURNAL, **81**, 5048 (1959).

(11) J. Hermans, Jr., and H. A. Scheraga, *Biochim. Biophys. Acta*, **36**, 534 (1959).

solutions of RNase-$H_{20}$ and RNase-all D in $D_2O$ were found to agree within 1%.

In order to check on possible effects of deuteration on the enzymatic activity of ribonuclease, the ratio between hydrolytic activity towards ribonucleic acid (as determined with the use of a *p*H-stat) and concentration was determined in $H_2O$ and compared with that of the original non-deuterated sample.[13] These ratios, expressed in arbitrary units, were 85 for RNase-$H_{20}$, 82 for RNase-all D and 87 for the untreated sample from lot 381-059. Since the assay is accurate within ±4%,[14] the figure of 82 differs from that of 87 for the untreated sample by an amount just possibly outside the limit of error. Therefore, we may conclude that deuteration at 38° and subsequent lyophilization do not produce any measurable denaturation of the enzyme and that denaturation occurs only to a very slight extent when the sample is deuterated at 60°. This slight amount of irreversible denaturation should not affect the validity of our results.

**Infrared Measurements.**—Near infrared absorption spectra were obtained both on Beckman DK and on Cary[15] Model 14 recording spectrophotometers. All the graphs shown here were based on data obtained with the Cary instrument. The stoppered quartz absorption cells had 10, 2 and 1 cm. light paths, respectively. The reference cell usually contained solvent; however, in measurements of difference spectra of ribonuclease, the reference cell contained RNase-all D in $D_2O$ and the other cell contained a solution of the same concentration of RNase-$H_{20}$ in $D_2O$.

In order to obtain a baseline (*i.e.* $D = 0$, where $D$ is optical density) the values of $D$ over the wave length range were first adjusted to a horizontal straight line with no cells in the light paths. Both cells, filled with $D_2O$, were then placed in the light paths and a constant value of $D$ was recorded in the wave length region below 1.8 $\mu$. Above 1.8 $\mu$, the $D$ values were not interpretable because of masking by the strong absorption by $D_2O$.

Previous measurements of difference spectra in the infrared have been carried out by Saroff, *et al.*[16]

## Results

**Model Compounds.**—In order to be able to identify the absorption peaks due to OH and NH groups and to see whether the peaks could be resolved, the near infrared spectra of 1% (v:v) solutions of methanol and aniline, respectively, in $CCl_4$ were determined using cells of 1 cm. path length. The absorption bands at wave lengths corresponding to twice the fundamental stretching frequency[6] are shown in Fig. 1A and 1B. The peaks occur at wave lengths[17] of 1.40 $\mu$ for the OH and 1.45 $\mu$ and 1.50 $\mu$ for the NH groups, respectively. These wave lengths presumably correspond to nonhydrogen-bonded OH and NH groups. If the concentrations of methanol and aniline are increased, the ratio of optical density at the peak to concentration decreases, *i.e.*, the band is depressed and the absorption at higher wave lengths (> 1.50 $\mu$) increases. This effect of increasing concentration on the spectrum probably is due to the formation of intermolecularly hydrogen-bonded aggregates.[6] Even at a concentration of 1% there is evidence that Beer's law does not hold, the deviations being greater for methanol than for aniline. These effects also have been discussed by Kaye.[6]

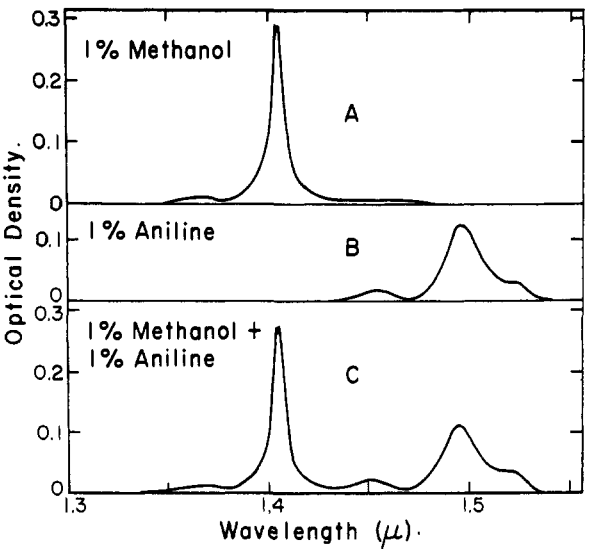


Fig. 1.—Near infrared spectra of model compounds in $CCl_4$ solution in 1 cm. quartz cells.

In a mixture of methanol and aniline these peak wave lengths are essentially unchanged, the spectrum being a superposition of those for the separate components (Fig. 1C). The slight deviations from additivity probably arise from the fact that Beer's law does not hold exactly for the separate components.

From the data of Fig. 1, it can be seen that nonhydrogen bonded OH and NH peaks can be resolved in the near infrared.

**Poly-γ-benzyl-L-glutamate.**—In order to obtain data on a *hydrogen bonded* NH group, measurements were carried out on PBG in DCE, a solvent in which PBG is in the helical configuration.[20] Chloroform would have been preferred to DCE as the solvent, in order to reduce the peaks due to CH-stretching overtones. However, at the concentrations required for observing the spectra, the viscosity of the solution in $CHCl_3$ was too high[21] for convenient work. The spectrum is shown (Fig. 2A) in the region where there is no interference from the absorption by the solvent. It should be noted that the wave length of maximum absorption has been shifted to 1.54 $\mu$, compared to 1.50 $\mu$ observed for aniline. This shift arises from the fact that the NH groups are hydrogen-bonded to CO groups in the helical configuration.[20,21] If this shift is indeed due to hydrogen bonding, then the

(12) The extinction coefficients were found to be 0.715 cm.²/g. for lot no. 381-059 and 0.790 cm.²/g. for lot no. 647-213.

(13) During the assay in $H_2O$, the hydrogen content of the proteins increases, the limit for RNase-$H_{20}$ being Rnase-all H and that for RNase-all D being RNase-$D_{20}$.

(14) J. Hermans, Jr., and H. A. Scheraga, unpublished results.

(15) We are indebted to Prof. R. L. Sproull of the Physics Department for making this instrument available to us.

(16) H. A. Saroff, N. R. Rosenthal, E. R. Adamik, N. Hages and H. A. Scheraga, *J. Biol. Chem.*, **205**, 255 (1953).

(17) The peak at 1.40 $\mu$ corresponds to OH stretching, the fundamental absorption occurring at 2.76 $\mu$[6]; those at 1.45 $\mu$ and 1.50 $\mu$ correspond to antisymmetrical and symmetrical stretching modes[6,18] of the $NH_2$ group, respectively.

(18) In reference 6, an alternative explanation is given for the origin of the peaks at 1.45 $\mu$ and 1.50 $\mu$. The former is assigned to the non-hydrogen bonded and the latter to the hydrogen bonded (due to association) NH-stretching modes. This alternative explanation cannot be correct for the following reasons: (1) The double-peak spectrum persists at lower concentrations where intermolecular hydrogen bonds would disappear, and (2) The NH-stretching mode of aniline gives rise to a double peak spectrum at 2.87 $\mu$ and 2.93 $\mu$ under conditions where no hydrogen bonds are formed[19]; the first overtones can therefore be expected at 1.44 $\mu$ and 1.47 $\mu$, in good agreement with our observations.

(19) A. M. Buswell, J. R. Downing and W. H. Rodebush, THIS JOURNAL, **61**, 3252 (1939).

(20) P. Doty and J. T. Yang, *ibid.*, **78**, 498 (1956).

(21) P. Doty, J. H. Bradbury and A. M. Holtzer, *ibid.*, **78**, 947 (1956).

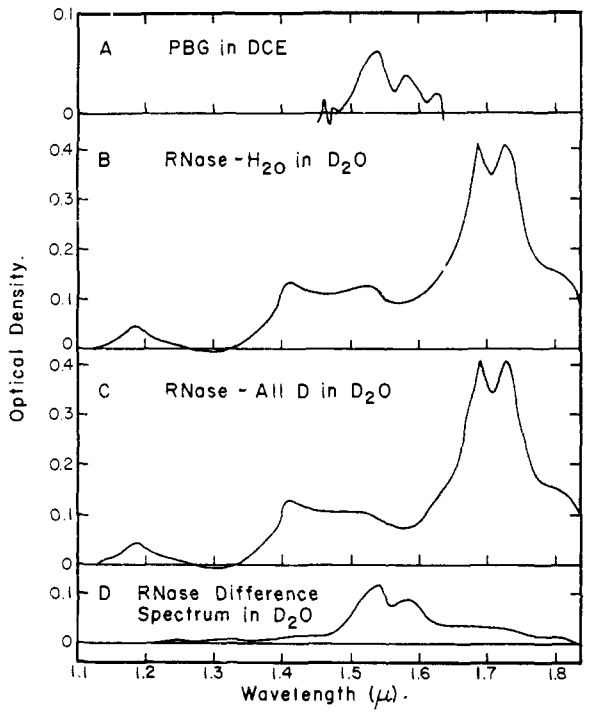


Fig. 2.—Near infrared spectra of PBG in DCE and of ribonuclease in $D_2O$. The light path was 10 cm. in A, 1 cm. in B and C, and 2 cm. in D. In D, RNase-all D in $D_2O$ is the reference for the measurement of RNase-$H_{20}$ in $D_2O$. Experiment 2 of Table I gave curve A, experiment 6 gave curves B and C, and experiment 5 gave curve D.

wave length 1.54 $\mu$ should be close to half that for the fundamental absorption of hydrogen-bonded NH groups. In support of this it may be noted that a value of 3.05 $\mu$ has been reported for a great variety of proteins[22]; this value is close to twice that found here for the overtone in PBG.

Spectral shifts, *i.e.* changes in the wave length at which the absorption has its maximum, vary with the nature of the acceptor and donor group forming the hydrogen bond. Absorption due to other hydrogen-bonded groups can, therefore, be expected at a large number of wave lengths, and we have not attempted to determine these from model compounds other than PBG.

**Ribonuclease.**—If ribonuclease were placed in $H_2O$, we would expect to observe free OH and NH frequencies at 1.40 $\mu$ and 1.50 $\mu$ and hydrogen-bonded OH and NH frequencies at somewhat higher wave lengths. However, $H_2O$ itself has a strong absorption band at 1.45 $\mu$, thus making observation difficult. If, on the other hand, $D_2O$ is used as the solvent, the solvent absorption becomes large only at wave lengths above 1.8 $\mu$. Thus, it is possible to detect OH and NH groups which do *not* exchange their hydrogens in $D_2O$. The samples RNase-$H_{20}$ and RNase-all D are, therefore, suitable for this type of experiment.

The spectra of RNase-$H_{20}$ in $D_2O$ and of RNase-all D in $D_2O$ (solutions of experiment No. 6 of Table I) are shown in Fig. 2B and 2C. There is a general absorption at all wave lengths in this region and the peaks are diffuse. This diffuseness arises because there are numerous groups in the ribonuclease molecule, each with its own fundamental frequency; therefore, there is a still greater variety of overtones and combinatory frequencies. In fact, this same problem arises in the interpretation of infrared measurements on proteins[22] in the longer wave length region (> 3 $\mu$).

In order to study the absorption of a single kind of group, it is necessary to eliminate the effects of absorption by the other groups. This can be accomplished by measuring a difference spectrum[16]; *i.e.* the spectrum of RNase-$H_{20}$ in $D_2O$ can be measured with respect to that of RNase-all D in $D_2O$, the protein concentration being the same in both solutions. The 20 hydrogens in RNase-$H_{20}$ should then appear as the only absorbing groups whereas the corresponding 20 deuteriums of RNase-all D will be masked by the OD absorption of the solvent.[23] The result of this experiment is shown in Fig. 2D, the solutions being those of experiment No. 5 of Table I. The only peak which occurs is a double line with maximum intensity at 1.54 $\mu$, the shape being practically the same as that of the NH band in PBG (Fig. 2A). The close similarly between the spectra of Figs. 2A and 2D supports the interpretation[4,5] that the 20 hydrogens of RNase-$H_{20}$ are parts of amide groups and, furthermore, are hydrogen-bonded to the oxygen atoms of carbonyl groups. It is presumed that these NH. . .OC bonds are involved in a helix. In other words, these 20 hydrogens are probably part of the backbone hydrogen system. This is reasonable since a cooperative set of hydrogen bonds, as exists in a helix, would be much stronger (accounting for their inability to exchange their hydrogens for deuterium) than the individual hydrogen bonds which one would encounter here and there among the side-chain groups.[24]

Further support for this interpretation may be obtained by comparing the extinction coefficients $\epsilon$ measured for the three compounds: aniline, PBG and ribonuclease. The values of $\epsilon$ were obtained by dividing the measured optical density at the maximum by the concentration, the latter being the number of moles of hydrogen atoms (in one liter) which contribute to the absorption. For ribonuclease the concentration was taken as 20 times the molar concentration of the protein. For PBG the concentration was taken as that of the monomer unit; this ignores the effects of the chain ends but this error is negligible since the degree of polymerization was very large (about 700). The results are shown in Table I. The baseline ($D$ = 0) for the optical density data was taken as the horizontal portion of the recording on the lower wave length side of the absorption band in order to compensate for instrumental artifacts at high optical density which appear as absorption by ribonuclease and $D_2O$ in the difference spectra and by DCE in the spectrum

(22) M. Beer, G. B. B. M. Sutherland, K. N. Tanner, and D. L. Wood, *Proc. Royal Soc. (London)*, **A249**, 147 (1959).

(23) In principle, the absorption at the overtones and the absorption at the frequencies which are produced by combining those of the 20 hydrogens with those of the rest of the molecule will be present. However, in practice, the intensities of the combinatory frequencies are so small that only the overtones are measured.[6]

(24) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

TABLE I

CHARACTERIZATION OF[a] THE NEAR INFRARED SPECTRA DUE TO THE FIRST OVERTONE OF THE NH-STRETCHING MODE

| Expt. no.[b] | Compound | Solvent | $d$ (cm.) | $M$ | $n$ | $c$ (g./ml.) | $D$[d] | $\epsilon$ (cm.$^2$/mole) | $\lambda_{max}$ ($\mu$) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Aniline | $CCl_4$ | 1 | 93 | 2 | 0.0102 | 0.13 | 590 | 1.50 |
| 2 | PBG | DCE | 10 | 219 | 1 | .0135 | .061 | 99 | 1.54 |
| 3 | PBG[c] | $CHCl_3$ | 10 | 219 | 1 | .0040 | .020 | 109 | 1.54 |
| 4 | RNase-$H_{20}$ (lot 647-213) | $D_2O$ | 1 | 13,683[e] | 20 | .13 | .014 | 74 | 1.54 |
| 5 | RNase-$H_{20}$ (lot 647-213) | $D_2O$ | 2 | 13,683[e] | 20 | .309 | .107 | 118 | 1.54 |
| 6 | RNase-$H_{20}$ (lot 381-059) | $D_2O$ | 1 | 13,683[e] | 20 | .194 | .025 | 88 | 1.54 |

[a] $d$ = length of light path; $M$ = molecular weight; $n$ = number of absorbing hydrogen atoms per group of weight $M$; $c$ = concentration; $D$ = optical density measured at $\lambda_{max}$, the wave length where the maximum occurs; $\epsilon$ = extinction coefficient defined by the equation $\epsilon = DM/ncd$. [b] Experiments 1, 2, 3 are direct spectra, and 4, 5, 6 are difference spectra. [c] This measurement was made with the Beckman DK, all others with the Cary spectrophotometer. [d] In comparing data for PBG and RNase-$H_{20}$, experiments 2 and 5, having the highest $D$-values, are the most accurate. [e] Value from ref. 25.

of PBG. For this reason it is difficult to evaluate the experimental error in the values of $\epsilon$ reported in Table I. If it were of the order of 10%, then the $\epsilon$-values of PBG and RNase-$H_{20}$ would be the same within the experimental error.

## Discussion

From the results reported here it appears that the "hard core" involving 20 hydrogen atoms in RNase-$H_{20}$ has a near infrared absorption spectrum which is of the same form, and very nearly the same intensity (within experimental error), as that of the NH groups in PBG.[26] The value of $\lambda_{max}$ is 1.54 $\mu$ in both cases. This is greater than the value of $\lambda_{max}$ for aniline (1.50 $\mu$), and this increase is in agreement with the observation that hydrogen-bonded NH groups in proteins absorb at 3.05 $\mu$,[22] whereas the non-hydrogen-bonded NH peak of aniline occurs at 2.93 $\mu$.[19] Therefore, the first overtones should, in first approximation, occur at half these wave lengths, i.e. at 1.53 $\mu$ for ribonuclease and PBG and at 1.47 $\mu$ for aniline. The slight discrepancies between these calculated values and our observed values are due to anharmonicity.[6]

Inspection of Table I reveals that the extinction coefficients per contributing hydrogen atom of the hydrogen-bonded NH group in PBG or ribonuclease are very much smaller than that of the free NH group of aniline. This phenomenon arises from two effects. In the first place, the integrated absorption, obtained by integrating $\epsilon$ as a function of frequency over the entire absorption band, is lower when the group is hydrogen-bonded than when it is not.[28] In the second place, hydrogen-bonded bands have a greater width than non-hydrogen-bonded ones.[28] From a comparison of the spectra of Fig. 1B with Figs. 2A and 2D, it can be seen that the aniline band is much narrower than those for PBG and ribonuclease. The areas under the peaks corresponding to those of Fig. 1B and Fig. 2A were determined by graphical integration on a scale of $\epsilon$ *vs.* $\lambda$.[29] The area under the peak corresponding to Fig. 1B was approximately 2.5 times as large as that corresponding to Fig, 2A. This means that the integrated absorption is about 2.5 times as large for the free NH group as it is for the hydrogen-bonded NH group, a result which is comparable to the lowering (on hydrogen-bond formation) which Mecke found for a series of other cases.[29] This provides additional evidence that the NH group is free in aniline and hydrogen-bonded in PBG and RNase-$H_{20}$ in our experiments.

The low value of $\epsilon$ for the hydrogen-bonded NH group sets limitations to the way in which near infrared spectroscopy can be applied to the study of protein structure. In order to obtain reliable results it is essential that there be a relatively large number of NH groups per unit weight of protein. The 20 groups of ribonuclease are probably the lower limit at which such measurements can yield useful information. Even at that, high concentrations (31%) and a long path length (2 cm.) were required to obtain an optical density as high as 0.1.

These experiments on ribonuclease were complementary to a kinetic study of deuterium-hydrogen exchange carried out previously.[3] It should also be feasible to use the near infrared technique to study the kinetics of deuterium–hydrogen exchange, *i.e.* of the reaction

$$NH + D_2O \rightleftharpoons ND + HDO$$

In such a measurement a difference spectrum should be used, *i.e.* the reference solution should contain the fully deuterated protein in $D_2O$ at the same concentration. The intensity of the hydrogen-bonded NH peak decreases and the HDO spectrum appears with time. The form of the HDO spectrum (not shown here) was determined with a mixture of $H_2O$ and $D_2O$, containing a trace of $H_2O$ (6 mg. $H_2O$ per ml.), measured against pure $D_2O$. On the basis of this spectrum, and those reported here, it is possible to detect both the NH and HDO peaks to follow the exchange reaction. Measurements of this kind should make it possible to distinguish hydrogens bound to nitrogen from those bound to oxygen or sulfur, and should be a useful adjunct to the method of Linderstrøm-Lang,[30] which detects the *total* number of exchangeable hydrogens.

The kinetics of the conversion of RNase-$H_{20}$ to

(25) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **219**, 623 (1956).

(26) The solvents used for PBG and RNase are different; nevertheless, the extinction coefficients are similar. This is a reasonable result since the absorbing groups are probably not accessible to the solvent. In support of this we cite these observations: (1) there are 20 hydrogens in ribonuclease which do not exchange in $D_2O$ below the transition temperature[8]; (2) the backbone hydrogens of PBG exchange extremely slowly with a deuterated solvent as long as the molecules are in a helical form.[27]

(27) A. Elliott and W. E. Hanby, *Nature*, **182**, 654 (1958).

(28) R. Mecke, *Discussions Faraday Soc.*, **9**, 161 (1950).

(29) The integration was carried out on a wave length instead of a frequency scale. This is justified since the variation in wave length over a band, or between two bands, is very small, and high precision is not required.

(30) K. Linderstrøm-Lang, "Symposium on Peptide Chemistry," Special Publications of the Chemical Society (London), *No. 2*, 1 (1955).

RNase-all D at 49° have been determined by the technique described above, and will be reported in the near future.

**Acknowledgment.**—We are indebted to Dr. W. D. Cooke for calling our attention to the usefulness of the overtone region.

---

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

# The α-Chymotrypsin Catalyzed Hydrolysis of *p*-Nitrophenyl Acetate[1a]

BY MARIO A. MARINI[1b] AND GEORGE P. HESS

RECEIVED AUGUST 28, 1959

By the criteria of kinetic, chemical and spectroscopic studies of the α-chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate, there appears to be at least two stable monoacetyl-α-chymotrypsin intermediates, AC-A and AC-I, in the catalytic reaction. AC-I is kinetically identical to the normal acyl intermediate at *p*H >6.2. At *p*H 5.0, AC-I can rearrange to give AC-A which is identical to the monoacetyl-α-chymotrypsin isolated by Balls and Wood but kinetically, chemically and spectroscopically different from AC-I. Experiments on the chemical reactivity of AC-A suggest that it is homogenous. On the assumption that the aliphatic hydroxyl group of serine and the imidazoyl group of a histidine residue participates in the catalytic reaction, a structure for the active site of AC-A is proposed which would account for all the data presented here.

The kinetics of the α-chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate has been interpreted by Gutfreund and Sturtevant in terms of three catalytic steps.[2] After the formation of an enzyme–substrate complex, a monoacyl enzyme capable of isolation[3] is formed, follwed by the decomposition or hydrolysis of this complex. The hydrolysis of specific substrates for α-chymotrypsin probably involves the same three step mechanism as the hydrolysis of *p*-nitrophenyl acetate.[4,5]

Many investigators have sought the nature of the group or groups involved in the catalytic reaction. Both an aliphatic hydroxyl group of serine[6] and an unprotonated imidazoyl group of a histidine[7] residue have been implicated. The evidence that serine is present[8,9] in a single active site[10] is fairly clear. The evidence for histidine is largely indirect[11,12] and of a more controversial nature.[13,14]

Direct nucleophilic attack by an unprotonated imidazoyl nitrogen on the substrate might form the N-acylimidazoyl derivative[15] on the enzyme surface. Evidence for this view is derived, in part, from studies of the phosphorylated derivatives of chymotrypsin.[16] The phosphorylated chymotrypsin is dephosphorylated with faster rates under acidic conditions which is easily explained if imidazole is the phosphorylated group. Objections to this mechanism have been raised. Dixon and Neurath[17] were unable to detect a species absorbing at 245 mμ, characteristic for N-acetyl imidazole,[18] during the formation of the monoacetyl enzyme in the reaction of chymotrypsin with *p*-nitrophenyl acetate at low *p*H. Cunningham[19] also objected to this mechanism on the basis that the heat of ionization of the rate determining group in the acylation of chymotrypsin is 11 kcal. mole$^{-1}$,[20] while only 6–7 kcal. would be expected if a direct attack of a normally ionizing imidazoyl nitrogen were involved. He proposes an alternate mechanism which proceeds by nucleophilic attack of a serine hydroxyl group which is activated by hydrogen bonding to imidazole. Hydrolysis of the acyl-enzyme occurs by attack of another nucleophilic reagent. This nucleophilic reagent may be the imidazoyl group of histidine which would then form an N-acyl imidazole and then hydrolyze rapidly in water.[21,22]

Dixon and Neurath,[21] from their spectroscopic studies, found that the isolated monoacetyl chymotrypsin was deacylated at high *p*H with an accompanying increase in absorption at 245 mμ and noted that the absorption maximum and the rate of de-

(1) (a) This work was aided by grants from the United States Public Health Service and the National Science Foundation.(b) Post-doctoral Fellow, National Heart Institute, United States Public Health Service.

(2) H. Gutfreund and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U. S.*, **42**, 719 (1956); *Biochem. J.*, **63**, 656 (1956).

(3) A. K. Balls and H. N. Wood, *J. Biol. Chem.*, **219**, 245 (1956).

(4) T. Spencer and J. M. Sturtevant, THIS JOURNAL, **81**, 1874 (1959).

(5) H. Gutfreund and B. R. Hammond, *Biochem. J.*, **73**, 526 (1959).

(6) N. K. Schaffer, S. C. May, Jr., and W. H. Summerson, *J. Biol. Chem.*, **202**, 67 (1953); N. K. Schaffer, L. Sinet, S. Harshman, R. R. Engle and R. W. Drisko, *ibid.*, **225**, 197 (1957); H. Gutfreund and J. M. Sturtevant, *Biochem. J.*, **63**, 756 (1956); R. A. Oosterbaan, P. Kunst and J. A. Cohen, *Biochem. Biophys. Acta*, **16**, 299 (1955); G. H. Dixon, S. Go and H. Neurath, *ibid.*, **19**, 193 (1956); R. A. Oosterbaan, H. S. Jansz and J. A. Cohen, *ibid.*, **20**, 402 (1956).

(7) B. S. Hartley, *Biochem. J.*, **64**, 27p (1956); L. A. Mounter, H. C. Alexander, 3rd, K. D. Tuck and L. T. H. Dien, *J. Biol. Chem.*, **226**, 867, 873 (1957); T. Wagner-Jauregg and B. E. Hackley, Jr., THIS JOURNAL, **75**, 2125 (1953); D. G. Doherty and F. Vaslow, *ibid.*, **74**, 931 (1952).

(8) J. A. Cohen, R. A. Oosterbaan, H. S. Janaz and F. Berends, *J. Cell. Comp. Physiol.* (Supp. 1), **54**, 231 (1959).

(9) R. A. Oosterbaan and M. E. van Adrichem, *Biochem. Biophys. Acta*, **27**, 423 (1958).

(10) E. F. Jansen, M. D. F. Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189 (1949); M. W. Loewus and D. R. Briggs, *ibid.*, **199**, 857 (1952); A. K. Balls and E. F. Jansen, *Adv. Enzymol.*, **13**, 321 (1952).

(11) B. R. Hammond and H. Gutfreund, *Biochem. J.*, **61**, 187 (1955).

(12) L. Weil, S. James and A. R. Buchert, *Arch. Biochem. Biophys.*, **46**, 266 (1953).

(13) J. R. Whitaker and B. J. Jandorf, *J. Biol. Chem.*, **223**, 751 (1956); V. Massey and B. S. Hartley, *Biochem. Biophys. Acta*, **21**, 361 (1956); B. S. Hartley and V. Massey, *ibid.*, **21**, 58 (1956).

(14) B. Atkinson and A. L. Green, *Trans. Faraday Soc.*, **53**, 1334 (1957).

(15) F. H. Westheimer, *Proc. Natl. Acad. Sci. U. S.*, **43**, 969 (1957).

(16) A. L. Green and J. D. Nichols, *Biochem. J.*, **72**, 70 (1959); B. J. Jandorf, H. O. Michel, N. K. Schaffer, R. Egan and W. H. Summerson, *Discussions Faraday Soc.*, **20**, 134 (1955); F. Hobbiger, *Brit. J. Pharmacol.*, **11**, 295 (1956).

(17) G. H. Dixon, W. J. Dreyer and H. Neurath, THIS JOURNAL, **78**, 4810 (1956).

(18) E. R. Stadtman, in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, eds. Johns Hopkins Press, Baltimore, Md., 1954, p. 581.

(19) L. W. Cunningham, Jr., *Science*, **125**, 1145 (1957).

(20) L. W. Cunningham, Jr., and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956).

(21) G. H. Dixon and H. Neurath, THIS JOURNAL, **79**, 4558 (1957).

(22) I. Tinoco, Jr., *Arch. Biochem. Biophys.*, **76**, 148 (1958).