1969

A Spectrophotometric Study Of Ribonuclease In The Middle Ultraviolet (220-250 M Mu)

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A SPECTROPHOTOMETRIC STUDY OF RIBONUCLEASE IN THE MIDDLE ULTRAVIOLET (220-250 mμ)

by

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Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Canada

October, 1968

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr. Charles Bigelow for his guidance and encouragement during the course of this study. Thanks are also due to Mrs. Sudha Sreenathan for performing the experiments with pepsin. The author wishes to thank Miss Janice Davidson for the typing of the thesis. Thanks are also due to Dr. Clifford L. Herzig and Dr. V. S. Ananthanarayanan for helpful discussions and advice during the course of this project. Lastly the author would like to thank his devoted wife Mary Ann and daughter Karen for their encouragement throughout the last four years.

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ABSTRACT

Spectral studies on Ribonuclease and small molecules related to the constituent chromophores of the protein have been carried out in the middle ultraviolet (220-250 mμ). The chief aims of the study have been:

a. to measure and interpret the spectral shifts which occur in this region when the solvent is changed;

b. to determine which groups are mainly responsible for the spectral shift which occurs in this region when the protein is denatured;

c. to see whether middle ultraviolet difference spectroscopy is a useful technique for structural studies of proteins.

In order to interpret the solvent effects observed with the protein it was necessary to measure the solvent effects which can also be observed for the constituent chromophores when they are small molecules. A considerable amount of numerical data of this sort, not hitherto available, was collected. The small molecules studied include tyrosine, histidine, phenylalanine, tryptophan and compounds

containing peptide bonds. The effects turn out to be qualitatively similar to the effects observed on protein absorption bands in the near ultraviolet (250-300 mμ), but the numerical data were required in order to understand the effects seen with the protein. Solvent effects were measured for ribonuclease both in the native state and in the completely denatured state. Calculations from the small compound data showed that the protein solvent effects can be accounted for within about ten percent which is very close considering some of the assumptions that are required.

In order to account for the solvent effects on the protein (which give a maximum extinction difference at 227 mμ) one must include the effect of the solvents on tyrosyl and histidyl residues and on the peptide bonds. On the other hand, the spectral effects caused by denaturation (which occurs maximally at 237 mμ) can be interpreted in terms of the tyrosyl residues alone. This is in part fortuitous -- histidine absorbs at 237 mμ, but in this particular protein all four of the histidyl residues appear to be exposed to the environment even in the native state, and hence do not undergo any change in their absorption properties when the protein is denatured. No contribution from peptide bonds seems to be required to account for the denaturation blue shifts at 237 mμ.

Our data also show that middle ultraviolet spectroscopy can be useful in studying protein denaturation. In particular, it has been

possible to show that three different denatured conformations of the ribonuclease molecule, previously studied by near ultraviolet spectroscopy, viscometry, and polarimetry, can also be characterized in the middle ultraviolet. This has permitted the determination of values of $\Delta\epsilon_{237}$ for the individual tyrosyl residues which are normalized in the different denaturation processes.

INTRODUCTION

It is of course well known that proteins absorb ultraviolet light. In the past few years many workers have carried out investigations aimed at explaining the general nature of protein spectra, and also at developing ultraviolet spectroscopy as a useful structural technique. This thesis reports on another such study, one designed to explore the feasibility of spectral studies in the region between 220 and 250 mμ. The results presented here make it look as if protein spectra in this region can be semi-quantitatively interpreted in terms of the spectra of the component chromophores; and further, they show that spectroscopy in this region can give structural information about protein molecules. There are both advantages and disadvantages to spectroscopy in this region. These will be discussed in the text that follows.

Throughout this thesis we will be dealing with common amino acids, and unconjugated proteins. Neither of these groups of compounds absorb light above about 300 mμ, so all work to be discussed involves absorption below this wavelength.

For convenience in writing, and it is hoped, in reading this

thesis, the ultraviolet region has been arbitrarily divided into four regions, named as follows:

250-300 mμ:	near ultraviolet
220-250 mμ:	middle ultraviolet
180-220 mμ:	far ultraviolet
below 180 mμ:	vacuum ultraviolet

This thesis is mainly concerned with the spectra of model compounds and bovine pancreatic ribonuclease (RNase) in the middle ultraviolet. It will however be necessary to make frequent references to the regions called "near" and "far".

The spectra of the important amino acid chromophores are shown in fig. 1, along with the spectrum of a fairly typical globular protein, RNase.

The protein spectrum has a maximum about 280 mμ, and a minimum about 250 mμ, and below that it rises again to a maximum at 190 mμ. As can be seen from the spectra of tryptophan, tyrosine, and phenylalanine, and as has been appreciated for nearly one hundred years, the 280 mμ absorption band in a protein is due to the presence of aromatic chromophores in the molecule.

Below about 250 mμ the absorption of the protein rises steeply. Here again the aromatic amino acids absorb strongly. But then so does histidine. Less important chromophores are cysteine,

FIGURE 1a

The absorption spectra of RNase and the chromophoric amino acids that are important in the middle ultraviolet. (a) RNase, (b) phenylalanine, (c) tyrosine, (d) tryptophan, and (e) histidine. The spectrum of histidine was reproduced; from the thesis of Dr. McDiarmid (1965), with the author's permission. The values for the RNase curve below 220 mμ were taken from Rosenheck and Doty (1961). The remaining values were determined by the author.

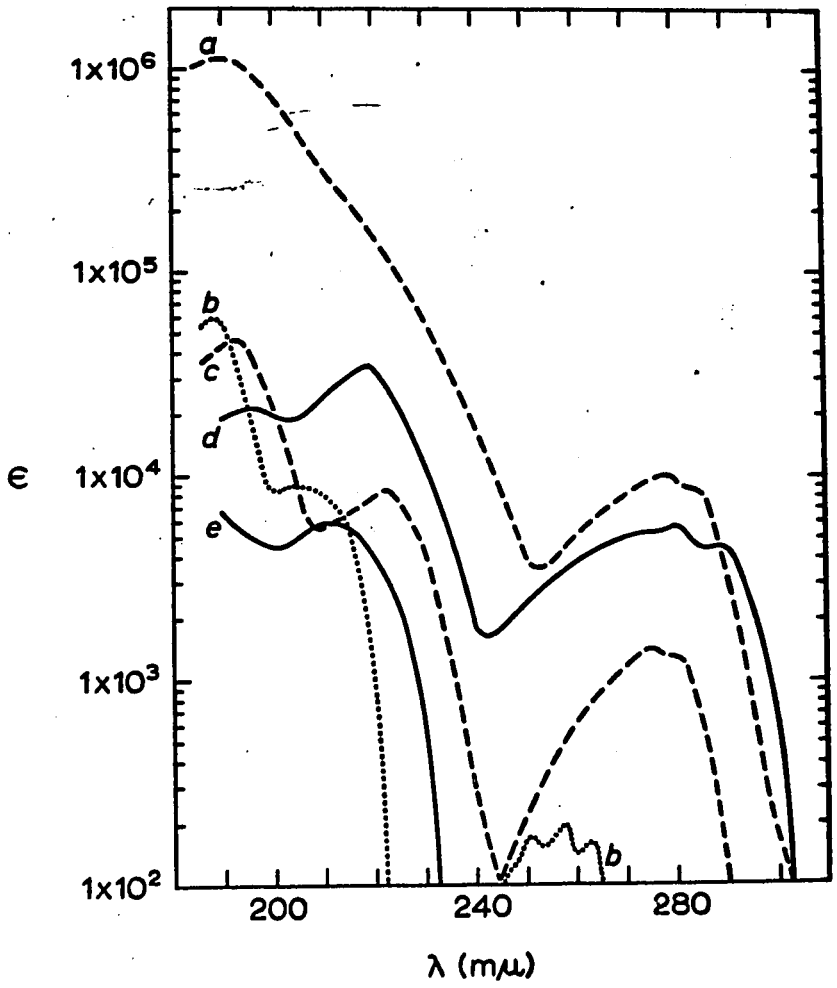
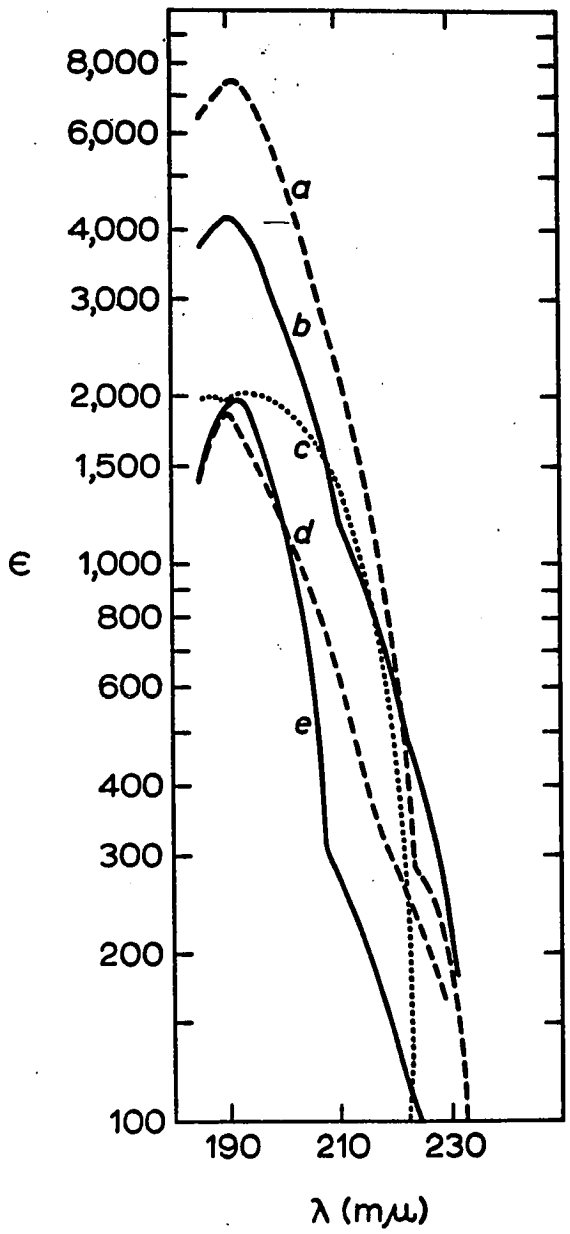


FIGURE 1b

The absorption spectra of the chromophoric amino acids that are important in the far ultraviolet. (a) peptide bond in random coil, (b) peptide bond in α-helix, (c) methionine, (d) cystine, and (e) cysteine. These spectra were taken from the thesis of Dr. McDiarmid (1965) with her permission.



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cystine, and methionine. The peptide bond with an intense absorption band at 190 mμ is also a very significant chromophore in the middle ultraviolet range.

THE NEAR ULTRAVIOLET

As is obvious from figure 1, the chief amino acids which contribute to protein absorption at 280 mμ are tryptophan and tyrosine. RNase, for which a spectrum is also shown in fig. 1, contains 6 tyrosine residues and no tryptophyl residues. Its molar extinction at 277.5 mμ, 9800, is only roughly equal to 6 times the molar extinction of tyrosine at 275 mμ, 1400. Proteins containing tryptophan of course have higher extinctions, but the general features of their spectra are qualitatively similar to that of RNase.

The spectra shown in figure 1 exemplify a commonly observed feature of protein spectra, which is that the magnitude and position of protein absorption bands can only be estimated approximately from the magnitudes and positions of the spectra of the constituent chromophores. The investigation of the origins of these discrepancies has provided valuable information about the conformation of proteins.

To understand these discrepancies we must digress to a simple review of the effects of environment on chromophores in the ultraviolet region. In the ultraviolet region due to the magnitude of the transitions, we know that they are electronic transitions.

Of course the electronic transitions are accompanied by changes in the vibrational and rotational states.

The electronic states are sensitive to changes in their environment. This can be seen if we take the vapor spectrum of a chromophore, we will produce a series of sharp peaks, clustered in a few regions of the spectrum. This is the closest that the absorption spectrum of a complex chromophore comes to the line spectra of metals. If we study the chromophore, dissolved in a hydrocarbon solvent, the sharp peaks are found to be broadened and the clustered peaks become merged into broad peaks with irregular prominences on their surfaces. Finally, if the chromophore is studied in a polar solvent, e.g., water, the prominences disappear under one broad band. The only remnant of the fine structure is the occasional shoulder seen in absorption bands as for example the 280 mμ shoulder in tyrosine. The effects seen with the chromophore are due to interactions between the solvent and states of the transition being studied. In the examples above the effects seen are due to the solvent blurring the vibrational and rotational states associated with this particular electronic transition.

The environment may effect the transition in three ways. First, it may raise or lower one of the states of the electronic transition thus causing the absorption band to shift up and down

the spectrum. Second, it may change the oscillatory strength of the band thus changing the magnitude of the absorption band. Third, it may interact with the rotational and vibrational transitions thus causing changes in the fine structure of the absorption band. The first of these three has proven the most valuable to protein chemist. The analysis of spectral shift has been developed into a precise conformational tool, because of the ease of measurement.

In proteins the formation of the peptide bond causes a small shift, but this shift does not account for the major effect observed. Therefore the folding of the polypeptide chain into the native protein must affect the local environment of the chromophores. If this is true then the unfolding of the protein during denaturation should cause a shift in the spectrum toward the predicted value. This is observed in the denaturation of globular proteins. Analysis of the shift has provided information on the environment in the interior of the protein.

THE MIDDLE ULTRAVIOLET

In the middle ultraviolet, as is obvious from fig. 1, the chief chromophores, which contribute to protein spectra are tryptophyl, tyrosyl, histidyl, phenylalanyl residue sidechains, and the peptide bond. RNase, for which a spectrum is also shown in fig. 1, contains 6 tyrosyl residues, 4 histidyl residues, 3 phenylalanyl

residues, 124 peptide bonds, and no tryptophyl resides, for a total of 137 chromophoric groups.

RNase has no maximum in the middle ultraviolet range, but its molar extinction increases in this range from a value of 3000, at 250 mμ to a value of 150, 000, at 220 mu. As can be seen from table 1 if one attempts to "predict" the absorption of RNase by summing extinctions for the constituent chromophores, a value of 119, 000 is obtained (at 223 mμ) which can be compared with the measured value of 130, 000. It should be noted that 91% of the absorption is approximately due to the presence in the protein of two types of chromophores, tyrosine and the peptide band. Even though the molar extinction of a single peptide bond is small, it is obvious that a large fraction of the absorption of a protein in the middle ultraviolet will be due to this chromophore, because every protein contains so many of them. Table I shows that the peptide bond causes about half of the molar extinction of RNase at 223 mμ, more than any other type of chromophore. Tyrosine is the second most important chromophore.

The discrepancies noted between the calculated and measured value of the molar extinction coefficient at 223 mμ have several origins. Several of the factors involved are similar to those found in the near ultraviolet range. They are the incorporation of the chromophoric amino acid in a polypeptide and also the

TABLE 1

CONTRIBUTION TO RIBONUCLEASE ABSORPTION AT 223 mμ

Chromophore	No. of Residues	ε 223	Contribution	Percent of Total
Tyrosine	6	8,200	49,200	41.0
Histidine	4	2,500	10,000	8.4
Phenylalanine	3	150	500	0.4
Peptide bonds	124	480	59,500	50.0
Totals	137		119,200	99.8

folding of the polypeptide chain into a protein. To these effects must be added one that was not found in the near ultraviolet. In fig. 2, taken from the thesis of McDiarmid (1965), it can be seen that the absorption bands of the chromophores change magnitude and position as they are moved from a random coil to a α-helix. It can be seen in fig. 1 that even the peptide bond is affected by the helix to coil transition.

THE FAR ULTRAVIOLET

The major chromophoric groups in the far ultraviolet range are the tyrosyl, tryptophyl, histidyl, phenylalanyl, methionyl, cysteinyl, cystyl, arginyl residue sidechains, and the peptide bond. RNase contains 6 tyrosyl residues, 3 phenylalanyl residues, 4 histidyl residues, 4 cystyl residues, 4 methionyl residues, 4 arginyl residues, 124 peptide bonds, no cysteinyl residues, and no tryptophyl residues, for a total of 149 chromophoric groups. As can be seen from fig. 1 RNase has a maximum at 190 mμ with a molar extinction of 1, 150, 000. As can be seen from table 2 two calculated values for RNase at 190 mu are 1, 300, 000 and 1, 120, 000; depending on which values are used for the absorption of the peptide bond at 190 mμ.

The peptide bond is the most important chromophore causing about 60% of the absorption. The aromatic chromophores (tyrosyl

TABLE 2

CONTRIBUTION TO RIBONUCLEASE ABSORPTION AT 190 mμ

Chromophore	No. of Residues	ε190	Contribution	Percent of Total*	Percent of Total**
Side Chain					
Tyrosine	6	35,500	213,000	16.0	19.0
Phenylalanine	3	53,300	160,000	12.0	14.0
Histidine	4	5,800	23,200	2.0	2.0
Cystine	4	3,800	15,200	1.0	1.0
Methionine	4	1,900	7,600	0.5	0.7
Arginine	4	12,450	49,800	4.0	4.0
Subtotal	25		468,800	35.5	40.7
Peptide bonds	124				
a. α-Helix(20%)	25	4,200	105,000	8.0	
Random Coil	99	7,300	722,700	56.0	
Total	149		1,300,000	99.5	
b. α-Helix(58%)**	72	4,100	295,200		26.0
Random Coil	52	6,900	358,800		32.0
Total	149		1,120,000		98.7

* Percent α-Helix taken from Kartha et al (1967), ε values taken from McDiarmid (1965).

** Percent α-Helix and ε values taken from Rosenheck and Doty (1961).

FIGURE 2a

The absorption spectra of the tyrosyl side chain in (■) an α-helix, (o) a random coil, and (---) the free amino acid. These spectra are reproduced from the thesis of Dr. McDiarmid (1965), with her permission.

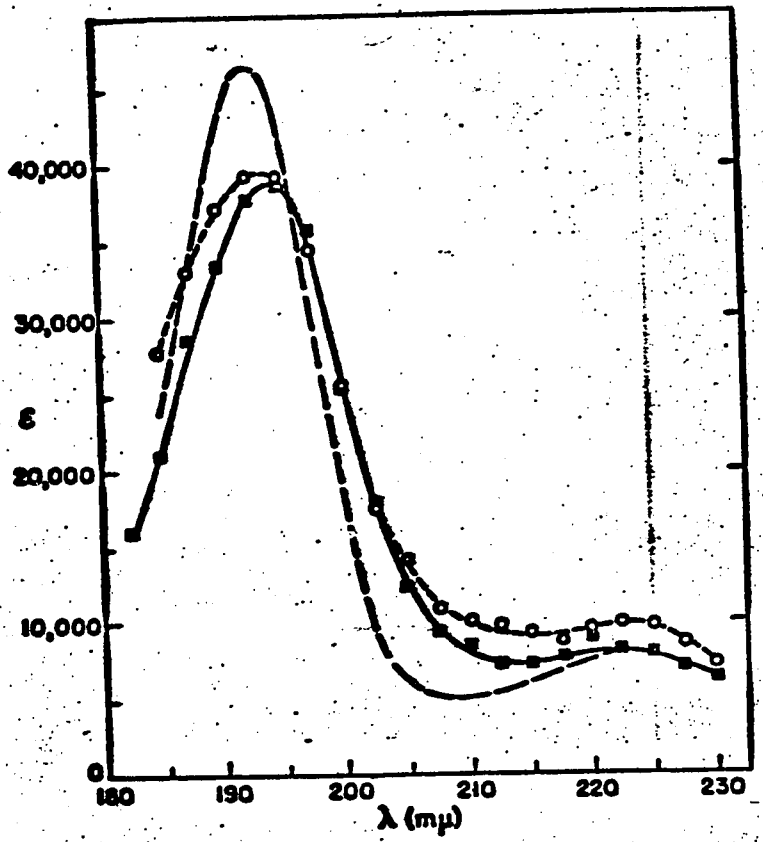
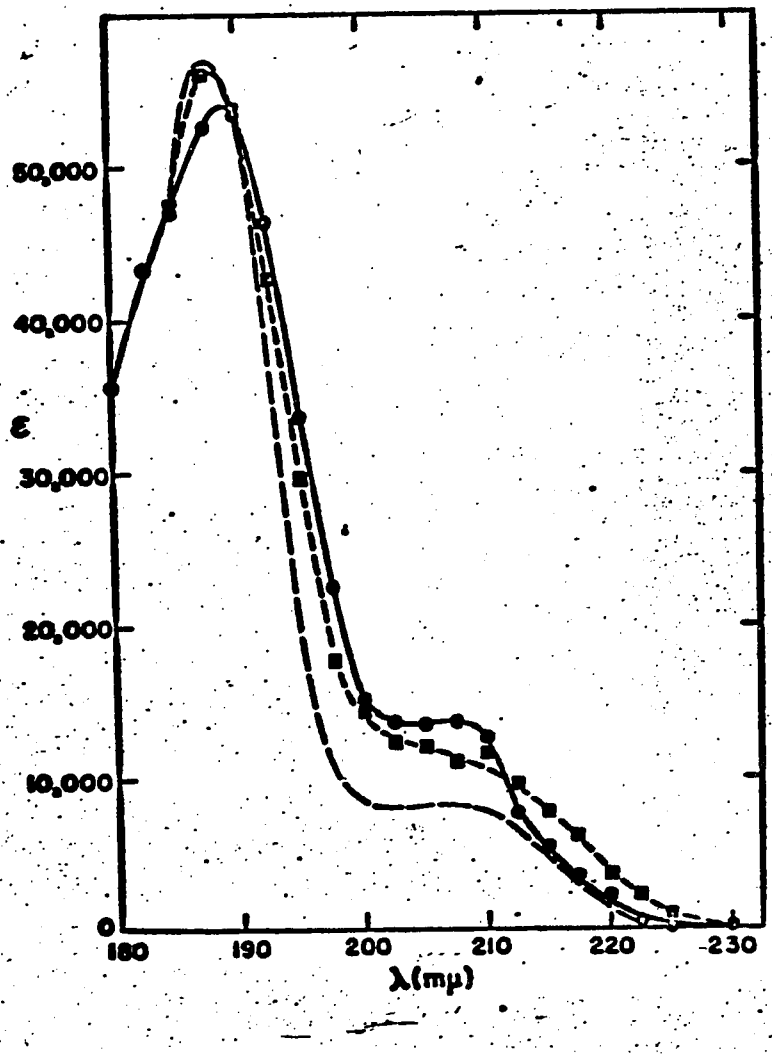

40,000
30,000
ε
20,000
10,000
0
180
190
200
210
220
230
λ (mμ)

FIGURE 2b

The absorption spectra of the phenylalanyl side chain in (o) an α-helix, (■) a random coil, and (----) the free amino acid. These spectra are reproduced from the thesis of Dr. McDiarmid (1965) with her permission.


50,000
40,000
ε
30,000
20,000
10,000
0
180
190
200
210
220
230
λ(mμ)

and phenylalanyl) are also important causing about 30% of the absorption. Together these three chromophores account for about 90% of the absorption at the 190 mu peak.

According to Wyckoff, Hardman, Allewell, Inagami, Johnson, and Richards (1967) 30% of the peptide bonds in RNase are in the β-structure. However the β-structure was ignored in this analysis even though it has been shown that the absorption band of β-structure differs from that of the random coil and α-helix. Insufficient data are available at present to evaluate this contribution, so in this analysis it has been treated as random coil, because its absorption band most closely resembles that of the random coil.

The factors which affect the chromophores and are responsible for the variation between the measured and calculated molar extinction coefficient are the same in the far ultraviolet range as the factors previously mentioned for the middle ultraviolet range, e. g., the helix-coil transition.

DENATURATION

During denaturation spectral shifts and other changes in the absorption bands of proteins are observed in all three ranges of the ultraviolet. As was previously mentioned, in the near ultraviolet range, the shift measured can be interpreted to obtain information about the interior of the protein molecule, for example the

types of chromophoric groups which are buried and maybe their numbers. Because the positions of the leading edge (the long wavelength side of the absorption band) of the tyrosine and tryptophan absorption bands are different, it is possible by studying the wavelengths of the maximum $\Delta\epsilon$ of the spectral shifts during denaturation to determine if the buried residues are tyrosyl, tryptophyl, or both. By studying the magnitude of the shifts it has been possible in a few cases to calculate the number of buried residues exposed during denaturation.

In the middle ultraviolet, it is not possible to separate the tyrosyl from the tryptophyl shifts during denaturation because the absorption bands and the leading edge of the bands of these two chromophores overlap. As a result it is not only impossible to separate the tyrosyl from the tryptophyl band shifts during denaturation but it is difficult to assign the magnitude of the shift to either chromophore.

To complicate this picture, in the middle ultraviolet, we must also take account of the absorption of the peptide bond and histidine. Even though their molar extinctions are small in this range, and therefore a spectral shift will produce a corresponding small value of $\Delta\epsilon$, still they must not be ignored. This is particularly true of the peptide bond because of the large number of peptide bonds present which may contribute to the observed spectral effects.

Because of these complications it is safe to say that denaturation studies in the middle ultraviolet range should be accompanied by similar studies in the near ultraviolet. Without the information available from studies in the near ultraviolet, it is most unlikely that a convincing interpretation of the middle ultraviolet data will be achieved.

The far ultraviolet even though it is complicated by the appearance of many chromophores is a very interesting range to work in. This is because of the large absorption of the peptide bond in this range and also the large difference, approximately 300 per peptide bond, between the absorption of the peptide bond in the α-helix and the random coil. This large difference makes the peptide bond an important chromophore to be considered during denaturation. Because of this large difference, the change in the absorption spectrum in the far ultraviolet has been used to calibrate the helix-coil transition even though 35 to 40% of the absorption in the far ultraviolet is due to other chromophores.

CONCLUSION

The middle ultraviolet range of the absorption spectrum is important because it will give us information that will support the work in the near ultraviolet range. It will also provide us with information on the histidyl residues. Since the histidyl residue

has been found in the active centers of many enzymes and implicated in their modes of action, it is important to study this residue to a greater extent than has been accomplished to date.

In this study we have attempted to study this range of the absorption spectrum of RNase and its chromophoric groups so that clear deductions can be drawn from its absorption spectrum in this range.

We began this study with a survey of the major chromophores and related models in this range. We then set out to study the effect of environment of the chromophores and related compounds. This is accomplished by adding additives that would simulate the environment conditions that we were trying to study.

We then studied the effects of various solvents on the chromophores on the surface of the RNase molecule. Protein denaturation studies were then undertaken to study the chromophores buried in the protein and also the environment in which they were buried. The project would thus explore completely the possibility of conformation analysis of protein structure in the middle ultraviolet.

HISTORICAL REVIEW

In the midfifties there was generated a great deal of interest in the subject of protein spectroscopy. This was due largely to two causes. The first of these was that it was found that protein denaturation could be followed spectrophotometrically in the near ultraviolet range. The second was the improvement in spectrophotometers e. g., the development of sensitive matched photomultipliers, improved light sources, and so on, which allowed the small spectral changes to be measured accurately.

With the availability of improved spectrophotometers protein chemists became able to measure a difference spectrum directly. A difference spectrum is simply the difference between two absorption spectra of a chromophore in different environments. The direct measurement of a difference spectrum presents a far more accurate evaluation of a small spectral shift than the individual measurement of the two absorption spectra followed by the subtraction of one from the other.

PROTEIN DENATURATION (1955-1960)

In the period between 1955 and 1960 several studies showed that the denaturation of proteins was accompanied by a shift of the near ultraviolet absorption peak to shorter wavelengths, "the denaturation blue shift," as it later was called. There appears to be two possible causes of such a blue shift in the near ultraviolet. One possible cause is the breaking of hydrogen bonds involving the chromophoric group. This appears to be a plausible explanation of some of the spectral shifts observed with tyrosyl containing proteins, but it cannot be said to be clearly proved even with them. Another possible cause is the effect of transferring residues from the nonpolar interior of the protein to an aqueous environment.

Chervenka (1959) showed clearly that the difference spectra observed in protein work, were due largely to shifts of the 280 mμ absorption band, and that they were not also caused by significant changes in the intensity of the bands. He did this simply by calculating the difference spectrum that would be produced by small shifts in the spectra of tyrosine and tryptophan. The resulting calculated difference spectra closely resembles those observed with proteins.

Scheraga (1957) presented the first analytical approach to

the factors responsible for the spectral shifts obtained during denaturation. Shugar (1952) and Tanford, Hauenstein, and Rands (1955) had previously shown that three of the six tyrosyl residues appear to be involved in interactions that modify their ultraviolet light absorption, and also that they are involved in some form of interaction that modifies their ionization behavior. Blumenfeld and Levy (1958) showed that in concentrated urea, all six tyrosyl residues ionize normally. It seemed a reasonable possibility that tyrosyl hydrogen bonds are responsible for the modified behavior of three tyrosyl residues.

Scheraga's experiments were motivated by the belief that the abnormal tyrosyl groups could be hydrogen bonded to carboxylate groups in the protein, and he reasoned that if so, titration of the carboxylates involved should be detectable by an affect on the tyrosyl spectrum. He therefore looked for a spectral shift resulting from the addition of acid to the solution, and he discovered a denaturation transition which occurred between pH 2. 5 and 1. 0. This evidence was therefore presented as supporting the existence of tyrosyl-carboxylate hydrogen bonds in the native molecule.

Williams and Foster (1959) proposed an alternative explanation for the spectral effects being observed on denaturation. They found that they could be accounted for reasonably quantitatively if

they were due to changes in dielectric constant of the medium surrounding the tyrosyl residues involved, that is, to a "solvent effect." The effects produced are complex and have two possible explanations, one based on the dipole moment of the medium (which they called the D effect), and the other based on the electric polarizability of the medium (the P effect) the latter of which appears to be more important. Both effects would operate in the same direction as rupture of hydrogen bonds, that is, they would cause a blue shift of the absorption band as the protein was denatured. This is important, because, while tyrosine may take part in hydrogen bonds, it is unlikely that tryptophan does. And since many of the effects observed with proteins are caused by tryptophyl chromophores, it is necessary to find an alternative explanation.

Other works have studied the effects of environment in causing the spectral shifts observed in protein denaturation and have advanced our understanding of this phenomenon, but it is still true that it is not possible to decide from spectral data alone whether a given tyrosine blue shift is caused by hydrogen bond-breaking or a solvent effect. Some of this work is discussed at length elsewhere in this thesis.

MODEL STUDIES

Because of the interest in protein spectra produced by the

reports just discussed, various investigators began studies of simpler systems in the hope that they would provide data which would help to explain the spectral effects observed with proteins. In the case of protein chromophores the natural starting point is the amino acids that contain the chromophoric sidechains. In the near ultraviolet range these would include tyrosine, tryptophan, and phenylalanine. The two effects mentioned in the previous section have been studied to a varying extent, with such model compounds. The studies will be reviewed in the chronological order in which they were investigated, first the medium effect, second the hydrogen bond effect.

The effect of solvent composition on amino acid chromophores was first studied by Bigelow and Geschwind (1960) and Yanari and Bovey (1960). Both groups studied many aspects of this problem, but only a few of their results will be cited here. Bigelow and Geschwind (1960) found that sometimes the value of the spectral shift observed when an additive was added to the solvent could be correlated very simply with the refractive index of the mixed solvent. For example almost identical difference spectra were observed for tyrosine using urea solutions and sucrose solutions of the same refractive index. They also showed that the value of the spectral shift (or, more strictly, the value of $\Delta\epsilon$ at the maximum of the

difference spectrum) was a linear function of the concentration of the additives they used. This linearity indicates that the effect is due to some bulk property of the solvent, such as refractive index or dielectric constant, and is not due to a specific interaction between the model compounds and the additive. This behavior is now commonly observed when organic solvents or solutes are used as the additives.

Bigelow and Geschwind also noted, however, that when salts were used as the additives they behaved in a different way quantitatively and qualitatively. Sodium salts, for example, did not obey the same quantitative relation to the refractive index as the organic compounds. Lithium salts actually caused blue shift solvent effects, while all the other compounds studied caused red shift solvent effects.

With O-methyltyrosine, sodium salts were in quantitative agreement with the organic solvents and the lithium salts gave red shifts, like the other compounds, even though smaller for the same refractive index. These results were interpreted to mean that tyrosine interacted specifically with sodium and lithium ions in some way to give a spectral blue shift. Presumably this would be independent of the red shift caused by the bulk effect. With the lithium salts the blue shift predominated, while with the sodium salts, the red shift predominated, indicating that the lithium ion interacts

more strongly with the phenolic chromophore than the sodium ion. The experiments with 0-methyltyrosine indicated that the specific interactions can be essentially abolished for sodium ions and much reduced for lithium ions, by 0-methylation, and this points to the phenolic hydroxyl as the group responsible for the specific interaction.

Yanari and Bovey (1960) also investigated the effects on amino acid chromophores caused by changing the medium. They also demonstrated that the magnitude of the spectral shift observed when amino acid chromophores are transferred from water into different hydrocarbons could be correlated with the refractive index of the hydrocarbon. This, they inferred, was due to the dispersive and dipole polarization forces of the solvent.

When the polar solutes indole and phenol are placed in salt solutions, for example in 4 M LiBr, it was found that the absorption band of indole did not shift while that of phenol shifted to the blue. Dispersive and dipole polarizations would predict shifts to the red in both cases, hence such causes cannot account for these results. Yanari and Bovey surmised that the orientation strain, due to the high degree of orientation of the solvent cage, nearly equals and opposes the previous two effects to provide the results observed.

Subsequently, Foss (1960) studied similar systems to those of Bigelow and Geschwind (1960) and Yanari and Bovey (1960). Foss's

conclusions agree in all essentials with those of the previous two papers.

Sarfare and Bigelow (1967) have recently carried out a further study of solvent effects in tyrosine caused by LiBr. As pointed out above, lithium salts gave unusual spectral shifts with tyrosine, and Sarfare and Bigelow found also that the effect was not a linear function of the LiBr concentration. The non-linearity is consistent with the specific interaction between lithium ion and the phenolic chromophore which was inferred earlier. A similar curved relationship was observed when LiBr was added to performic acid oxidized RNase.

Hydrogen bond formation was briefly discussed by Yanari and Bovey (1960). They found that the absorption bands of benzene, phenol, and indole all undergo blue shifts of about 1 mμ on passing from isooctane into water. "This result makes it appear probable that neither orientation strain nor hydrogen bonding are important in determining the spectral peak positions for phenol or indole in water." Ethanol has a refractive index that is between that of isooctane and water. On transferring a solute from isooctane to ethanol one would then expect a shift to the blue, slightly smaller than that of transferring the solute from isooctane to water. Benzene conforms to this rule and for benzene we draw the conclusion that

refractive index is the important factor in determining spectral shift when it is transferred to ethanol. But phenol and indole do not follow this pattern when they are transferred from isooctane to ethanol; instead their absorption bands are shifted to the red. This indicates that in ethanol, phenol and indole are affected by another influence besides refractive index. Such an influence could be hydrogen bonding.

Grinspan, Birnbaum, and Feitelson (1966) carried out a more thorough study of the effects of hydrogen bonds on the absorption spectra of amino acids. They used phenylalanine as the model for a non-hydrogen bond former and tyrosine for a hydrogen bond former, and reasoned that any difference in shifts of the bands between tyrosine and phenylalanine must be due to the hydroxyl group. These differences could then be explained as either interactions of the polar solute with a polar solvent, or effects due to hydrogen bonding, or both. They found, as usual, that red shifts are observed when the solvent is made less polar. Theory predicts that they should shift to the blue when the solvent is made less polar if the interactions are polar solute-solvent interactions. Thus the data point to the conclusion that the differences observed are due to hydrogen bond formation. This conclusion is strengthened by the finding that a curved line is found if the wavelength of the absorption band maximum

is plotted against concentration of organic solvent. The curvature indicates that some specific interaction, like bond formation or breakage, is involved in the process.

Grinspan et. al. found that the solvents tested could be separated into three groups. The first group consisted of acids, including water; the members of this group caused equal spectral shifts with tyrosine and phenylalanine. The second group consisted of the alcohols, and these caused a shift with tyrosine which was more than twice the size of the shift observed with phenylalanine. The third group consisted of ethers, and these caused a shift with tyrosine which was almost three and a half times the shift observed with phenylalanine.

Grinspan et. al. found that they could separate the shift due to the hydroxyl group by subtracting the phenylalanine spectral shift from that of tyrosine. When this correction had been performed it was found that the ethers had twice the effect for the same concentration as did the alcohols.

The fundamental equation for calculating the energy changes associated with a spectral transition is the Planck equation:

$$E = h\nu$$

where E is the energy change in ergs per molecule, h is Planck's constant, and ν is the frequency at which the transition is observed.

On conversion to kcal per mole and cm^{-1} the equation becomes:

$$E = hcN\nu$$

This equation can also be written in a form suitable for making calculations from spectral shifts:

$$\Delta E = hNc\Delta\nu' \text{ or } E = 2.86 \times 10^{-3} \Delta\nu' \qquad \text{(kcals/mole)}$$

Here ΔE is the difference in the energy of the transition under two different conditions and $\Delta\nu'$ is the spectral shift in cm^{-1}.

The shift produced by the interaction of alcohols with the hydroxyl group according to Grinspan et. al. is 145 cm^{-1} in the near ultraviolet and when this is substituted into the equation a value of .41 kcal per mole for the shift is calculated, (the authors report a value of .5 kcal) and .45 kcal for the same shift in the middle ultraviolet. The comparable values for the ethers are .9 and 1.3 kcal.

THE MIDDLE ULTRAVIOLET RANGE

The results of protein conformational studies carried out by difference spectroscopy in the middle ultraviolet range must still be interpreted with great caution because of the many chromophores which absorb in this range. In the near ultraviolet range protein studies were relatively simple because there are only two major chromophores, tyrosine and tryptophan, which absorb in this range. But in the middle ultraviolet we must also take account of absorption by histidine, the peptide bond, and possibly phenylalanine.

Glazer and Smith (1960) were the first investigators to

demonstrate the existence of a large blue spectral shift which occurs in the middle ultraviolet during the denaturation of proteins. In the near ultraviolet range, as explained in the preceding section, spectral shifts found during denaturation could be explained as being due to the exposure of buried sidechain chromophores. This effect may also be important in the middle ultraviolet, as Glazer and Smith showed that tryptophan, tyrosine, and histidine have large acid difference spectra in this range. However, they also showed that in the middle ultraviolet a second general effect may be important; this is the effect of secondary structure e. g., α-helix and random coil, on the absorption band of the peptide bond. This conclusion was based on studies they carried out with poly-l-glutamic acid. In these studies they found a difference spectrum on comparing the helical form of the molecule to the random coil form. The difference spectrum exhibited a peak at 225 mμ with $\Delta\epsilon_{225}$ per residue of 85.

In 1961, Glazer and Smith extended their studies on the effect of denaturation on the spectral difference peak at 230 mμ. They found, among other things, that while most proteins produced a peak in this region, on denaturation, pepsin did not, even though a difference spectrum was observed for pepsin in the near ultraviolet.

They also studied the time-dependence of the denaturation of ovalbumin by urea and guanidine hydrochloride and they found there

was a difference between the rate constant for $\Delta\epsilon$ 232.5 on the one hand and $\Delta\epsilon$ 287.5 or $\Delta\epsilon$ 292.5 on the other. The last two were essentially synchronous.

These results led Glazer and Smith to the conclusion that the 230 to 240 mμ difference spectrum peak could not be attributed solely to changes in the environment of the aromatic amino acid sidechains. This work was the first which pointed to the possible utility of the middle ultraviolet range in studies of protein structure.

In 1963, Scott and Scheraga (1963) studied the kinetics of the acid and temperature denaturation of RNase. They found that the form of the rate curves at 235 mμ is the same as at 287 mμ but the rates are generally slower than those at 287 mμ. The difference in rates indicated to them that although they were observing the same portions of the protein being unfolded at both wavelengths, they were monitoring the unfolding by following changes in the absorption of two different groups of chromophores. They believed that the groups involved were the tyrosyl residues in the near ultraviolet and the peptide bonds in the middle ultraviolet.

In 1964, Kabacoff and Laken (1964) studied the kinetics of chymotrypsin denaturation by middle ultraviolet difference spectroscopy. They found that the change in the 231.5 mμ difference peak during heat inactivation was synchronous with the change in the 293 mμ difference peaks, and with the inactivation of the enzyme.

This would obviously imply that the 231. 5 and 293 mμ difference peaks are due to the same chromophores, rather than different ones. This was the first investigation to challenge the conclusion of Glazer and Smith (1961) that the two difference peaks had different origins.

Martin (1964a) reinvestigated the kinetics of ovalbumin denaturation in urea. He attempted to verify the conclusion of Glazer and Smith (1961) that the kinetics of denaturation in the middle ultraviolet were different from those in the near ultraviolet, but he found that the 233. 5 mμ difference peak was synchronous with the 287 and 293 mμ peaks. He also found that two reactions were occurring, and that both could be observed in both regions of the ultraviolet spectrum. The rate constant for the fast reaction, which was first order, was the same at both wavelengths.

McDiarmid (1965) studied the important chromophores in the middle and far ultraviolet from 185 to 230 mμ and the effect of various conditions on them. Copolymers of glutamic acid and tyrosine or phenylalanine were used to study the influence of the formation of the peptide bond on the absorption of the tyrosyl and phenylalanyl sidechains. McDiarmid's results on the effect of peptide bond formation on the sidechain absorption are shown in figure 2. From these results it can be seen that a small intensification results from this process.

McDiarmid (1965) also attempted to estimate the change in

extinction of the peptide bond during the helix to coil transition in the following way. Performic acid oxidized RNase was taken as a model for a random coil molecule and paramyosin as a model for a completely helical molecule. McDiarmid measured the extinctions of these proteins and subtracted from them contributions for the absorption of the sidechain chromophores, to obtain values for the absorption of the peptide bond in the random coil and α-helix (see fig. 1). From her results, one can subtract, one set of values from the other to obtain a difference spectrum which is found to have a maximum at 225 mμ. The maximum has a value of 110 extinction units, which compares favorably with the value of 110 obtained by Glazer and Rosenheck (1962), and with the value of 85 measured by Glazer and Smith (1961).

The effects of denaturation on the absorption bands of chromophoric sidechains in this region has been studied in a preliminary way by several investigators. After the study of Glazer and Smith (1960) the next paper to appear was that of Hermans (1962). He was interested in studying the denaturation of various proteins in the alkaline pH range. In the alkaline pH range the tyrosine sidechains ionize producing a large change in the absorption spectrum of tyrosine and tyrosyl-containing proteins in the near ultraviolet. This ionization process has been used to measure tyrosyl content even in the presence of large amounts of tryptophan (Chervenka, 1959).

Hermans found that ionization of the tyrosyl sidechain also produced a large change in absorption in the middle ultraviolet range, giving a difference peak with a maximum at 245 mμ. He was able to study the denaturation of the heme proteins by following this difference peak. The value of the difference peak for tyrosine at 245 mμ was 10, 000. The value obtained for the various heme proteins varied from 9, 700 to 12, 100 per tyrosyl residue.

Eisenberg and Edsall (1963) studied the alkaline and acid spectrophotometic titration of human serum albumin and reduced carboxymethylated albumin in the middle ultraviolet range. They found that in all of the acid titrations the ratio between the 236 mμ and the 287 mμ peak was 3. 3 $\pm$ 0. 25. For the alkaline denaturation the peaks were at 244 and 295 mμ, but here the ratio was 4. 6 $\pm$ 0. 15. The ratio in the alkaline denaturation process compares favorably with ratios found by Riddiford, Stellwagen, Mehta, and Edsall (1965) working with human carbonic anhydrases B and C. These workers found the ratio to vary between 5. 2 and 5. 5. According to Eisenberg and Edsall, these results provide evidence that the 236 and 287 mμ peaks in the acid denaturation, and the 244 and 295 mμ peaks in the alkaline denaturation have the same origin, that is, they are both caused by changes in the environment of the aromatic amino acids, in this case tyrosine.

In a series of papers, Martin and Bhatnagar (Martin, 1964b;

Martin and Bhatnagar, 1966, 1967) have explored the denaturation of chymotrypsin and some derivatives in the middle ultraviolet range. Martin found that besides the large trough at 231 mμ there was a large peak at 224 mμ in the difference spectrum accompanying the urea denaturation of α-chymotrypsin. This difference spectrum was found to disappear on dilution of the urea, that is, the denaturation was reversible.

Martin and Bhatnagar showed that a model mixture of the amino acid chromophores present in chymotrypsin produces a large red shift centered at 228 mμ when the spectrum in 8 M urea is compared with its spectrum in water. N-methylacetamide used as a model compound for the peptide bond, produces a small red shift centered at 220 mμ under the same conditions.

Martin and Bhatnagar also studied the denaturation of α-chymotrypsin and DIP-chymotrypsin in urea and guanidine hydrochloride. They found that in concentrated solutions of either of these denaturants unfolding occurs at lower concentrations in α-chymotrypsin than in DIP-chymotrypsin. In urea or guanidine hydrochloride at low concentrations, for example up to 3. 5 M urea, the spectrum of either protein was shifted to the red and $\Delta\epsilon_{231}$ was found to be a linear function of the denaturant concentration. At higher concentrations, up to 6 M urea, of denaturant this red

shift was reversed until it became a blue shift which reached its largest value in 6 M urea, after which another red shift was found. This behavior is of course qualitatively identical with the behavior typically observed in the near ultraviolet. Martin and Bhatnagar plotted $\Delta\epsilon_{231}$ against the denaturant concentration and corrected it for solvent effects by extrapolating to zero denaturant concentration using the method of Bigelow (1960). This procedure gave a value for the denaturation blue shift at 231 mμ of 35, 000 for both proteins in both denaturants. At 293 the corrected value of the denaturation blue shift is 10, 000, so the ratio of the values $\Delta\epsilon_{231}/\Delta\epsilon_{293}$ is 3. 5, quite close to the value observed by Eisenburg and Edsall (1963) in their work with serum albumin.

Martin and Bhatnagar found that if one plotted the fraction of molecules denatured against concentration of urea at any pH, the change at 293 mμ develops in phase with that for 231 mμ. They showed that the rate constants for the unfolding of DIP-chymotrypsin in 8 M urea are the same over the pH range 4 to 9 at 231 mμ and 293 mμ. All of these facts support the argument that both difference peaks arise from the same chromophores.

Martin and Bhatnagar also estimated the value of $\Delta\epsilon_{293}$ per tryptophyl residue produced by transferring the tryptophyl residue from a medium of the same refractive index as the interior

of a protein into water. If this value is multiplied by four, the number of buried tryptophyl residues exposed during denaturation, (Williams, Herskovits, and Laskowski (1965), and Williams and Laskowski (1965)) it accounts for the blue shift observed in the near ultraviolet during denaturation. In the middle ultraviolet according to their values the shift due to the four tryptophyl residues explains 90% of the blue shift observed during denaturation at 231 mu. The method of calculation and results found in this paper will be examined later in this thesis, however it is enough to say that their conclusion, that at least 90% of the denaturation blue shift at 231 mu is due to the aromatic amino acids, is correct.

McDiarmid (1965) carried out a study of the effect on their middle ultraviolet spectra of moving amino acid chromophores from an aqueous environment into the interior of detergent micelles. In this study she measured the absorption spectra of various amino acid chromophores in the presence of 0. 1 M sodium dodecyl sulfate. The detergent in this molarity forms micelles, the interior of which McDiarmid used for a model of the interior of protein molecules, and the nonpolar amino acids are selectively absorbed into the micelles. She found, as one would expect, that the absorption spectra of these chromophores shift to the red when they are moved from water to the detergent medium. Thus, the same explanation used for the near ultraviolet spectral shifts, namely that

they are essentially due to solvent effects, can also be applied to the middle ultraviolet shifts.

Subsequently, Edsall, Mehta, Myers, and Armstrong (1966) studied the structure and denaturation of human carbonic anhydrases B and C in urea and guanidine hydrochloride by middle ultraviolet spectroscopy. In all the difference spectra measured no solvent effect red shift was observed, even at very low concentrations of the denaturant, there was either no shift (at the lowest concentration), or a denaturation blue shift (at higher concentrations). This indicated to them that all aromatic amino acid chromophores are buried in the native protein. This conclusion is supported by the earlier results of Riddiford et. al. (1965) and Riddiford (1965).

Edsall and his colleagues also found in kinetic experiments that the peak at 235 mμ in the difference spectrum develops synchronously with those at 285 and 292 mμ.

As discussed in the section on tyrosine, two factors account for the magnitude of a difference spectral extremum. One is the v alue of the slope ($d\epsilon/d\lambda$) of the spectrum and the other is the magnitude of the spectra shift ($\Delta\lambda$). Edsall et. al. (1966) determined from the literature the values of $d\epsilon/d\lambda$ for the free amino acids tryptophan, tyrosine, and histidine at 230 mμ. Since the absorption bands of the chromophores will be shifted to the red when they are buried, they assumed that the $d\epsilon/d\lambda$ values for the free amino

acids at 230 mμ would correspond to the $d\epsilon/d\lambda$ values for the same chromophores at 235 mμ in the protein. They assumed that all tyrosyl, tryptophyl, and histidyl residues in carbonic anhydrases B and C are buried. They then summed up the $d\epsilon/d\lambda$ values for the various residues in both proteins. This provided them with a value for that part of the slope of the protein spectrum which is accounted for by the sidechain chromophores alone (that is, excluding the peptide bonds). By dividing this value into $\Delta\epsilon_{235}$, observed in a denaturation difference spectrum they were able to estimate the value of $\Delta\lambda$ (see the discussion section on tyrosine) needed to account for the desired difference spectrum. They found that a shift of 2.5 mμ could be enough, and pointed out that this is well within the range expected during denaturation. They purposely ignored the contribution of the peptide bonds because they were attempting to demonstrate that the sidechain chromophores could account for most of the denaturation blue shifts observed in the middle ultraviolet. They concluded that their results justified such interpretation.

In all the work just discussed, the attention had been concentrated on the aromatic sidechains and peptide bond. In 1965, Donovan carried out the first significant study of imidazole absorption in proteins.

As can be seen in Figure 1, histidine has an absorption peak

at 212 mμ with a molar extinction of 6, 000. As can be seen from Table 1, the four histidyl residues in RNase account for about 8. 4% of the absorption at 223 mμ. While this is small compared to the absorption due to tyrosyl residues and the peptide bonds in RNase, which together account for 91% of the absorption at 223 mμ, it is the third most important chromophore at this wavelength and for an accurate analysis it should not be ignored. However because of its small size very careful experiments must be designed if one is to measure the effect of changes of the medium on the imidazole absorption in proteins. Such changes can only be studied if the background is constant (that is, where the medium changes do not affect the other chromophores) or if the background changes are very small, and can be corrected for very accurately. This obviously means that it will be difficult to study the effect of changing solvents on imidazole absorption.

Donovan (1965) showed that in spite of these difficulties it was possible to carry out a spectrophotometric study of the titration of the four imidazole groups in RNase. The reasons for this are, of course, that the imidazole groups titrate between pH 4 and 7, which is well below the pH range where the tyrosyl residues ionize, and is also a pH range in which the native protein structure is unaltered by changes of the pH. Donovan found that his experimental data were consistent with two of the imidazole groups having pH's

of 6.2 and two of 5.6. This is the only work to date to deal with the imidazole residues of a protein by spectroscopy.

This section has dealtwith those investigations which have made significant contributions to our understanding of the origins of the middle ultraviolet difference spectra observed with proteins. For the sake of completeness, several other investigations, in which middle ultraviolet difference spectroscopy has been employed in an empirical fashion, but in which little or no effort was expended on interpretation are listed here: Donovan (1964), Rickli, Ghazanfar, Gibbons and Edsall (1964), Riddiford (1964, 1965), Libor, Elodi, and Nagy (1965), Polet and Steinhart (1968), and Reynolds, Herbert, and Steinhart (1968).

RIBONUCLEASE

The physical chemical properties of bovine pancreatic ribonuclease have been intensively studied for the past fifteen years. Much of this immense volume of work has been found to agree reasonably well with the structure of RNase A determined by X-ray crystallography by Kartha, Bello, and Harker (1967) and the structure of RNase-S completed by Wyckoff et. al. (1967).

In the following pages we will review some of the important physical-chemical studies that preceded the structural studies just mentioned.

It was first noted by Shugar (1952) who studied the spectrophotometric titration of RNase in the alkaline range, that three of the six tyrosyl residues titrate only after irreversible denaturation above pH 12, and he concluded that they must be buried inside the molecule, or otherwise prevented from easy titration. These experiments were repeated by Tanford, Hausenstein, and Rands (1956) whose results agree with those of Shugar.

In 1958, Blumenfeld and Levy (1958) carried out the first spectral titration of a protein in 8 M urea and they reported that all six of the tyrosyl residues in RNase titrate reversibly with a pK of 10.67. These results demonstrate that in 8 M urea all of the tyrosyl residues are normal, that is, exposed to the environment.

ACID-TEMPERATURE DENATURATION

The acid and temperature denaturation of RNase and their inter-relationship is now a well explored area of RNase denaturation. Many studies have also demonstrated the effects that changes in other parameters, e.g., ionic strength, can have on this denaturation transition.

In 1956, Harrington and Schellman using optical rotation measurements, showed that RNase undergoes a reversible heat-induced transition with a midpoint at 58°C at pH 6.5.

In 1957, Scheraga showed that there is a denaturation transition,

which he followed by difference spectroscopy at 287 mu, in the acid range between pH 2. 5 and 1. 0. This acid transition occurs over a quite narrow pH range, and is reversible.

In 1961, Bigelow demonstrated an inter-relationship between acid and temperature denaturation. Scheraga (1957) had shown a plot of $\Delta\epsilon$ versus pH over the acid transition gave a sigmoid shaped curve. Bigelow showed that this low pH transition does not occur in solutions of RNase which had previously been boiled, and in which the RNase had been irreversibly denatured. This demonstrates that the same region of the protein molecule is unfolded in both processes and that temperature has an effect on the acid transition.

Bigelow also compared the difference spectra produced when he compared irreversibly acid- and alkali-denatured protein with the native protein. He found that the irreversibly alkali-denatured protein produced a larger denaturation blue shift than the irreversibly acid-denatured protein. One can also correlate the size of the denaturation blue shift with the number of tyrosyl residues exposed during the denaturation. Since he had demonstrated by alkaline titration that the irreversibly acid-denatured protein has 5 exposed tyrosyl residues, then the irreversibly alkali-denatured protein must have all three previously buried tyrosyl residues exposed.

The interrelationship of temperature and pH on the denaturation process in RNase was further studied by Hermans and Scheraga (1961a, b). They found that temperature had two effects on the acid transition. The first of these is that increasing temperature increases the transition pH and the second effect is that the magnitude of the acid transition was strikingly dependent on the temperature. Below 35°C the magnitude of the transition increases as the temperature increases. Between 35 and 40°C the magnitude is independent of the temperature. And finally, above 40°C the magnitude decreases as the temperature increases. These results can be interpreted as follows. The molecule can be caused to unfold (as shown by Bigelow (1961)) to the same extent either by raising the temperature at neutral pH or by lowering the pH at T = 35-40°C. If the acid transition is studied below 35°C, one does not achieve the same extent of denaturation, so that below 35°C $\Delta\epsilon$ increases as T increases. Above 40°C the reference solution, at "neutral pH" (6. 5), begins to unfold too, with the result that $\Delta\epsilon$ apparently decreases as T increases.

Hermans and Scheraga also studied the effect of pH on the heat denaturation of RNase. When $\Delta\epsilon_{287}$ is plotted against the temperature in an experiment like this one, one obtains a sigmoid shaped curve with values of $\Delta\epsilon$ rising in the temperature range where denaturation is occurring and reaching a plateau value when the

denaturation is complete. Such a curve is called a "melting curve." Hermans and Scheraga found that the height of the melting transition was constant and that the transition was reversible between pH 2.9 and 6.9, but that below pH 2.9 the value of $\Delta\epsilon_{287}$ did not return to zero even if the solution was cooled below 10°C. That is, the transition became irreversible below pH 2.9 as discovered independently by Bigelow (1961). After cooling the final value of $\Delta\epsilon_{287}$ increased as the pH was lowered below 2.9 indicating that irreversible denaturation was more significant at lower pH values. However they found that at low temperature the acid transition was smaller than at high temperature. This is due to the fact that one portion of the molecule containing one tyrosyl residue is sensitive to low pH at both low and high temperature so it unfolds at all temperatures above about 15°C (justification of this statement requires consideration of some work by Bigelow which will be discussed in a later section). However another portion of the molecule containing another tyrosyl residue is sensitive to low pH (below pH 3.0) only at temperatures above 15°C. Thus at low temperature and low pH only one buried tyrosyl residue is exposed. As the temperature is raised the second portion of the molecule becomes sensitive to low pH and begins to unfold exposing a second tyrosyl residue. This causes an increase in ΔC_{287} that reaches a maximum when the second tyrosyl residue is completely exposed so that the molecule contains two newly

exposed tyrosyl residues that had been buried in the native protein.

Hermans and Scheraga (1961a, b) found essentially identical results by both of the optical techniques used in their study. Specifically, if the fraction of molecules unfolded at any pH is plotted against the temperature by either difference spectroscopy or by ORD, the agreement is quantitative.

In 1964, Bigelow and Krenitsky (1964) demonstrated that raising the ionic strength of the solution could bring about a decrease in the magnitude of $\Delta\epsilon_{287}$ in the acid transition apparently due to a difference in the extent of denaturation. At high ionic strength, above 0.5 M, only one of the buried tyrosyl residues became exposed in the transition. This demonstrates that the region of the molecule in which the second tyrosyl residue is located is extremely sensitive to ionic strength.

UREA AND GUANIDINE HYDROCHLORIDE

Urea and guanidine hydrochloride will be the only two organic denaturants to be discussed in this historical section on RNase and there are two reasons for their selection. In the first place they are the most thoroughly studied of the organic denaturants and, in the second, they cause a larger change in the RNase molecule during the denaturation transition than does the acid-temperature transition. This last point will be discussed further in the section

on the denaturation states of RNase.

Harrington and Schellman (1956) studied the effect of concentrated urea on the RNase molecule. They found that 8 M urea had brought about an unfolding of the molecule, as evidenced by a large increase in the intrinsic viscosity and by a spectral shift in the near ultraviolet. They found also, however, that 8 M urea did not significantly affect the hydrodynamic and spectral properties of performic acid oxidized RNase. The optical rotation of RNase in 8 M urea decreased linearly with increasing temperature from 10 to 50°C, that is, once the molecule was unfolded in 8 M urea no evidence could be found for a thermal transition indicating that the transition caused by urea was at least as great as that caused by high temperature in the absence of urea.

The first study of the guanidine hydrochloride denaturation of RNase was published by Sela, Anfinsen, and Harrington (1957). They demonstrated that the optical rotation and intrinsic viscosity of RNase are slightly larger in 6 M guanidine hydrochloride than in 8 M urea. They also demonstrated (fortuitously as it later turned out) that 6 M guanidine hydrochloride caused the same size blue shift during denaturation of RNase as did 8 M urea. In 1964, Bigelow applied a correction to the difference spectra for the solvent effects and found that in fact the denaturation blue shifts caused by these two denaturants really were of the same size.

Nozaki and Tanford (1967) studied the hydrogen ion titration curve of RNase in 6 M guanidine hydrochloride, a solvent in which proteins exist essentially as random coils, according to Tanford, Kawahara and Lapanje (1967). They found that RNase titrated reversibly between pH 2 and 10, and the titration of RNase in the tyrosyl pH range produced a curve that showed 17 groups being titrated. The 17 protons titrated came from six tyrosyl residues, 10 lysyl residues, and one from a β-elimination reaction (this reaction leads to the dissociation of a proton from the β-carbon of the cystine, which causes a rearrangement of the cystine to form one dehydroalanyl and one thiocysteinyl residue). The spectrophotometric titration carried out at 295 mμ gave a value of close to what would be expected for six phenolic groups with a $\Delta\epsilon$ value of 2450 per phenolic group titrated.

SALT DENATURATION

Neutral salts have become an important denaturant for the investigation of protein structure. This is partly due to the fact that salts differ remarkably among themselves in their effect on the structure of protein molecules.

Bigelow and Geschwind (1960) and Bigelow (1960) studied the effect of LiBr on the spectral properties of RNase. They found that blue shifts were produced at all concentrations of LiBr and that

the protein went through a reversible transition between 3 and 5 M LiBr, as had been previously reported by Harrington and Schellman (1957). It was also concluded, incorrectly as it later turned out (Sarfare and Bigelow (1967)), that a second spectral step occurred between 8 and 10 M LiBr. This second "transition" had no effect on the intrinsic viscosity which remained unchanged between 7 and 10 M LiBr (Bigelow (1960)). The second step appeared to be due to the exposure of the last buried tyrosyl residue.

Bigelow and Geschwind (1961), stimulated by the earlier work of Harrington and Schellman (1959), carried out a study of the effect of salts on RNase. They demonstrated that a sharp transition characterised by a rapid increase in levorotation occurred between 3 and 5 M LiBr, but that optical rotation showed no apparent transition between 5 and 10 M LiBr. They also showed that outside of the transition region the levorotation of the solution at the sodium D line decreased linearly with increasing LiBr concentration. They went on to demonstrate that the decrease in levorotation was a general medium effect and that similar effects were seen with all the salts they tried on a suitable model compound, namely oxidized RNase. The solvent effect was a linear function of the salt molarity and a curved function of the water activity. The change in optical rotation for a 1 M increase in the salt concentration varied from salt to salt; it was found that iodides caused a larger change than

bromides which in turn caused a larger change than chlorides.

Mandelkern and Roberts (1961) studied the effect of LiBr on the thermal transition of RNase. They found that increasing concentrations of LiBr caused a lowering of the transition temperature, and a decrease in the magnitude of the transition. In 4. 5 M LiBr no transition between 10 and 70°C can be seen, indicating that the transition caused by the salt is at least as great as that caused by elevated temperatures in the absence of salt, the same conclusion had earlier been reached by Bigelow (1960).

Sarfare and Bigelow (1967) continued the study of the effects of LiBr on the RNase transition. They found, as did Mandelkern and Roberts (1961) that a rise in temperature facilitated the transition at 5 M LiBr but seemed to inhibit the supposed second transition that Bigelow (1960) believed he had observed at 10 M LiBr. Attempts to produce the second transition by melting solutions in which the LiBr was more concentrated than 6 M failed to produce any transition. This, coupled with the fact that no optical rotation or intrinsic viscosity change could be seen (Bigelow (1960)), cast doubt on the validity of the earlier conclusion that there was a second transition at high LiBr concentration. The conflict in these data was removed by a study of the spectral shift caused in model chromophores by LiBr. It was found that the concentration dependence of this spectral s hift, caused by LiBr when it interacts with phenolic chromophores,

was not linear as it is with most other denaturants, and as had previously been assumed. By measuring the concentration dependence accurately, data were provided which allowed accurate interpretation of the solvent effects observed with RNase in concentrated LiBr. When the nonlinearity in the solvent effect is taken into account and the proper correction made the supposed second transition is no longer observed.

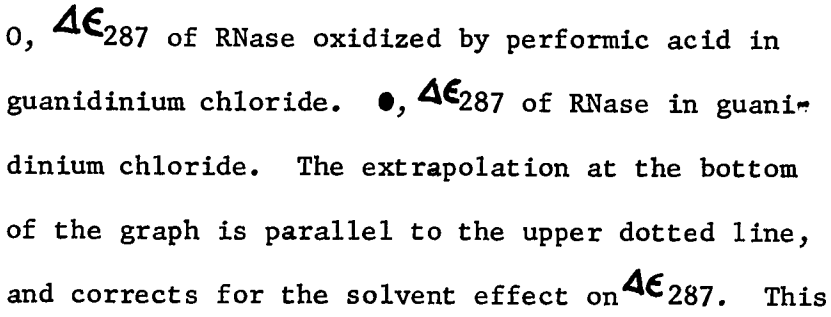
In studying protein denaturation by spectroscopy there are two different effects that one will observe. These are the spectral shift due to denaturation (the denaturation blue shift) and the spectral shift due to the changes in solvent medium on the chromophores. To obtain accurate values for the denaturation blue shift some method must be used to correct for the "solvent effect" on the chromophores. Bigelow (1960, 1964) has studied this problem extensively. He demonstrated that in some cases the effect can be corrected for effectively by obtaining values for the solvent effect on a completely unfolded molecule. These values are then subtracted from the extinction changes obtained with the protein to produce a corrected value for the denaturation blue shift. Bigelow (1964) demonstrated that the same correction for the solvent effect could be obtained, at least in the case of guanidine hydrochloride, by plotting $\Delta\epsilon$ versus denaturant concentration and extrapolating the straight line found for the solvent effect above the denaturation concentration back to

zero concentration. Fig. 3 demonstrates this method (Bigelow (1964)). Bigelow and Geschwind (1961) have demonstrated that the same sort of correction can be made for solvent effects observed by optical rotation.

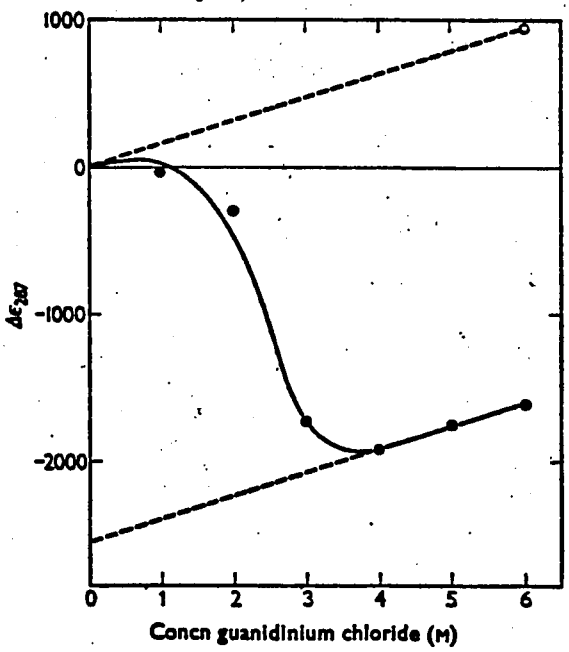
THE DENATURED STATES OF RNASE

Since denaturation is commonly believed (Haurowitz (1963, Tanford (1969)) to be caused by the production of any molecular conformation which is grossly different from the particular conformation defined as the "native state," it would not be surprising if different denatured conformations or states could be produced for a given protein in different denaturing media. Bigelow and Sarfare, in a series of papers (Bigelow (1960, 1961, 1964) and Sarfare and Bigelow (1967)) have investigated the production of different denatured states of RNase. Bigelow found that on denaturation each of the three buried tyrosyl residues which become exposed contributes a certain amount to the denaturation blue shift at 287 mu. The value of denaturation blue shift caused by the exposure of tyrosyl A, B, and C are about 1000, 700 and 1000 extinctions units respectively. Since the sizes of the blue shifts for each residue are known, it was possible to show that only the three denatured states could be observed under the denaturing conditions mentioned in this thesis. These Bigelow calls states I, II, and III, and it was shown that in

FIGURE 3



O, $\Delta\epsilon_{287}$ of RNase oxidized by performic acid in guanidinium chloride. ●, $\Delta\epsilon_{287}$ of RNase in guanidinium chloride. The extrapolation at the bottom of the graph is parallel to the upper dotted line, and corrects for the solvent effect on $\Delta\epsilon_{287}$. This figure is reproduced from the work of Dr. Bigelow (1964), with the permission of the author and the Journal of Molecular Biology.


1000
0
-1000
-2000
$\Delta\varepsilon_{287}$
0
1
2
3
4
5
6
Concn guanidinium chloride (M)

these states, respectively, tyrosyl residues B, B and A, and B, A, and C are exposed during denaturation. It was shown by Bigelow that molecules in state II produced by different denaturants also possess the same values of optical rotation and intrinsic viscosity, and similarly for state III molecules. In table 3 can be seen a compilation of the results of various workers from which Bigelow identified the denaturation states attained by RNase in various media. It need not be inferred that only three denaturation states can exist in RNase, but it is apparently true that to date only three denaturation states have been found. Scheraga's research group, in a series of papers (Broomfield, Riehm, and Scheraga (1965), Cha and Scheraga (1963), and Fujioka and Scheraga (1965), Riehm and Scheraga (1965, 1966), and Li, Riehm, and Scheraga (1966)), have applied chemical methods in an effort to identify tyrosyl residues A, B, and C. They have tentatively identified A as residue 25, B as 92, and C as 97. They have also attempted to identify the carboxyl groups which they believe interact with the abnormal tyrosyl residues of the native protein. The pairings they found were tyr 25-asp 14, tyr 92-asp 38, and tyr 97-asp 83. The pairings proposed for tyr 25 and tyr 92 are quite consistent with the possible interactions that one would suggest from studying the X-ray structure of Kartha et. al. (1967).

TABLE 3

DENATURATION STATES OF RIBONUCLEASE *

State	Denaturant	$\Delta \epsilon$ 287	[n] (dl./g)	$\Delta[\alpha]$ (%)	Residues Normalized
Native	--------	0	.033	0	none
I	pH 1 at 15°C	-700	.035	7	B
II	pH 6 at 60°C	-1700	.065	21-23	B and A
	pH 1 at 40°C	-1700	.072	24	B and A
	5 M LiBr	-1700	.073	23	B and A
III	8 M urea	-2700	.093-.095	46	B, A and C
	6 M guanidinium chloride	-2700	.094	51	B, A and C

* Taken from Bigelow (1964).

METHODS AND MATERIALS

MATERIALS

Ribonuclease

Three samples of RNase were used in this research. All were products of Mann Research Laboratories with lot numbers: R3779, S1684, and T1746. Mann claimed their product was homogeneous and produced by the method of Crestfield, Stein and Moore (1963), with the only change in procedure being that the sample was ethanol precipitated.

Further purification was carried out according to the methods of Herzig (1967), and will be described in a later section.

Model Compounds

L-tyrosine and L-histidine were products of Nutritional Biochemical Corp. L-tryptophan was a product of Fisher Scientific Co. The DL-phenylalanine was a product of Eastman Organic Chemicals.

The N-acetyl-L-phenylalanine ethylester and N-acetyl-L-tyrosine ethylester were products of Mann Research Laboratory. The tetraglycine was a product of Nutritional Biochemical Corp. The

poly-L-glutamic acid was a product of Pilot Chemical Inc. The copolymer of L-tyrosine and L-glutamic acid was a gift from Dr. Paul Doty.

The phenol, p-cresol, imidazole, indole, benzene, and toluene were reagent grade.

The purity of all of these compounds was checked spectrophotometrically.

Other Reagents

In the early stages of this study the urea used was purified by the method of Levy and Margolish (1962). In the later stages Mann Ultra Pure Urea was used. The Mann product was found to have less absorbance than the 3 x recrystallized urea.

Ethanol was purified by two methods. In the beginning it was purified by redistillation and the fraction boiling at 77°C was collected and used in the experiments. Later it was found that if ethanol was run through an activated charcoal column it had less absorbance than the redistilled product and that procedure was used in the later experiments.

The guanidine hydrochloride used was Mann Ultra Pure Guanidine hydrochloride. The methanol was Fisher spectranalyzed grade. The p-dioxane was Matheson, Coleman, Bell spectrograde.

All other chemicals were of reagent grade and used without further purification.

METHODS

Instrumentation

The Cary 15 automatic recording spectrophotometer was used for the recording of all difference spectra and also for the recording of kinetic data. The instrument was modified by attaching a second cell holder in both reference and sample compartments, which permitted subtraction of any solvent absorption by the instrument.

In the case of difference spectra these were automatically recorded by the spectrophotometer from a point outside the range of chromophore absorption down to the range of interest. With the exception of the experiment in which complications occurred (e. g. the results for RNase in methanol and ethanol), all experiments could be reproduced to within .1% of the values we reported for the difference spectra.

When kinetic measurements were made on the spectrophotometer the chart was moved at a fixed speed with the wavelength fixed at the minimum of the difference spectrum, in the 230 to 240 mu region. Values would then be taken from the graph at definite intervals and plotted against time.

The 1 cm. cells used for the thermal measurements were from Quaracell Products Inc. They had permanently cemented block tops, which accepted thermometers. For the other experiments a set of six matched Suprasil Quartzglass cells manufactured by Hellma Cells Inc. were used.

The optical rotation measurements were taken on a Jasco ORD-VU 5 recording spectropolarimeter. While the Cary 15 was used

to study both equilibrium and kinetic experiments in RNase, the Jasce ORD-UV 5 was used only for kinetic experiments. The reason for this is that it was felt that the kinetic experiments required corroborating data, measured by a second physical technique. The chart drive was calibrated at three of the available speeds with a stopwatch and these were the speeds at which runs were performed. A 10 cm. pathlength cell was used for the ORD readings.

The pH and thermal denaturations of RNase were studied in thermostatted cells. All other experiments were performed at ambient temperatures. Ambient temperature for both instruments varies between 28 and 30°C.

In the pH denaturation the temperature of both cuvettes containing RNase was controlled by means of special cell holders through which water at the desired temperature could be circulated. The circulating water was kept at the desired temperature and circulated by a Haake pump.

For the melting curves the same instrument system was used as in the low pH work. The melting curves were measured following a heating schedule in which the thermostatwas raised from one temperature to the next temperature and allowed to equilibrate for 15 minutes before a reading was taken.

Measurements of pH were made with a Radiometer pH Meter 22 or a Radiometer Titrater TTT1C.

Purification and Preparation of Samples

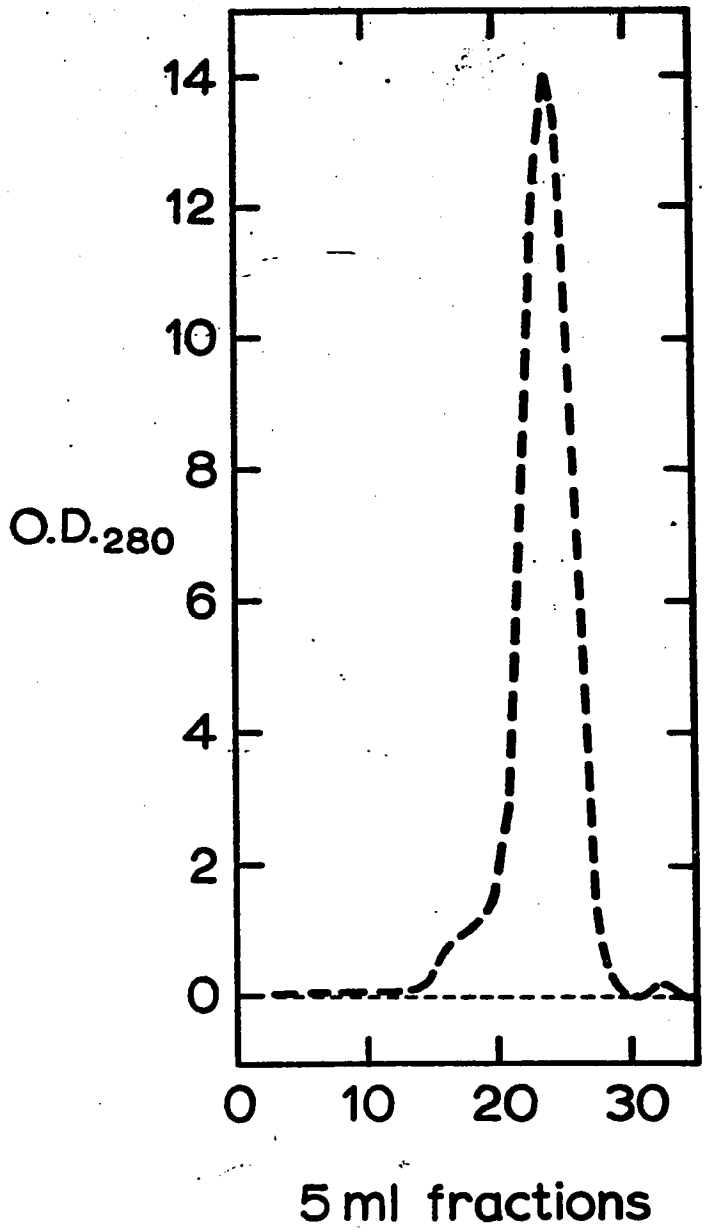
Ribonuclease

The same purification procedure for RNase was used throughout this entire study. The procedure adopted was Herzig's (1967), modification of the procedure of Crestfield et. al. (1963), which had been shown by them to produce a protein solution that was more than 97% pure RNase A. The RNase sample was dissolved in 0. 1 M KCl in concentrations varying from 5 to 15% by weight. The solution was passed through a 0. 45 μ Millipore filter in a Swinnex 25 filter holder mounted on a syringe. The protein solution was heated to 65°C and kept at that temperature for 10 minutes after which it was cooled. It was then eluted from a Sephadex G-75 column. A 2. 4 x 35 cm column was used and the protein eluted at the rate of either 30 or 60 mls per hour using a 0. 1 M KCl solution. The eluate was monitored at 280 mμ and was found to produce a chromatogram similar to that shown in fig. 4. Over 90% of the protein absorption is found in one peak. The fractions of this peak with an optical density over 2 were saved and stored. Some fractions with optical density between 1 and 2 from the tail of the peak were saved and used for solvent effect work.

The RNase was stored frozen in 0. 1 M KCl. The protein was thawed before use and refrigerated at 4°C. If the thawed solution

FIGURE 4

The gel filtration of Mann RNase-A using a G-75 Sephadex column and a 0.1 M KCl solution. For other conditions, see text.



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had been at 4°C for longer than three days or had been frozen for more than one month it was discarded. These precautions were taken to avoid the possibility of deterioration.

The concentration of RNase was determined by its absorbancy at 277.5 mμ. The molar extinction coefficient used to determine concentration was 9800 (Bigelow, 1961).

Model Compounds

The model compounds were made up in stock solution fresh every month and stored at 4°C. They were compared bi-weekly with fresh solutions for deterioration by comparing the absorption in the 250 to 270 mμ range to that of fresh solutions.

The concentration of the stock solutions was adjusted so that after dilution to the experimental concentration the optical density of the absorption band maximum in the region of measurement was always greater than 1.0.

All solutions were filtered through a 0.45μ Millipore filter before being used.

The concentrations of the model compounds were determined by optical density at an absorption band maximum where values had been reported in the literature. These values and references are given in table 4. The concentrations of model compounds for which no reliable extinction coefficient could be found were determined by weight.

TABLE 4

ε VALUES FOR SIMPLE MODEL COMPOUNDS*

	λ	ε	λ	ε	λ	ε
Tyrosine	275	1400a	223	8200	193	44,000
Phenol	269	1400	211	5450	189	53,500
p-Cresol	277	1650	220	5700	192	45,000
Tryptophan	280	5550b	218	34000	196	21,000
Indole	270	5400	215	33000	195	21,000
Phenylalanine	258	195c	206	8000d		
Toluene	261	225e	206	7000e		
Benzene	254	204e	203	7400e		
Histidine (pH = 4.7)	211	5600f				
Histidine (pH = 9.0)	208	5350f				
Imidazole (pH = 4.7)	207	5000e				
Imidazole (pH = 9.0)	207	4600				

* The ε values without references were determined by the author (see text.

a. Nozaki and Tanford (1963) give a value of 1370.
Beaven and Holliday (1952) give a value of 1340.

b. Beaven and Holliday (1952) give a value of 5500.

c. Beaven and Holliday (1952).

d. Wetlaufer, page 310 (1962).

e. Jaffe and Orchin (1962).

f. Saidel (1954).

Preparation of Experimental Solutions

The solutions to be measured were prepared by pipetting directly from stock solutions into the cuvette. The cuvette was stoppered with parafilm and the solution in the cuvette was mixed by inverting the cuvette several times very rapidly.

All solutions for the kinetic portion of the thesis were made up according to the methods outlined so far in this thesis, with one difference. All of the components except the denaturant were added and mixed. The denaturant was then added and the time between this addition and the readings was measured with a stopwatch.

pH Control of Model Compound Solutions

When pH difference spectra were being taken the pH was adjusted by adding measured amounts of stock HCl solutions. The pH was measured on the same model compound solution after the difference spectra had been recorded. For histidine and imidazole all difference spectra were taken in solutions for which the pH had been regulated by adding to the cuvette either acetate buffer at pH 4.7 or glycine buffer at pH 9.0. The dilutions were made so that the final buffer concentration in both cases was 0.01 M. For polyglutamic acid and the copolymer of tyrosine and glutamic acid the pH was adjusted to pH 4.0 by adding acetate buffer and to pH 7.0 by adding glycine buffer. No attempt was made to control the pH of the other model compounds during the model studies, because it

was found that between pH 5. 5 and 7. 0, pH had little effect. However they were checked periodically to ensure that they were between pH 5. 5 and 7. 0.

pH Control of Ribonuclease Solutions

In all cases the pH of the RNase solutions was controlled. This was done by adding measured amounts of HCl to each RNase solution. The pH of the solution was then taken after the difference spectra had been recorded. When an organic reagent was added to the RNase solution the pH recorded was that which the same amount of RNase and HCl would have in water.

Corrections for Light Scattering

In the kinetic runs to be described later, a second reaction occurred which appeared to be due to aggregation of RNase molecules. Aggregation causes an increase in the light scattering of a solution. Light scattering is generally observed in a spectrophotometer as an apparent increase in absorption that is a function of wavelength. To obtain true values for the denaturation blue shift in the middle ultraviolet some procedure for the correction of light scattering is therefore required. In the near ultraviolet it has been common practice to record the light scattering outside of the region of interest (for RNase in the 370 to 300 mμ region). Then the optical density has been plotted against the logarithm of the wavelength

(Gratzer (1967) and Schauenstein and Bayzein (1955)). This procedure produces a straight line that can be extrapolated into the range of interest. This procedure is very risky in the middle ultraviolet because the extrapolation is twice as long as that in the near ultraviolet. However Gratzer (1967) recommends this procedure even for the far ultraviolet. To evaluate this procedure we attempted to compare the results obtained by this method, with the results obtained by kinetic measurements.

In the kinetic measurements as mentioned, we observed two stages in the absorption changes. In the first stage, rapid decrease in absorption occurs, which is due to the denaturation blue shifts. This is followed by an increase in absorption, which is assumed to be due to the aggregation reaction. After a kinetic run was completed a value for the scattering increase could be obtained by extrapolating the second reaction back to zero time and calculating the increase in absorption due to aggregation during the run. The difference spectra of the same solution after the completion of the kinetic run was then taken. The optical density was plotted versus the logarithm of the wavelength and a value obtained by this correction for the light scattering. In Table 5 are compared the values obtained for the increase in absorption due to light scattering calculated by the two methods and it can be seen that there is good agreement between them.

TABLE 5

CORRELATION OF LIGHT SCATTERING BY TWO METHODS
(See text for methods, experimental conditions)

Sample No.	Δ OD_{240} kinetic	ΔOD_{240} semilog	Scattered Light kinetic	Scattered Light semilog
1	1.01	1.06	.64	.65
2	1.05	1.09	.73	.79
3	1.02	.75	.77	.50

RESULTS AND DISCUSSION

The results of this thesis will fall naturally into two sections, the first dealing with model compounds and the second dealing with protein studies. It will therefore be convenient to discuss the model compounds first, and the protein work afterwards.

Furthermore, the discussion of the model compounds will be divided into two major subsections, the first dealing with simple amino acids and structurally related compounds, and the second dealing with the more complex model compounds, namely compounds in which the carboxyl and amine groups of the amino acid are blocked by peptide formation. The second subsection on the model compounds includes work on the peptide bond.

The second major section of the thesis deals with proteins. It is also divided in two subsections. The first deals with the study of solvent effects on the exposed chromophores of RNase under conditions when denaturation does not occur, and the second deals with the denaturation studies of this protein. In the first subsection reporting of the protein studies, the exposed chromophores of RNase are studied both when the protein is in its native conformation

(pH 3. 2 and ambient temperature), and also in its completely unfolded state (4 M guanidine hydrochloride, pH 3. 2).

After the results and discussion section we have included two appendices in which results are presented that are peripheral to the main project. The first deals with kinetic studies on the processes which occur when RNase is subjected to high ethanol or methanol concentrations at low pH. The second appendix deals with an attempt to calculate RNase difference spectra in the middle ultraviolet from the spectra of the constituent chromophores. This work is not included in the body of the thesis because, although its results are very interesting, they are very definitely preliminary, and it is difficult to reach any unequivocal conclusion from them.

SIMPLE MODEL COMPOUNDS

Tyrosine, P-Cresol, and Phenol: Solvent Effects

It is useful to be able to compare the spectral shifts observed when amino acid chromophores are studied to those observed with related compounds which do not have titratable carboxyl and amino groups. Phenol was originally selected as the appropriate compound to compare to tyrosine, but it was soon found that it behaved unexpectedly differently from tyrosine, whereas p-cresol did not. Some results for both these compounds will be reported below.

In what follows we will want to compare measured difference

spectra produced by changes in the environment with the difference spectra we expected. When one attempts to "predict" the nature of a difference spectrum, the assumption is usually made that the difference is due to a simple shift in the spectrum, that is a shift which is not accompanied by changes in intensity or shape of the absorption band (Chervenka (1959); Donovan, Laskowski, and Scheraga (1961)). As Donovan and his colleagues showed, a symmetrical absorption band for which these assumptions are valid gives a very simple difference spectrum with a maximum and a minimum of equal size. For a red shifted spectrum the maximum occurs at the longer wavelength; for a blue shifted spectrum it occurs at the shorter wavelengths. If the absorption band is not symmetrical the maximum and minimum are no longer of equal size. Tyrosine and the related compounds exhibit absorption bands at 230 mμ which are not symmetrical, chiefly because of the influence of a very intense band which occurs at about 190 mμ. In the difference spectra of these compounds the essential result of this asymmetry is that the difference extremum at the longer wavelength (220-230 mμ) is consistently larger than that at the shorter wavelength (200-210 mμ). Because of this it has been necessary to limit any quantitative measurements of these difference spectra to the extrema at the longer wavelengths.

In the presence of organic reagents, for example the urea and

ethylene glycol, phenolic absorption spectra in both the near and middle ultraviolet are shifted to the red. As can be seen from Figure 5 the difference spectra found in the near ultraviolet for tyrosine and p-cresol have two maxima, and that found for phenol has three. In the middle ultraviolet the red shift produces a maximum and minimum for all three compounds and the difference spectra for the three compounds are very similar, with the exception that that of phenol occurs about 10 mμ towards shorter wavelengths than those of tyrosine and p-cresol. In Table 6 it can be seen that the values for the maxima in the near and middle ultraviolet are quantitatively similar for the three compounds in urea or in ethylene glycol. If the value of the maximum is plotted against the concentration of urea or ethylene glycol straight lines are produced. This indicates that the spectral shifts are caused by a bulk effect, for example changes in the refractive index or the dielectric constant.

In the presence of most of the salts used in this study (LiCl, $CaCl_2$, $NaClO_4$, $LiClO_4$, and $Ca(ClO_4)_2$), the spectra of tyrosine, phenol and p-cresol are shifted to shorter wavelengths in both the middle and near ultraviolet ranges; other salts e.g. NaCl, KCl, and Na-Acetate, caused red shifts in the near ultraviolet but were unsuited for work in the middle ultraviolet. As can be seen from Figure 6, this results in two minima in the near ultraviolet for tyrosine and p-cresol; and three minima for phenol. In the middle ultraviolet

TABLE 6

$\Delta \varepsilon$ VALUES FOR TYROSINE, p-CRESOL AND PHENOL

Additive	Concentration	λ **	Sign	Tyrosine $\Delta\varepsilon$ /M*	p-Cresol $\Delta\varepsilon$ /M*	Phenol $\Delta\varepsilon$/M*
HCl	8.3 M	231	+	590	-35	-25
		283	-	173	20	18
LiCl	7.5 M	230	-	91	25	24
		285	-	19	20	17
$CaCl_2$	2.5 M	223	-	47	12	0
		285	-	19	12	16
$Ca(ClO_4)_2$	3 M	230	-	168	100	67
		285	-	40	43	50
$NaClO_4$	5 M	230	-	125	50	34
		285	-	19	19	31
$LiClO_4$	4 M	227	-	159	37	83
		285	-	47	30	33
Urea	7.5 M	227	+	110	85	95
		288	+	26	25	23
Ethylene Glycol	15 M	227	+	142	128	121
		287	+	38	41	42

* This value is $\Delta\varepsilon$ divided by the moles of additive at which $\Delta\varepsilon$ was measured, in the case of HCl, the value of $\Delta\varepsilon$ for tyrosine was not divided by the moles of HCl present.

** λ refers to the wavelength of the maximum or minimum for the tyrosine difference spectrum.

FIGURE 5

The difference spectra produced by the addition of 83.3% ethylene glycol to (a) p-cresol, (b) phenol, and (c) tyrosine.

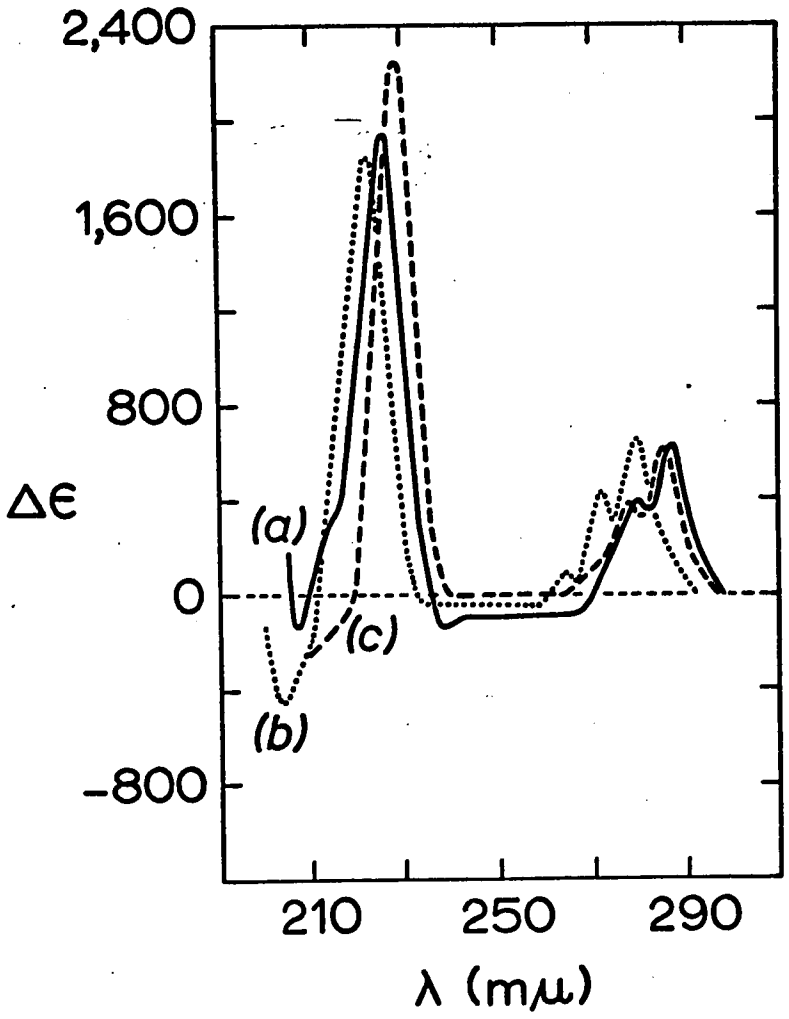
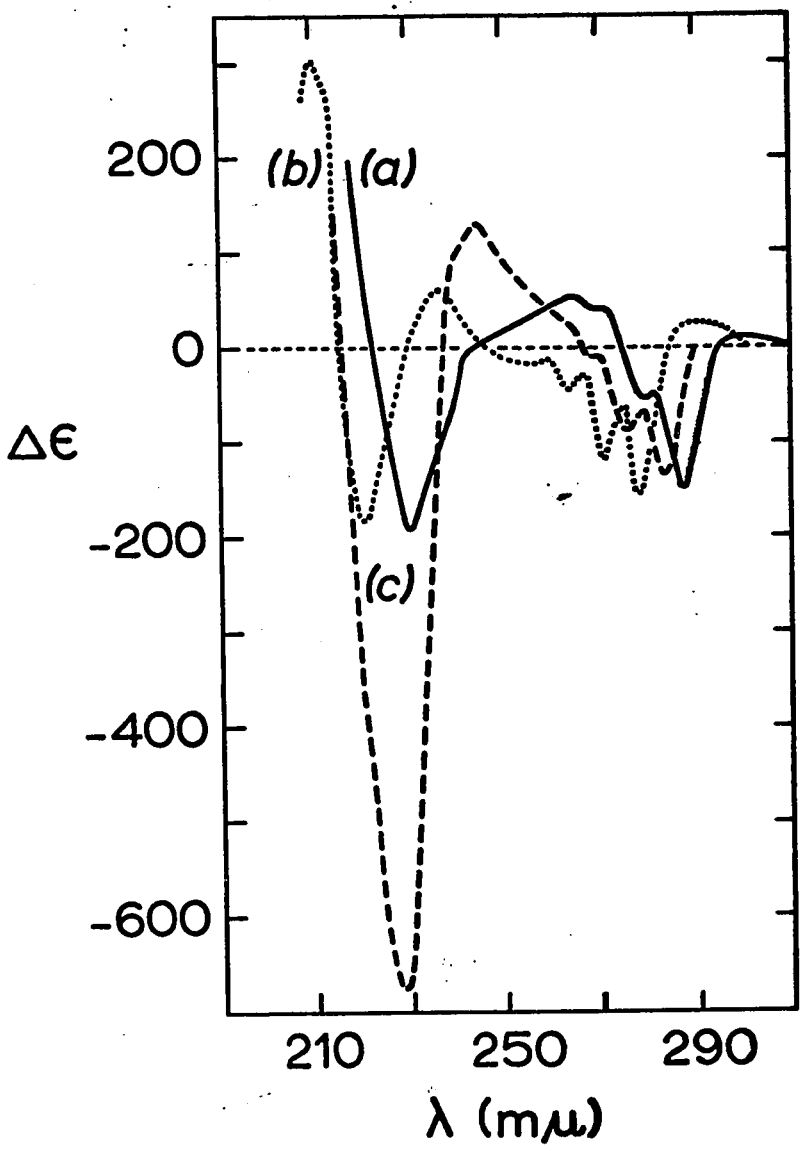

2,400
1,600
800
0
-800
Δε
(a)
(b)
(c)
210
250
290
λ (mμ)

FIGURE 6

The difference spectra produced by the addition of 7.5 M LiCl to (a) p-cresol, (b) phenol, and (c) tyrosine.


200
0
-200
-400
-600
Δε
(b)
(a)
(c)
210
250
290
λ (mμ)

all three have a maximum and a minimum. It can also be seen in Figure 6 and Table 6 that in the presence of salts that cause a blue shift, the minimum for tyrosine is twice as large as that produced for phenol and p-cresol in the same concentration of salts. When the minimum for tyrosine is plotted against salt concentration as in Figure 7 a curved line was produced for all salts used. It can be seen in Figure 8 that even the plot of p-cresol versus LiCl concentration is curved, but that the curvature is much less apparent than for tyrosine, and this is also true for phenol.

Effect of Low pH: Titration of the Carboxylate Group

A study of the effect of the titration of the carboxylate group of tyrosine on the spectrum of the compound in the middle ultraviolet was carried out. As can be seen in Figure 9 the titration of the carboxylate group causes a blue shift in the near ultraviolet and a red shift in the middle ultraviolet. Figure 9 also shows that the addition of acid to solutions of phenol and p-cresol caused blue shifts in both wavelength ranges and this is expected since these compounds have no titratable groups and they would therefore be expected to act as they did in salt solutions. When the maximum of the tyrosine difference spectrum (at 231 mu) is plotted against pH the result is the expected sigmoid shaped titration curve, with an apparent pK of 2.2 and a $\Delta\epsilon_{max}$ of 590. Below pH 1.0 the

FIGURE 7

$\Delta\varepsilon_{230}$ for tyrosine plotted against the concentration of $Ca(ClO_4)_2$, $NaClO_4$, $LiClO_4$, and LiCl.

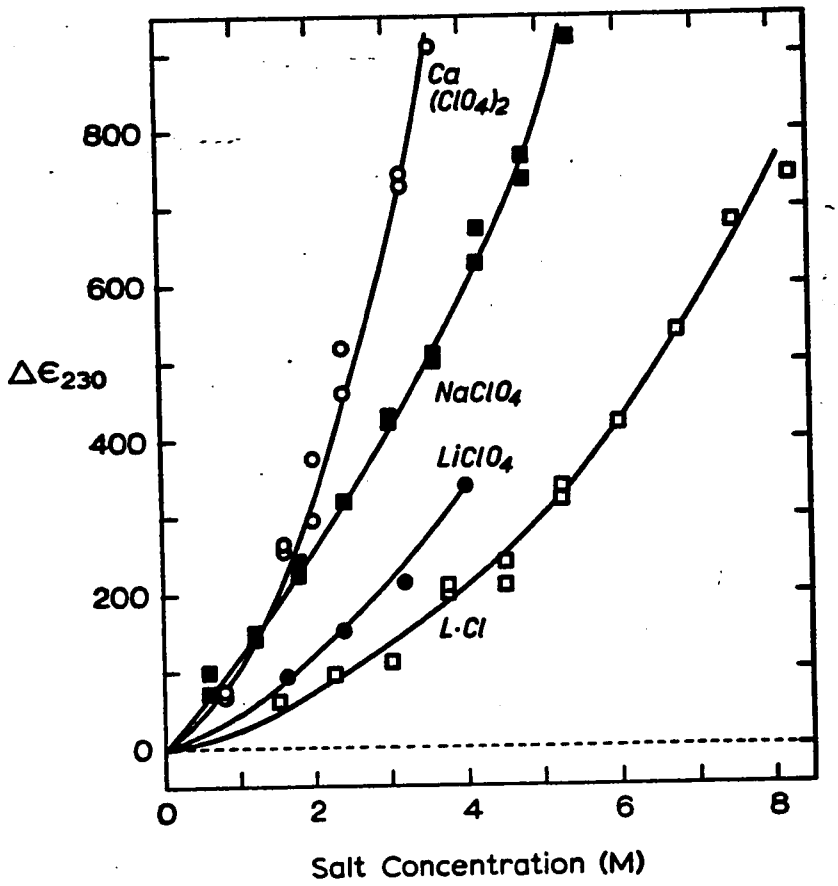

Ca
(ClO4)2
NaClO4
LiClO4
L·Cl
Δε230
800
600
400
200
0
0
2
4
6
8
Salt Concentration (M)

FIGURE 8

$\Delta\epsilon_{230}$ for tyrosine and p-cresol plotted against the concentration of LiCl.

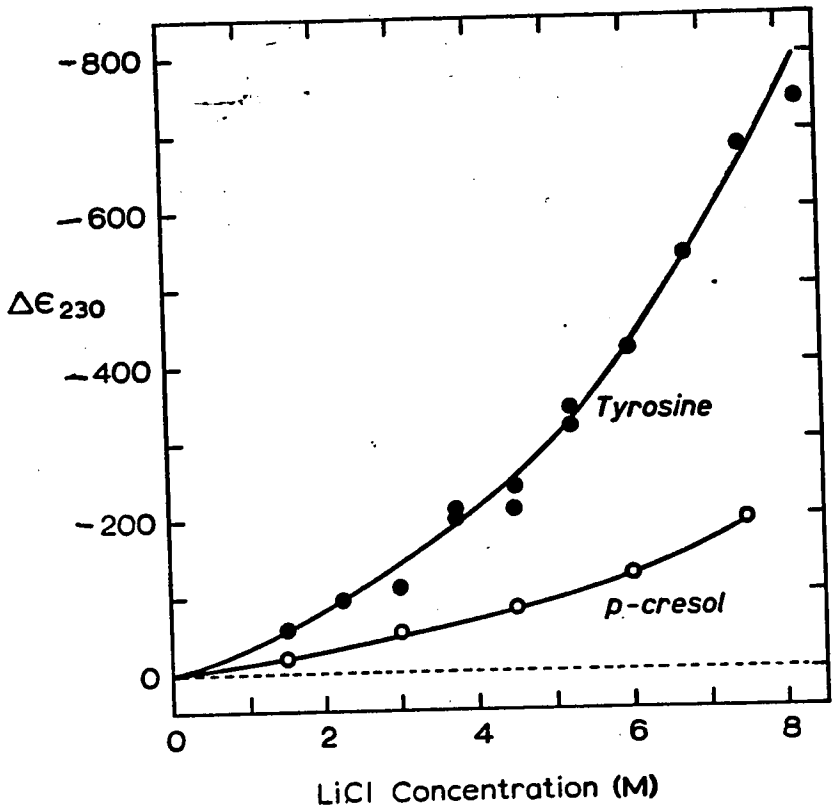
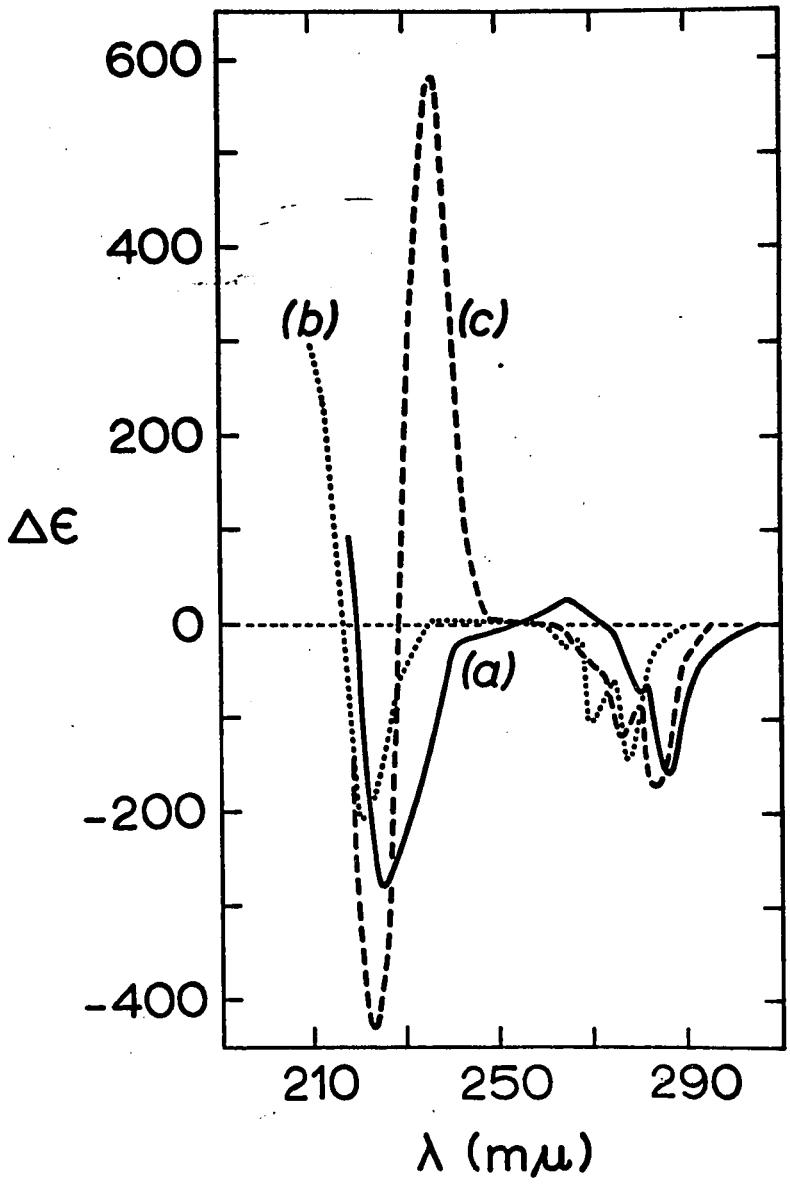

-800
-600
$\Delta\epsilon_{230}$
-400
-200
0
Tyrosine
p-cresol
0
2
4
6
8
LiCl Concentration (M)

FIGURE 9

The difference spectra produced by the addition of 8.33M HCl to (a) p-cresol, (b) phenol, and (c)tyrosine.


600
400
200
0
-200
-400
Δε
(b)
(c)
(a)
210
250
290
λ (mμ)

maximum levels off and remains constant to a concentration of HCl of 8.3N. When similar plots were made using the minima in the near ultraviolet and at 220 mμ in the middle ultraviolet it was found that the titration curves are complicated by the fact that $\Delta\epsilon_{max}$ continues to increase in magnitude below pH 1.0.

Discussion

The two extrema that were quantitatively studied are the ones that appeared in the difference spectrum on the long wavelength side of the two absorption peaks. For the 220 mμ peak this extremum was generally between 220 and 230 mμ and in the near ultraviolet it generally occurred about 285 mμ for tyrosine and p-cresol, and 275 mμ for phenol. In Table 7 are presented values of the ratio of the $\Delta\epsilon$ values at these two extrema. These ratios are of some interest and will be discussed below.

If a difference spectrum arises from a simple band shift of $\Delta\lambda$ then the value of the extrema can be approximated by $\Delta\epsilon = -\Delta\lambda(d\epsilon/d\lambda)_\lambda$ (Bigelow (1960) and Chervenka (1959)), where $(d\epsilon/d\lambda)_\lambda = S_\lambda$, the slope of the spectrum at λ, which is the wavelength both of the extremum in the difference spectrum and of the maximum slope of the spectrum. It is assumed here that we can measure the extremum at a wavelength where there are no complications from shifts in other absorption bands; and this is probably reasonable on the upper wavelength side of both spectral peaks. In

TABLE 7

$\Delta\varepsilon$ RATIOS FOR TYROSINE, P-CRESOL AND PHENOL

Additive	Concentration	Tyrosine	P-Cresol	Phenol
		$\frac{\Delta\varepsilon\ 231}{\Delta\varepsilon\ 285}$	$\frac{\Delta\varepsilon\ 228}{\Delta\varepsilon\ 286}$	$\frac{\Delta\varepsilon\ 222}{\Delta\varepsilon\ 280}$
HCl	0.1 N	- 9.9		
HCl	8.3 N	- 3.4	1.7	1.5
LiCl	7.4 M	4.9	1.3	1.4
$CaCl_2$pH=5.5	2.5 M	2.5	1.0	0.0
$NaClO_4$	5 M	6.7	2.6	1.1
$Ca(ClO_4)_2$	3 M	4.2	2.3	1.3
$LiClO_4$	4 M	3.4	2.9	2.5
Urea	7.5 M	4.2	3.4	4.1
Ethylene Gycol	15 M	3.7	3.1	2.9

	Tyrosine	P-Cresol	Phenol
$\frac{\text{S Lower}}{\text{S Higher}}$	5.5	3.1	2.9
$\frac{\varepsilon\ 223}{\varepsilon\ 275}$	5.9	3.5	3.9

comparing two different bands, it is seen that if $\Delta\lambda$ is the same for both, then $\Delta\epsilon_\lambda$ is proportional to S_λ, and $\Delta\epsilon_{\lambda_1}/\Delta\epsilon_{\lambda_2} = S_{\lambda_1}/S_{\lambda_2}$. Values of $S_{\lambda_1}/S_{\lambda_2}$ are given in Table 7, where λ_1 is the wavelength of maximum negative slope of the lower peak and λ_2 is the wavelength of maximum negative slope of the upper peak.

Comparison of the measured ratios and slope ratios shows that usually the measured values are within a factor of 2 of the expected value. The results observed from the addition of HCl to tyrosine and $CaCl_2$ to all three compounds are exceptions. These data provide reason for thinking that $\Delta\epsilon$ of the two spectral peaks is approximately the same.

The value of the $\Delta\epsilon$ ratios for tyrosine in HCl are negative, reflecting the fact that on titration of the carboxylate group the lower peak shifts to the red and the upper to the blue. The larger value observed in 0.1 N HCl (-9.85) shows that most of the change at 231 mμ is connected with the titration. The lower value observed in 8.3 N HCl (-3.4) shows that after the titration is completed the upper peak continues to shift, whereas the lower peak does not.

The effects observed here can be qualitatively explained in the usual fashion. The organic reagents give linear relations between $\Delta\epsilon$ and the additive concentration. This linearity is typical of spectral shifts which are caused by changes in bulk properties of the solvent. The curvature and the blue shifts observed

with the salts is probably indicative of a specific interaction between the salt and the chromophoric group. Since the blue shift and curvature is observed with both phenol and p-cresol whereas O-methyltyrosine exhibits no blue shift (Bigelow and Geschwind (1960)), this would indicate that this effect can be traced to the interaction of the hydroxyl group and the salt. The carboxyl or amino group must in some way assist the interaction since tyrosine has a larger effect per mole of salt than phenol or p-cresol. Using space-filling atomic models we constructed a model of the tyrosine molecule, from which it could be seen that the carboxyl group could be oriented in such a way that the carboxyl and hydroxyl groups are within 4 Å of each other. Presumably such an arrangement of these two groups could offer a fairly strong attraction to metal ions which could account for the difference observed between tyrosine and phenol or p-cresol.

In the case of the titration experiments the evidence and arguments are not very conclusive. The results with organic reagents and salts would tend to indicate that the transitions in the near and middle ultraviolet are of the same sort. However the protonation of the carboxylate group causes two very large and different effects in both regions. And after the protonation is complete the resulting species reacts differently at both wavelengths to additional "salt" effects, that is, the near ultraviolet absorption

bands are sensitive to the addition of further HCl and the 200 mμ difference peak indicates a sensitivity to solvent that cannot be interpreted unequivocally because of the nearness to the 190 mμ band. The 231 mμ difference peak appears to be unchanged by the addition of further HCl.

These results would tend to invalidate the conclusion that only $\pi \rightarrow \pi^*$ transitions occur in the middle and near ultraviolet range for tyrosine (Dr. D. M. Graham, Private Communication). This leaves us with two possible alternatives to consider. The first is that while the transition in the near ultraviolet is $\pi \rightarrow \pi^*$, the transition in the middle ultraviolet range is $n \rightarrow \pi^*$ transition. This alternative is quite implausible because of the low oscillatory strength of $n \rightarrow \pi^*$ transitions and the resulting low intensity in such absorption bands. The second possibility is that the middle ultraviolet band really is the result of two transitions, one a $\pi \rightarrow \pi^*$ transition, and the other an $n \rightarrow \pi^*$ transition. Some indirect evidence in support of this suggestion is already available (Jaffe and Orchin (1962)). Jaffe and Orchin mention that while the spectrum of phenol does appear to have only one transition in the middle ultraviolet, the corresponding band in the spectrum of thiophenol has a shoulder which they identify as due to an $n \rightarrow \pi^*$ transition. It is therefore quite reasonable to expect phenol to have a similar transition; if it has, the absorption band it causes must be hidden under the more intense $\pi \rightarrow \pi^*$

band. This would explain the results we have observed here, if the $n \rightarrow \pi^*$ transition is not very sensitive to changes in the solvent but is extremely sensitive to protonation of the carboxylate group and vice versa for the $\pi \rightarrow \pi^*$ transition.

Tryptophan and Indole: Solvent Effects

Both organic reagents and salts cause red shifts of the spectra of tryptophan and indole. As can be seen in Figures 10 and 11 this results in the three well-known maxima in the near ultraviolet difference spectrum and one maximum and minimum in the middle ultraviolet. The value of $\Delta\epsilon$ at the various difference maxima are given in Table 8. These results with these chromophores are more self-consistent than those found for the phenolic chromophores, which presumably indicates that specific interactions do not occur between any of the additives and the indoles.

It can be seen that in general that there is good qualitative and even quantitative (except for $LiClO_4$ and HCl) agreement between the results for tryptophan and indole. No obvious reason for the quantitative difference observed with $LiClO_4$ presents itself; one would have thought that it must be caused by a difference in the interaction of one or the other ion with one of the compounds, but both LiCl and $NaClO_4$ give good quanitative agreement for indole and tryptophan. It would therefore seem necessary to conclude that both ions, interacting together, and with one or other of the compounds,

TABLE 8

$\Delta\varepsilon$ VALUES FOR TRYPTOPHAN AND INDOLE

Additive	Concentration	λ **	Sign	Tryptophan $\Delta\varepsilon$ / M*	Indole $\Delta\varepsilon$/M*
HCl	.83 M	225	-	2980	+277
		235	+	626	0
		291	-	230	+ 12
LiCl	7.5 M	226	+	680	587
		292	+	53	53
$CaCl_2$	2.5 M	227	+	1860	1600
		290	+	148	160
$LiClO_4$	4 M	220	+	275	85
		285	+	33	10
$Ca(ClO_4)_2$	3 M	227	+	467	467
		285	+	40	50
$NaClO_4$	5 M	225	+	234	234
		285	+	28	40
Urea	7.5 M	226	+	747	680
		292	+	80	80
Ethylene Glycol	15 M	226	+	760	753
		292	+	100	120

* This value is $\Delta\varepsilon$ divided by the moles of additive at which $\Delta\varepsilon$ was measured, in the case of HCl, the value of $\Delta\varepsilon$ for tryptophan was not divided by the moles of HCl present.

** λ refers to the wavelength of the maximum or minimum for the tryptophan difference spectrum.

FIGURE 10

The difference spectra produced by the addition of 7.5 M LiCl to (a) indole, and (b) tryptophan.

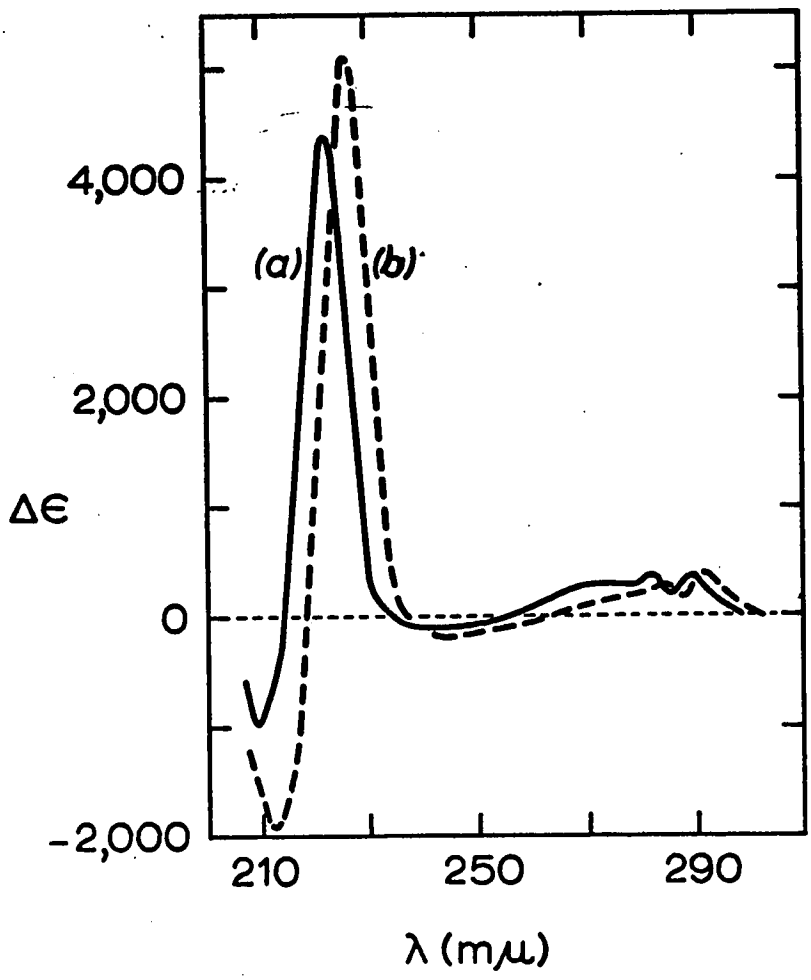
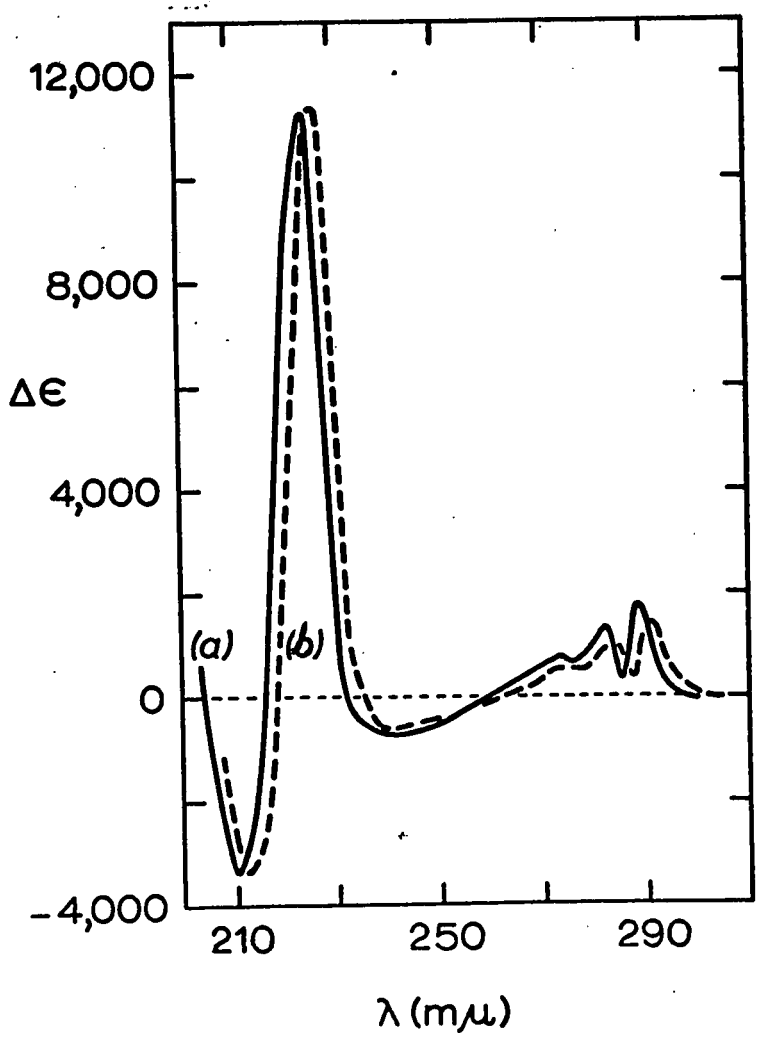

4,000
2,000
0
-2,000
Δε
(a)
(b)
210
250
290
λ (mμ)

FIGURE 11

The difference spectra produced by the addition of 83.3% ethylene glycol to (a) indole, (b) tryptophan.


12,000
8,000
Δε
4,000
0
-4,000
(a)
(b)
210
250
290
λ (mμ)

caused the observed differences.

Effect of Low pH: Titration of the Carboxylate Group

A study of the effects of the titration of the carboxylate group of tryptophan on the spectrum of the compound in the middle ultraviolet was carried out. In HCl indole gave red shifts ((as a salt would) at all concentrations studied, but the behavior of tryptophan was more complicated.

The behavior of the tryptophan spectrum on titration with HCl is more complicated even than that of tyrosine. Difference spectra at selected concentrations are shown in Figure 12. It can be seen that at the lowest concentration shown (0. 083 N) that blue shifts occur both in the near ultraviolet range around 290 mμ and in the middle ultraviolet around 225 mμ. This is clearly connected with titration of the carboxyl group. However after the titration is completed and more HCl is added the blue shift near 230 mμ is overcome by a larger red shift, which is accompanied by a distortion of the near ultraviolet blue shift. The resulting difference spectra we will call anomalous to distinguish them from the normal ones. In Figure 13 are shown the values of $\Delta\epsilon$ at 225 and 235 mμ as functions of pH. Here it is clearly seen that titration causes a very large negative value of $^{\Delta\epsilon}225$, but that this becomessmaller when more HCl is added. On the other hand, $^{\Delta\epsilon}235$ undergoes a small increase during titration followed by a larger increase

FIGURE 12

The difference spectra of tryptophan in (a) 8.3 M, (b) 0.083 M HCl, and (c) 0.83 M HCl.

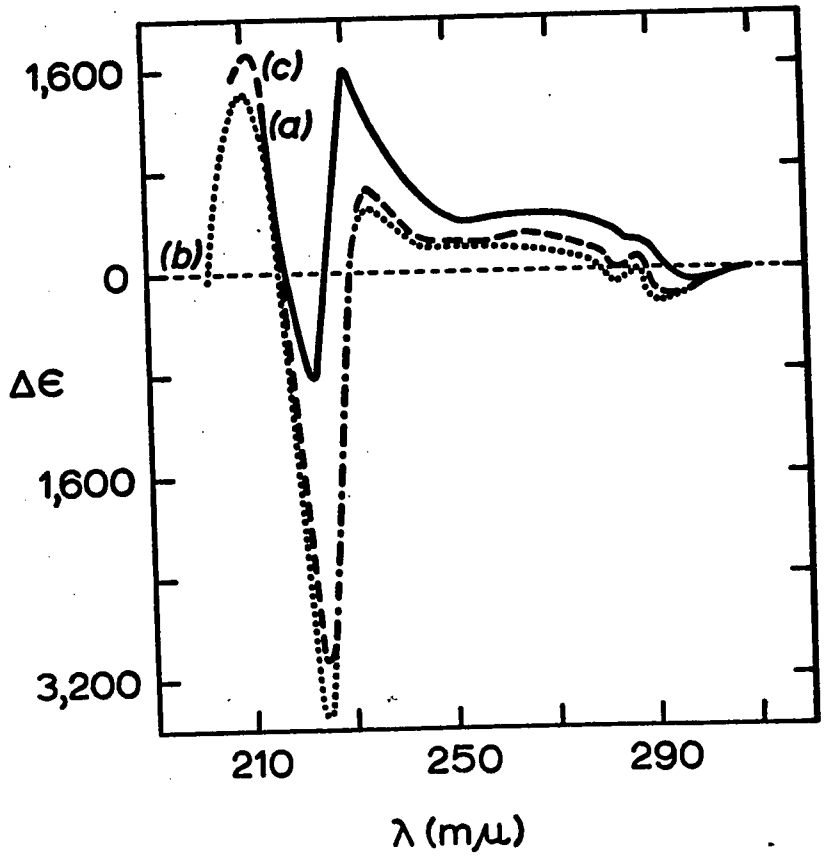

1,600
0
Δε
1,600
3,200
(c)
(a)
(b)
210
250
290
λ (mμ)

FIGURE 13

$\Delta\epsilon_{234}$ and $\Delta\epsilon_{225}$ for tryptophan at different pH's. The reference solution was at pH 6.4.

1,000
Δε234
0
-1,000
-2,000
Δε225
-3,000
-2
0
2
4
pH

afterwards. This behavior is quite different from that of tyrosine which was largely unaffected by the addition of HCl past the titration end point.

Discussion

While it has not been shown in a figure it is true that all of the additives studied (except HCl) give linear relations between $\Delta\epsilon$ at the lower wavelength extremum and the solvent concentration. This is true even for the lithium salts; another difference in the behavior of indole and phenol chromophores.

In Table 9 are calculated some values of the $\Delta\epsilon$ ratios for the two extrema; one at about 225 mμ and one at about 290 mμ. These are compared to ratios of the slopes of the spectra at the same wavelengths, as was done for tyrosine earlier. It can be seen that usually the $\Delta\epsilon$ ratio is larger than the slope ratio, indicating that $\Delta\epsilon$ at the lower peak is larger than at the upper peak, or else that a band intensification occurs. The values of the $\Delta\epsilon$ ratios for tryptophan and indole were quantitatively very similar; they were much closer to each other than the $\Delta\epsilon$ ratios of tyrosine on the one hand and p-cresol or phenol on the other were. They were even closer than was observed in some cases between p-cresol and phenol.

In a general way all of the results reported here for the various additives studied (except HCl) can be easily interpreted as the

TABLE 9

$\Delta\varepsilon$ RATIOS FOR TRYPTOPHAN AND INDOLE

Additive	Concentration	Tryptophan $\frac{\Delta\varepsilon 226}{\Delta\varepsilon 291}$	Indole $\frac{\Delta\varepsilon 226}{\Delta\varepsilon 289}$
Ethylene Glycol	15 M	7.6	6.3
Urea	7.5 M	9.3	8.5
HCl	.083 M	14.0	
	0.83 M	13.0	23.0
	8.3 M	9.3	
LiCl	7.5 M	12.7	11.0
$CaCl_2$ pH=5.5	2.5 M	12.6	10.0
$NaClO_4$	5 M	8.4	5.9
$Ca(ClO_4)_2$	3 M	11.7	9.4
$LiClO_4$	4 M	8.5	8.5

	Tryptophan	Indole
$\frac{S \text{ lower}}{S \text{ higher}}$	6.4	5.5
$\frac{\varepsilon 218}{\varepsilon 280}$	X 6.2	X 6.1

consequences of changes in the polarizability (or refractive index) of the medium. It is clear that they are bulk effects, from the fact that the relation between $\Delta\epsilon$ and additive concentration is linear.

Phenylalanine, Toluene, and Benzene

Only a few investigations, of a rather qualitative nature, were carried out with these compounds. Their difference spectra at 230 mμ are about one or two orders of magnitude smaller than those of tyrosine and tryptophan. In order to get good quantitative results it is necessary to work closer to 210 mμ. This is difficult with most of the additives we are using, and in any case it is not very relevant to the main aim of this thesis, that is, a study of the chief factors that contribute to the difference spectra of proteins in a quantitatively significant way around 230 mμ.

Difference spectra are shown in Figure 14 for the three compounds phenylalanine, toluene, and benzene, in 7.5 M LiCl. It can be seen that these become negligible above 230 mμ, but that they are appreciable at 220 or 210 mμ. The same can be said for the difference spectra of phenylalanine in 83.3% ethylene glycol, Figure 15. Thus below 225 mμ solvent effect red shifts of the phenylalanine absorption spectra cause difference spectra with two maxima between 210 and 220 mμ. These effects are typical of normal solvent effects on $\pi \rightarrow \pi^*$ transitions. With the exception

FIGURE 14

The difference spectra produced by the addition of 7.5 M LiCl to (a) phenylalanine, (b) toluene, and (c) benzene.

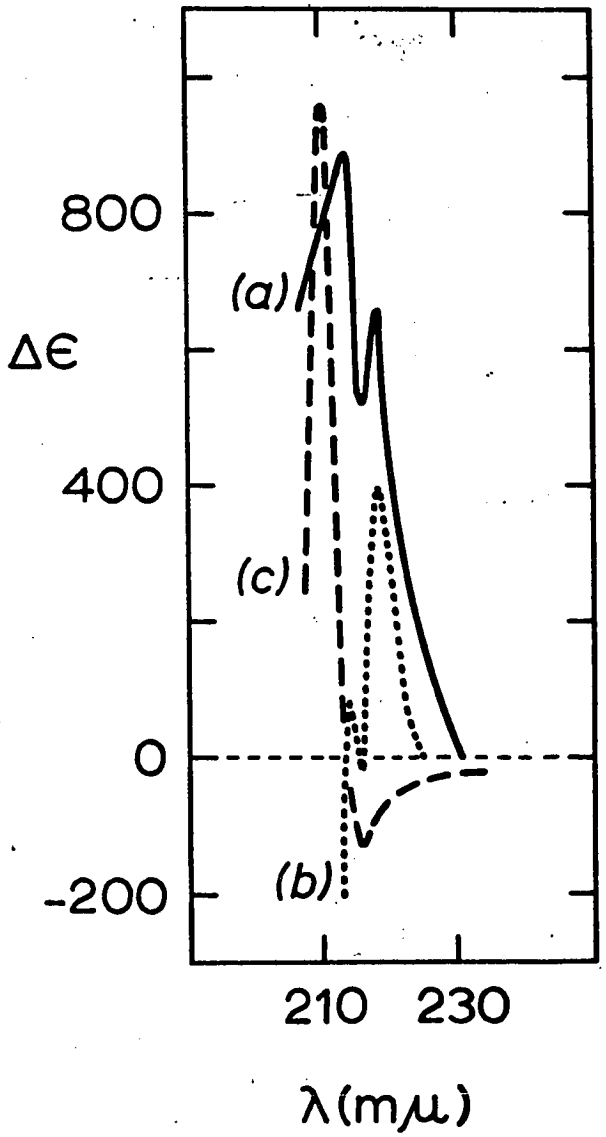
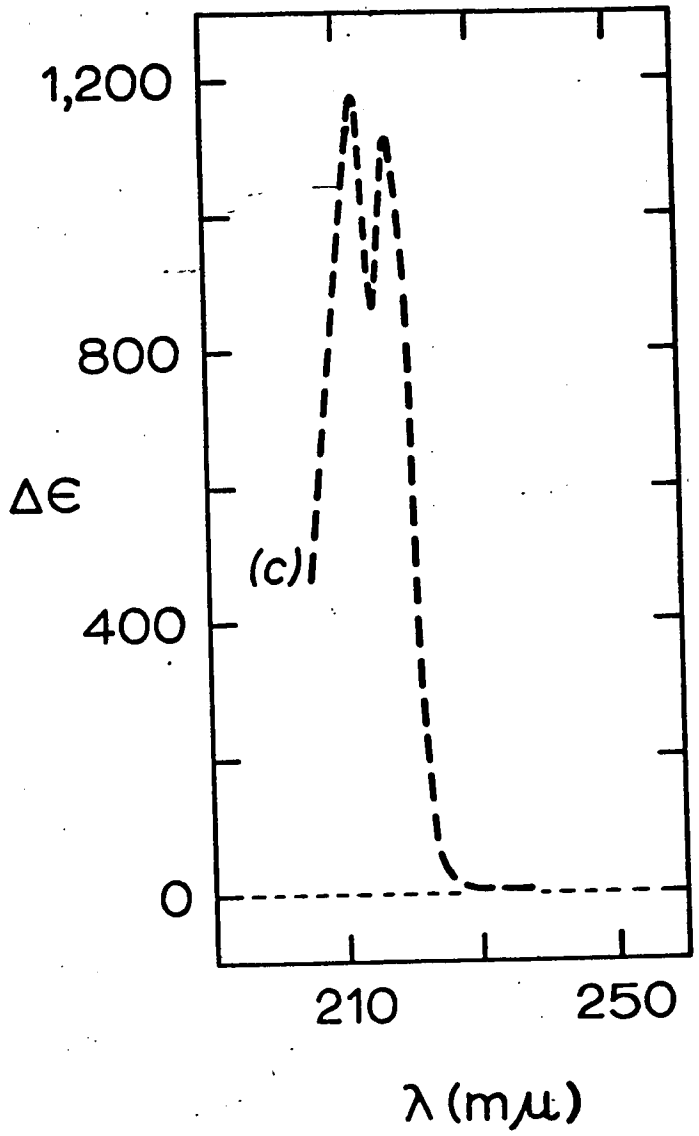

800
400
0
-200
Δε
(a)
(c)
(b)
210
230
λ(mμ)

FIGURE 15

The difference spectrum produced by the addition of 83.3% ethylene glycol to phenylalanine.


1,200
800
Δε
400
0
(c)
210
250
λ (mμ)

of HCl all additives caused red shifts with all three compounds. $\Delta\epsilon$ was a linear function of the concentration of solvent for ethyleneglycol and LiCl. The titration of phenylalanine with HCl gives results which are qualitatively similar to those observed with tryptophan: a blue shift during titration, followed by a red shift after the titration is complete.

$\Delta\epsilon$ values for the three compounds are given in Table 10. Because of the many maxima produced in the middle ultraviolet only the significant ones are recorded. It can be seen in Table 10 that although there is qualitative agreement between the three compounds, the quantitative agreement is not very good. The red shifts and the linear relationship between $\Delta\epsilon$ and the concentration of additives would indicate that the solvent effects observed (with the exception of that caused by HCl) are due to general bulk properties of the solvent, that is refractive index or dielectric polarizability of the medium, and not due to a specific interaction between the solute and the solvent.

Histidine and Imidazole

Owing to the fact that imidazole, and the imidazole side chain of histidine have pKs of about 6.5, both were studied at two pHs, namely pH 9.0 where the group is unprotonated, and pH 4.7 where it is protonated. There were significant differences in the results observed at the two pHs, and each set of results will therefore be

TABLE 10

$\Delta\varepsilon$ VALUES FOR PHENYLALANINE, TOLUENE AND BENZENE

Additive	Concentration	λ **		Phenylalanine $\Delta\varepsilon$ /M*	Toluene $\Delta\varepsilon$/M*	Benzene $\Delta\varepsilon$/M*
Ethylene Glycol	15M	218	+	75	87	109
		213	+	83	153	70
Urea	7.5 M	216	+	210	53	---
LiCl	7.5 M	218	+	89	53	123
		213	+	119	13	---
HCl	8.5 M	220	+	510**	41	84
		215	+		27	---
$NaClO_4$	5 M	218	+	26	36	149
		213	+	48	68	130
$Ca(ClO_4)_2$	3 M	219	+	13	167	273
		212	+	57	240	350
$CaCl_2$	2.5 M	219	+	412	80	400
		212	+	516	---	---

* This value is $\Delta\varepsilon$ divided by the moles of additive at which $\Delta\varepsilon$ was measured, in the case of HCl, the value of $\Delta\varepsilon$ for phenylalanine was not divided by the moles of HCl present.

** λ refers to the wavelength of the maximum or minimum for the phenylalanine difference spectrum.

considered separately.

Histidine and Imidazole at pH 9.0

Additives to histidine and imidazole, both salts and organic reagents, caused red shifts in the middle ultraviolet range. As can be seen in Figures 16 and 17 a red shift in the absorption band of either histidine or imidazole causes a lone maxima in the middle ultraviolet around 220 mμ. The values for $\Delta\epsilon$ at the maxima are presented in Table 11. It is readily apparent that there is good qualitative agreement between histidine and imidazole at pH 9.0, in LiCl and $LiClO_4$, however there is not good agreement in ethylene glycol, $CaCl_2$, and $NaClO_4$. No explanation is evident for the large difference between the histidine and imidazole results in the last three solutions. It can be seen from the table that all of the additives studied show a linear dependence on the value of $\Delta\epsilon_{max}$ on the concentration.

Donovan et. al. (1961) have reported the difference spectrum produced by the titration of the imidazole group on histidine down to 220 mμ, and Donovan (1965) has reported the titration difference spectrum for imidazole down to 200 mμ. The difference spectra measured by us (Figure 18) are in all essentials identical to those of Donovan. As can be seen in Figure 18 the chief features of the difference spectrum produced by titration of the imidazole group in histidine or imidazole are a maximum around 225 mμ and a minimum

FIGURE 16

The difference spectra produced by the addition of 80% ethylene glycol to histidine at (a) pH 9.0 and (b) pH 4.7.

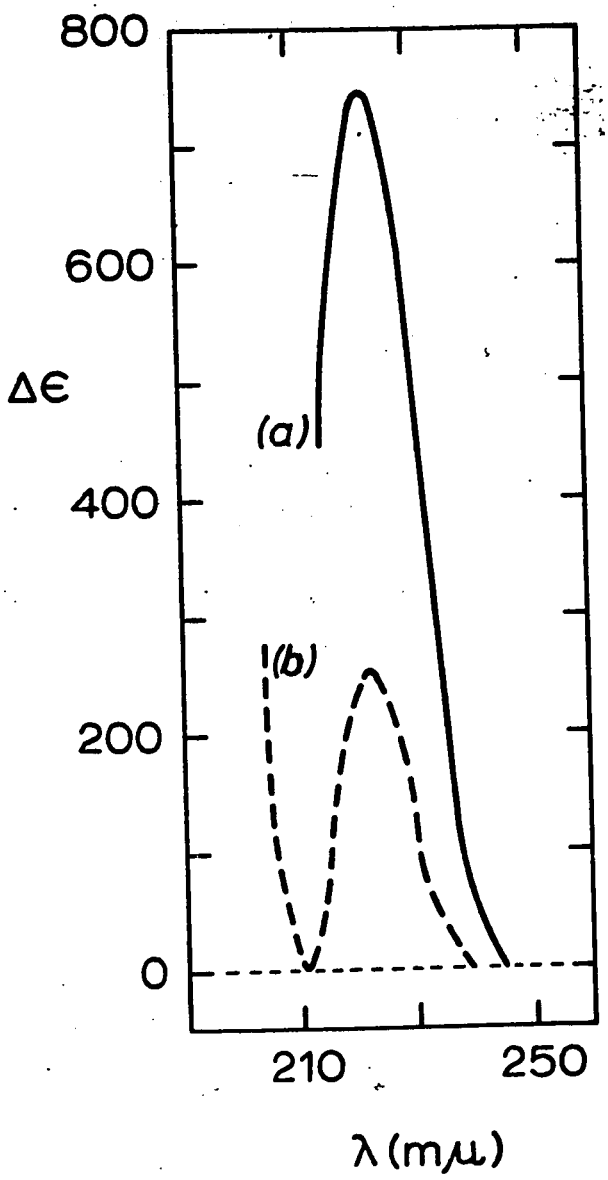

800
600
Δε
400
(a)
(b)
200
0
210
250
λ (mμ)

FIGURE 17

The difference spectra produced by the addition of 7.2 M LiCl to (a) histidine at pH 9.0, (b) histidine at pH 4.7, (c) imidazole at pH 9.0, and (unlettered) imidazole at pH 4.7.

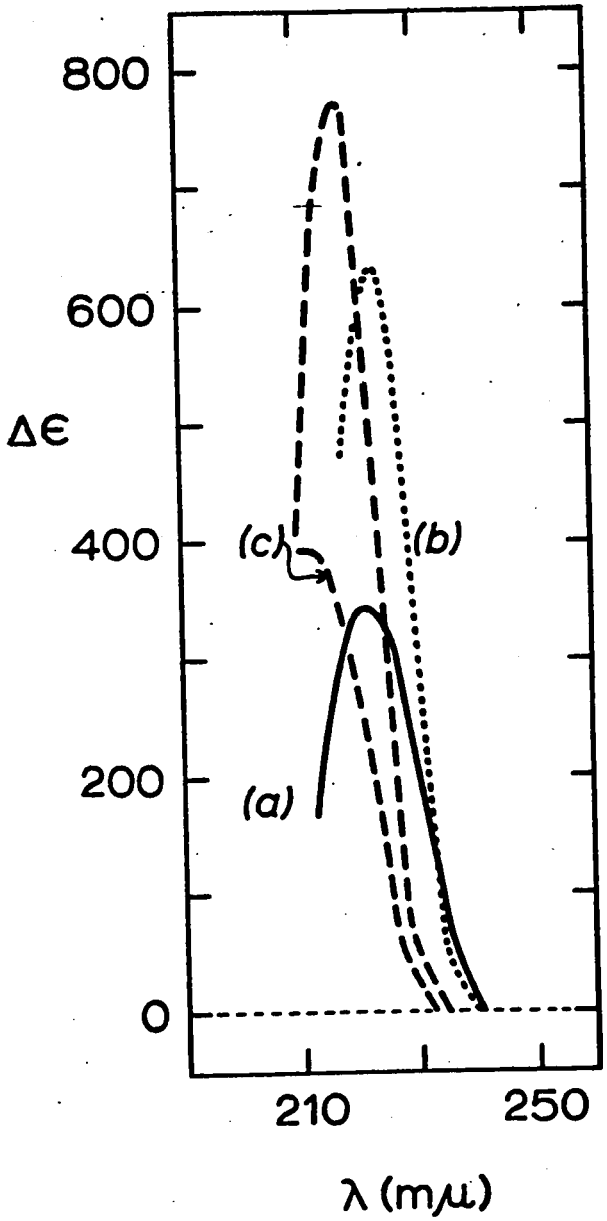
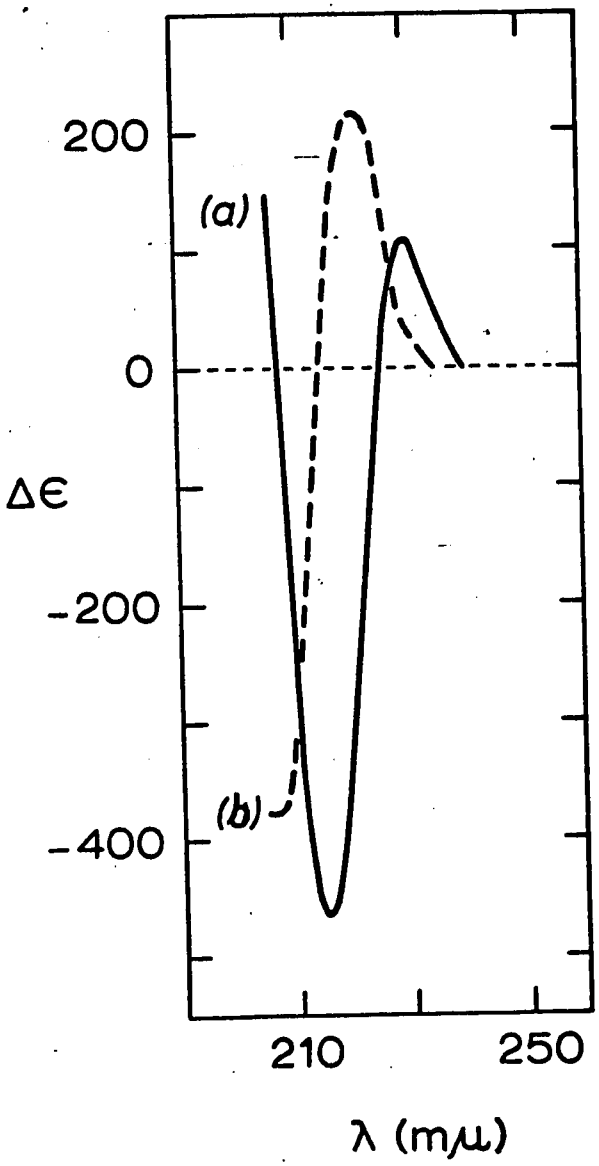

800
600
Δε
400
200
0
(c)
(b)
(a)
210
250
λ (mμ)

FIGURE 18

Acid difference spectra of (a) histidine and (b) imidazole. Sample pH 4.7, reference pH 9.0.


200
0
-200
-400
Δε
(a)
(b)
210
250
λ (mμ)

around 210 mμ. The values for $\Delta\epsilon$ at the maximum and minimum presented in Table 11 are in close agreement with those reported by Donovan (1965).

Histidine and Imidazole at pH 4.7

The addition of additives, salts and organic reagents, caused red shifts of the absorption bands of histidine and imidazole at pH 4.7. Red shifts of the absorption bands of histidine and imidazole in the middle ultraviolet produced difference spectra with one maximum near 220 mμ (Figures 16 and 17). As can be seen from Table 12 there is fairly good quantitative agreement between the values for histidine and imidazole.

As can be seen from Table 12 none of these solvent effects is a linear function of the concentration of additive. Figure 19 shows the histidine data from Table 12 for ethylene glycol plotted against the glycol concentration. This non-linearity is interesting, especially since good linearity was observed at pH 9.0.

Discussion

The electronic transition in histidine and imidazole that gives rise to the absorption band at 210 mμ has, as far as the writer knows, not been identified in the literature. The solvent effects observed here are qualitatively similar to those observed with phenylalanine and tryptophan and this fact would lead one to assign

TABLE 11

$\Delta\varepsilon$ VALUES FOR HISTIDINE AND IMIDAZOLE AT pH 9.0

Additive	Concentration	λ **	Histidine $\Delta\varepsilon$ / M*	Imidazole $\Delta\varepsilon$ / M*
Ethylene Glycol	7.2 M		49	23
	14.4 M	223	53	23
Urea	3.6 M		28	
	7.2 M	223	27	
LiCl	3.6 M		48	50
	7.2 M	220	48	54
$CaCl_2$	1.2 M		120	204
	2.4 M	218	133	177
$LiClO_4$	3.2 M	220	16	16
$NaClO_4$	4.8 M	227	28	9
pH 4.7		230	110	210
		215	-460	-380

* This value is $\Delta\varepsilon$ divided by the moles of additive at which $\Delta\varepsilon$ was measured in the case of HCl, the value of $\Delta\varepsilon$ was not divided by the moles of HCl present.

** λ refers to the wavelength of the maximum or minimum for the histidine difference spectrum.

TABLE 12

$\Delta\varepsilon$ VALUES FOR HISTIDINE AND IMIDAZOLE AT pH 4.7

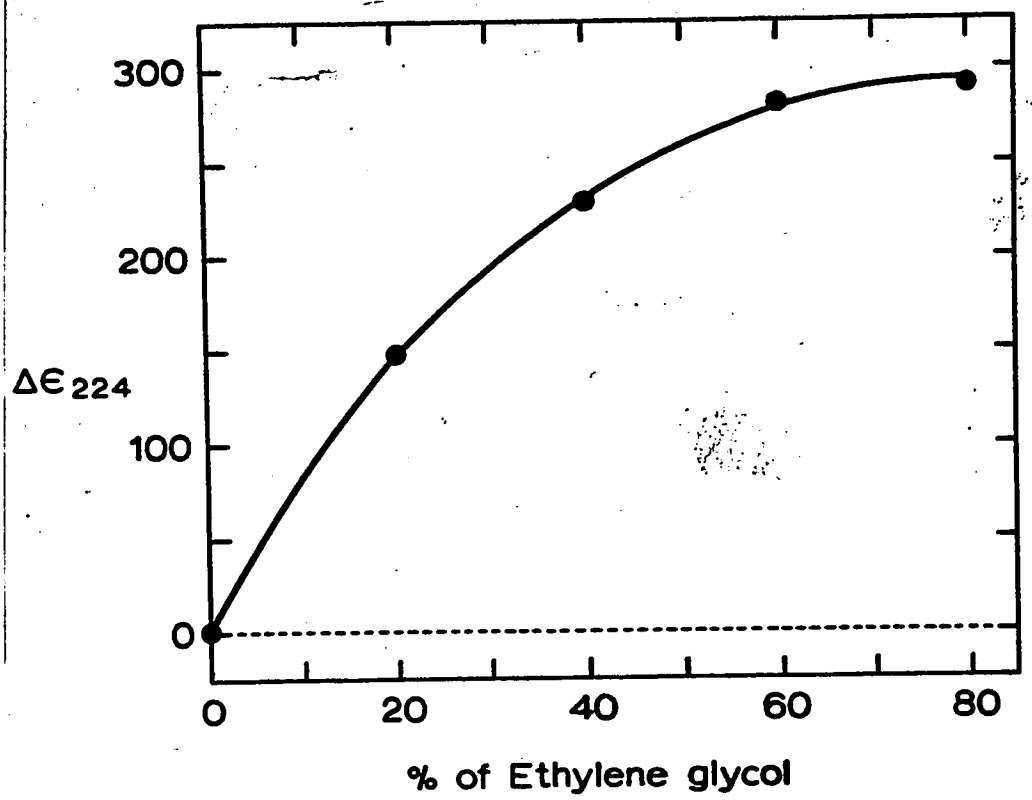
Additive	Concentration	λ **	Histidine $\Delta\varepsilon$ /M*	Imidazole $\Delta\varepsilon$ /M*
Ethylene Glycol	13.6 M		41	
	7.2 M		32	38
	10.8M		27	
	14.4 M	224	20	33
Urea	3.6 M		46	
	7.2 M	224	40	
LiCl	3.6 M		126	128
	7.2 M	223	93.8	108
$CaCl_2$	1.2 M		308	319
	2.4 M	223	252	260
$Ca(ClO_4)_2$	1.6 M		74	
	3.2 M		25	
$LiClO_4$	3.2 M	215	9.4	33
$NaClO_4$	4.8 M	220	19	35
$NaSO_4$	2.4 M		55	

* This value is $\Delta\varepsilon$ divided by the moles of additive at which $\Delta\varepsilon$ was measured.

** λ refers to the wavelength of the maximum or minimum for the histidine difference spectrum.

FIGURE 19

$\Delta\epsilon_{224}$ for histidine at pH 4.7 plotted against the concentration of ethylene glycol added.


300
200
100
0
$\Delta\epsilon_{224}$
0
20
40
60
80
% of Ethylene glycol

the electronic transition a $\pi \rightarrow \pi^*$ transition. However the protonation of the imidazole does not produce the shift expected for a $\pi \rightarrow \pi^*$ transition, and they do not rule out a $n \rightarrow \pi^*$ transition. The change in solvent effects from linear to nonlinear with protonation of the imidazole group can not be explained at this time. However the curvature seen at pH 4.7 would seem to indicate that some interaction is taking place between the protonated histidine and solvent additive. But it is certainly noteworthy that the non-linearity was observed with all the additives and it is therefore difficult to ascribe the non-linearity to any particular sort of interactions, for example hydrogen bonding or electrostatic interactions.

It should be noted that the value of $\Delta\epsilon$ for the unprotonated imidazole compound is always smaller than the corresponding value for the protonated imidazole compounds (by about fifty per cent). The one exception to this is histidine in ethylene glycol. This would indicate that the charged histidine or imidazole species is more sensitive to salts than the uncharged species and also that the uncharged species is more sensitive to organic reagents than the charged species, as one might have expected.

COMPLEX MODEL COMPOUNDS

The purposes of this section are threefold: first, to study the effects of peptide bond formation on the chromophoric amino acid sidechains; second, to study the solvent effects of additives on the peptide bond; and third, to obtain a value for the change in absorption of the peptide bond due to the helix-coil transition. By doing this we can discover whether the information obtained with simple compounds can be used in a study of the same chromophores in proteins and effects of peptide bond formation on the chromophores. We will also obtain values for the solvent effects and conformational effects on the peptide bond.

Tyrosine Containing Peptides and Polypeptides

Two compounds containing both tyrosine and peptide bonds were used in this research. N-acetyltyrosine ethylester (NATEE), was used to study the effect of peptide bond formation on the absorption bands and their solvent effects, and a copolymer of L-tyrosine and L-glutamic acid was used to study the effects of the helix-coil transition on the absorption bands of tyrosine.

The absorption bands of NATEE are shifted 0.5 mμ to longer wavelengths in both the near and middle ultraviolet. Therefore the formation of the peptide bond has not greatly altered the position of thetryrosyl residue absorption band in the middle ultraviolet but it has shifted the maximum in the difference spectrum,

measured in various additives, 2 to 5 mμ, as can be seen in Table 13: The addition of ethylene glycol or urea to NATEE results in a red shift in both the near and middle ultraviolet range (Figure 20). The red shift results in a difference spectrum very similar to the one caused by these additives in tyrosine, namely one with two maxima in the near ultraviolet and a maximum and minimum (the maximum being at longer wavelengths) in the middle ultraviolet range. The only major difference, which is a remarkable one, is between the difference spectra with tyrosine on the one hand, and NATEE on the other is found in those caused by urea (Figure 20). The NATEE difference spectra were found to have shoulders on the long wavelength sides of the maxima in both the near and middle ultraviolet. The shoulder was found to occur in various concentrations of urea. It does not seem likely that those shoulders arise from impurities in the NATEE, since the shoulder was not found in ethylene glycol difference spectra. The values for NATEE and tyrosine are compared in Table 13, and it can be seen that for the same concentration the maxima are the same size in either tyrosine or NATEE. This would indicate that the peptide bond has little effect on the magnitude of the maximum, but that it does have some effect on the tyrosyl sidechain interaction with urea.

The copolymer of L-tyrosine and L-glutamic acid was used to study the effects of the helix-coil transition on the absorption

TABLE 13

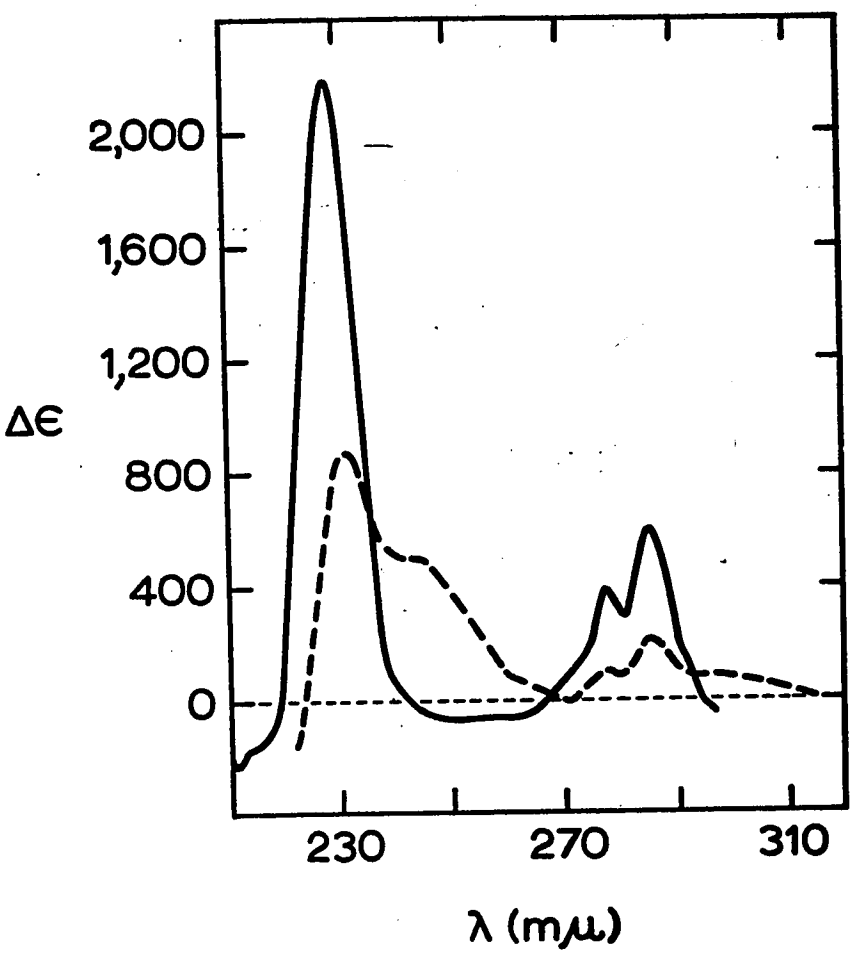
$\Delta\varepsilon$ VALUES FOR NATEE* AND NAPEE**

Chromophore	Additive	Concentration	λ max	$\Delta\varepsilon$	$\Delta\varepsilon$ /Concentration***
Tyrosine	Urea	7.5 M	227	825	110
NATEE	Urea	8.1 M	232	881	110
Tyrosine	Ethylene Glycol	83.3 %	227	2130	256
NATEE	Ethylene Glycol	90.0 %	229	2200	244
Phenylalanine	Urea	7.5 M	220	1580	210
NAPEE**	Urea	3.2 M	219	300	94
Phenylalanine	Ethylene Glycol	83.3 %	213	1240	149
NAPEE	Ethylene Glycol	40.0 %	213	740	185

* N-acetyltyrosine ethylester
** N-acetylphenylalanine ethylester
*** $\Delta\varepsilon$ /concentration gives the values observed in 1M urea or in 10% ethylene glycol.

FIGURE 20

The difference spectra produced by the addition of 90% ethylene glycol (solid line) and 8.1 M urea (dashed line) to NATEE.



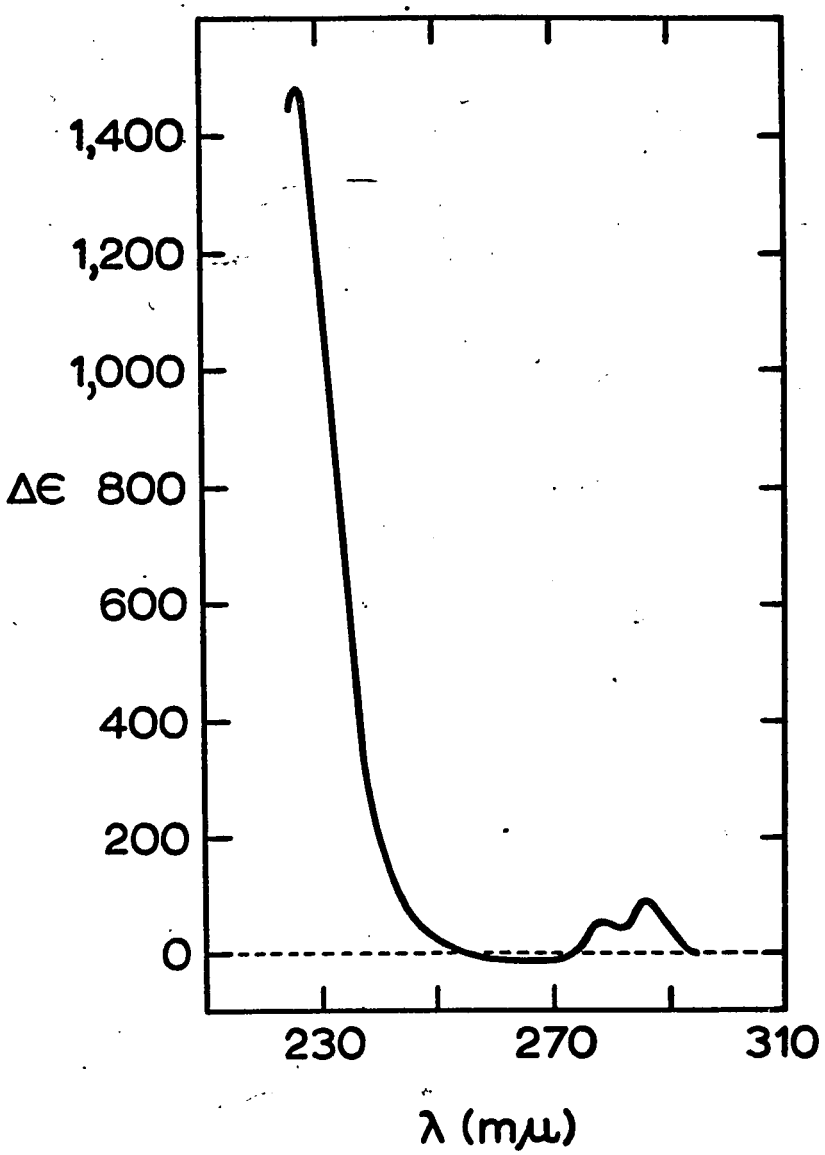
bands of tyrosine. It can be seen in Figure 21 that when the co-polymer undergoes the transition from a random coil to the helical form (pH 7.0 to 4.0) the spectrum of tyrosine is shifted to the red in both the near and middle ultraviolet. In the near ultraviolet this results in the expected two maxima and in the middle ultraviolet it results in one large maximum. Since the peptide bond is the most numerous of the middle ultraviolet chromophores in the copolymer (it outnumbers the tyrosyl residues 20 to 1), it would be expected that the maximum for the helix-coil transition difference spectrum which is at 225 mμ would be a major contributor to the maximum for the copolymer difference spectrum at 227 mμ. This will be discussed further in the section on the peptide bond. The red shift of the tyrosyl difference spectrum is what one would expect for the partial burial of the tyrosyl residues. An alternative explanation is that the helix-coil transition itself will shift the absorption band of the tyrosyl residue to the red. At present we can not choose between either alternative.

Phenylalanine Containing Peptides

N-acetyl phenylalanine ethylester (NAPEE) was studied to observe the effects of peptide bond formation on the absorption bands of phenylalanine. It is found that the formation of peptide bonds to the phenylalanyl residue causes large changes in the

FIGURE 21

The difference spectrum produced when a copolymer of L-glutamic acid and L-tyrosine undergoes the helix-coil transition (from pH 4.0 to pH 7.0) $\Delta\epsilon$ was calculated per tyrosyl residue, assuming $\epsilon_{275} = 1400$.



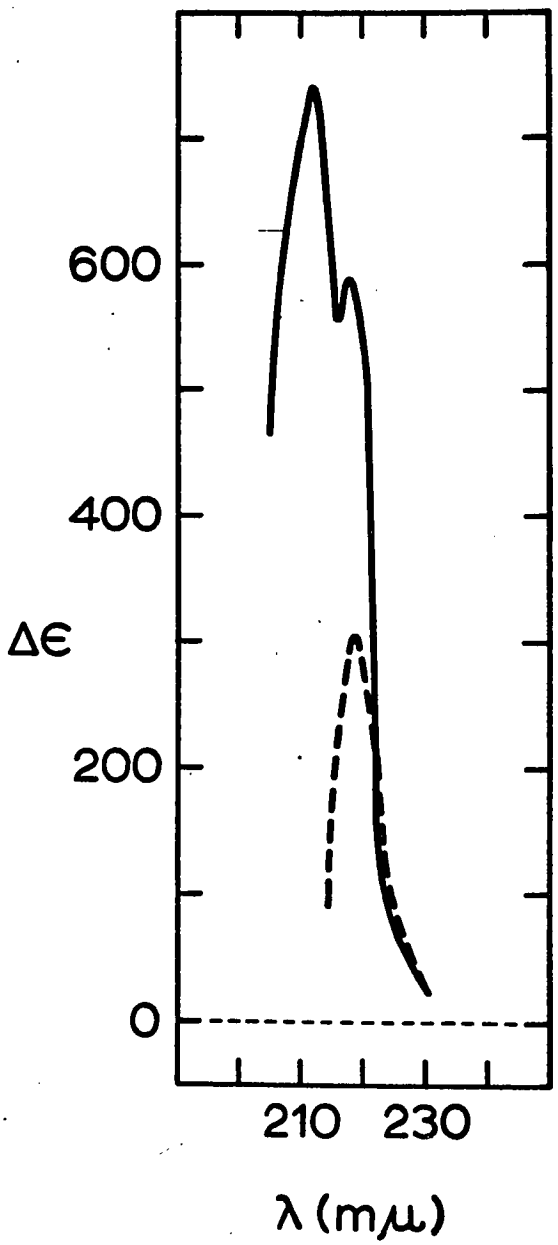
magnitude and shape of the phenylalanine absorption bands, as can be seen in Figure 2b. The addition of urea or ethylene glycol caused red shifts in the spectrum of NAPEE, which produced difference spectra (Figure 22) that resembled the difference spectra of phenylalanine caused by the same additives (Figure 15). The difference spectra included the two maxima in the middle ultraviolet range previously noted for phenylalanine. The positions of the difference maxima in NAPEE were the same as the corresponding maxima in phenylalanine (see Table 13). Thus, the difference spectra of NAPEE qualitatively resemble those of phenylalanine in the same additives. However when we compare the magnitude of the maxima produced by a fixed amount of additive (Table 13) it is found that there are large differences between NAPEE and phenylalanine. The data is not very consistent because, while NAPEE gives a <u>smaller</u> value of $\Delta\epsilon$/concentration in urea than phenylalanine does, it gives a <u>larger</u> value of $\Delta\epsilon$/concentration in ethylene glycol than phenylalanine. It must be concluded that while the formation of the peptide bond does not affect the wavelength at which the maximum of the difference spectrum occurs, it appears to have a very significant quantitative effect on the absorption band of the benzyl sidechain.

<u>Peptide Bond Models: Solvent Effects</u>

Tetraglycine was used to study the effect of solvents on the absorption bands of the peptide bond. Tetraglycine was used, instead

FIGURE 22

The difference spectra produced by the addition of 40% ethylene glycol (solid line) and 3.2 M urea (dashed line), to NAPEE.



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of higher polymers of other amino acids because there is no danger of its undergoing a helix-coil transition, and because it has no chromophoric groups other than the peptide bond.

The addition of ethylene glycol and urea caused red shifts in the absorption bands of the peptide bond. A red shift, as can be seen in Figure 23, produces a very broad peak in the difference spectrum that appears to have a maximum at 199 mμ. The maximum is so broad that $\Delta\epsilon$ is still significant even at 235 mμ. When $\Delta\epsilon$ is plotted against concentrations of urea or ethylene glycol (Figure 24) it is found to be linear at 220, 227.5 and 235 mμ. The absence of maxima or minima in the region of 215 to 235 mμ is very surprising. It was assumed that since there is a weak $n \rightarrow \pi^*$ transition at 225 mu (Gratzer (1967)), its presence would be observed in the difference spectrum. This was not the case and it can only be concluded that the $n \rightarrow \pi^*$ transition is not solvent-sensitive or else it is very weakly sensitive to solvent and the small shift produced by solvent on its absorption band is hidden under the large solvent effect of the $\pi \rightarrow \pi^*$ transition at 190 mμ.

Peptide Bond Models: The Helix-Coil Transition

Studies of a preliminary nature were carried out on PGA (poly-L-glutamic acid) and the copolymer of L-tyrosine and L-glutamic acid. The object of the studies was to demonstrate that correction of the observed difference spectra could be made for the

FIGURE 23

The difference spectrum produced by the addition of 40% ethylene glycol to the peptide bond. The compound used was tetraglycine and $\Delta\epsilon$ was calculated per peptide bond.

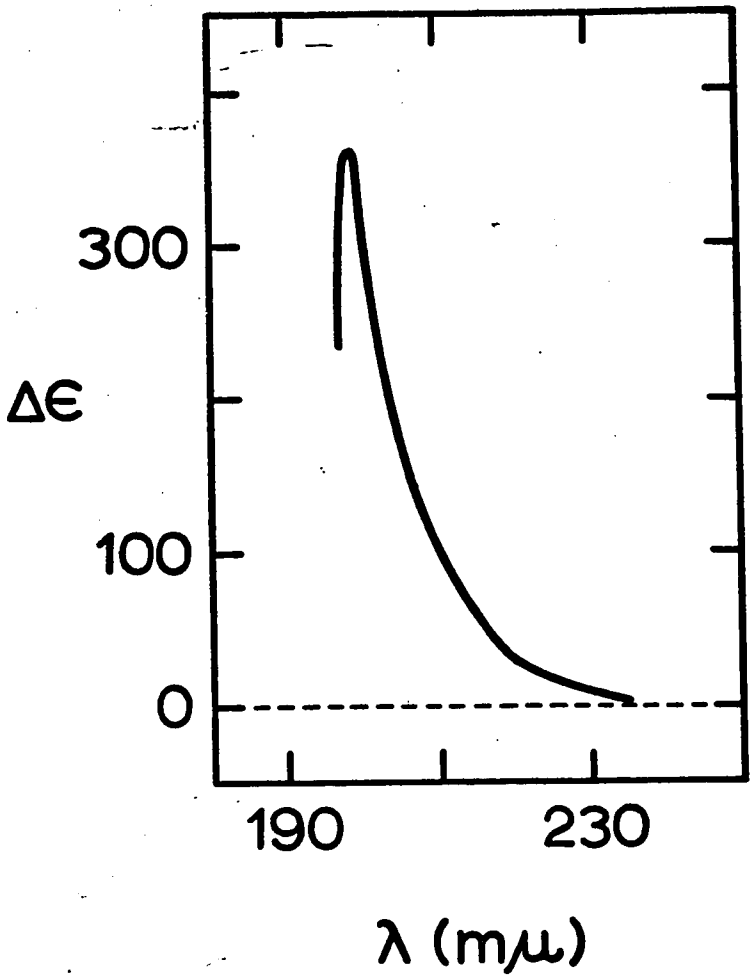
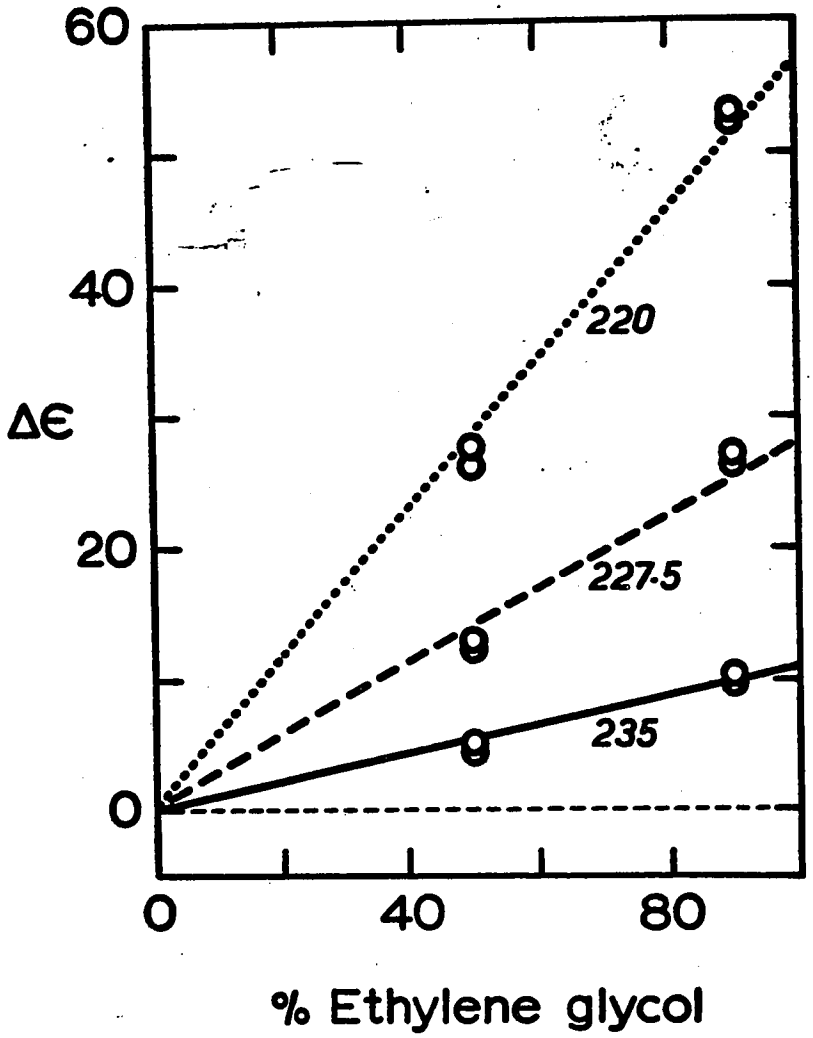


FIGURE 24

Effects of ethylene glycol on the absroption of the peptide bond in the middle ultraviolet range.



difference spectrum produced by the chromophoric amino acid sidechains. The helix-coil transition in PGA has been studied spectroscopically by Glazer and Smith (1961), Glazer and Rosenheck (1962), and McDiarmid (1965), but no such corrections were attempted by them. The coil-helix transition causes a red shift in the spectrum of the peptide bond in the middle ultraviolet. The difference spectrum according to Glazer and Rosenheck (1962) has a single maximum at 225 mμ and no corresponding minimum. We found in our experiments that the value calculated for $\Delta\epsilon_{225}$ was found to be 55 per residue in PGA (the method of calculation is that used by Rosenheck and Doty (1961)). The value of the maximum at 227 mμ observed with the copolymer (Figure 21) can be corrected for the $\Delta\epsilon$ due to the tyrosyl residues present. This was done by multiplying the value of $\Delta\epsilon_{286}$ by 3.5 (this value is taken from Table 7). This value should equal that part of $\Delta\epsilon_{227}$ which is due to the tyrosyl residues. The remainder is then caused by the effect of the coil-helix transition on the peptide absorption. Since the difference spectrum in Figure 21 was calculated per mole of tyrosyl residues the resulting value of $\Delta\epsilon_{227}$ caused by the peptide bond undergoing a helix-coil transition is per twenty peptide bonds, because there are twenty peptide bonds for every tyrosyl residue. After making the appropriate correction we get a value of 55 per mole of peptide bonds for the helix-coil transition. Although

this does not agree very closely with the values reported earlier by other researchers (85 found by Glazer and Smith (1961), 110 found by Glazer and Rosenheck (1962), and 110 found by McDiarmid (1965)), there is agreement for the two polymers used in this study. This proves that the difference spectrum for the chromophoric sidechains can be effectively corrected for in the middle ultraviolet.

PROTEIN STUDIES

Quantitative spectroscopic studies of proteins in the near and middle ultraviolet must be interpreted with caution because, as has been explained earlier, there are two general effects occurring at the same time, one due to denaturation, and one due to the solvent effects on the exposed chromophores. In the middle ultraviolet, furthermore, there are more kinds of chromophores absorbing than in the near ultraviolet. As can be seen in Figures 25 and 26, at concentrations of denaturant below those at which denaturation transitions occur, 70% ethylene glycol and 40% ethanol, the difference spectra of RNase appear very simple. In the near ultraviolet there are the expected two maxima for the effect of additives on the exposed tyrosyl residues and in the middle ultraviolet there is one large maximum at 227 mμ which is due primarily to the effects of solvent on the exposed tyrosyl and histidyl residues and also peptide bonds. But at higher concentrations of denaturant, denaturation

FIGURE 25

The difference spectra produced by the addition of (a) 70% ethylene glycol, and (b) 80% ethylene glycol to RNase.

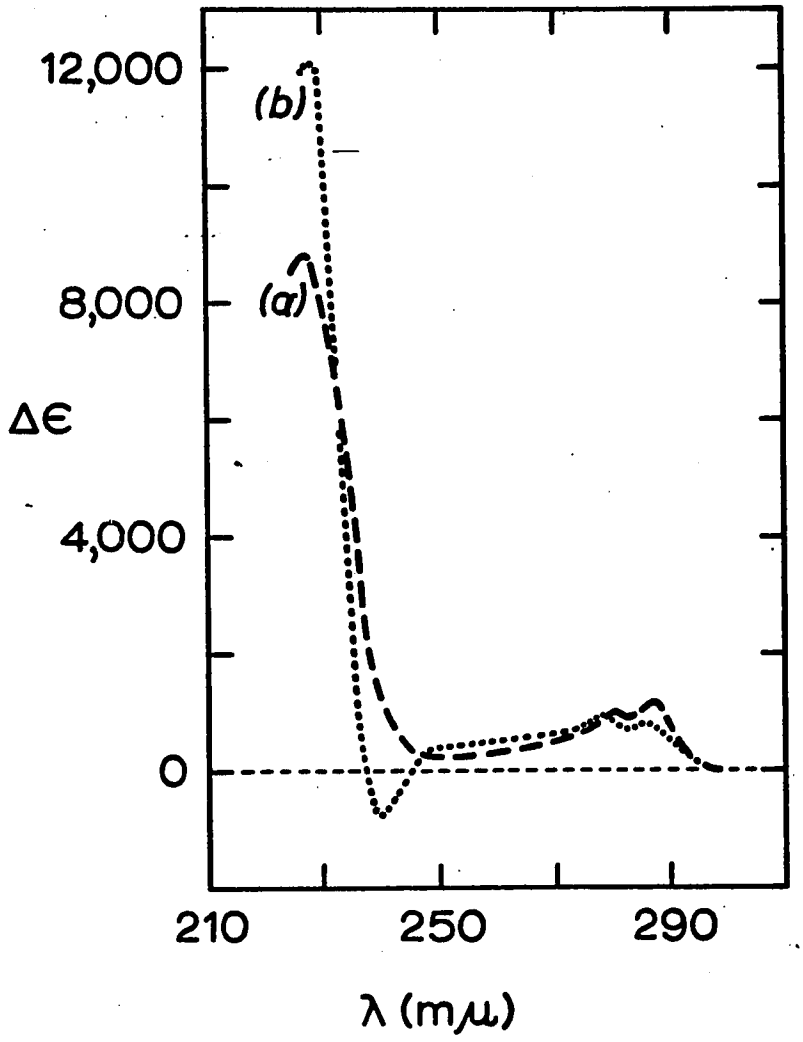
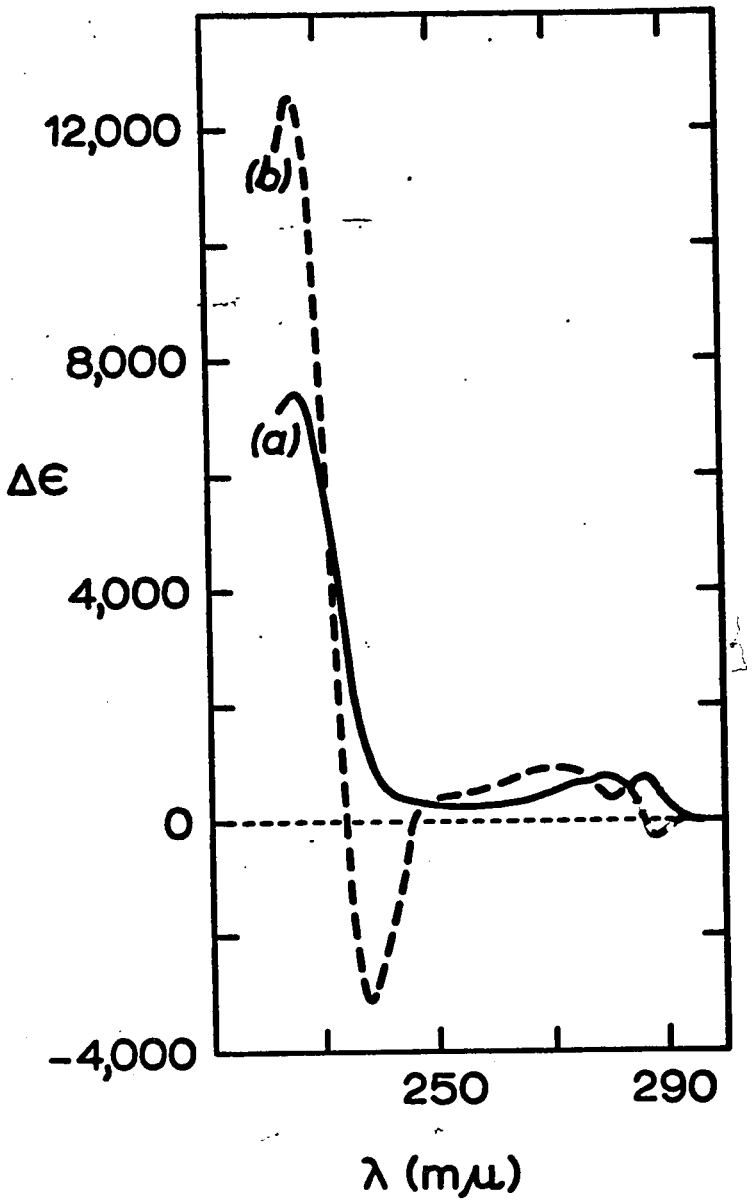

12,000
8,000
4,000
0
Δε
(b)
(a)
210
250
290
λ (mμ)

FIGURE 26

The difference spectra produced by the addition of (a) 40% ethanol, and (b) 70% ethanol to RNase.


12,000
8,000
4,000
0
-4,000
Δε
(b)
(a)
250
290
λ (mμ)

begins and this simple picture becomes complex. As can be seen in Figure 25, partial denaturation of RNase in 80% ethylene glycol results, in the near ultraviolet, in the lowering of the maxima. In ethanol, as the concentration of denaturant is raised to 70% the difference spectra produced by the additional increase in denaturation (Figure 26) results in the appearance of a minimum in the near ultraviolet. In both cases in the middle ultraviolet partial denaturation results in the appearance of a minimum on the long wavelength side of the maximum and an increase in the magnitude of the maximum. When urea was used as the denaturant the denaturation was complete by 6.9 M, and then a much simpler difference spectrum was produced with the expected two minima in the near ultraviolet and large minimum around 235 mμ in the middle ultraviolet and a large maximum at 227 mμ.

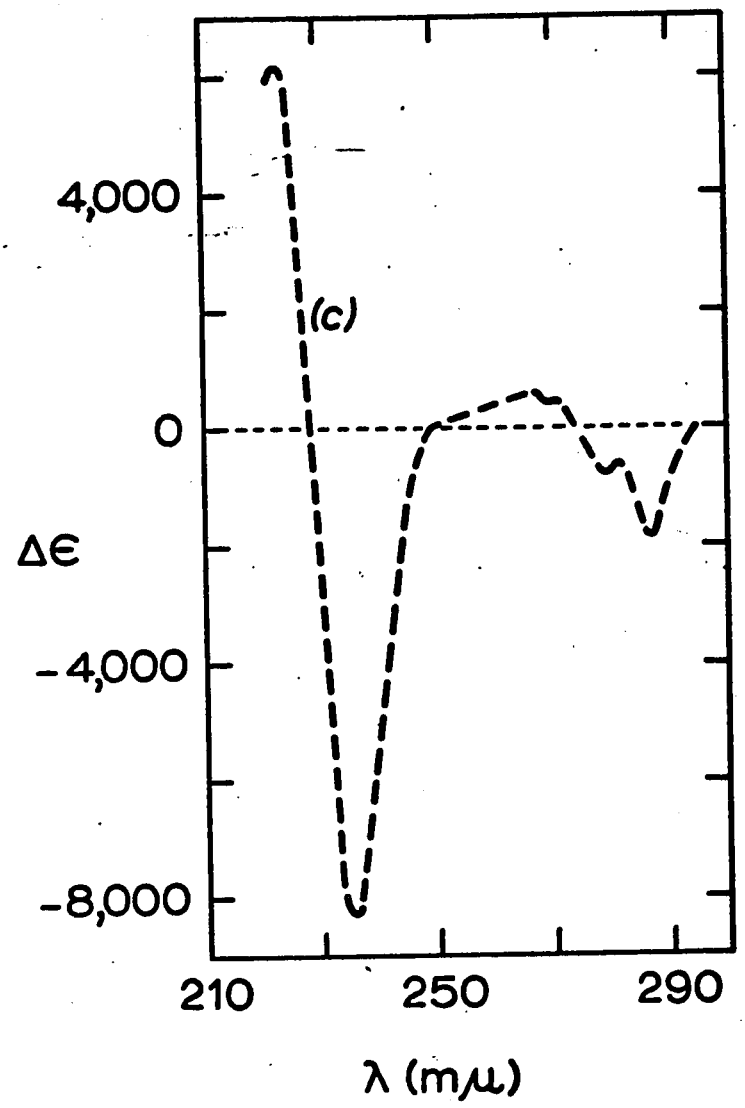
Ribonuclease: Solvent Effects

In this thesis we define the native state of RNase as the conformation present at pH 3.2, ambient temperature (28-30°), and ionic strength 0.01. In some of the experiments that follow these conditions may be changed but the conformation studied will be the same. These experiments will allow us to evaluate solvent effects on the chromophores normally exposed in the native state.

In other solvent effect investigations, the effect of additives on State III molecules was studied. State III molecules can be

FIGURE 27

The difference spectrum produced by the addition of 6.9 M urea to RNase.


4,000
(c)
0
Δε
-4,000
-8,000
210
250
290
λ (mμ)

produced, at pH 3.2, by the addition of 4 M guanidine hydrochloride. It is highly probable that State III molecules are as close to random coil molecules as they can be, considering the restraints imposed by the four intact disulfide bonds (Nozaki and Tanford (1967)). Thus by adding another solvent component to RNase in 4 M guanidine hydrochloride we can evaluate solvent effects measured when all the chromophoric groups should be exposed.

Native State

When the value of $\Delta\epsilon_{227}$ is plotted against concentration of additive a plot similar to the one in Figure 28 is produced. It shows that at low concentrations of ethylene glycol, below 70%, $\Delta\epsilon_{227}$ is a linear function of the additive concentration. Above 70% ethylene glycol there is a sudden rise in the value of $\Delta\epsilon_{227}$, which, combined with the appearance of the minimum at 235 mμ, indicates that denaturation is taking place. Values of $\Delta\epsilon_{227}$ taken from the linear portion of the curve may be used to study the effects of solvents on the native state of the RNase molecule.

The values of $\Delta\epsilon_{227}$ measured in ethylene glycol and urea at two difference pHs are given in Table 14. It can be seen that there is a fairly wide difference between the two values for urea. The results obtained at pH 5.9 disagree with the results obtained at pH 3.2. It seems reasonable to assume that the difference in the urea results is probably due to the change in pH, but it is

TABLE 14

SOLVENT EFFECTS FOR RIBONUCLEASE
AT 227 mμ

State	Additive	Sample pH	Δε /conc.* measured	Tyrosine No.	Tyrosine Δε/conc.
Native	Urea	3.2	1300	3	330
Native	Urea	5.9	880	3	330
Native	Ethylene Glycol	3.2	1300	3	770
State III**	Urea	3.2	1200	6	660
State III**	Ethylene Glycol	3.2	1900	6	1530

* Concentration for urea is M and for Ethylene glycol is 10%

** State III was produced by adding 4M guanidine hydrochloride at pH 3.2 to RNase.

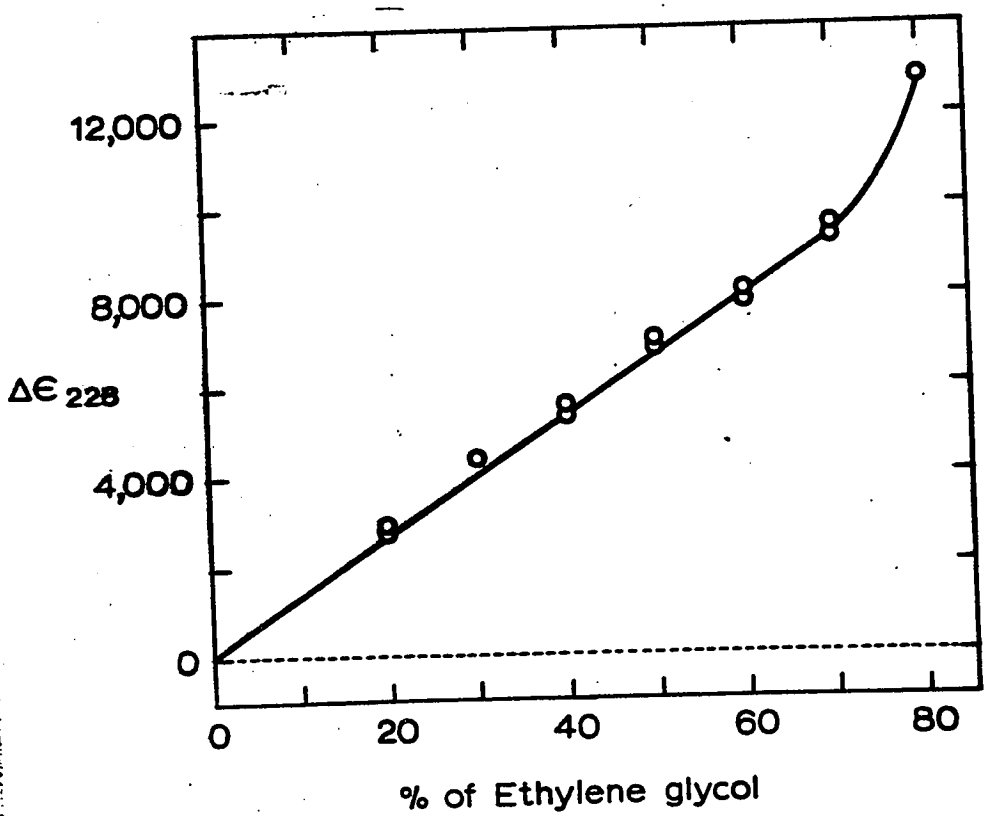
TABLE 14

SOLVENT EFFECTS FOR RIBONUCLEASE
AT 227 mμ

Histidine		Peptidebonds		$\Delta\varepsilon$/conc. theoretical	$\frac{\Delta\varepsilon \text{theoretical}}{\Delta\varepsilon \text{measured}}$
No.	$\Delta\varepsilon$/conc.	No.	$\Delta\varepsilon$/conc.		
4	190	94	180	700	0.53
4	190	94	180	700	0.81
4	170	94	260	1200	0.90
4	190	124	240	1100	0.91
4	230	124	360	2100	1.13

FIGURE 28

The effect of ethylene glycol on the absorption of RNase at pH 3.2.


12,000
8,000
4,000
0
$\Delta\epsilon_{228}$
0
20
40
60
80
% of Ethylene glycol

difficult to see why changing the pH should have this effect. The experimental value of $\Delta\epsilon$ at pH 3.2 is higher than it is at pH 5.9 but this cannot be attributed to a conformational change (which would expose more chromophores to the solvent) because many studies, by various physical techniques, have shown that no such change occurs in this pH range. In particular difference spectroscopy shows no change in the native structure (Scheraga (1957)) over this pH range. While further study of this question is indicated, our results may mean, that for reasons not now clear, urea interacts more powerfully with a protein at low pH than at a higher pH, so that the protein experiences a local urea concentration higher than that of the bulk of the solvent. One word of caution is needed before we leave the urea results. Because of the experimental difficulties in studying the 227 mμ peak (high optical density of proteins and solvent), it was often necessary in this study to study difference optical densities of the size of 0.04 to 0.05 and the possibility of the occurrence of a large error is increased over the results with ethylene glycol.

In Table 14 an attempt was made to compute from the model studies (data in Tables 6 and 12) the theoretical value of $\Delta\epsilon_{227}$ that the three major chromophores, tyrosyl and histidyl residues, and peptide bonds, would produce if they were placed in the medium used for the solvent effects studied (phenylalanine was not included because the additive difference spectra for phenylalanine and NAPEE

were shown in a previous section to be negligible at 227 mu). The numbers of each residue to be used in the calculation of the solvent effect was derived in the following manner; for tyrosyl residues it has been known since Shugar's experiments (1952) that three of the six tyrosyl residues are exposed (Herskovits and Laskowski (1968) have proposed a model recently in which two tyrosyl residues are fully exposed, two are partially exposed and two are buried; however for our purposes the calculation is unchanged, even if they are correct). Donovan (1965) showed that all four of the histidyl residues are exposed in the native protein. Both of the foregoing facts are seen to be reasonable from the X-ray data of Kartha et. al. (1967). As for the peptide bond, a simple geometrical calculation based on the assumption that the molecule is spherical showed that 94 of the 124 peptide bonds would be within five Angstroms of the surface of the molecule, presumably, that is, exposed to the environment. It can be seen in Table 14 that the solvent effects calculated as described give values for the native molecule which are (except for urea at pH 3.2) between 81 and 90% of the measured values.

The foregoing calculations draw to our attention the fact that the peptide bonds, although they account for 36% of the absorption in the native state, apparently account for only 26% of the solvent effect at 227 mμ. Similarly the histidyl residues, which cause 23% of the absorption at 227 mμ, account for 27% of the solvent effect

at 227 mμ, and the tyrosyl residues account for 42% of the absorption and 40% of the solvent effect. There are two reasons for the differences between the percentages of the absorption and the percentages of the solvent effects. The first is that different fractions of the different chromophores are exposed in the native molecule, and the second is the ratio of the chromophores solvent effect to its extinction at the same wavelength. This ratio, of course, differs from one chromophore to another. A very simple calculation allows us roughly to calculate the percentage contributions to the solvent effect from the percentage contributions to absorption. From the numbers of exposed chromophores used earlier, we can see that the fractions of the chromophores which are exposed are: histidine, 1.00; tyrosine, 0.5; and peptide bonds, 0.76. Ratios of solvent effect at 227 mμ (for urea) to extinction at 227 mμ can be determined from Tables 6 and 12, which give $\Delta\epsilon_{227}/M$ for tyrosine (110), and histidine (43) and the peptide bond (1.95), respectively, and Figure 1 from which ϵ_{227} for the chromophores can be estimated: tyrosine, 5600, histidine, 4600; and the peptide bond, 234. The ratios, $\Delta\epsilon_{227}/M\ \epsilon_{227}$ then are: for tyrosine, 19.7×10^{-3}; for histidine, 9.4×10^{-3}; and for the peptide bond, 8.3×10^{3}.

The amount a particular chromophore contributes to the solvent effect, relative to tyrosine, let us say, then can be calculated by multiplying its percentage contribution to the absorption by

the fraction of the residues exposed and by the ratio of ($\Delta\epsilon$ /Mϵ) chromophore/($\Delta\epsilon$ /Mϵ)tyrosine. For example tyrosyl chromophores in RNase should contribute 42 x 0.5 = 21 (arbitrary units). The histidyl chromophores will contribute 23 (1.0) (9.4/19.7) = 11, and the peptide bonds 36 (0.76) (8.3/19.7) = 11.5. The total solvent effect would then be 43.5 (arbitrary units), with percentage contributions tyrosyl residues, 48%, from histidyl residues 25%, and from peptide bonds, 27%. That these percentage contributions are the same as can be calculated from Table 14 is not surprising -- the two calculations should lead to the same result. We have gone through them here mainly because they serve to indicate why the various chromophores can contribute differentlyto the absorption of a protein and to the solvent effects observed with it.

Considering the sizable number of chromophores involved and the assumptions made in these calculations the agreement between the theoretical solvent effects and experimental ones is very good, though one of course wonders why it is not better. One explanation which may help to account for the differences between the calculated and measured values of $\Delta\epsilon_{227}$ stands out. This is the well-known change in intensity of the absorption bands of the chromophores as they are placed in the protein and rigidly fixed in a microenvironment that can not be reproduced with model compounds. It is generally observed (Wetlaufer (1962)) that extinction values for

proteins are larger (usually by 10-15%) than the sum of the extinction values of the constituent chromophores. Although the reasons for this fact are not clearly understood, it obviously will tend to make $\Delta\epsilon$, as well as ϵ, larger than one would calculate from model compounds in a given experiment. Bigelow and Geschwind (1960) showed that differences occurred in their solvent effect data obtained with tyrosine models and those obtained with oxidized-RNase, in the near ultraviolet. In short, what we are saying here, is that in order to study solvent effects accurately, one may have first to evaluate the applicability of different sorts of model compounds. Our estimation of $\Delta\epsilon_{227}$ from model compounds data gives very good agreement with the experimental value observed, probably as good as can be expected at present.

State III

Solvent effects on State III molecules were studied, as mentioned before, by denaturing the RNase molecule with 4 M guanidine hydrochloride, and then adding increasing concentrations of a third solvent compound. In these experiments, one expects $\Delta\epsilon$ to be an uninterrupted linear function of the additive concentration, because the State III molecule appears to be completely denatured with no residual secondary or tertiary structure. Values for such solvent effects, measured on State III molecules at 227 mu, are given in Table 14.

A value for urea and one for ethylene glycol are recorded in Table 14. The urea experiments were not as reproducible as one would like. The measured urea solvent effects on the State III molecule is, on the strength of the results in this study, rather erratic with an error of $\pm$ 15% at all concentrations of urea. We have at this time no explanation for this erratic behavior with the possible exception of the instrumental difficulties encountered in this work.

Because it has been shown that State III molecules are in the random coil conformation (Nozaki and Tanford (1967)), the calculation of $\Delta\epsilon_{227}$ for the model data was done on the assumption that all chromophores present would contribute. It can be seen that the calculated value of $\Delta\epsilon$ varies from 91 to 113% of the measured value at 227 mμ. This is excellent agreement between theoretical and experimental results and a closer agreement between the two values could not be expected. The basis for the small variation between the experimental and the theoretical values lies in the assumption that all residues would contribute, for local environments may prevent some residues from contributing fully. Support for this theory comes from the work of Herskovits and Laskowski (1968) who found that by using the solvent perturbation technique in the near ultraviolet on RNase in State III, they measured a smaller effect than they had calculated. They then pointed out that two of the tyrosyl residues are adjacent to disulfide bridges which might restrict

their ability to be solvated by the additive. However even considering these factors the results must be considered to be in excellent agreement with theory.

Ribonuclease: Denaturation

Denaturation studies were conducted on RNase using a wide range of denaturants which were reasonably transparent in the middle ultraviolet. It will be convenient to discuss the results of these studies one denaturant at a time. Not only do different denaturants cause different processes to occur, but the methods of solvent correction are often particular to a class of denaturants. The final subsection on the denaturation results will deal with the correlation of the data found in the near and the middle ultraviolet for the various denatured states of RNase.

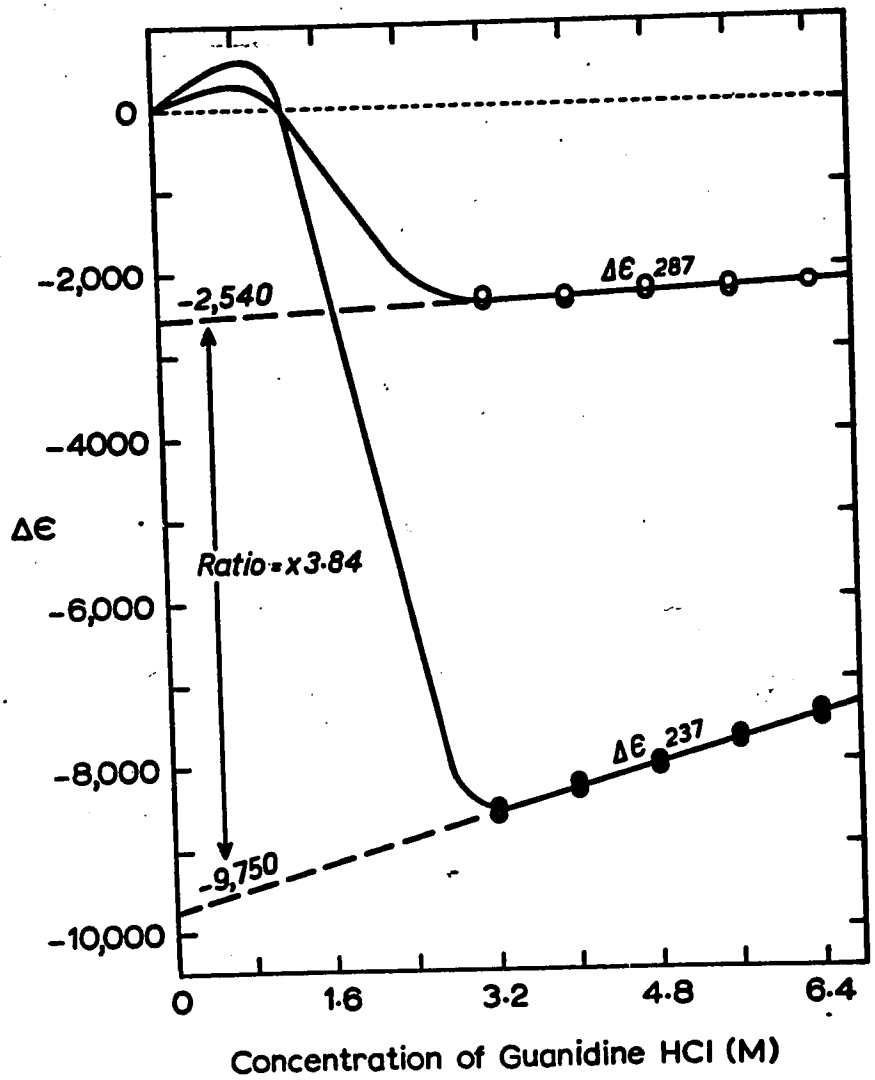
Guanidine Hydrochloride

Guanidine hydrochloride along with urea causes the largest near ultraviolet denaturation blue shift measured for RNase (Bigelow (1964)) and according to Nozaki and Tanford (1967) RNase in guanidine hydrochloride exists as a random coil. It was naturally important to study the middle ultraviolet denaturation blue shift of RNase in guanidine hydrochloride and fortunately it is transparent enough to permit this.

As can be seen in Figure 29 at pH 3.2, there is the expected

FIGURE 29

Effect of guanidine hydrochloride on the absorption of RNase at 237 and 287 mμ. This figure demonstrates the extrapolation solvent effect correction of Bigelow (1960,1964).


0
-2,000
-4000
-6,000
-8,000
-10,000
Δε
-2,540
-9,750
Ratio = x 3·84
Δε 287
Δε 237
0
1·6
3·2
4·8
6·4
Concentration of Guanidine HCl (M)

red shift at 237 mμ at low concentrations of guanidine hydrochloride followed by the denaturation blue shift at intermediate concentrations and then another red shift at high concentrations of guanidine hydrochloride. The denaturation transition under the same conditions is also shown at 287 mμ, in the near ultraviolet. It is readily apparent that the plot of $\Delta\epsilon_{287}$ and $\Delta\epsilon_{237}$ change together as the concentration of guanidine hydrochloride changes. The curve for $\Delta\epsilon_{287}$ is qualitatively and quantitatively the same as that of Bigelow (1964). The extrapolation to zero concentration of guanidine hydrochloride demonstrated in Figures 3 and 29 and used to correct the denaturation blue shift for solvent effects was first suggested by Bigelow (1960) and this method will be used in all the experiments to follow. As can be seen in Table 15, denaturation of RNase with guanidine hydrochloride at pH 6.0 or 3.2 causes a denaturation blue shift, $\Delta\epsilon_{237}$, of 10,000. This demonstrates that the magnitude of the denaturation blue shift is unaffected by pH and also that the RNase molecule becomes destabilized by lowering the pH, because the denaturation is completed at pH 3.2 in 3.2 M guanidine hydrochloride while at pH 6.0, 4.8 guanidine hydrochloride is needed. The red shift at low concentrations of guanidine hydrochloride can not be used to compare to solvent effect data on the model compounds as was done for other denaturants in the preceding section, because the data in the Figures are for $\Delta\epsilon_{237}$, not

TABLE 15

DENATURATION BLUE SHIFT FOR RIBONUCLEASE AT 237 mμ

Denaturant	pH	Ref. pH	Denaturant Conc.	State	$\Delta\varepsilon$ 287	$\Delta\varepsilon$ 237	$\frac{\Delta\varepsilon\ 237}{\Delta\varepsilon\ 287}$
EtOH	3.2	3.2	80%	III	?	10,500	-
Urea	2.1	3.1	6M	III	2700	10,000	3.7
Guanidine HCl	6.0	6.0	4.8M	III	2700	10,100	3.7
Guanidine HCl	3.2	3.2	3.2M	III	2700	9,750	3.6
P-Dioxane	3.1	3.1	55%	****	2000	7,550	3.8
Temperature*	3.2	3.2	48°C	****	1950	7,600	3.9
$LiClO_4$	3.2	3.2	2.8M	II	1700	6,600	3.9
pH/temp.**	0.55	3.2	40.5°C	II	1760	6,300	3.6
pH/temp.***	0.55	3.2	16.5°C	I	690	3,100	4.5

* Ionic strength .01

** Reference temperature was 25°C, ionic strength 0.1

*** Reference temperature was 16.5°C, ionic strength 0.1

**** Apparently intermediate between states II and III

$\Delta\epsilon_{227}$. The ratio of $\Delta\epsilon_{237}/\Delta\epsilon_{287}$, also given in Table 15, is 3.7.

Urea

Urea is also able to produce the same large denaturation blue shift in RNase that is found in guanidine hydrochloride (Bigelow (1960)) and the shape of the plot of $\Delta\epsilon_{237}$ against concentration of urea resembles that for RNase in guanidine hydrochloride. Thus it is important to see whether these two denaturants also give the same values of $\Delta\epsilon_{237}$. As can be seen from Table 15, the denaturation blue shift in urea is the same size as that found in guanidine hydrochloride, and the ratio of $\Delta\epsilon_{237}/\Delta\epsilon_{287}$ is again 3.7.

p-Dioxane

p-Dioxane was chosen because it produces an intermediate unfolded state in RNase assumed to be State II by Bigelow and Krenitsky (1964). It was found that when the p-dioxane plot was corrected for solvent effects the values listed in Table 15 were produced. The value of 2000 found at 287 mμ is larger than the value of 1700 reported by Bigelow and Krenitsky (1964), but when their data was replotted and the proper extrapolation to correct for solvent effects made it was found that a value of 2000 was also produced. The corrected value of $\Delta\epsilon_{237}$ was found to be 7550, which leads to a ratio, $\Delta\epsilon_{237}/\Delta\epsilon_{287}$, of 3.8. The similarity of the value

of thisratio to the values observed for urea and guanidine hydrochloride supported the conclusion that the value of $\Delta\epsilon_{287}$ is 2000, and not 1700. If these values are in fact correct, there seem to be two possible explanations. The first is that we are observing a new denaturation state, State IV, in which tyrosyl residues A and C ($\Delta\epsilon_{287}$ = 1000 for each of these, but $\Delta\epsilon_{237}$ = 3400 for each) have become exposed. The second possibility is that the RNase molecules are in State II but that some shift or intensification has affected the tyrosyl absorption band producing an increment to the normal absorption shift. This possibility is supported by the optical rotation and intrinsic viscosity data which agree with other measurements on State II molecules, and by the fact that it is difficult to see, from the crystallographic results of Kartha et. al. (1967) how tyrosyl A (tyr. 25) and tyrosyl C (tyr. 97) could be exposed without also exposing tyrosyl B (tyr. 92)

Ethanol

Ethanol was used in the hope that another organic reagent would reproduce the intermediate state found in p-dioxane. However, as can be seen in Table 15, $\Delta\epsilon_{237}$ is 10, 500, which would mean that ethanol, like urea and guanidine hydrochloride produces State III molecules. Because State III was already well studied the investigation of RNase denaturation in ethanol was discontinued; however, some additional information on RNase in ethanol can be

found in the first appendix.

pH and Temperature Denaturation

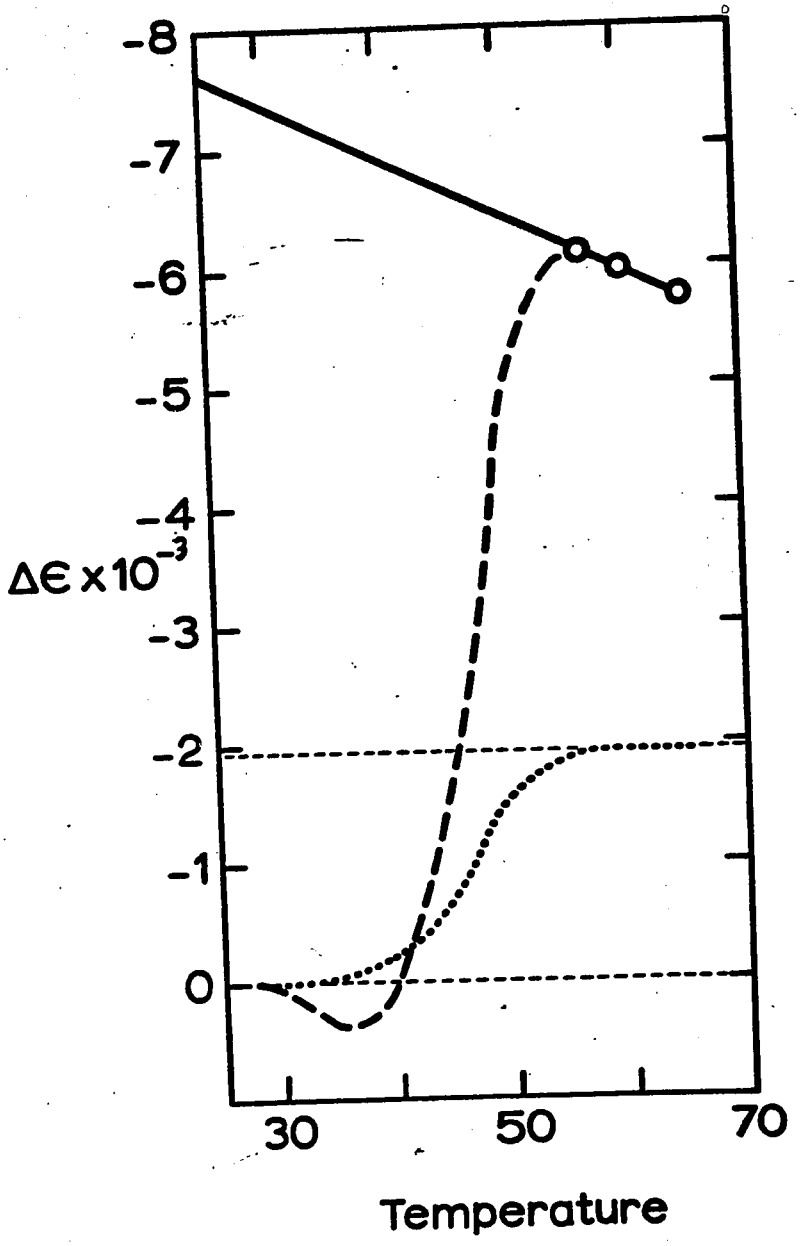
The pH and temperature denaturation must be considered together because of the interrelations which have been shown to exist between them (Bigelow (1961) and Hermans and Scheraga (1961a, b).

Melting curves for RNase, measured at both 287 and 235 mμ are shown in Figure 30. It is clear that both $\Delta\epsilon_{287}$ and $\Delta\epsilon_{235}$ change together, with a transition temperature of about 48°C. The curves in Figure 30 were measured at pH 3. 2, ionic strength 0. 01, but other measurements were made at ionic strength of 0. 11.

As can be seen in Figure 30, the data for $\Delta\epsilon_{235}$ are temperature-dependent even outside the range where denaturation is occurring -- there is a red shift of the middle ultraviolet band as the temperature is raised. Although it cannot be seen from Figure 30, a similar, but much smaller, effect can also be detected at 287 mμ. The temperature effects have been corrected for in all the melting curve experiments by extrapolating to 25°C, as shown in Figure 30. Thus the denaturation blue shift, $\Delta\epsilon_{235}$, after correction for the temperature effect, has a value of 7600, and $\Delta\epsilon_{287}$ has a value of 1950. The latter value is higher than found by either Bigelow (1961) or by Hermans and Scheraga (1961a, b) and this is apparently due to the low ionic strength at which the data in Figure 30 were measured.

FIGURE 30

Effect of temperature on the absorption of RNase at 237 mμ (dashed line) and 287 mμ (dotted line). This figure demonstrates the temperature correction.



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Values measured at the higher ionic strength were, for $\Delta\epsilon_{235}$, 6300 $\pm$ 100 and for $\Delta\epsilon_{287}$, 1760 $\pm$ 50. The latter value is in excellent agreement with the values of the earlier workers.

In both cases, it is interesting to note that the ratio, $\Delta\epsilon_{235}/\Delta\epsilon_{287}$ is 3.75 $\pm$ 0.15, the same value as observed in the other denaturants.

It appears that although temperature denaturation produces State II (Bigelow, (1961, 1964)) there is an additional effect observed at very low ionic strength, which makes $\Delta\epsilon$ slightly larger than it would be at the higher, more usual, values. Presumably this had to do with the interaction of the ions present in the solution with the tyrosyl chromophores of the proteins.

Next the pH was lowered to 0.55, at which changing the temperature causes a transition between State I and II (Bigelow, 1961). It was then possible to study both State I and State II, namely by changing the temperature from 16.5°C to 40°C. When the experiment was carried out at 40°C (when all the molecules are in State II), it was found that $\Delta\epsilon_{235}$ was 6300, and $\Delta\epsilon_{287}$ was 1760. The latter value is in excellent agreement with those previously observed (Bigelow (1961) and Hermans and Scheraga (1961a, b)). The ratio $\Delta\epsilon_{235}/\Delta\epsilon_{287}$ is 3.6. When the experiment was carried out at 16.5°C (when all the molecules are in State I) the values obtained were, for $\Delta\epsilon_{237}$, 3100, and for $\Delta\epsilon_{287}$, 690. The latter value

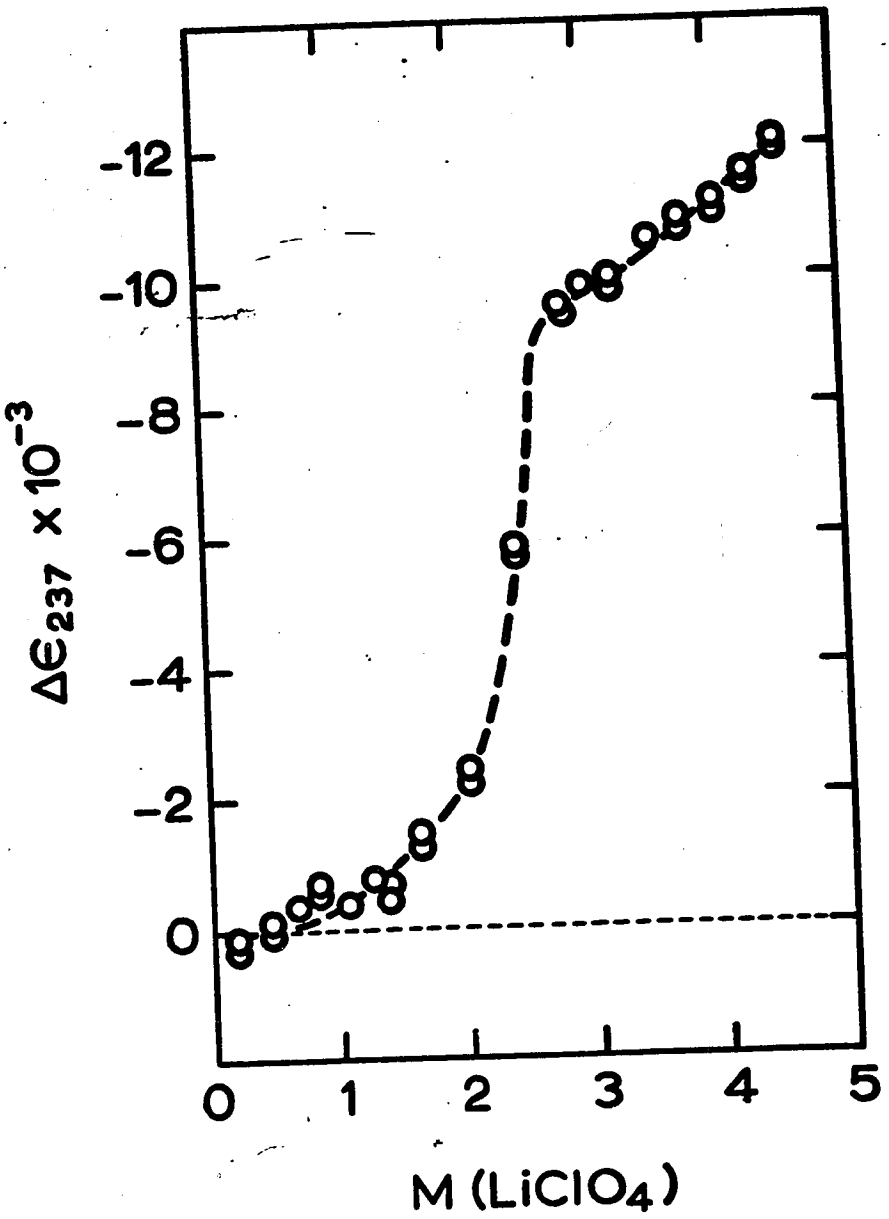
is in excellent agreement with the value of 700 reported by Bigelow (1964). It should be noted that the State I data give a ratio of $\Delta\epsilon_{237}/\Delta\epsilon_{287}$ of 4.5, appreciably higher than those found with the other denaturants. The point will be discussed further below.

$LiClO_4$

Lithium salts produce an intermediate state of unfolding that has been identified as State II (Bigelow (1960, 1964)), and they were therefore used in this study to obtain a value for $\Delta\epsilon_{237}$ that could be compared with the temperature data. Bigelow used LiBr in his study, but this salt is not sufficiently transparent for work in the middle ultraviolet, and we have therefore used $LiClO_4$ instead. As can be seen in Figure 31, $\Delta\epsilon_{237}$ is always negative at all concentrations of $LiClO_4$, that is there is a blue shift solvent effect, as well as a denaturation blue shift. The same sort of behavior is observed at 287 mμ by us with $LiClO_4$ and with LiBr by Bigelow (1960). The denaturation transition occurs between 1.5 and 2.8 M $LiClO_4$, but owing to the nonlinear solvent effects (Sarfare and Bigelow (1967)) the plot is curved even where only the solvent effects are occurring. To obtain accurate values for the denaturation blue shift, the solvent effect must be removed. It was found by Sarfare and Bigelow (1967) that the curvature of the LiBr results could be corrected for by using a semilorgithmic plot in which the log $\Delta\epsilon_{287}$ was plotted against the molarity of LiBr. When this was done for our data in

FIGURE 31

Effect of $LiClO_4$ on the absorption of RNase at 237 mμ.



$LiClO_4$ the plot in Figure 32 was obtained. It is readily apparent that the solvent correction does provide a linear extrapolation which will provide the desired correction. When this correction is effected, the value of $\Delta\epsilon_{287}$ was found to be 1700 and that of 237, 6,600. They agree well with the values obtained in the melting curve experiments. The ratio of $\Delta\epsilon_{237}/\ \Delta\epsilon_{287}$ is 3.9 which also agrees well with those measured in the other denaturants which produce State II and State III molecules.

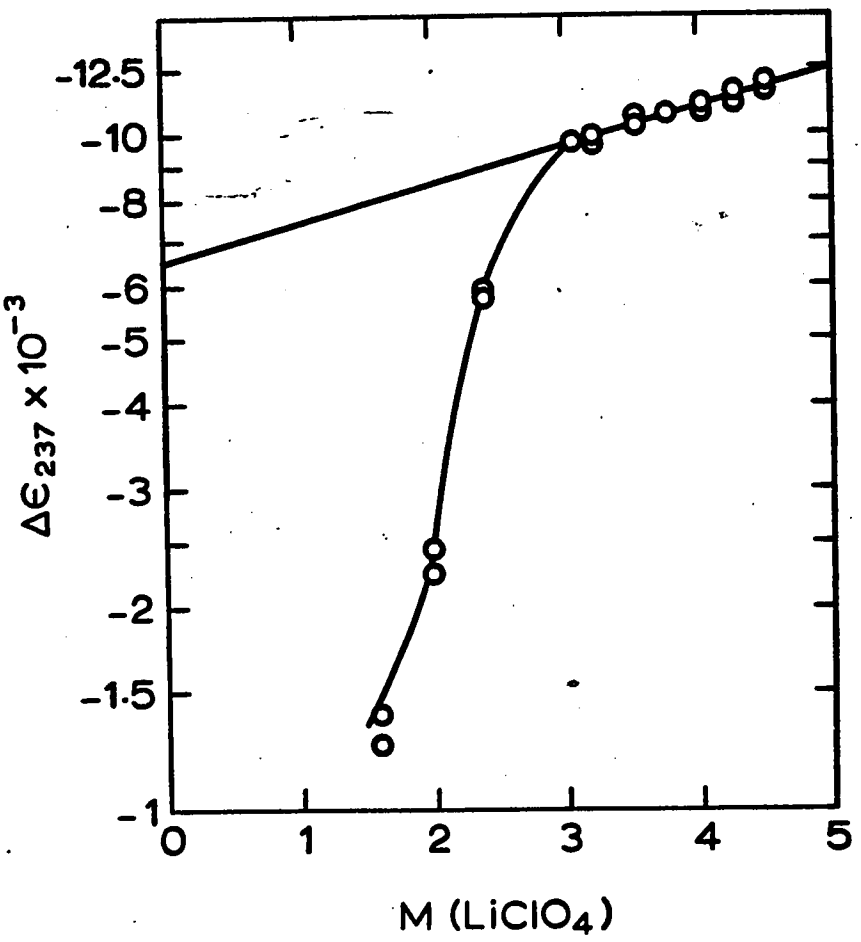
DENATURED STATES OF RIBONUCLEASE

As was previously shown in the historical section, three denaturated states of RNase have been clearly characterized by Bigelow (1964) using the techniques of difference spectroscopy at 287 mμ, optical rotation, and intrinsic viscosity. This study was initiated partly to see if the denatured states could also be distinguished by characteristic changes in the middle ultraviolet and if they could be quantitatively studied at 237 mμ. In what follows we will discuss the data we have collected for RNase, and it will be shown that the middle ultraviolet data verify the earlier conclusions reached by Bigelow.

As can be seen in Table 15, the production of State III molecules is accompanied by a denaturation blue shift, $\Delta\epsilon_{235}$, equal to 10,000, for both the denaturants used. The ratio of $\Delta\epsilon_{235}/\Delta\epsilon_{287}$

FIGURE 32

Semilogarithmic plot of data from Figure 31.



varies between 3.6 and 3.7 for the three studies of State III molecules. There is clearly excellent agreement among these three sets of data.

State II denaturation was caused either by the addition of $LiClO_4$, or by melting at pH 0.55 and ionic strength 0.45. The results of these experiments are also given in Table 15. The values measured for $\Delta\epsilon_{287}$, 1730 $\pm$ are in excellent agreement with those of Bigelow (1964). The value of $\Delta\epsilon_{237}$ was found to be 6450 $\pm$ 150. The ratio of $\Delta\epsilon_{235}/\ \Delta\epsilon_{287}$ varies from 3.6 to 3.9 in agreement with ratios found in the other denaturants.

Unusual values of $\Delta\epsilon$ were found when RNase was denatured at an ionic strength of 0.01, or when it was melted at very low ionic strength (0.01 M). $\Delta\epsilon_{287}$ was found in these experiments to be 2000, and $\Delta\epsilon_{237}$ was 7600. Both of these are intermediate between the values found for State II molecules and State III molecules. In both of these experiments an increase in the ionic strength caused the denaturation blue shifts to decrease to the values normally found for State II molecules.

There are two possible models that could explain these results. The first is that we are causing the molecules to take up a fourth denatured conformation in which tyrosyl residues A and C ($\Delta\epsilon_{287}$ = 1000 each) are exposed and B is buried. And the second possibility is that these denaturants are really reproducing State II molecules

but that because of low ionic strength in the denaturing environment, $\Delta\epsilon$ has a value slightly larger than it usually has. The second possibility is favored for several reasons. In the first place it is impossible to see, from a study of the crystallographic results of Kartha et. al. (1967) how tyrosyl A (Tyr-25) and tyrosyl C (Tyr-97) could be exposed in a denaturation process without also exposing Tyrosyl B (Tyr-92). Secondly if we take the values calculated for A, B, and C (see Table 16) and compare the two possible models we find that for A and B at 287, $\Delta\epsilon$ is 1700, and at 237 it is 6500 so that an increment is needed at both wavelengths in accordance with the second model. Now if we take the first model -- the one in which tyrosyl residues A and C are being exposed and there is no increment -- we obtain the value of 2000 for $\Delta\epsilon_{287}$ which agrees with the experimental value, but at 237 mμ, A and C would be expected to give a $\Delta\epsilon$ value of only 6900, which is not equal to the experimental value. Therefore, this model does not explain the $\Delta\epsilon_{237}$ value observed. We therefore prefer the second alternative which indicates that some shift or intensification of the tyrosyl absorption bands has taken place causing $\Delta\epsilon$ to be slightly larger than normal, with this behavior sensitive to ionic strength. The ratio of $\Delta\epsilon_{237}/\Delta\epsilon_{287}$ of 3.8 to 3.9 shows good agreement between the results and also indicates a general trend of the ratio increasing as the unfolding decreases.

State I denaturation has so far been observed under only one set of denaturation conditions, namely low pH (about 1), and low temperature (below about 17°C). When these conditions were studied in the middle ultraviolet the value of $\Delta\epsilon_{237}$ (3100) was obtained while in the near ultraviolet the value of $\Delta\epsilon_{287}$ (690) was in excellent agreement with the value of 700 reported by Bigelow (1964). The ratio of $\Delta\epsilon_{237}/\Delta\epsilon_{287}$ is 4.5, significantly larger than observed for State II and III molecules.

The reason for the change in the ratio can be seen in Table 16, which presents the average values of $\Delta\epsilon_{287}$ and $\Delta\epsilon_{237}$ for the different denatured states in RNase. It also includes the values for the change in $\Delta\epsilon_{287}$ or $\Delta\epsilon_{237}$ between the different states. This value should be the value of $\Delta\epsilon$ due to the individual tyrosyl residue which is exposed in the transition from one state to the next. As was shown in the historical section and as can be seen in Table 16, the values of $\Delta\epsilon_{287}$ are 1000, 700 and 1000 for residues A, B, and C respectively. As can be seen also in Table 16, the values of $\Delta\epsilon_{237}$ are 3400, 3100, and 3500. This means that the ratio for the tyrosyl residues are 3.4, 4.5, and 3.5. This variability of this ratio for different tyrosyl residues is the basis for the variability of the ratio between the different denatured states in RNase. It should also be noted that the values of $\Delta\epsilon_{237}$ are almost the same for each of the three residues, the value for residue B is almost 90%

TABLE 16

VALUES OF $\Delta\epsilon$ OBSERVED IN THE VARIOUS DENATURATED STATES OF RIBONUCLEASE

State	$\Delta\epsilon$ 287	$\Delta(\Delta\epsilon$ 287)	$\Delta\epsilon$ 237	$\Delta(\Delta\epsilon$ 237)	$\frac{\Delta(\Delta\epsilon\ 237)}{\Delta(\Delta\epsilon\ 287)}$
I	690	690	3,100	3,100	4.5
II	1700	1000	6,500	3,400	3.4
III	2700	1000	10,000	3,500	3.5

of the values for A and C, whereas at 287 mμ the value for B is about 70% of the values for A and C.

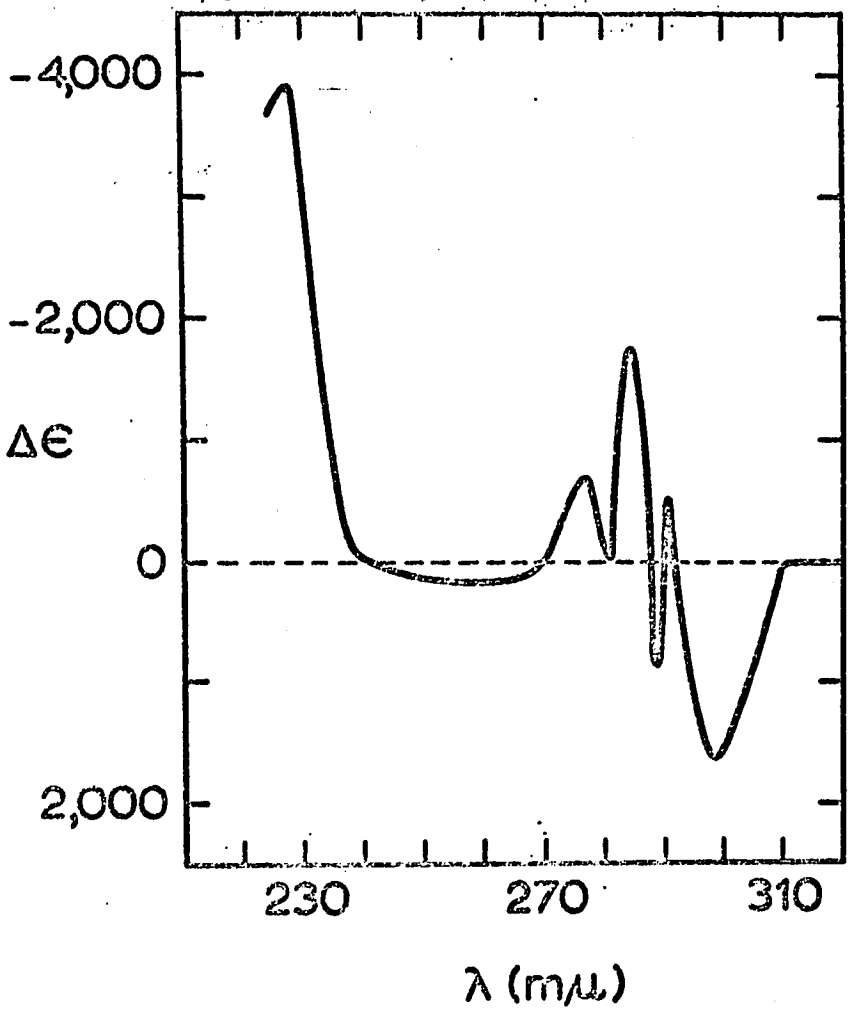
GENERAL DISCUSSION (OTHER PROTEINS)

In the work presented in this thesis we have shown as others before us have shown, that the denaturation blue shifts in the near ultraviolet are accompanied by similar denaturation blue shifts in the middle ultraviolet (in the 230 to 240 mμ range), and that both are due to the sidechains of the aromatic amino acids. The only outstanding piece of evidence which seems to contradict this conclusion can be seen in the work of Glazer and Smith (1961) on the unfolding of pepsin at pH 7.3. They found, on unfolding pepsin under alkaline conditions (pH 7.3), that a near ultraviolet difference spectrum developed, but it was not accompanied by a middle ultraviolet difference spectrum above 230 mμ. We felt that it was important to see if the denaturation blue shift might not be found below 230 mμ. Though this would be an unusually low wavelength judging from our RNase results, pepsin, unlike RNase, contains trytophan, and its blue shifts are found at lower wavelengths than those of tyrosyl residues. As can be seen in Figure 33, when the experiment was repeated a large blue shift was found at 227 mu.

In order to compare our values of $\Delta\epsilon_{235}/\Delta\epsilon_{287}$ to values in the literature, we have collected as many of these as possible. In some cases the values were not reported by the original authors, and

FIGURE 33

Acid difference spectrum of pepsin. Sample pH 7.3, reference pH 5.7. This work was performed by Mrs S. Sreenathan.



we have therefore calculated them from data presented in figures or tables wherever that was possible. All of these results are compiled in Table 17. It should be pointed out that many of these data have not been corrected for solvent effects, and therefore they do not represent correct values of the ratio of denaturation blue shifts. They are included in Table 17 for the sake of completeness, but they will not be discussed further.

It can be seen from Table 17 that only Eisenburg and Edsall (1963) and Martin and Bhatnagar (1966, 1967) have corrected their denaturation blue shifts for solvent effects. Their data therefore can be used to determine ratios $\Delta\epsilon_{237}/\Delta\epsilon_{287}$ to compare with those we have measured for RNase. As Table 17 shows, these ratios are remarkably constant for the proteins considered. If we exclude the denaturation of RNase to State I, all the ratios from these investigators are 3.4 ± 0.1, that is, the ratio of the two extinction changes is the same, within a few percent, regardless of the protein.

The value observed for the denaturation of RNase to State I is somewhat larger than the others, namely 4.5. This is interesting but it is not easy to see why such a variability is observed. It appears from our experiments, that the denaturation blue shifts observed with RNase at 237 mμ are, like the ones at 287 mμ, due solely to tyrosyl residues, that is, to the exposure of the three that are buried in the native molecule. Peptide bonds do not absorb significantly at 237 mμ

TABLE 17

RATIOS FOR DENATURATION STATES OF PROTEIN

Reference	Proteins	Denaturant	$\Delta\varepsilon\lambda 1/\lambda 2$	$\lambda 1/\lambda 2$
Glazer and Smith (1960)	BSA	pH	4.3	234/288
	Pepsin	pH	8.5	230/286
	Papain	pH	4.7	235/288
	Ovalbulmin	NaLauryl SO_4	11.4	232/293
	Chymotryspinogen	Alkaline pH	4.3	230/293
	Chymotrypsinogen	Acid pH	4.3	230/293
Glazer and Smith (1961)	HSA	pH	3.9	233/288
	RNase	pH	5.8	235/288
	lysyzyme	Alkaline pH	3.6	231.4/293
	Oval	Urea	13.9	233/287
	Oval	GuHCl	7.9	233/288
Kabacoff and Laken (1964)	Chymotrypsin	Temperature	3.1	232/292
Hermans (1962)	Myoglobin	Alkaline pH	3.9	245/295
	Horse globin	Alkaline pH	5.0	245/295
Eisenberg and Edsall* (1963)	HSA	low-high pH	3.3	236/288
	RCA	low-high pH	3.3	236/288

Martin (1964)	-chymotrypsin	Urea	3.4-3.2	231/293
Martin and Bhatnagar* (1966)	DIP-chymotrypsin	Urea	3.5	231/193
Edsall et al (1967)	CAB	Urea, GuHCl	4-7	235/293
	CAC	Urea, GuHCl	4-7	235/293
Martin and Bhatnagar* (1967)	Chymotrypsin	Urea, GuHcl	3.5	235/293
	DIP-Chymotrypsin	Urea, GuHcl	3.5	235/293
A'Zary* (this Thesis) (1968)	RNase	State I (see text)	4.5	237/288
	RNase	States II and III (see text)	3.4-3.5	237/288

* Corrected for solvent effects by method of Bigelow (1960).

(see Figure 1), and as mentioned earlier, all the histidyl residues seem to be exposed in the native molecule (Donovan (1965); Kartha et. al. (1967)). Thus the ratio of $\Delta\epsilon_{237}/\ \Delta\epsilon_{287}$ for RNase seems to be really a ratio of two tyrosyl spectral shifts, uncomplicated by contributions from other chromophores. The variability in the ratio cannot therefore, be explained away as arising in contributions, say, from histidyl residues. No really plausible explanation for this variability presents itself.

It is also interesting that the ratios are relatively constant from one protein to another. The proteins used by Eisenberg and Edsall (1963) and by Martin and Bhatnagar (1966, 1967) contain tryptophan, of course, which RNase does not, and tryptophan residues certainly should be involved in determining the values of $\Delta\epsilon_{237}$ and $\Delta\epsilon_{287}$. Indeed, from what we now know about tryptophan, we might expect that the ratio would increase as more tryptophan residues became involved in the denaturation transition. It would obviously be worthwhile to correct $\Delta\epsilon$ ratios for more tryptophyl containing proteins.

CONCLUSION

In this thesis we have been concerned with the middle ultraviolet (220-250 mμ) spectra of proteins and their constituent chromophores. We have attempted to show that the difference spectra which are observed when proteins are transferred from one solvent to

another or when they are denatured can be interpreted qualitatively and quantitatively in terms of the properties of the constituent chromophores. In this endeavor we have been guided by many similar studies which had previously been carried out in the near ultraviolet (250-300 mμ).

In pursuit of the goal mentioned in the preceding paragraph, we have carried out extensive studies of the effects of solvent additives (organic solvents, inorganic salts, guanidine hydrochloride, urea) on the middle ultraviolet spectra of chromophoric amino acids, peptide bonds, and related compounds, and RNase. These studies with small compounds were carried out to collect numerical data from which we could estimate the importance of the various chromophores in the proteins. RNase, which contains no tryptophan, was the protein studied most extensively.

Solvent effects on the spectrum of Ribonuclease (both in the native state and in one of the possible denatured states) cause a maximal value of $\Delta\epsilon$ to occur about 227 mμ, and our studies show that their numerical values observed can be estimated (within about 10%) from the model compound data if one includes the chromophores tyrosine, histidine, and the peptide bond. In the course of the study of the amino acids and derivatives we have also observed a number of interesting facts about the spectra and difference spectra of these compounds which are incidental to the chief aim, but nevertheless of some importance. They will not be listed here.

The second major aim of the study was to see whether and how usefully protein denaturation can be followed by middle ultraviolet difference spectroscopy. For this study, the enzyme RNase, already extensively studied by other methods, was used. The results show that denaturation can be followed just as well in the middle ultraviolet as in the near ultraviolet and that the interpretation of the observations is just as simple as it was at the higher wavelengths. This is partly fortuitous and some of the problems to be expected with other proteins are discussed.

RNase had previously been shown to reach different states dependent on the denaturant used, by the techniques of near ultraviolet difference spectroscopy, optical rotation, and intrinsic viscosity. Our results show these changes are accompanied by spectral change (denaturation blue shifts) with maxima at 237 mμ. At this wavelength, as our model compound data show, only tyrosyl and histidyl residues should contribute significantly to the observed differences. However data of other investigators show that all the histidyl residues and three of the six tyrosyl residues are exposed to the environment even in the native molecule. Our results therefore can be interpreted in terms of the three buried tyrosyl residues, just as the near ultraviolet data could be.

Our results in the middle ultraviolet show:

(a) that denaturation of proteins can be followed quantitatively;

(b) that, at least in this protein, values of $\Delta\epsilon$ can be assigned to individual tyrosyl residues as they are exposed in denaturation; and

(c) that, in agreement with data measured by other methods, RNase is capable of forming three different denatured states, dependent on the denaturant used.

It will, of course, be of great interest to apply middle ultraviolet difference spectroscopy to other proteins in a quantitative fashion. Our data appear to indicate that denaturation difference spectra, with maxima at 237 mμ will in general, require interpretation in terms of tyrosyl, histidyl and tryptophyl residues, but not the peptide bond.

POTENTIAL FUTURE INVESTIGATIONS

1. It would be of interest to continue a study of this type including model studies, protein denaturation studies and protein solvent effect studies into the far ultraviolet range, that is the 185 to 220 mμ range.

2. A continuation of this study to other tyrosyl containing proteins would provide additional information now lacking, in particular on whether the contribution due to each tyrosyl residue can be separated to obtain the $\Delta\epsilon_{237}$ and $\Delta\epsilon_{287}$ values for each individual tyrosyl residue.

3. No work on complex tryptophan models has been carried out here and such work will be needed in the future. Furthermore there remain some unexplained phenomena that were observed with some of the amino acids studied here, although it appears that these problems do not prevent a consistent semi-qualitative interpretatation of the middle ultraviolet difference spectrum observed with RNase.

4. It will obviously be essential to extend this sort of investigation to tryptophyl containing proteins. The presence of that chromophore in proteins will naturally be of great importance, and it is not possible to say, on the strength of the present work, whether such good quantitative relationships will be found

between data for such proteins and their constituent chromophores.

5. A detailed study of the effects of methanol, ethanol, and other alcohols on RNase is needed to demonstrate if the peculiar effects we observed with methanol (appendix I) are particular to methanol or are general effects of alcohol on the denaturation process of RNase.

6. Continuation of work designed to calculate the absorption spectra of proteins from those of the constituent chromophores is indicated (appendix II). The difference between the calculated and measured spectra may provide useful information on the interior of proteins.

7. It would be interesting to try to extend the calculation of protein spectra into the far ultraviolet where they may bring the most benefit not only because of the presence of many chromophores but also because the effects of conformation on the peptide bond absorption will show up in the calculated difference spectrum.

8. The 227 mμ peak should be studied during denaturation because some effect of the denaturation blue shift is felt at that wavelength and it may be possible, because of the nearness of the 225 mμ maximum in the difference spectrum for the helix- coil transition to estimate changes in helical content during denaturation.

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APPENDIX I

THE UNFOLDING OF RIBONUCLEASE IN METHANOL AND ETHANOL

It was found in the course of this study that at high concentrations of ethanol and methanol the optical rotation was dependent on time. The time dependency could be observed even after the reaction mixture was an hour old, and it could not be reconciled with the apparent fact that at the concentrations of methanol and ethanol being studied, RNase should have completed the unfolding transition in a shorter time. The logarithm of the optical rotation was then plotted against time, because if the process occurring was denaturation such a plot should be linear. The plot was however, found to be nonlinear, that is, it had two regions that were linear connected by a transition region. This fact, combined with the fact that when the same reaction was studied by difference spectroscopy at 239 mμ it was found to have a rapid first step that was a blue shift due to denaturation, followed by a slow second step that was a red shift, indicating two processes are taking place.

The pseudo-order of the second process was found to vary

between 2 and 14, and it could be assumed that the order of the second process was at least two and subsequent results were calculated as though it was second order. When the optical rotation and ultraviolet results are plotted as a second order process (see Figures 34 and 35) it can be seen that the plot is linear from 3-9 minutes to 30 minutes under the conditions used. Changing the methanol and ethanol concentration while keeping the concentration of protein constant (0. 15%) has little effect on the second reaction with the exception of changing the transition time between the two reactions.

Lowering the pH was found to speed up the transition from the first to the second reaction. When the concentration of the protein was increased from 0. 15 to 0. 2% the first reaction was completed when the first reading was taken (after 0. 5 minutes) and the pseudo-order of the second reaction increased to 14.

To study the first reaction it is necessary to correct the results for the second reaction, this can be done by extrapolating the plot of the data from the second reaction back to zero time and calculating the difference between the extrapolated line and the experimental values. When this is done the logarithm of the difference was plotted against time and this produces linear plots similar to Figures 36 and 37. This demonstrates that the first process (denaturation) is first order, as one would expect. The second reaction is second order or higher, and since it causes intense light

FIGURE 34

Time dependence of $1/\alpha_{350}$ for RNase in 70% methanol at pH 1.12.

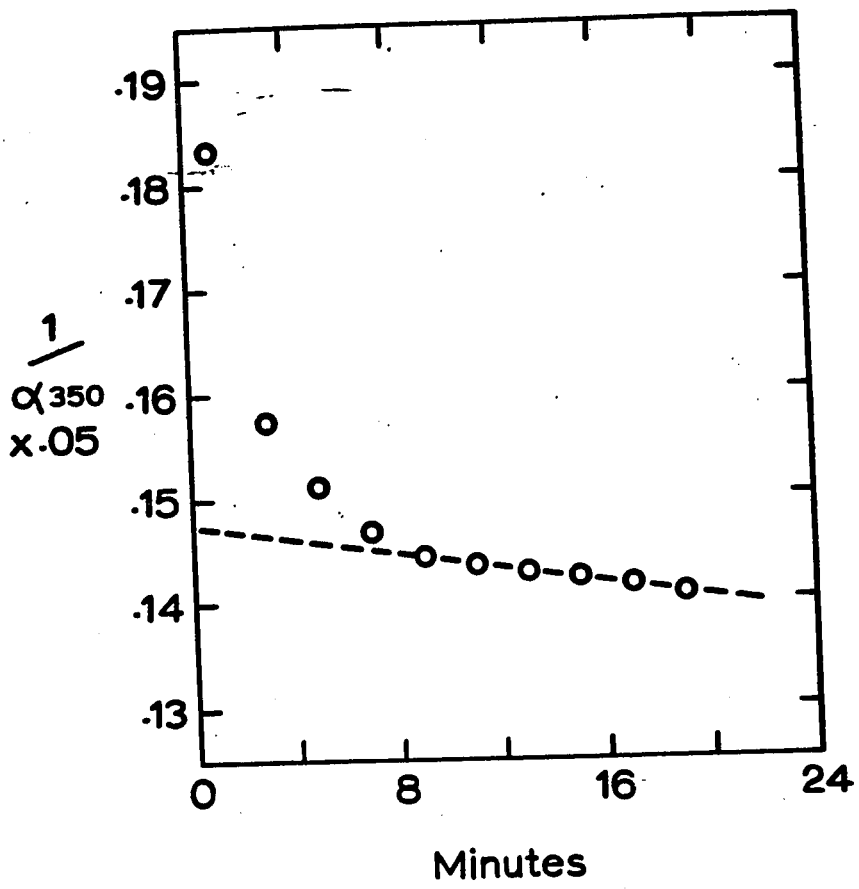


FIGURE 35

Time dependence of $1/\Delta$ O.D.$_{239}$ for RNase in 85% ethanol at pH 3.2.

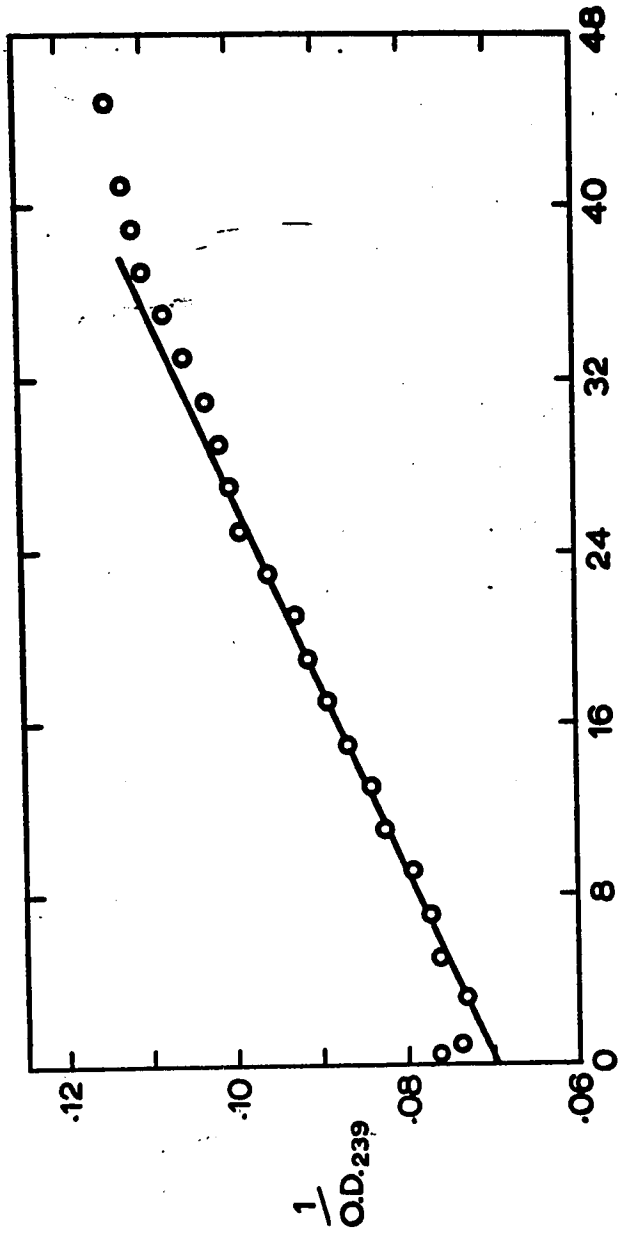

48
40
32
24
16
8
0
Minutes
.12
.10
.08
.06
1/O.D.239
Art Service U.W.O.
London Ontario

FIGURE 36

Time dependence of $\log \Delta\alpha_{350}$ for RNase in 70% methanol at pH 1.12. For explaination of $\Delta\alpha$ see text.

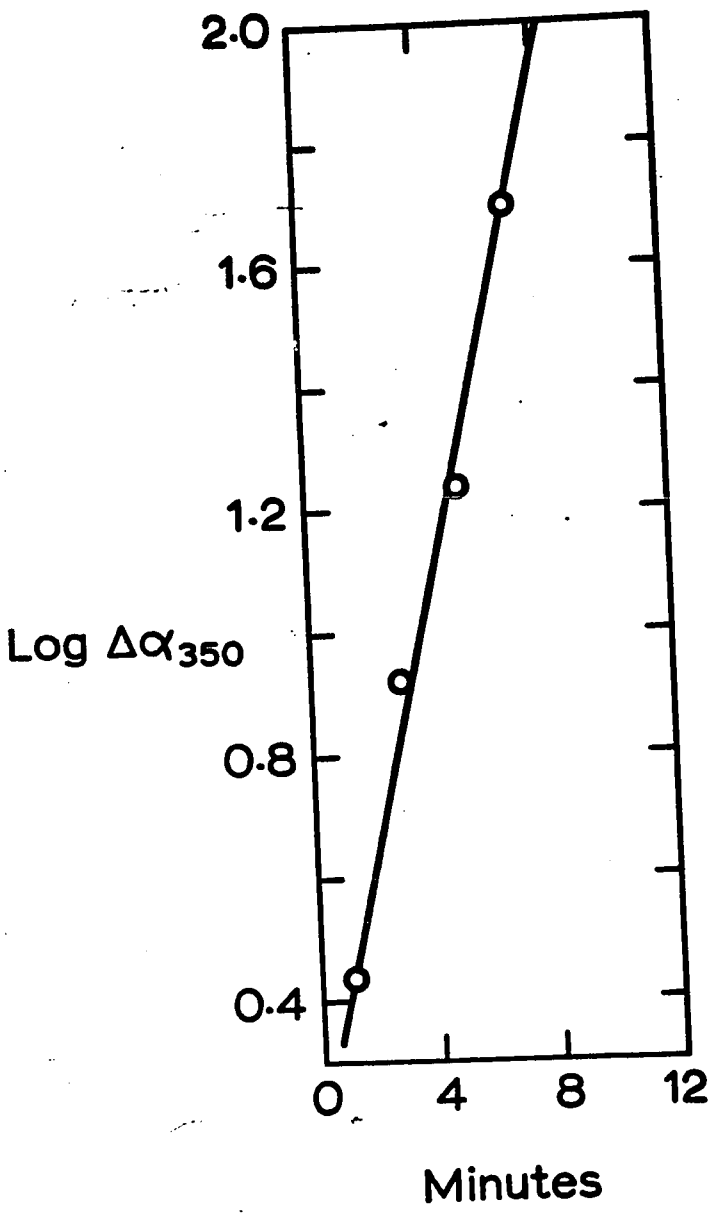
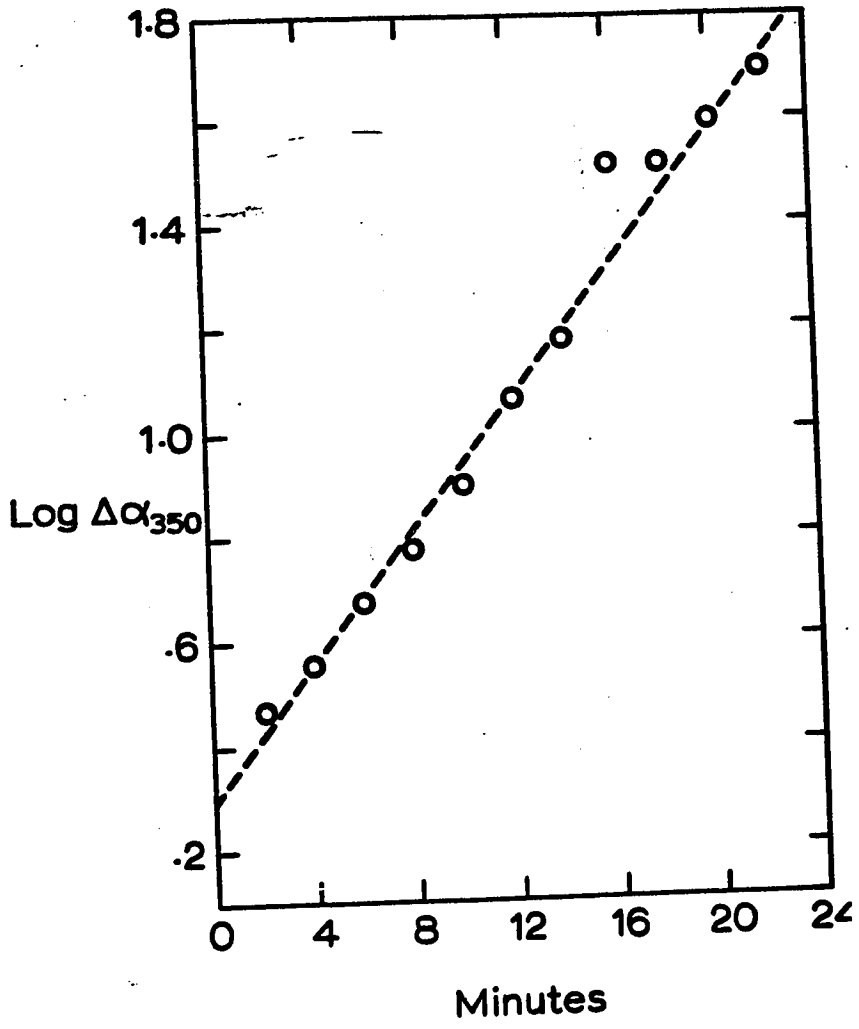


FIGURE 37

Time dependence of $\log \Delta \alpha_{350}$ for RNase in 85% methanol at pH 3.2. For explaination of $\Delta \alpha$ see text.

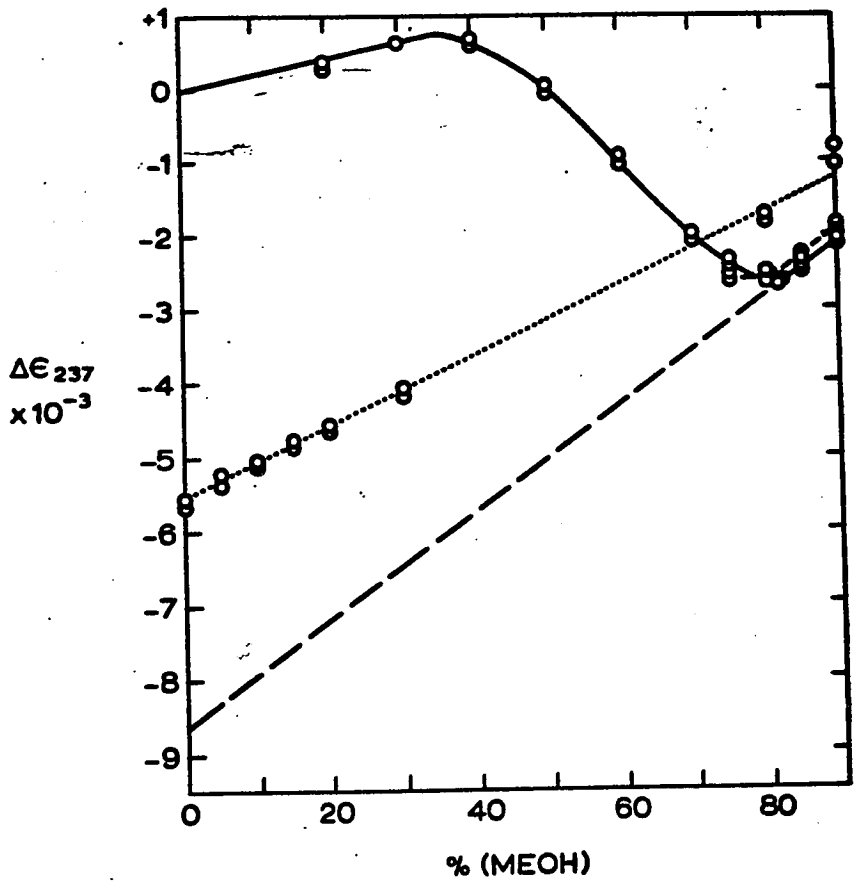


scattering which is a symptom of aggregation it is logical to conclude that the second reaction is due to aggregation of two or more unfolded RNase molecules. This conclusion is supported by the rapid increase in the order of the second reaction when the protein concentration is increased.

Thus we can correct the difference spectral results, measured for the denaturation of RNase in methanol or ethanol for the results of secondary time dependent aggregation. The correction allows us to determine a denaturation blue shift which can then be studied as a function of the alcohol concentration. The dependence of the denaturation blue shift on methanol concentration is shown in Figure 38 at three pH's. At pH 3. 2 we find a usual denaturation curve, that is a linear red shift at low concentrations that is linear with concentration of methanol, followed by the denaturation blue shift and then the solvent red shift at high concentrations of methanol. At pH 2. 2 only a few points, at high concentrations of methanol, were measured, and it was found they are shifted upward to slightly smaller values of $\Delta\epsilon_{287}$ than were observed at pH 3. 2 and the same concentrations of methanol. Thus the behavior of RNase at pH 2. 2 is qualitatively similar to its behavior at 3. 2. It appears from the data that methanol causes a corrected denaturation blue shift, $\Delta\epsilon_{237}$ of about --8600, intermediate between the values observed for State II and State III molecules. But it is clear from Figure 38 that

FIGURE 38

Effect of methanol on the absorption spectra of RNase at 237 mμ, at pH 1.12 (dotted line), 3.2 (solid line with the dashed extrapolation to zero concentration of methanol), and 2.2(dashed line with four values above 70% methanol).



the extrapolation of the solvent effect is very risky, and it is not possible to be certain it is correct. We believe that methanol (and ethanol) cause RNase to denature to State III, but the intuitive nature of this conclusion must be emphasized.

The behavior of RNase in methanol at pH 1. 1 is anomalous at this pH, of course the molecule is already partially unfolded, even without methanol present. Our results show that methanol even up to 90%, causes only a linear solvent effect -- there is no apparent additional denaturation blue shift observed (Figure 38). Optical rotation results lead to the same conclusion. This is most surprising behavior -- at pH 3. 2 or 2. 2 methanol causes more unfolding than at pH 1. 1. We are unable to propose any convincing explanation of these results at this time, and they obviously require more investigation.

APPENDIX II

CALCULATED DIFFERENCE SPECTRA IN THE MIDDLE ULTRAVIOLET

An attempt was made to shed some light on the middle ultraviolet spectra of the constituent chromophores. The results of this computation while they are preliminary and fairly inconclusive, are nevertheless of some peripheral interest, and we are therefore including a few figures showing some of these results.

Middle ultraviolet spectra of RNase were calculated by summing contributions from six tyrosyl residues, four histidyl residues, three phenylalanyl residues and 123 peptide bonds (we assumed 20% of these were in the helical conformation, and 80% in random coil conformation). The spectra used for the constituent chromophores are those of Figure 1. It should be noted that the sulfur-containing amino acids were not included in this calculation, and the relevance of the results is limited to the region between about 225 and 250 mμ. The calculations were done by the computer, which produced extinction curves at 11 wavelengths between 220 and 250 mμ (250, 245, 240, 237.5, 235, 232.5, 230, 227.5, 225, 222.5, and 220 mμ) though the

figures show continuous curves. In all of the spectra calculated, the constituent spectra for histidyl residues, phenylalanyl residues, and the peptide bonds, were taken just as they are in Figure 1. Various assumptions about the position of the tyrosyl spectra were made, and the various results depend significantly on these assumptions.

Once the spectra were computed they were subtracted from the measured spectrum of RNase, and the results that will be shown represent the resultant "theoretical difference spectra."

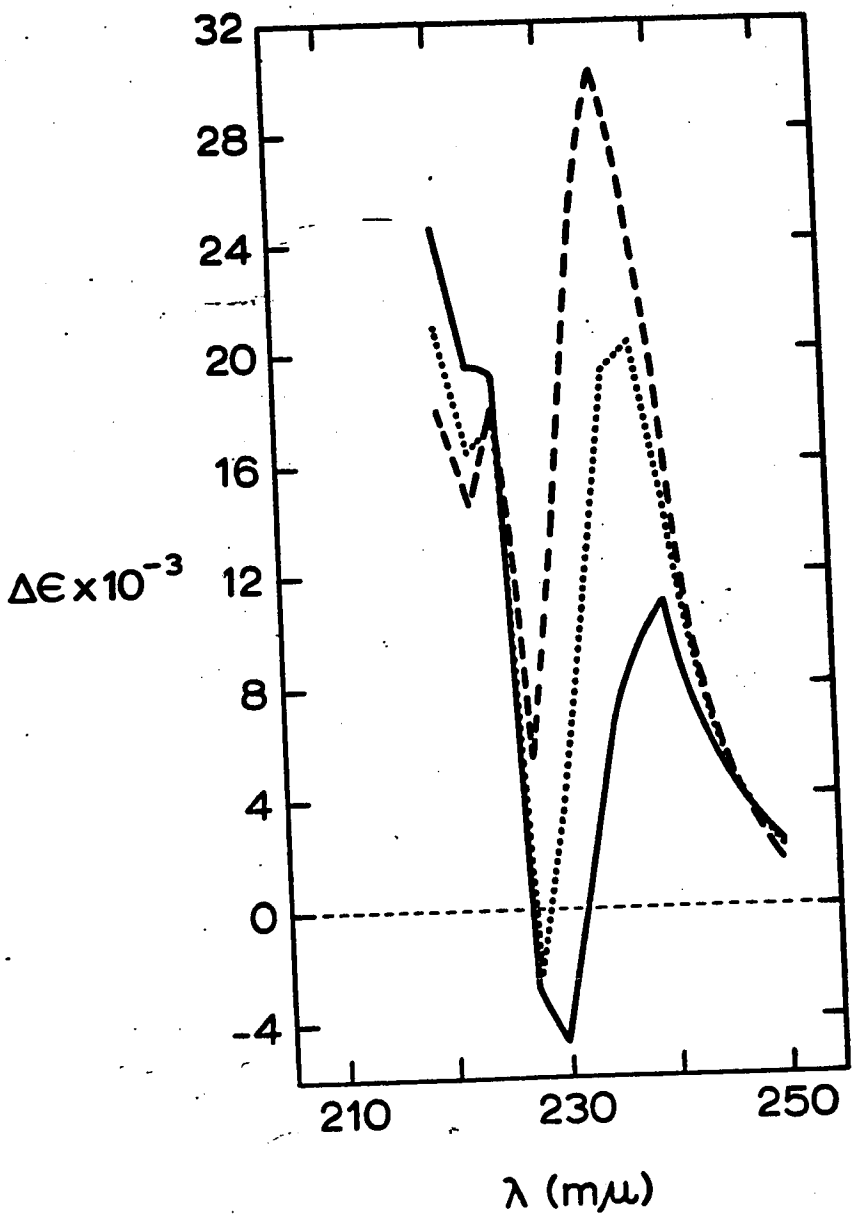
In figure 39 are shown theoretical difference spectra calculated on the assumption that the spectra of all six tyrosyl residues are shifted 0. 2. 5, or 5 mμ to longer wavelengths compared to the spectrum of the free amino acid. As can be seen the third curve (5 mμ shift) resembles, both qualitatively and quantitatively many of the measured difference spectra found elsewhere in this thesis. There is a positive value of $\Delta\epsilon$ around 235 or 240 mμ, rather than a negative one, as observed on denaturation, but this is mostly the result of subtracting the theoretical value from the measured value, rather than the other way around.

The theoretical difference spectra calculated assuming 0 and 2. 5 mμ shifts of the tyrosyl spectra only resembles the observed difference spectra in a qualitative fashion.

It would seem at first glance that the theoretical difference spectra calculated for the 5 mμ shift of the tyrosyl spectra satis-

FIGURE 39

Theoretical difference spectra for RNase. (see text for method of calculation) The absorption of the six tyrosyl residues has been assumed to be: unshifted (dashed line), shifted 2.5 mμ (dotted line), and 5.0 mμ (solid line).



factorily accounts for the difference spectra observed with State III molecules (if we judge from $\Delta\epsilon_{235}$, which is about 10, 000 in both cases). However we have had to assume a 5 mu shift of the spectra of exposed tyrosyl residues, which is obviously much too large.

A few other calculations were carried out with different assumptions about the contributions of the exposed and the buried tyrosyl residues, in an effort to see whether something resembling the spectrum of the native protein could be effected.

In some of these it was assumed that the three exposed residues had undergone no shift, while the buried ones had undergone shifts of 7. 5, 10 and 12. 5 mμ (Figure 40). In others, the exposed residues were assumed to have undergone shifts of 2. 5 mμ with shifts of 7. 5, 10, and 12. 5 mμ for the buried ones (Figure 41). If we were really able to calculate spectra accurately by this very elementary approach, the correct theoretical difference spectra would of course have the value of 0 at all wavelengths. It can be seen in Figure 41 that the approximation to this value (above about 225 mu, of course) is much better than it was on the assumptions underlying Figure 40. It seems therefore, that a passable spectrum of RNase in the middle ultraviolet can be calculated on the assumption that the three exposed residues are shifted 2. 5; and the three buried ones 10-12. 5 mμ, to longer wavelengths compared to the spectrum of the free amino acid.

FIGURE 40

Theoretical difference spectra for RNase. (see text for method of calculation) The absorption of the three exposed tyrosyl residues has been assumed to be unshifted and those of the other three are shifted 7.5 mμ (dashed line), 10 mμ (dotted line), and 12.5 mμ (solid line).

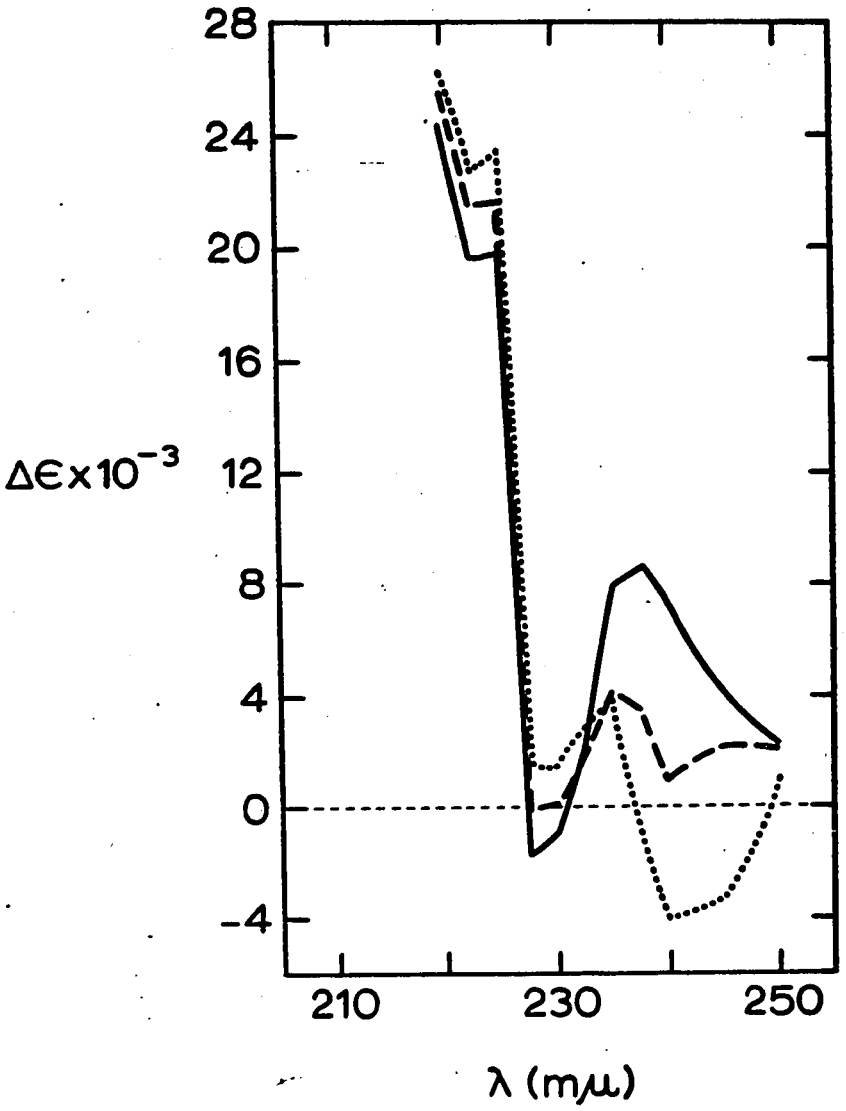
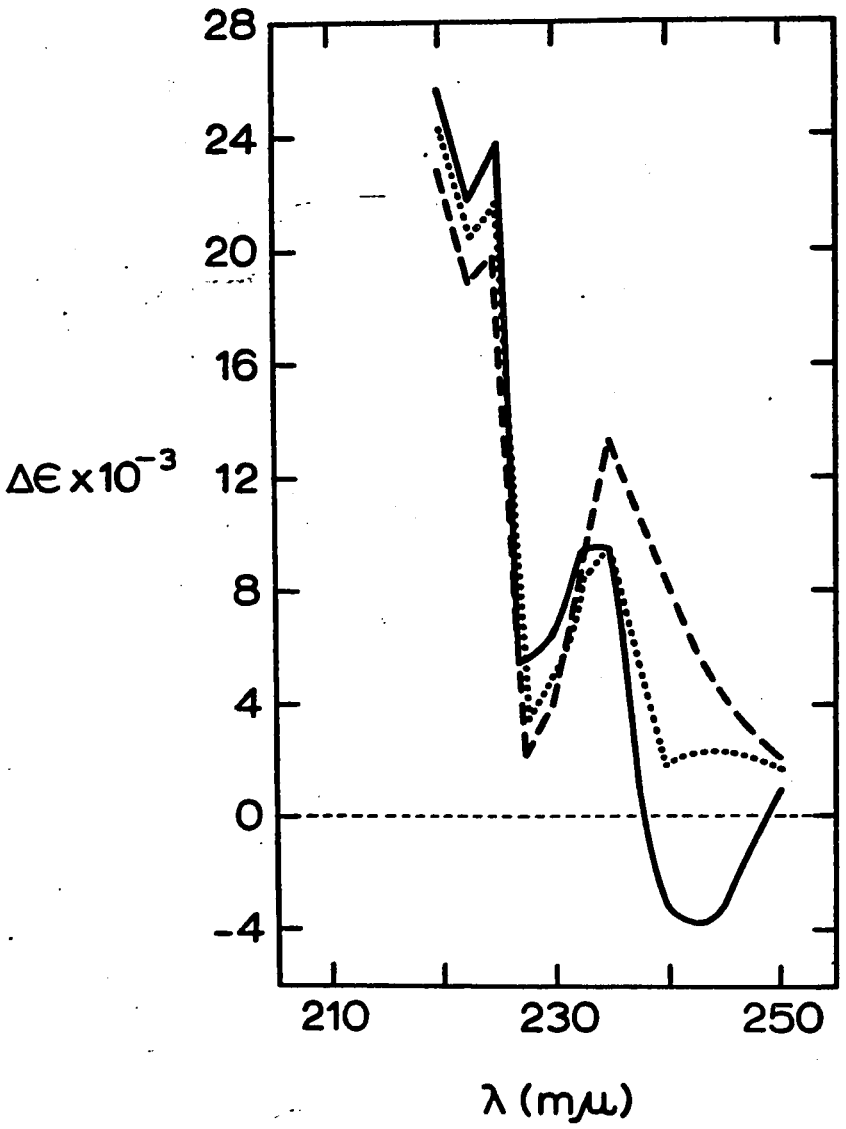


FIGURE 41

Theoretical difference spectra for RNase. (See text for method of calculation) The absorption of the three tyrosyl residues has been assumed to be shifted 2.5 mμ, and that of the other three are shifted 7.5 mμ (solid line), 10 mμ (dashed line), and 12.5 mμ (dotted line).



It should be obvious that the procedure adopted here is too elementary for us to expect anything better than approximations to the measured spectra. We have not, for example, attempted to include any measure of the increase in intensification of the absorption bands. However it would not be justifiable or profitable to do so on the strength of data now available. In spite of the oversimplifications involved, the work discussed in this appendix does serve to support one of the major conclusions of this thesis, namely that the denaturation blue shifts observed with RNase at 235 mμ are due completely to changes in the environment of tyrosyl residues.