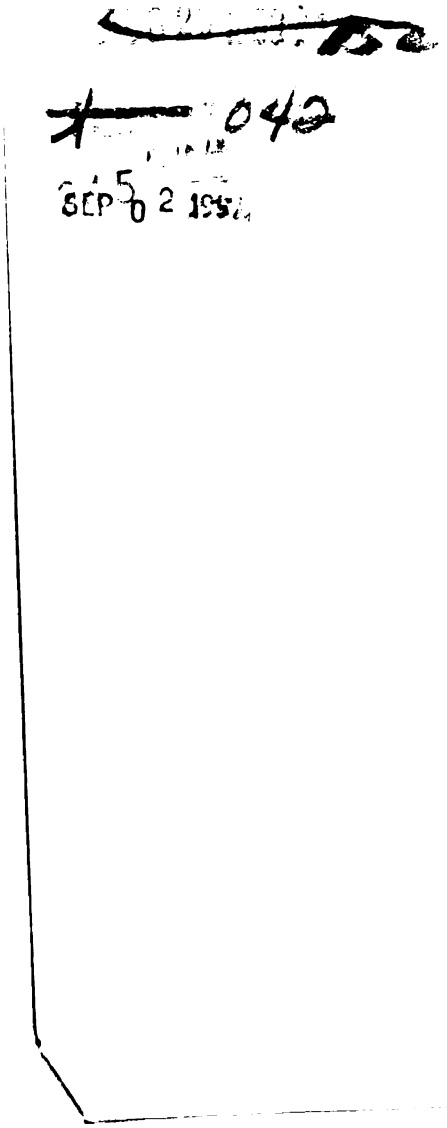


ABSTRACT

REACTION OF RETINAL WITH IMINES AND SECONDARY AMINES

By Jean Toth

The U.V. and visible spectra of unprotonated and protonated solutions of retinal with various imines and secondary amines (e.g., pyrrole, indole, pyrrolidine and indoline) were measured in carbon tetrachloride. Subsequent to this initial survey, quantitative measurements of the various chemical properties of solutions of retinal with the imine indole (a precursor of the amino acid tryptophan) were undertaken. A striking similarity to the properties of metarhodopsin---an intermediate in the photodegradation of rhodopsin, the pigment of the rods---is noted. Its stability is extremely temperature dependent; it possesses indicator-like properties; and is readily hydrolyzed. These factors and the marked bathochromic shift evident subsequent to protonation (resulting $\lambda_{max} \approx 580$ mμ), indicate its possible relevance to the chemistry of visual pigments.

From the experimental evidence and the work of several Russian scientists on condensation products of aldehydes and cyclic amines, a proposal for the structure of the protonated species of these retinal compounds is made---that of an "enamine salt." This proposed structure corresponds to the retinylene ammonium structure postulated by Morton and Pitt in their hypothesis for the nature of the rhodopsin linkages. The failure of rhodopsin and its degradation products above metarhodopsin to react with such reagents as borohydride and hydroxylamine

indicates that the proposed C = N linkage is shielded in some way. If the linkage in rhodopsin were analogous to that of an "enamine salt," this "shielding" would be provided. Splitting off of one residue of opsin from the nitrogen involved in this linkage, during the degradation process, would result in reactivity with the above mentioned reagents. Such a linkage could also satisfactorily account for the extent of bathochromic shift evidenced in the various visual pigments ---the exact extent of shift being dependent on the nature of the amino acid residues bound to the nitrogen atom of the linkage.

REACTION OF RETINAL WITH IMINES AND SECONDARY AMINES

by

Jean Toth

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biophysics

1967

To my parents

ACKNOWLEDGEMENT

The author wishes to thank Dr. Barnett Rosenberg for his inspiration and guidance. In addition, the author wishes to acknowledge the personal support of the Traineeship Grant GM 01422 of the National Institutes of Health and the support of this research by Grant NB 04145 of the National Institute of Neurological Disease and Blindness of the National Institutes of Health.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Chemistry of Visual Pigments

The visual pigments consist of a protein of the opsin family combined with the chromophore vitamin A aldehyde (retinaldehyde). Opsin is a high molecular weight protein, species-specific in composition; the chromophoric portion exists in nature in two forms, vitamin A_1 aldehyde (retinal) and vitamin A_2 aldehyde (3-dehydroretinal). Retinal in a water solution possesses a λ_{max} at 385 mμ. When combined with an opsin, a large bathochromic shift into the visible region of the spectrum (about 500 mμ) is evidenced. Since 3-dehydroretinal contains an additional double bond in its structure, the bathochromic shift produced on union with an opsin is even greater.

Evidence for the chemical composition of these pigments has been derived through extensive experimentation with rhodopsin---the visual pigments of the rods. Numerous attempts have been made to extract cone pigments, without success. The usual explanation for these repeated failures is that cones contain only very small amounts of pigments. However, work with pure cone retinae, by the method of in vivo bleaching, has shown the magnitude of maximum density change to be similar to that observed in other species containing both rods and cones. By the method of ophthalmoscopic densitometry, it was also shown that the density of pigment in human cones is approximately the same as that of human rhodopsin.[1] It has thus been postulated that failure to extract cone pigments may possibly result from the destruction or dissociation of the pigments by the commonly used extraction procedures. Despite the fact that all present evidence

concerning the nature of visual pigments has been obtained from work with various species of rhodopsin, the generality of the proposed reactions has not been seriously questioned.

When exposed to light, rhodopsin undergoes photodegradation---"bleaches." The breakdown and formation processes of this pigment have been elucidated through the efforts of Wald, Hubbard and their colleagues. They ascertained that the reaction between retinaldehyde and opsin is stereospecific---requiring the hindered 11-cis isomer of retinaldehyde for the formation of rhodopsin.[2] It was discovered that opsin will also react with the 9-cis isomer of retinaldehyde, forming compounds different from rhodopsin, which has been named isorhodopsins. Present evidence indicates, however, that these isopigments do not occur naturally.[3,4]

Elucidation of the nature of the linkage (or linkages) resulting between retinaldehyde and opsin in forming rhodopsin is of great biological importance. One possible method of attaining information concerning this linkage(s) is studying intermediate products in the synthesis or degradation of the pigment. Since it has not yet been possible to detect any intermediates in the synthetic process,[5] attention was turned to the photochemical breakdown. As previously noted, identification of the intermediates of this process was accomplished chiefly through the efforts of Wald and his associates.[6,7] Only the first step in the decomposition of rhodopsin by light is photochemical. The subsequent steps are thermal reactions, resulting in hydrolysis of the compound into all-trans retinaldehyde and opsin (Figure 1). The photochemical step involves isomerization of the

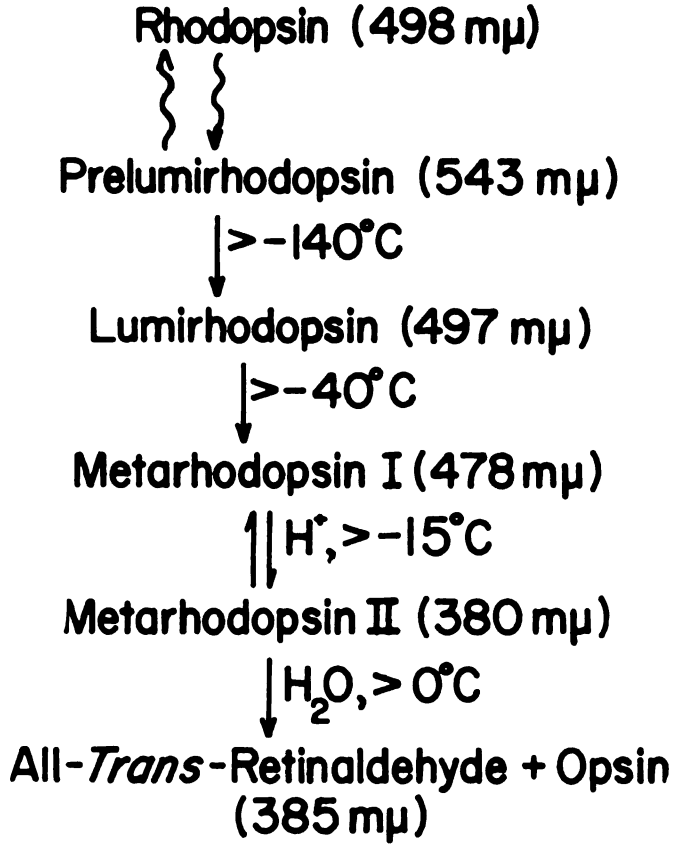


FIGURE I. Photochemical Degradation of Rhodopsin

chromophore from the 11-cis to the all-trans configuration.[8] The detailed chemical nature of these intermediates is obscure.

The structure of N-retinylideneopsin, a compound which, under certain conditions, is formed from metarhodopsin II,[9] has been identified as a Schiff's base formed by union of the aldehyde group of retinaldehyde and an amino group on opsin.[10-12]

$$C_{19}H_{27}CHO + NH_2\text{-opsin} \rightleftarrows C_{19}H_{27}CH = \text{N-opsin} + H_2O$$

(retinaldehyde) (N-retinylideneopsin)

There is evidence that the terminal carbon of retinaldehyde (C_{15}) (Figure 2) is linked in some way to an amino group of opsin.[11-13] The postulate of a conjugate acid form of a Schiff's base (Figure 2) can account for only roughly half the required spectral shift from the 385 mμ λ_{max} of a water solution of retinal alone to the region of 500 mμ on union with opsin. Hubbard has suggested that opsin stabilizes one resonance form of the conjugate acid.[6] Conventionally, the positive charge is shown on the nitrogen atom (Figure 2). A number of resonance forms exist with the positive charge localized on carbon atoms in the polyene chain. If one of these unstable resonance forms were to react with a side group on the protein moiety, for example, a carboxylate group, the absorption maximum would probably shift to longer wavelengths.

Dartnall also suggested a formula for rhodopsin involving a Schiff's base, but did not consider the conjugate acid form.[14] He suggested that when the fit between the chromophore and protein is suitable, there is an overlapping and interaction of the π electron cloud of the polyene chain with the electrons of the amino acid resi-

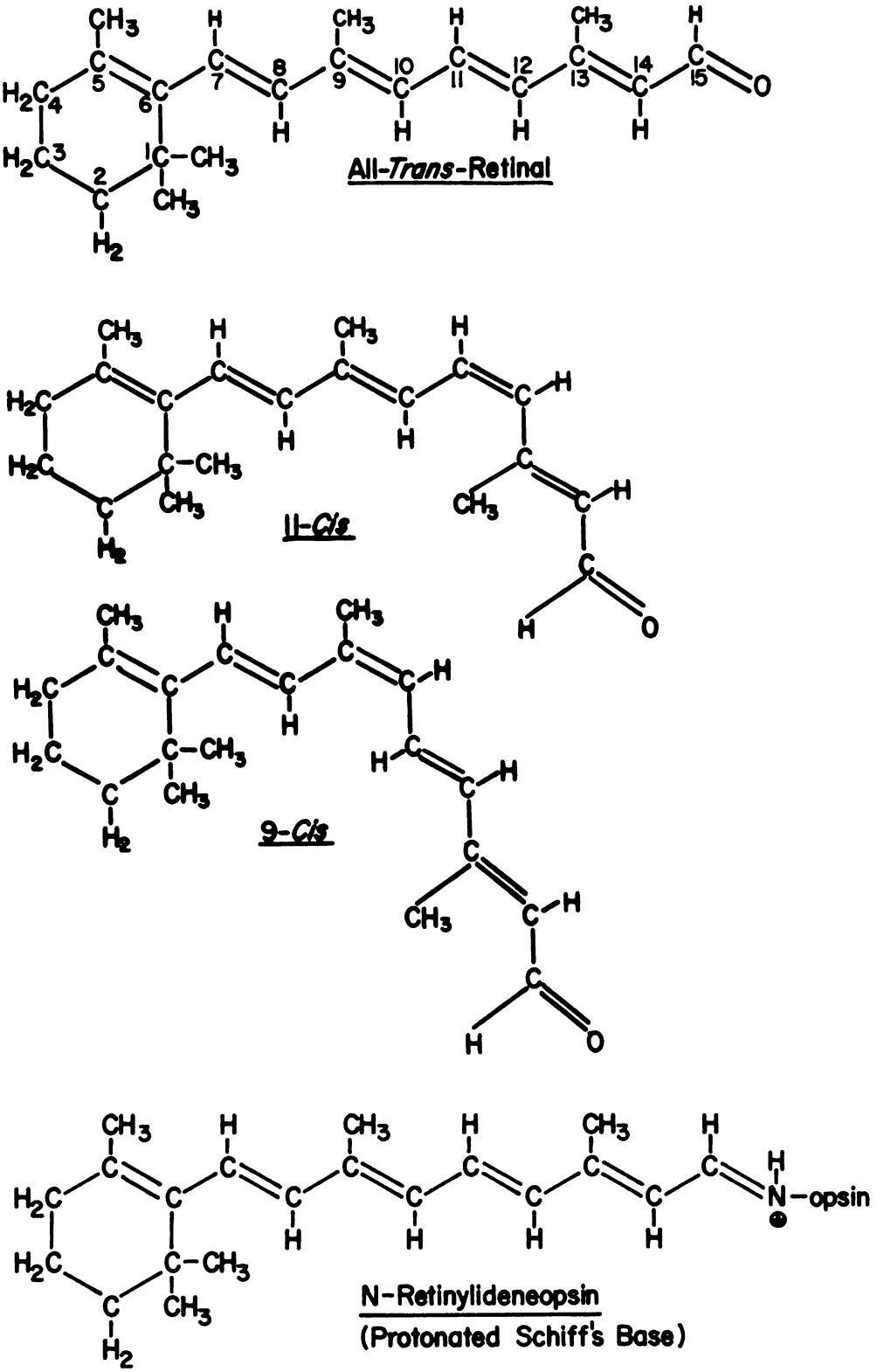


FIGURE 2. Structures of Retinal Significant to Rhodopsin Chemistry and of N-Retinylideneopsin

dues of the protein forming new orbitals. If this were true, the number of interactions of retinaldehyde and opsin would be indeterminable and any attempt to write one particular structure formula for the compound would be futile.

Both of the above suggestions rely on close fitting between the polyene chain and the protein moiety to explain the large bathochromic shift on formation of rhodopsin. The discovery in recent years of prelumirhodopsin, in which the polyene chain now in the all-trans configuration, surely has a poorer fit with the protein than in rhodopsin but yet gives a λ_{max} at longer wavelengths, severely weakens such hypotheses.

The Transduction Mechanism of Vision

Besides the problem of elucidating the actual structure and linkages of the compound, the nature of the transduction mechanism for vision is of major concern. The classical photochemical theory of Wald[15] supposes that the conformational changes of the protein moiety during the photodegradation, or "bleaching", process leads to the triggering of a nervous impulse. The quantum efficiency for bleaching of rhodopsin in solution is 0.5 to 0.6.[16] This suggests that light possibly initiates some other process as well, and the significance of this other process cannot be ignored as it could well have at least an equal quantum efficiency in vivo.

In recent years a more physical than chemical theory of the photoreceptor process has been proposed---a photoconduction process---the direct generation of charge carriers by optical excitation.[17] The ordered, quasi-crystalline structure of the outer segments of the

rods and cones,[8,18] the structural portions containing the visual pigments, suggests such functional properties, more closely related to solid state phenomena than classical "wet" chemistry. Rosenberg, working with sandwich cell configurations of β-carotene samples (a dimer of retinal with the aldehyde group split out), was able to demonstrate both semiconductivity and photoconductivity in this system---the cis and hindered cis isomers being the most efficient photoconductors.[19,20] Moreover, the β-carotene samples evidenced a strong spectral sensitivity---the ability to function as true color discriminators.[21] Of greater interest, their spectral response curves agreed with those recorded from the Müller fibers of the teleost fish by Svaetichin---the chromatic S potentials.[22] Dried receptors of sheep eyes likewise proved to be photoconductive.[23]

Photoisomerization and photoconductivity may be competitive processes initiated by illumination. Photoisomerization of the pigments may serve as one of the adaptive mechanisms of the eye, allowing vision to operate over the widest dynamic range.[18] The release from the excited chromophore of a mobile electronic charge---the photoconductive process---may well be the direct antecedent of the nervous impulse.

Model Systems

Rhodopsin is extracted into water solution with the aid of the detergent digitonin, which results in the formation of micelles---the pigment molecules being completely surrounded by molecules of the detergent. This configuration renders the extract unsuitable for photoconductivity measurements. Because of this and the fact that

cone pigments have not been successful extracted to date, photoconductivity studies in the past have been limited to model systems, such as β-carotene, lycopene and Schiff's base complexes of retinal and various substituted aniline compounds. In an attempt to produce a model system somewhat closer to reality, complexes of retinal with various amino acids were produced and acidified (protonated). Visible spectra of both the unprotonated and protonated species were measured. As earlier reported by Ball, et. al.,[9] the λ_{max}'s of protonated solutions of these complexes ranged from approximately 430 to 460 mμ. However, on preparing a complex with tryptophan, two species were evidenced when precipitation of the protonated species was attempted. Since tryptophan contains an imino group as well as a primary amine, it was postulated that the second product was a result of interaction between retinal and the imino group. Its λ_{max} was at approximately 500 mμ.

Subsequently, attempts were made to react retinal with various imines and secondary amines and to measure their spectra before and after protonation. The experiments reported here are the results of such spectral measurements. The earliest work involved a survey of the reaction with various imino and amino compounds. More extensive quantitative studies were then pursued with one specific compound, indole, a precursor of tryptophan.

EXPERIMENTAL METHODS

Carbon Tetrachloride---Solvent Employed

Originally, complexes of retinal (all-trans isomer) and various imines and secondary amines were prepared in such solvents as ethanol, acetone, dioxane and chloroform, and then protonated. (All protonation was accomplished by bubbling anhydrous hydrogen chloride through the solutions.) It was soon discovered, however, that retinal alone in these solvents turned blue to purple on protonation. (An unprotonated retinal solution is a pale to golden yellow, depending on concentration.) Its spectrum was markedly red shifted from that of the unprotonated solution. The color faded in time and its spectrum returned slowly to the blue region. To avoid ambiquity, spectro-grade carbon tetrachloride (CCl_4) was subsequently used for all spectral studies. A protonated solution of retinal in CCl_4 is not significantly different as far as visible coloration or spectrum, from an unprotonated solution for as long as 24 hours after protonation.

Survey of Imine and Secondary Amine Reactions

All-trans retinal was obtained from Distillation Products Industries and used without further purification. For the initial crude survey of the reaction of retinal with imines and secondary amines, an unmeasured amount of all-trans retinal was dissolved in CCl_4 and an excess of the imine or amine added. The mixture was allowed to react on a magnetic stirrer, sometimes overnight, depending on the solubility of the reactant in CCl_4. If necessary, the solution was filtered to remove any undissolved $>NH$ compound. Visible spectra of these

solutions, both unprotonated and protonated, were then measured in 1cm pathlength, stoppered quartz cuvettes, using dilutions which gave reasonable O.D.'s. Both the Beckman D.B. Spectrophotometer and the Bausch and Lomb Spectronic 505 were used for these measurements.

Studies with Indole

More quantitative studies were then undertaken with indole. 2.5×10^{-3}M solutions of both all-trans retinal and indole were prepared in volumetrics. Since solutions of retinal readily isomerize in light and indole is known to undergo autooxidation in the presence of oxygen and light, the samples were weighed out and the solutions prepared in the dark. The volumetrics were kept wrapped in aluminum foil to prevent subsequent exposure to light. Equal aliquots of both solutions were mixed and diluted to give a 2.5×10^{-5}M concentration, assuming a one-to-one combination of retinal and indole. This mixture was likewise prepared and maintained in darkness.

Visible spectra were then measured at 0°C, room temperature and 38°C, following the reaction, subsequent to protonation, in time. These spectra were measured on the Beckman D. B. as it is equipped to allow for adequate temperature control of the sample chamber. Kinetic studies were also done on the protonated reaction, at both 0°C and room temperature. The spectrophotometer was set at 610 mμ, the wavelength originally believed to be the λ_{max} of the fully protonated solution, and the process followed in time

Visual pigments characteristically are photosensitive---are decomposed by exposure to light ("bleached"). It is known that the photochemical effect is photoisomerization of the chromophoric portion of the molecule. It was of interest to investigate the possi-

bility of some type of photosensitivity in the retinal-indole system. Using a Bell and Howell 300 watt projector lamp for irradiation, the unprotonated solutions were irradiated at intervals, directly in the cuvette, and their spectra measured after each period of irradiation, using a Bausch and Lomb Spectronic 505 for these measurements. Irradiation of protonated solutions (solutions in which the reaction, subsequent to protonation, was allowed to go as far as possible to completion), was accomplished with the same source and spectra measured after each period of illumination. The irradiation was accomplished with the cuvette maintained in an ice bath and all spectra measured at 0°C, allowing the temperature of the sample to re-equilibrate in the sample chamber for a fixed interval after irradiation. (These measurements were taken using the Beckman D. B.).

Indole itself is known to undergo autooxidation in the presence of oxygen and light. As a result, solutions of indole (1×10^{-4}M) were prepared in the dark and irradiated at intervals with a 2537 Å light source. Spectra were measured after each period of irradiation.

The λ_{max} of various of the intermediates of rhodopsin photodegradation is known to be pH dependent. Although pH has no real significance in reference to organic solvents, an attempt was made to investigate effects of "alkaline" conditions on a previously protonated solution of retinal and indole. Since HCl was usually present in excess, a precipitate of ammonium chloride (NH_4Cl) was initially formed (when NH_3 was bubbled through the solution). To avoid the complications such a precipitate could produce, a 5.0×10^{-5}M solution of the complex was protonated and allowed to react in an ice bath. It

was then diluted to 2.5×10^{-5}M with methanol. Methanol was used since NH_4Cl is fairly soluble in this solvent and the protonated species of the compound appeared stable in the mixed solvents, when maintained in an ice bath.

Anhydrous ammonia was bubbled through this solution just until a color change was evident. The spectrum was then measured and HCl bubbled through until a color change was again apparent, and the spectrum remeasured. The above sequence of bubbling gases was again repeated and the respective spectra measured.

Visible spectra at 0°C as well as photochemical studies were also undertaken with a different isomer of retinal, specifically the 9-cis isomer, as it is known to be capable of reacting with opsins to form the isopigments. The 9-cis isomer was also obtained from Distillation Products Industries, and used without further purification.

Structural Studies

From the original work with tryptophan it was postulated that complexes of retinal with imines and secondary amines most probably involved reactions between the aldehyde group of the retinal and the imine or amine. To check this hypothesis and possibly determine the nature of the resulting linkage, infra-red measurements were made. Using a Unicam Spectrophotometer 220, measurements were made on all-trans retinal and an unprotonated solution of retinal and pyrrole, an imine whose reaction was investigated in the original survey. All spectra were measured using CCl_4 as the solvent.

Using a Perkin-Elmer Infra-red Spectrophotometer, measurements were made on all-trans retinal, indole and an unprotonated solution of

retinal and indole, all in CCl_4. Since, at the concentrations required for infra-red measurements, the protonated complex of retinal and indole precipitates out of solution, a KBr pellet of a precipitated and dried sample of a protonated 13-cis retinal-indole complex was prepared and its infra-red spectrum measured.

In an effort to ascertain if the resulting complex of retinal and indole is a one-to-one reaction, as the proposed structure of the complex and nature of the resulting linkage would require (see discussion), a concentrated solution of indole and retinal was prepared in CCl_4 and protonated. The temperature of the solution was maintained at approximately 0°C in an ice bath, throughout the preparatory process. The precipitated compound, after suction filtration through a Millipore filter, was dried and sealed in an evacuated tube. It was subsequently sent to Spang Microanalytical Laboratory for analysis.

To allow for purification and recrystallization of the protonated compound, the solubility and stability of the compound was investigated with various solvents at room temperature and 0°C. The protonated compound is practically insoluble in the preparatory solvent, CCl_4.

RESULTS

Spectral Measurements with Various Imines and Secondary Amines

All-trans retinal in CCl_4 solution shows a λ_{max} at 380 mμ (Figure 3). The spectral changes resulting from complexing retinal with imines or secondary amines and protonating the resulting solutions are varied. The λ_{max}'s for both the unprotonated and protonated solutions investigated are summarized in Table I. The structural formulae of these imines and amines are shown in Figure 4. No attempt was made to control concentrations, temperature or extent of reaction subsequent to protonation for the above measurements. As a result, the λ_{max}'s listed for the protonated species may not represent the final, exact values, but are at least indicative of the degree of bathochromic shift which results on protonation of the various complexes. The protonated spectrum of the carbazole system was measured at 0°C, as the stability of this compound is extremely temperature dependent and cannot be maintained in the typical protonated form at room temperature for the time required to take a spectral measurement.

The extent of bathochromic shift subsequent to protonation is dependent on the degree of conjugation in the structure of the reacting compound. This effect is evident in Figure 5 which shows the visible spectra of protonated solutions of retinal with pyrrolidine and pyrrole. Pyrrolidine is a fully saturated 5-membered heterocyclic compound with nitrogen as the hetero-atom; pyrrole is its conjugated analogue (see Figure 4). Comparison of the λ_{max}'s of the protonated species of the various other compounds investigated shows the same general effect of conjugation on extent of bathochromic shift.

It was also discovered that the above complexes are readily hydrolyzed. The addition of several drops of water to the protonated solution results in dissociation of the complex.

Compounds containing two nitrogen atoms in a conjugated ring structure, with one N atom sharing a double bond, fail to show any marked spectral shift subsequent to protonation. Figure 6 shows the structural formulae of such compounds investigated; Figure 7 is a typical set of spectral measurements on such a system.

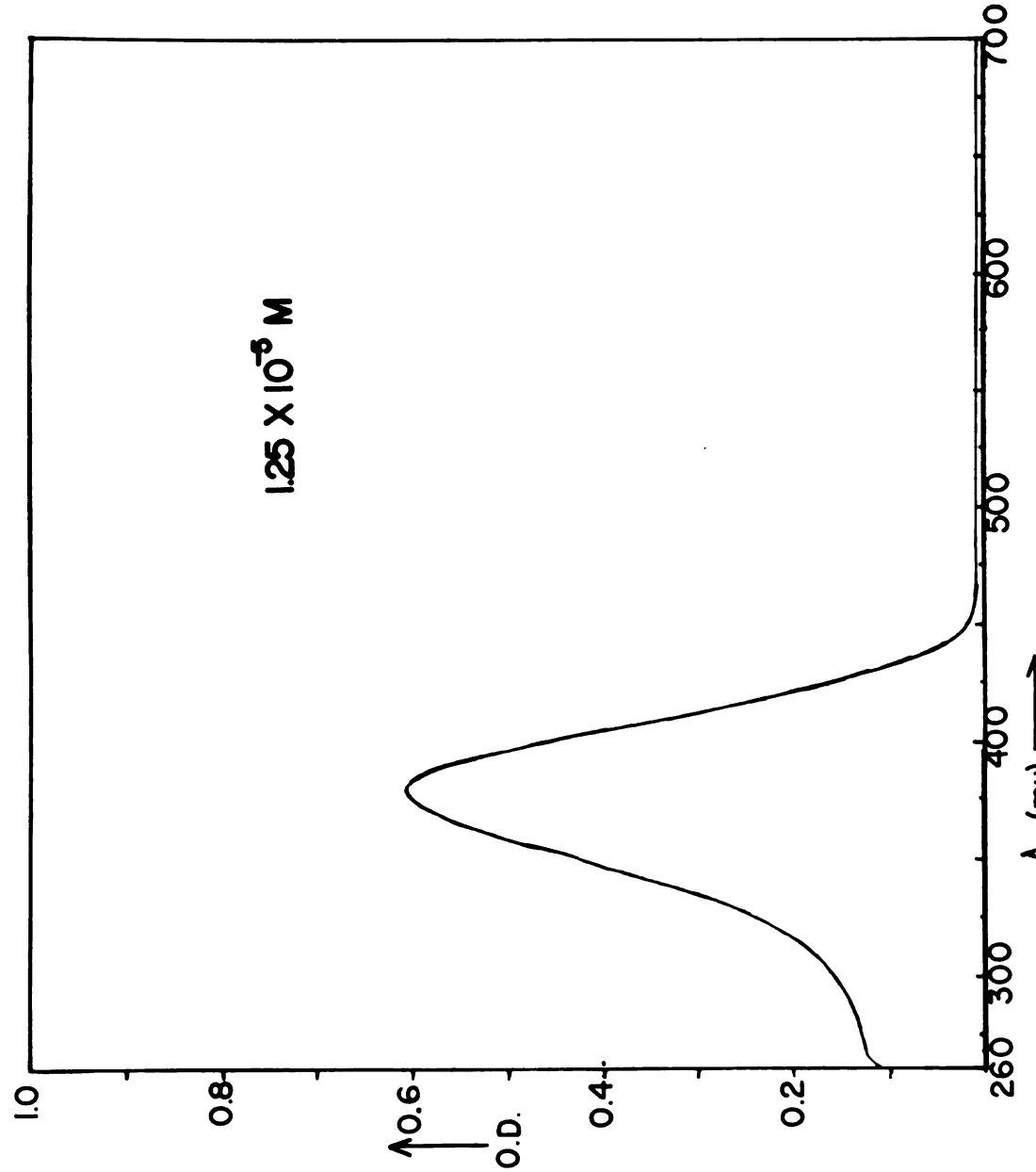


FIGURE 3. Absorption Spectrum of All-Trans Retinol in CCl_4

Table I: Summary of spectral measurements of reactions with secondary amines and imines

Secondary amine or imine	λ_{max}-unprotonated solution (mμ)*	λ_{max}-protonated solution (mμ)
piperidine	342	440
pyrrolidine	357	445
piperazine	350	470
indoline	376 508 (indoline peak)	510 shoulder ca 570
3-pyrroline	330	475 - main peak 424,398,378 - subsidiary peaks
diphenylamine	380	530
pyrrole	380	655
indole	380	610
carbazole	380 360	620

*(U.V. peaks typical of the secondary or imine are not included)

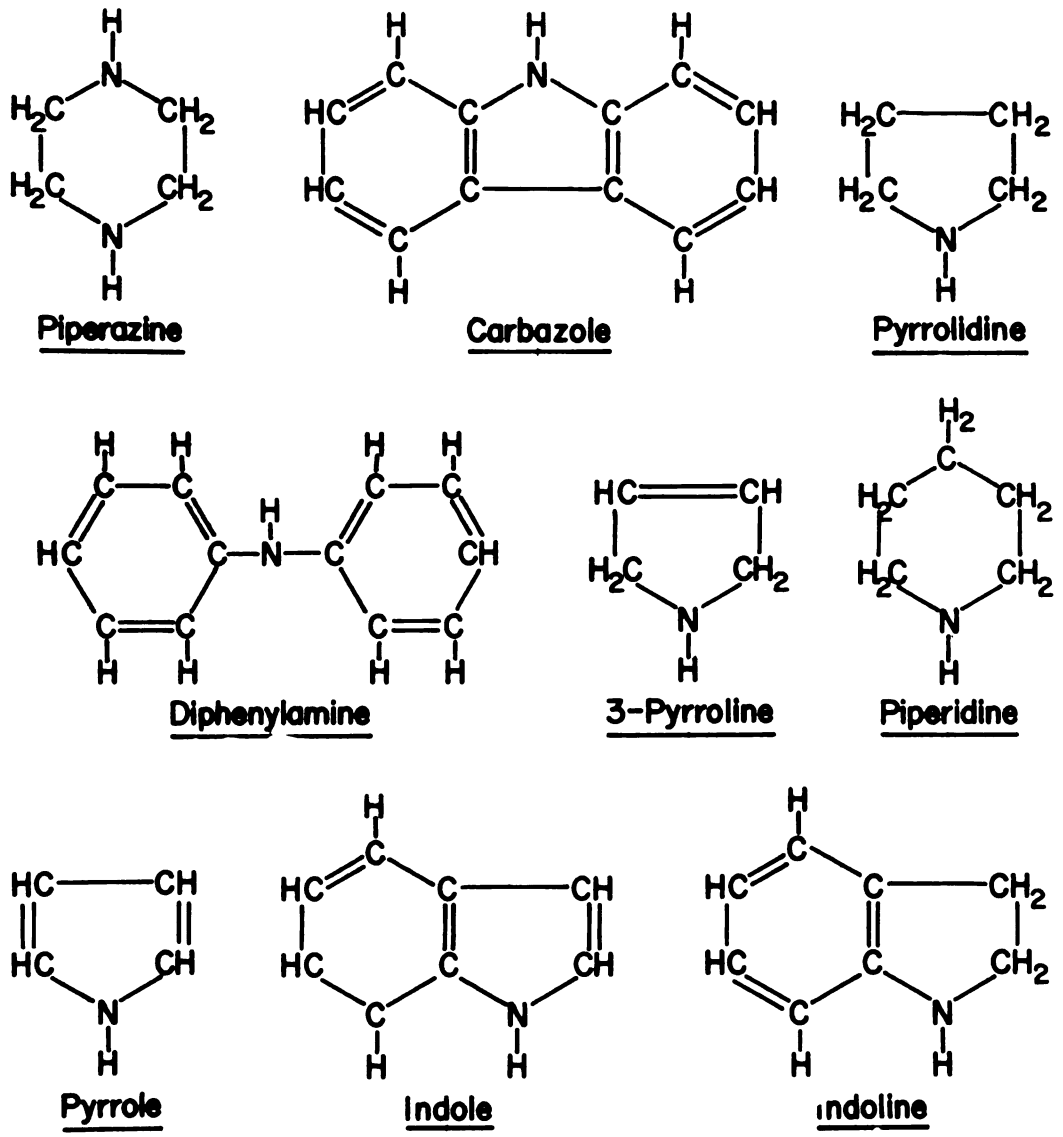


FIGURE 4. Structural Formulae
of the Secondary Amines and Imines

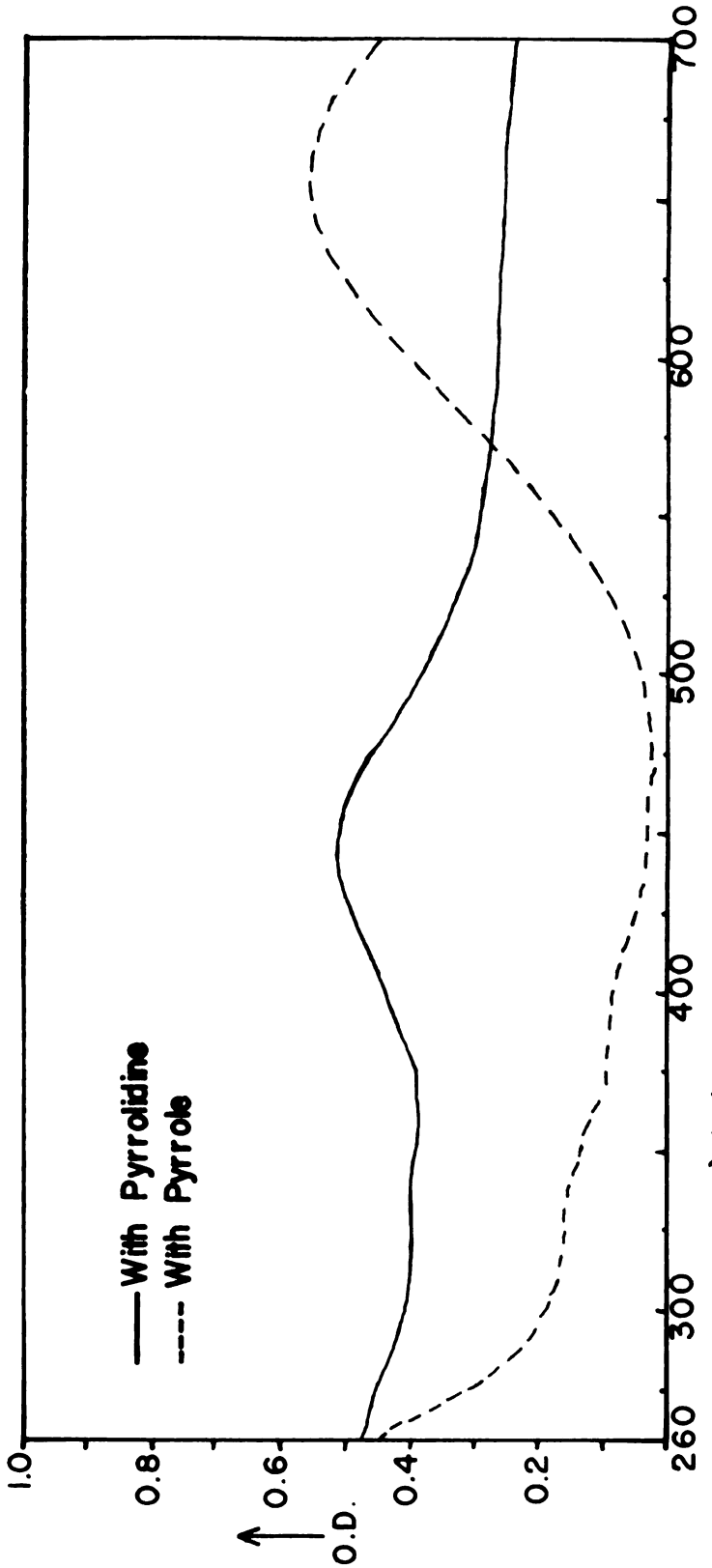
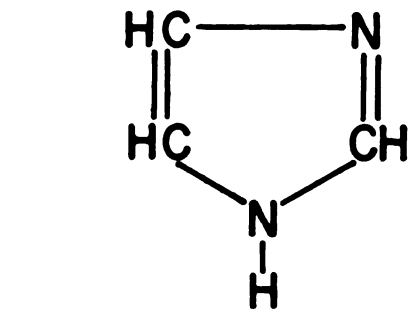
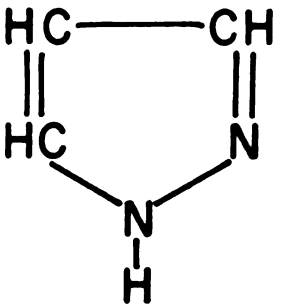


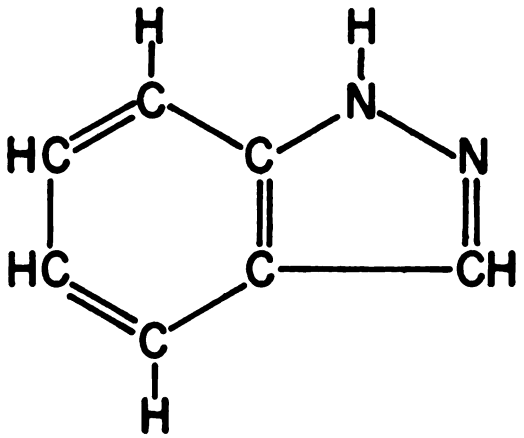
FIGURE 5. Absorption Spectra of Protonated Solutions of All-Trans Retinal with Pyrrolidine and Pyrrole



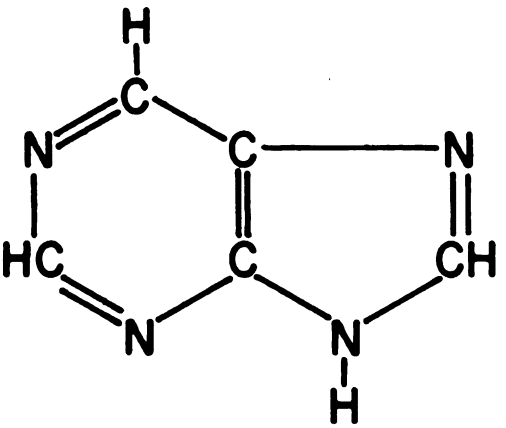
Imidazole



Pyrazole



Indazole



Purine

FIGURE 6. Structural Formulae of Compounds Containing Two N Atom in a Conjugated System

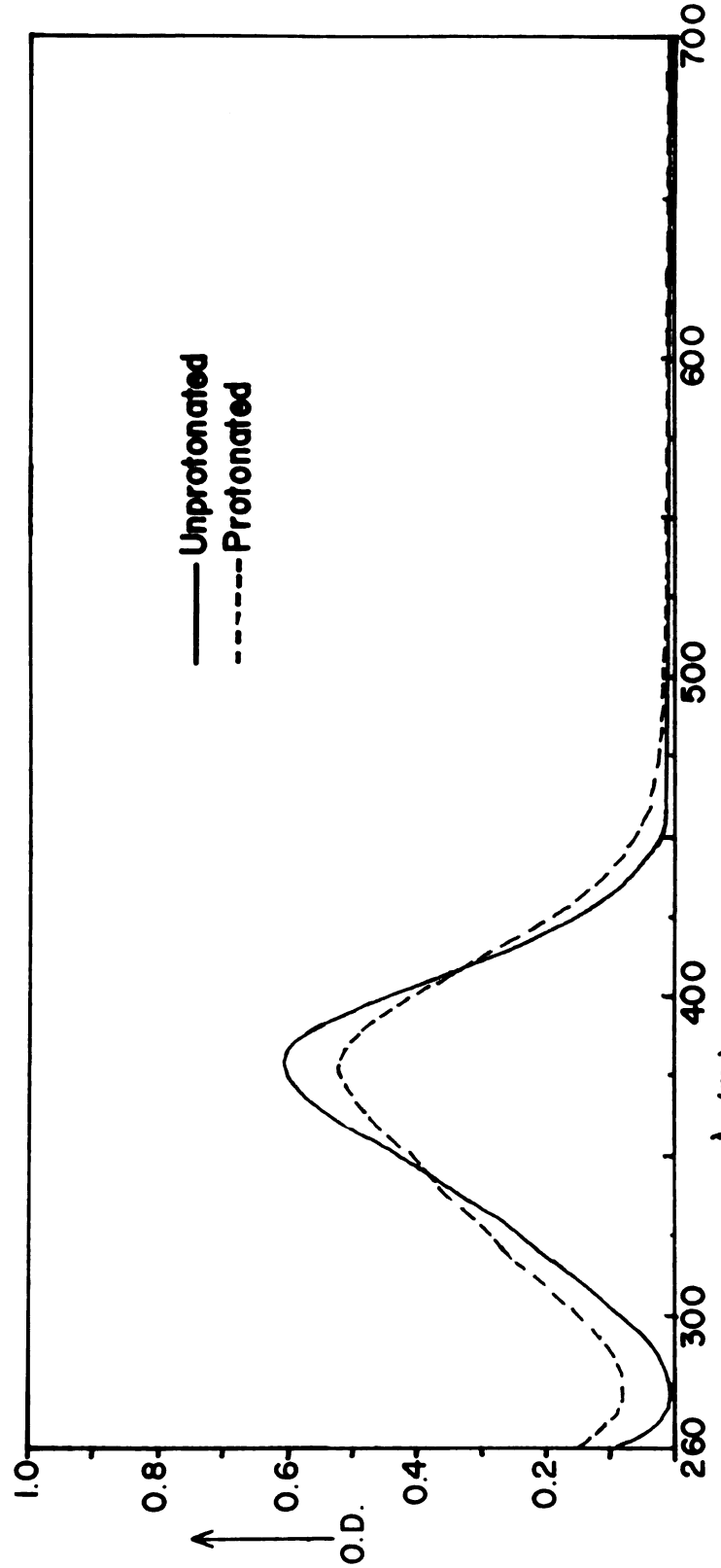


FIGURE 7. Absorption Spectra of Solutions of All-Trans Retinal with Pyrazole

Quantitative Measurements of the Retinal-Indole System

Temperature Effects

The stability of the protonated solution of retinal and indole proved to be extremely temperature dependent. If, subsequent to protonation, the solution (2.5×10^{-5}M) is allowed to react at 0°C and the reaction followed spectrally with time, an isosbestic point is clearly evident, at approximately 463 mμ (Figure 8). The λ_{max} characteristic of the unprotonated solution is slightly red-shifted on protonation, from 380 mμ to approximately 387 mμ. The O.D. of this peak decreases progressively as the reaction proceeds toward completion. The peak representative of the protonated species initially appears as an extremely broad band in the red region of the spectrum, appearing to peak in the vicinity of 610 mμ. As the reaction progresses, the O.D. of this band increases and its shape and placement become more clearly defined. A solution of the protonated solution in which the reaction has gone to completion shows a λ_{max} at 580 mμ, with U.V. peaks at approximately 324 and 276 mμ (Figure 9). Visibly, the solution changes from the pale yellow coloration of the unprotonated solution, to a green intermediate mixture, to a final purple-blue.

The final position of the λ_{max} of the protonated species of this compound at 580 mμ, rather than at 610 mμ as evidenced in the initial survey, indicates, as previously noted, that the λ_{max}'s listed in the third column of Table I are merely indicative of the regions of the spectrum in which these bands lie and are not exact positions.

If the protonated solution is maintained at room temperature or

higher, the resulting reaction is markedly different from that at 0°C. The first difference evident is the failure of the band characteristic of the unprotonated solution to red-shift on protonation. The O.D. of this band, however, progressively decreases in time, much the same as it does when the reaction proceeds at 0°C (Figure 10). The band characteristic of the protonated species is initially broad and its O.D. increases for a time. It soon reaches a maximum, however---far below that of the completed reaction---and then decreases in O.D. with time, its λ_{max} shifting toward the blue region of the spectrum (Figure 10). Visibly, the solution takes on the green intermediate color as at 0°C, but then fades toward colorless, eventually becoming red-orange. The spectrum of this final form of the compound is markedly different from that of both the unprotonated and protonated solutions (Figure 11).

The difference in the reactions at 0°C and room temperature (or higher) is also evident from the kinetic studies at 610 mμ (Figure 12). At 0°C, the O.D. and this wavelength increases with time and eventually levels off to a steady value. At room temperature, however, the O.D. increase is not as sharp and begins to level off much more rapidly, at a lower value. The O.D. then decreases with time, rather than remaining level.

When the protonated solution, which has reacted to completion at 0°C, is allowed to warm up, it fades toward colorless and eventually becomes red-orange. If the solution is recooled before it fades completely, the blue coloration typical of the protonated species is retained. Consecutive warming and cooling periods, however, do not continuously result in the reformation of the blue form of the com-

pound. With each recooling, the blue color becomes less and less evident, the solution eventually remaining colorless and proceeding to the red-orange compound. All results seem to indicate an equilibrium condition existing between the protonated form of the compound and some unknown colorless form, which proceeds irreversibly to a red-orange compound, whose nature is also unknown. The irreversibility of this final compound is indicated by the inability to continuously reverse the first reaction by consecutive periods of warming and cooling. The proposed reaction sequence can be summarized as follows:

$$\text{unprotonated species} \xrightarrow[0^\circ C]{HCl} \text{protonated species} \underset{\text{cooling}}{\overset{\text{warming}}{\rightleftarrows}} \text{colorless compound}$$

$$\text{protonated species} \rightarrow \text{red-orange compound}$$

The complex formed between retinal and carbazole shows the same type of temperature effect. The stability of the protonated species of this system demands even lower temperatures, closer to those of dry ice, at which temperature the solvent, CCl_4, "freezes."

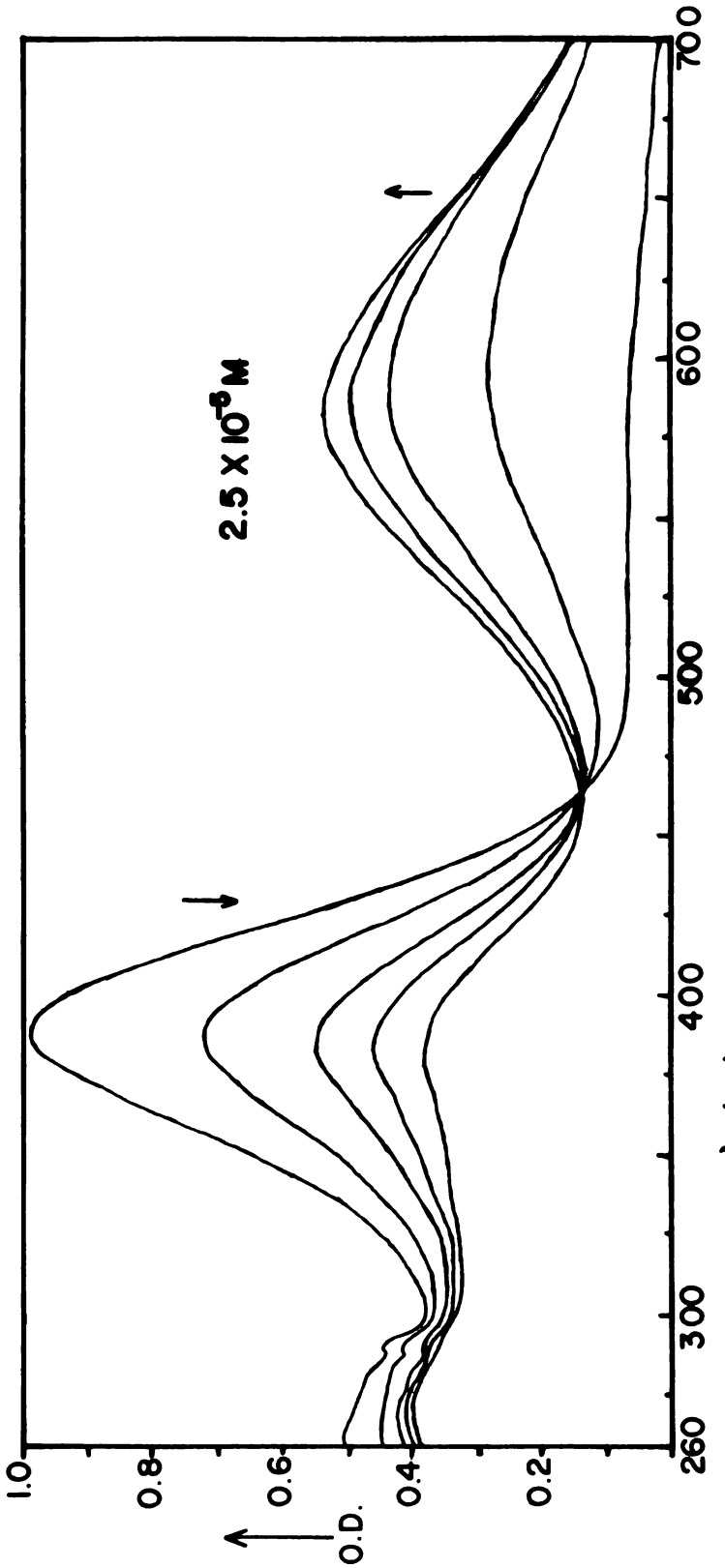


FIGURE 8. Absorption Spectra Showing Reaction of a Protonated Solution of All-Trans Retinal with Indole at 0°C

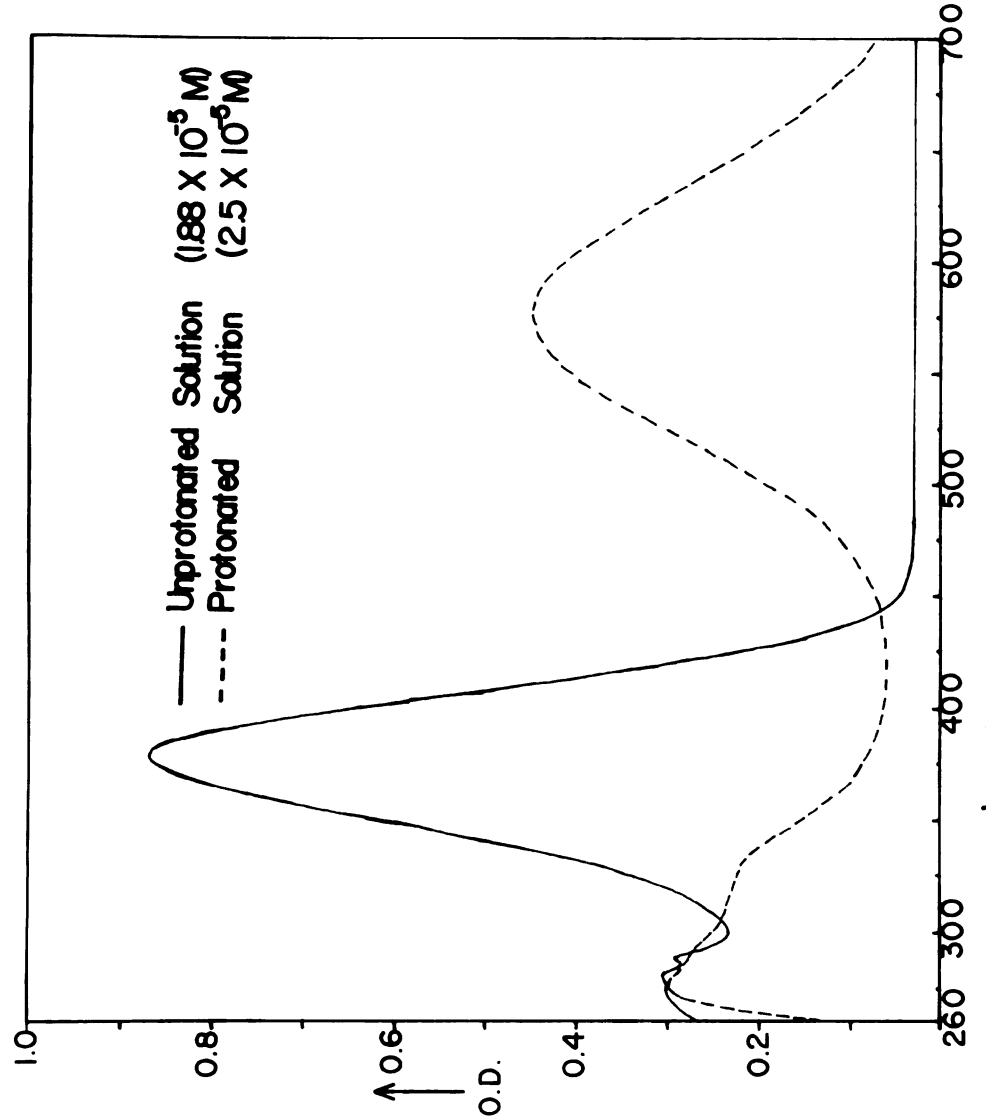


Figure 9. Absorption Spectra of Unprotonated and Protonated Solutions of All-Trans Retinal with Indole

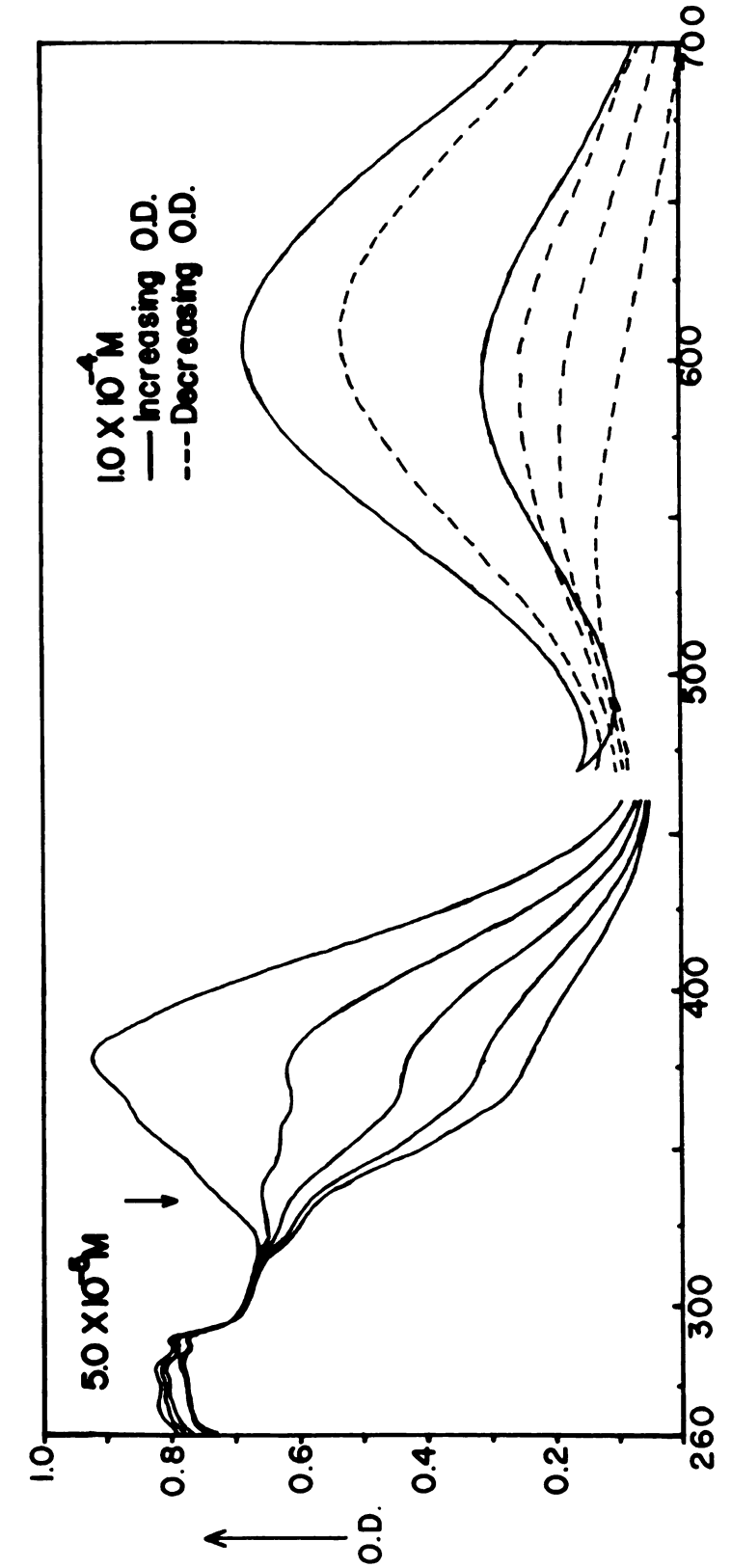


FIGURE 10. Absorption Spectra Showing Reaction of a Protonated Solution of All-Trans Retinal with Indole at 38°C

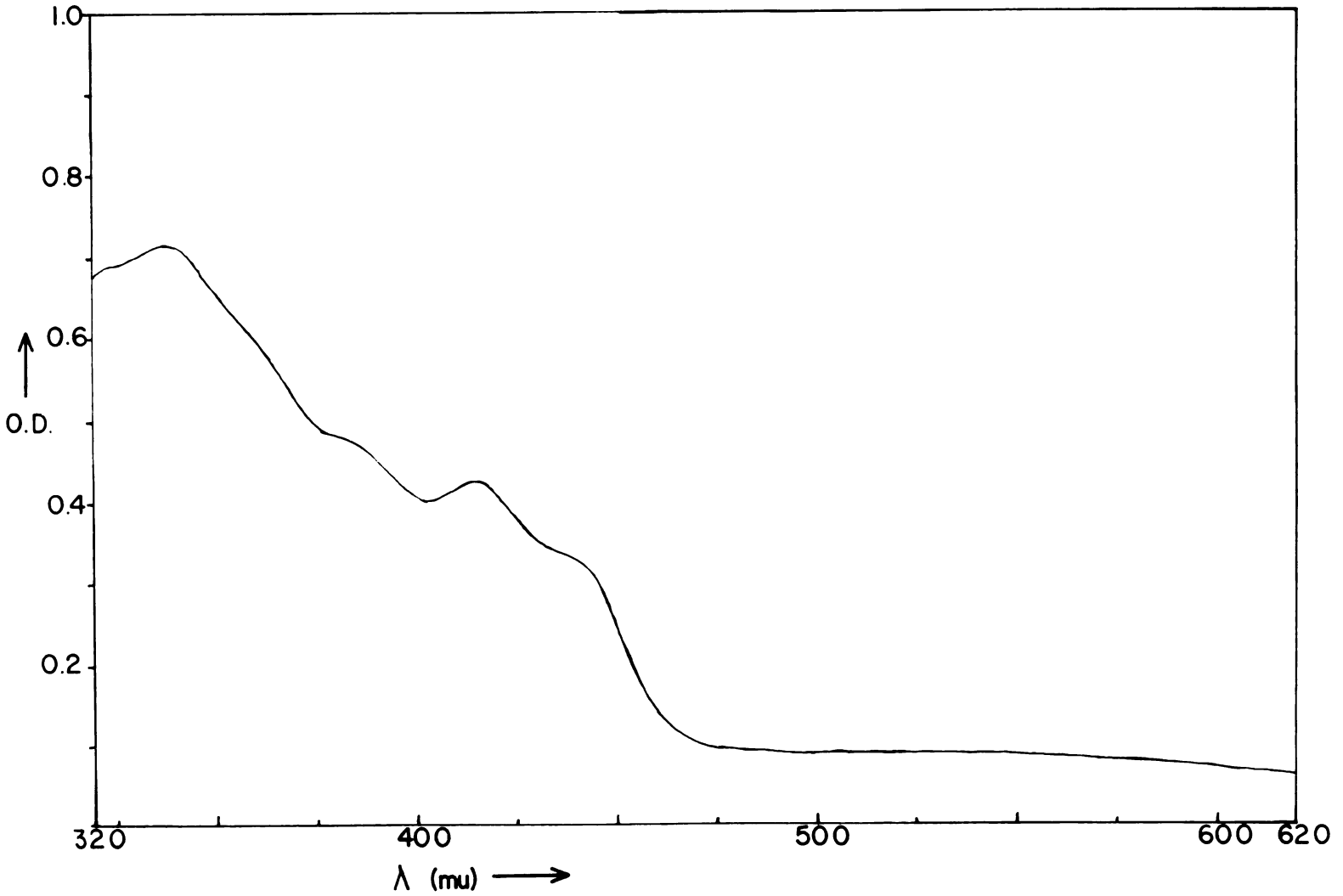
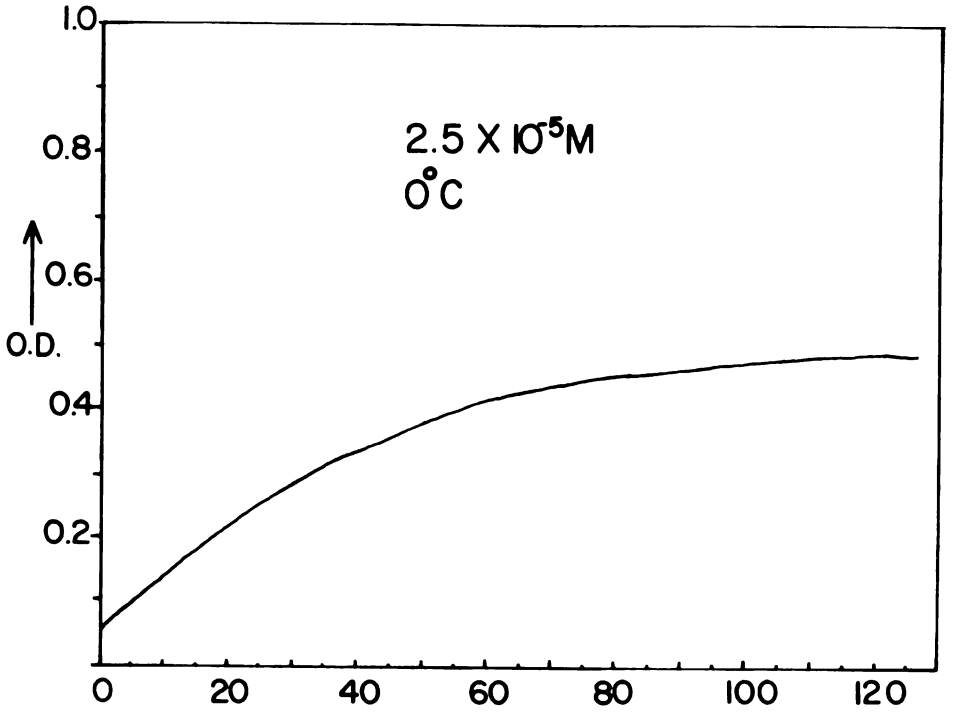


FIGURE II. Absorption Spectrum of Final Form of Compound at Room Temperature



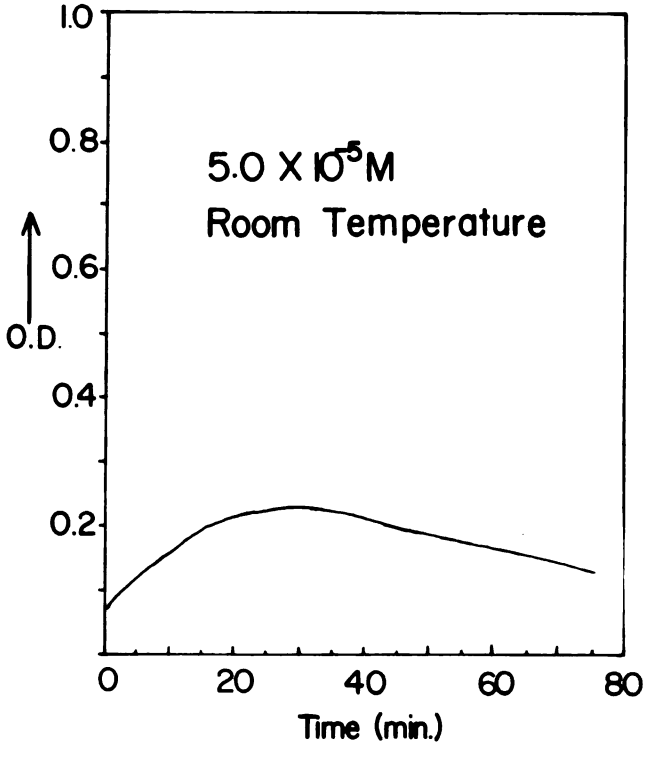


FIGURE 12. Kinetic Studies at 610 mu

Photosensitivity

Both unprotonated and protonated solutions of retinal and indole proved to be "photosensitive"---their characteristic visible spectra were significantly altered by intervals of illumination. Figure 13 shows the typical spectrum of the unprotonated solution (2.5×10^{-5}M) and the changes resulting from one minute intervals of illumination. Alteration of the bands characteristic of the indole moiety raised the possibility of energy transfer from the retinal portion of the molecule to the indole, and a subsequent reaction typical of the indole nucleus. Direct irradiation of a solution of indole (1×10^{-4}M) with 2537 Å U.V. light produced the spectral changes shown in Figure 14. The distinctive sharp peak at approximately 290 mμ disappears following irradiation of indole itself, while it remains definite and distinct for irradiated solutions of the unprotonated complex. The approximately 280 mμ shoulder typical of indole becomes a distinct peak in the spectra of the illuminated complex, while it decreases markedly and eventually becomes indistinguishable for irradiated solutions of indole. Moreover, a definite red band emerges in irradiated indole solutions (Figure 14), while the spectra of the illuminated complex remain virtually at the base line from 460 mμ on out. Although these results do not definitely rule out the possibility that the spectral changes evident in the unprotonated complex result from alterations of the indole nucleus, they at least indicate that any such changes are not those typical of uncomplexed indole.

To insure that any observed spectral changes of the protonated system following irradiation are light-induced and not merely the

result of a warming of the solution, all irradiation was carried out with the cuvette in an ice bath. After each interval of illumination, the solution was allowed to re-equilibrate for a fixed time in the sample chamber of the spectrophotometer, which was maintained at approximately 0°C. This was done to insure that all spectra were measured at the same temperature. Figure 15 shows the results of such irradiation.

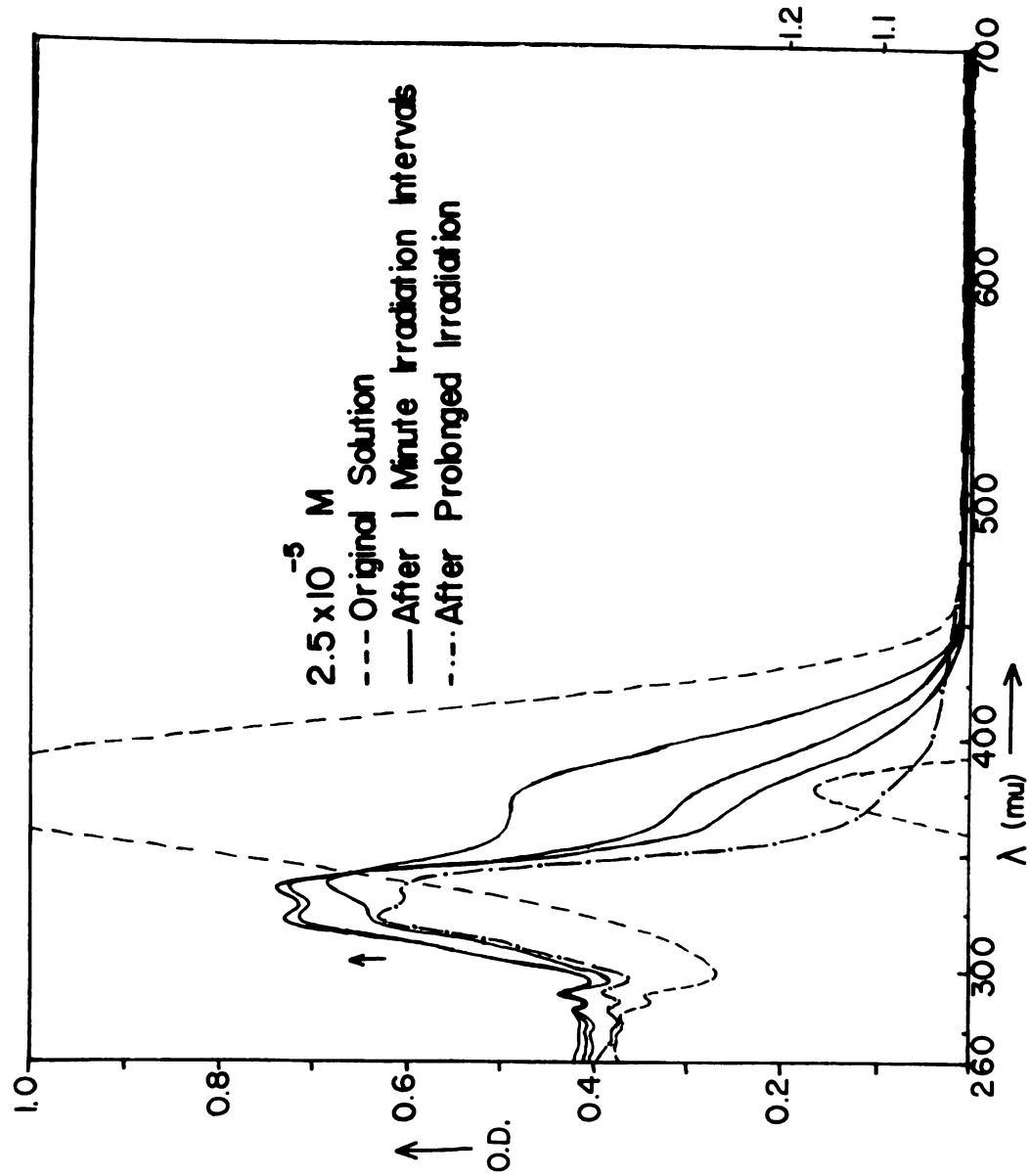


FIGURE 13. Absorption Spectra Showing Effect of Irradiation of the Unprotonated Solution

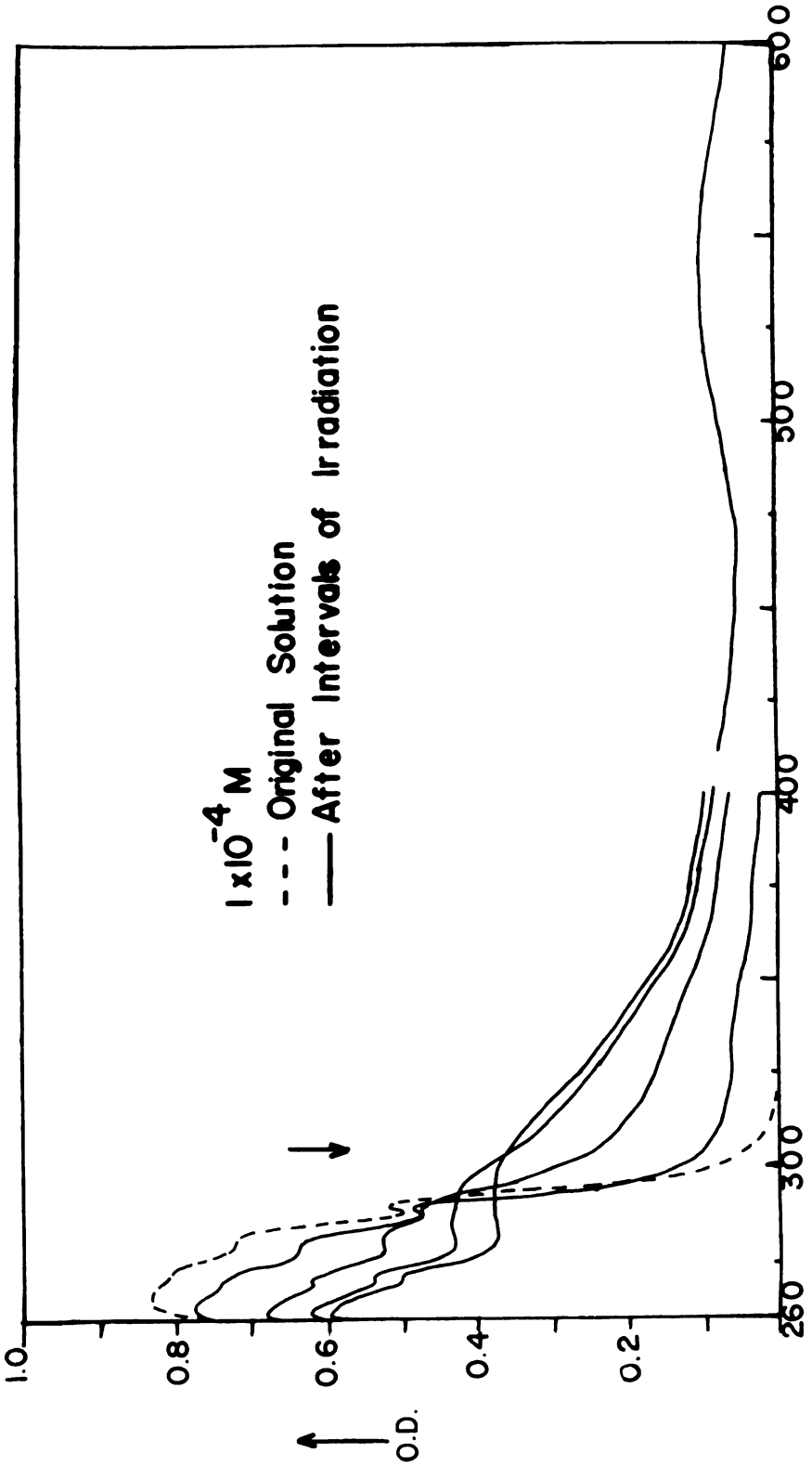


FIGURE 14. Absorption Spectra Showing Effect of Irradiation of an Indole Solution

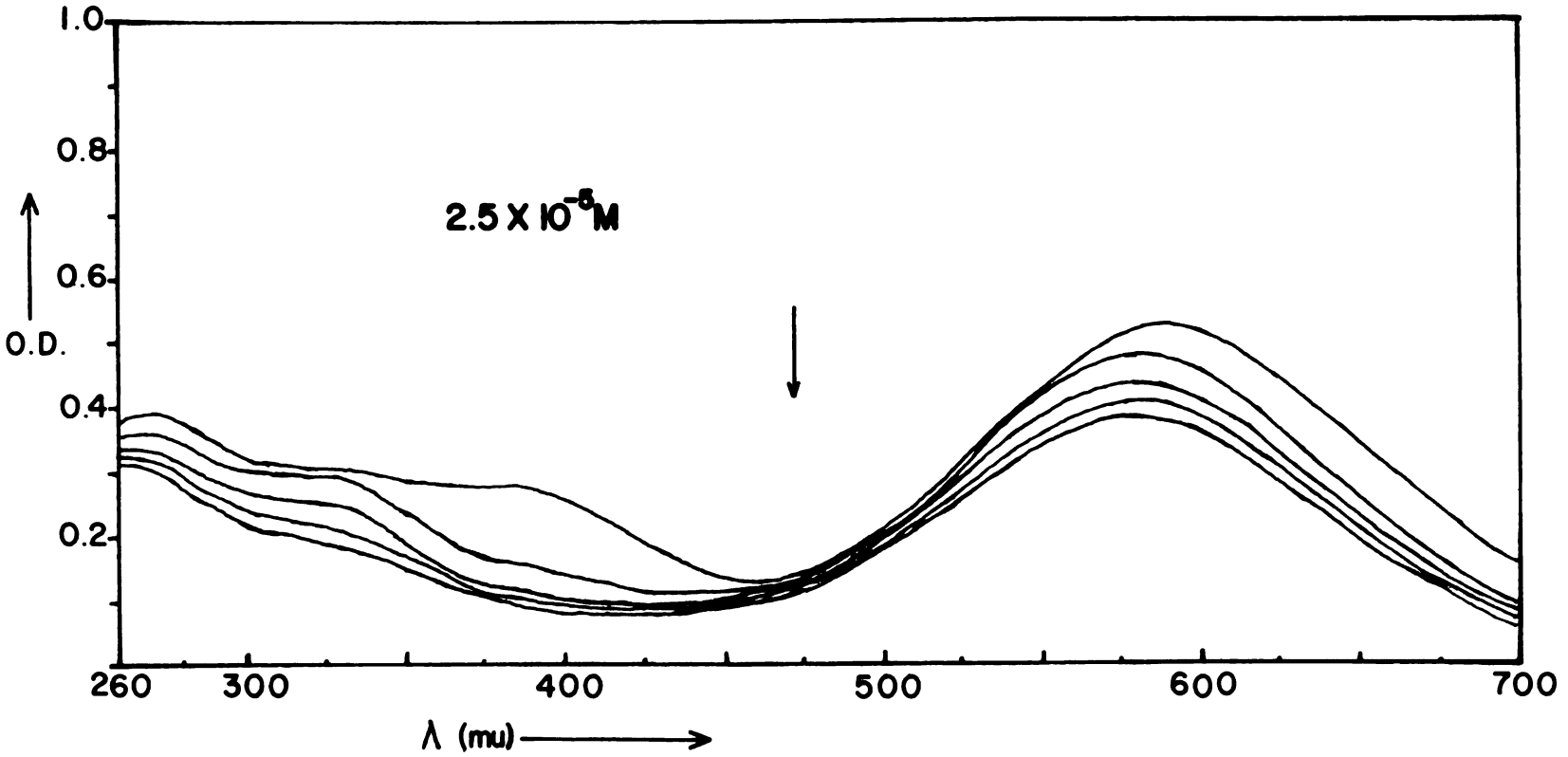


FIGURE 15. Absorption Spectra Showing Effect of Irradiation of the Protonated Solution

Indicator Properties

As noted above, bubbling anhydrous ammonia through a protonated solution of the compound resulted in the initial formation of a precipitate of NH_4Cl. In the initial studies of this process, bubbling was continued beyond this point, resulting in a bright yellow solution. Suction filtration successfully removed the precipitate and allowed spectral measurements to be made. When HCl was bubbled through this solution, a precipitate of NH_4Cl again formed and the system returned to the initial blue color of the protonated compound. Filtration, however, resulted in a colorless solution, as the protonated compound was adsorbed on the NH_4Cl precipitate. In one instance, the amount of precipitate formed on bubbling HCl was either insignificant or nonexistent, and a spectrum of the solution was measured (Figure 16). However, the spectrum of the reprotonated solution was not again attainable in pure CCl_4.

To eliminate the problem of precipitation and adsorption, a protonated solution (5.0×10^{-5}M) was diluted by half with methanol, a solvent in which NH_4Cl has a limited solubility. In some cases, it was still necessary to dilute the solution with several drops of methanol after bubbling either NH_3 or HCl to attain a completely clear solution. As a result, the O.D.'s of the resulting spectra are not truly significant. Figure 17 shows the spectra measured for the compound in this mixed solvent after several consecutive bubblings of NH_3 and HCl. They clearly indicate the indicator-like nature of the protonated species. It is interesting to note that the "alkaline" solutions of the compound evidenced the same type of temperature dependence as the pro-

tonated solutions---fading in time on standing at room temperature. (The displacement of the various λ_{max}'s of this system from their values in pure CCl_4 was not unexpected as methanol is a somewhat polar solvent.)

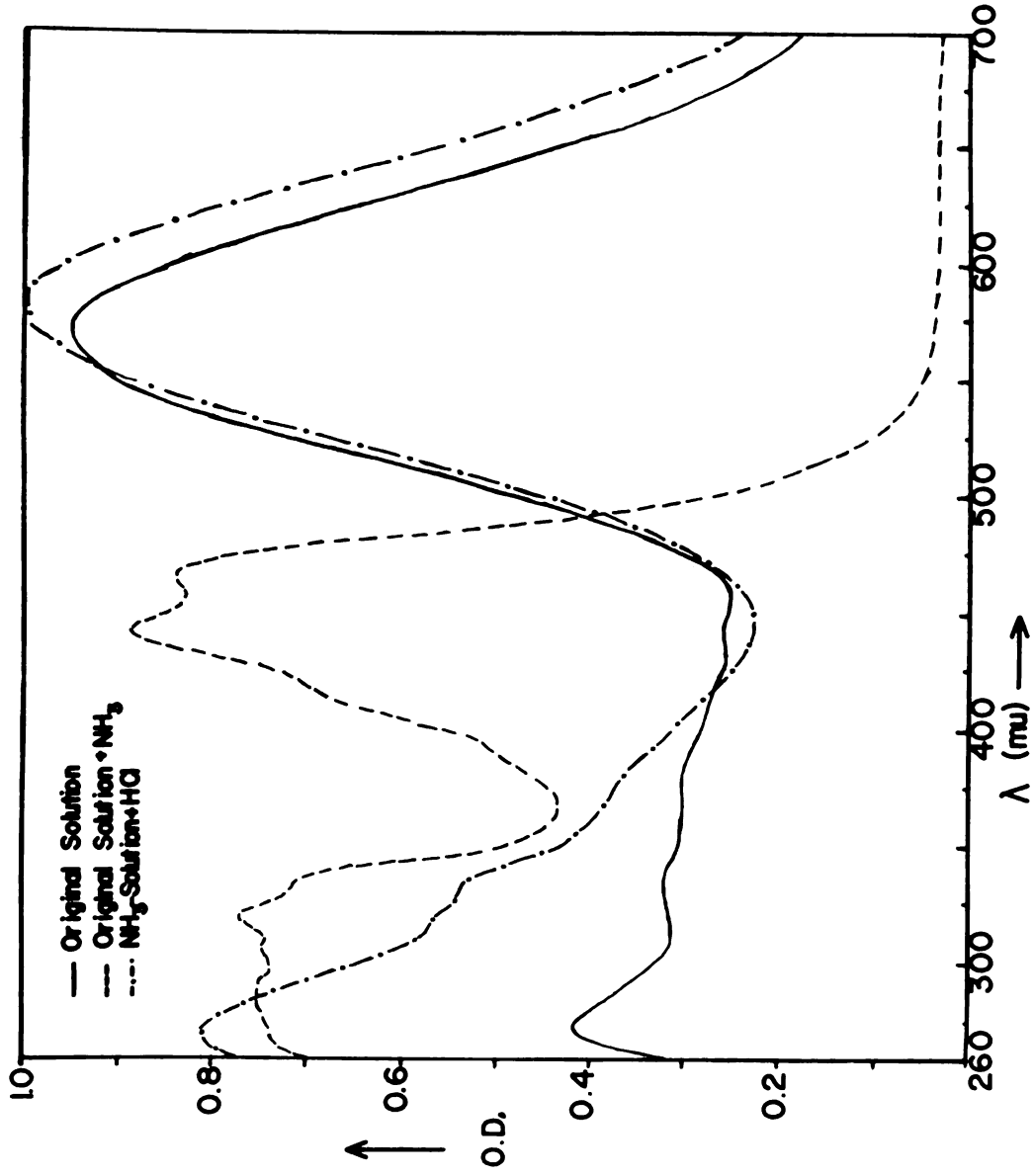


FIGURE 16. Absorption Spectra of Indicator Studies in CCl_4

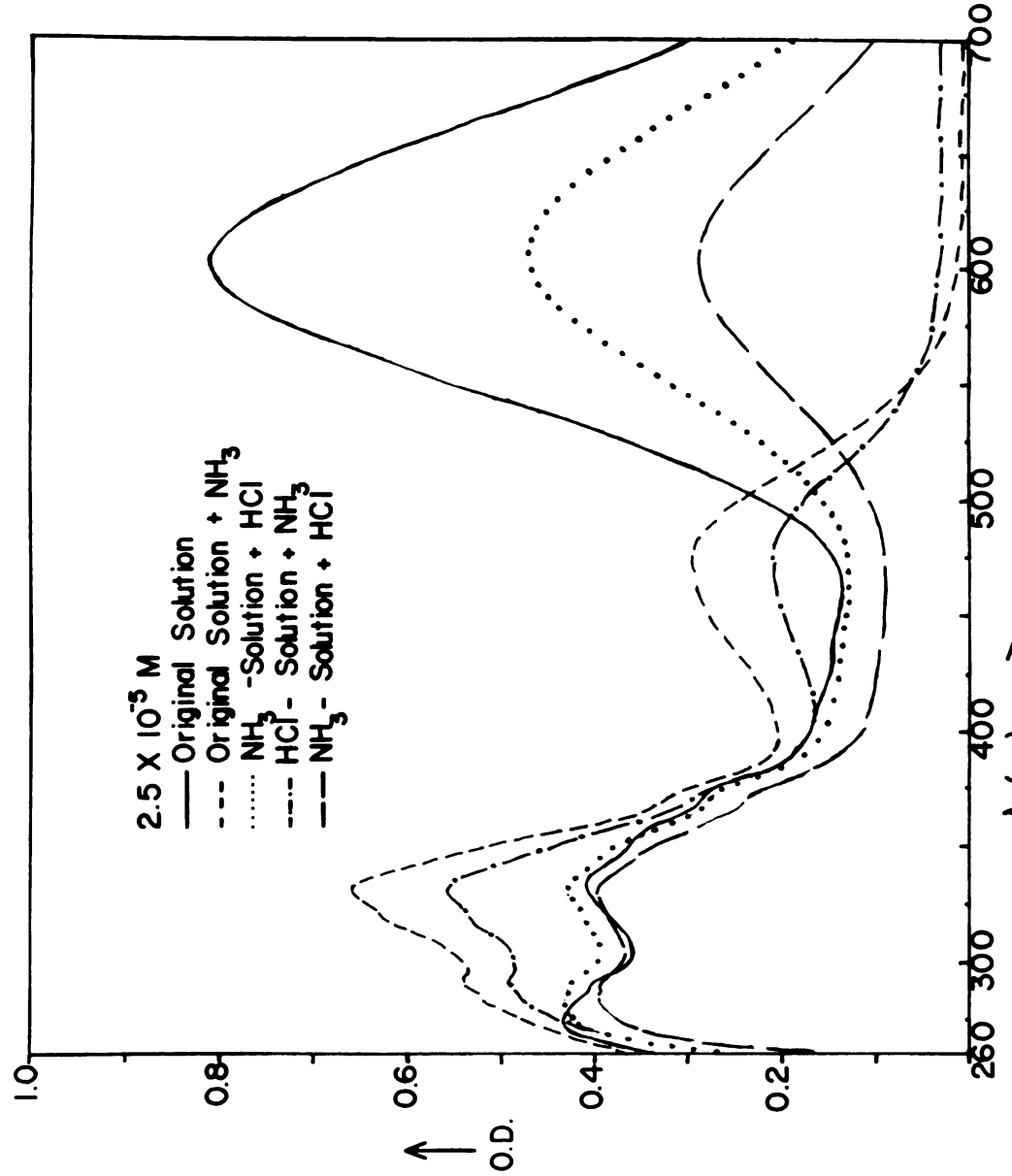


FIGURE 17. Absorption Spectra of Indicator Studies in CCl_4 - Methanol System

9-cis Isomer of Retinal

Spectral measurements of solutions of 9-cis retinal and indole gave results similar to those with the all-trans isomer. The spectrum of the unprotonated solution is approximately a combination of the spectra typical of the particular isomer of retinal and of indole (Figure 18). On protonation, the band characteristic of the unprotonated solution red-shifts from 372 mμ to approximately 385 mμ. As the reaction is followed at 0°C, the band representative of the protonated species initially appears as a broad band in the red region of the spectrum with an apparent λ_{max} at 600 mμ. The spectra evidenced an isosbestic point at approximately 462 mμ, the O.D. of the "unprotonated band" decreasing with time as that of the "protonated band" increases and becomes more defined (Figure 19). The final λ_{max} of the protonated species is situated at approximately 585 mμ, with U.V. peaks at 334, 392 and 272 mμ.

Illumination studies of the unprotonated and protonated solutions show significant spectral changes. The changes evident for the unprotonated solution are identical with those for the all-trans isomer. The peak characteristic of the unprotonated solution decreases rapidly as a doublet of peaks at approximately 320 and 336 mμ rises; the 280 and 290 mμ peaks characteristic of indole become more defined. The changes in the spectrum of the protonated solution, however, are somewhat different from those evidenced with the all-trans isomer (Figure 20). The region of the spectrum between approximately 370 and 520 mμ rises in O.D. progressively with each interval of irradiation, resulting in what approximates to two isosbestic points at 520 and 370 mμ.

For the all-trans compound, the entire expanse of the spectrum decreases in O.D. with continued intervals of irradiation.

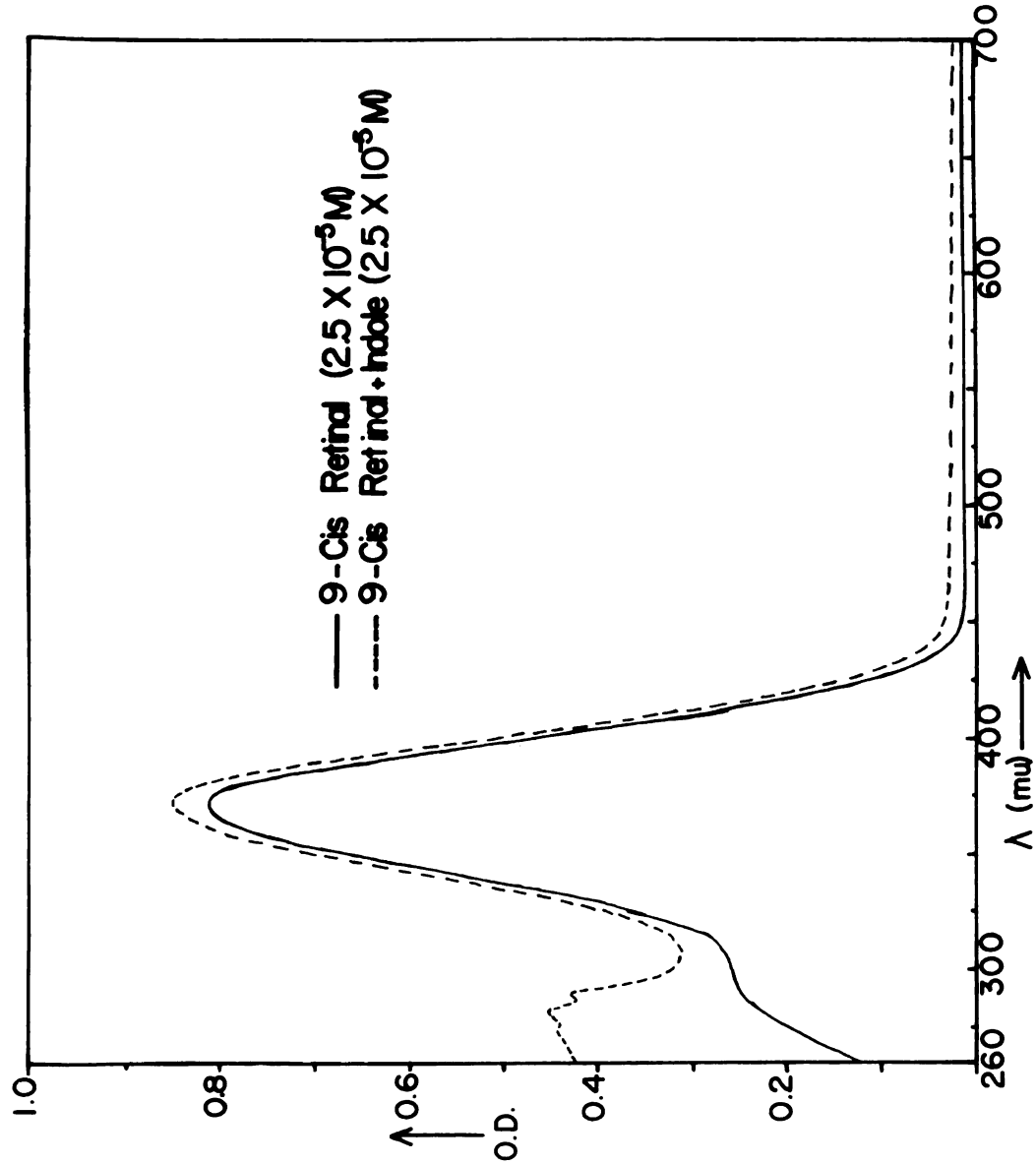


FIGURE 13. Absorption Spectra of 9-Cis Retinal and a Solution of 9-Cis Retinal with Indole

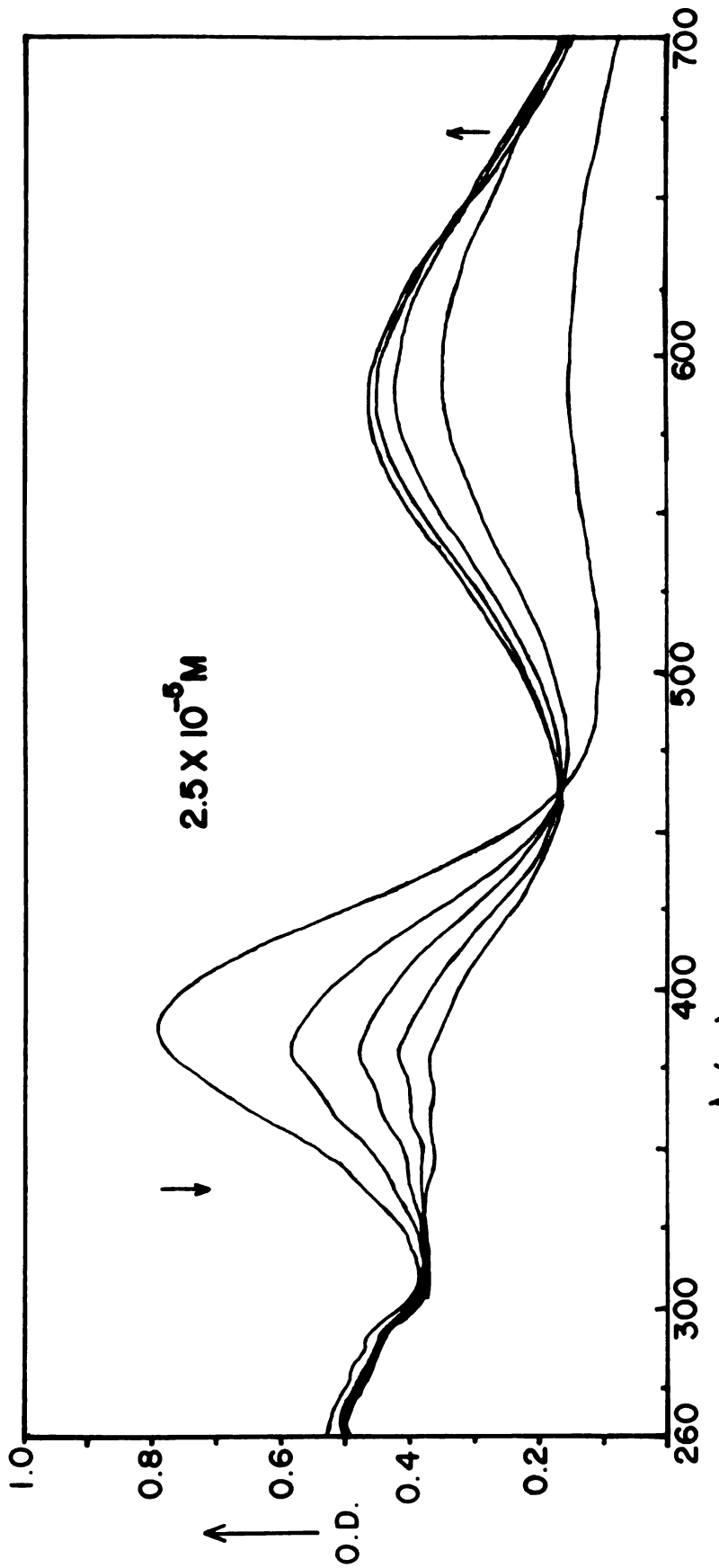


FIGURE 19. Absorption Spectra of the Reaction of a Protonated Solution of 9-Cis Retinal with Indole at 0°C

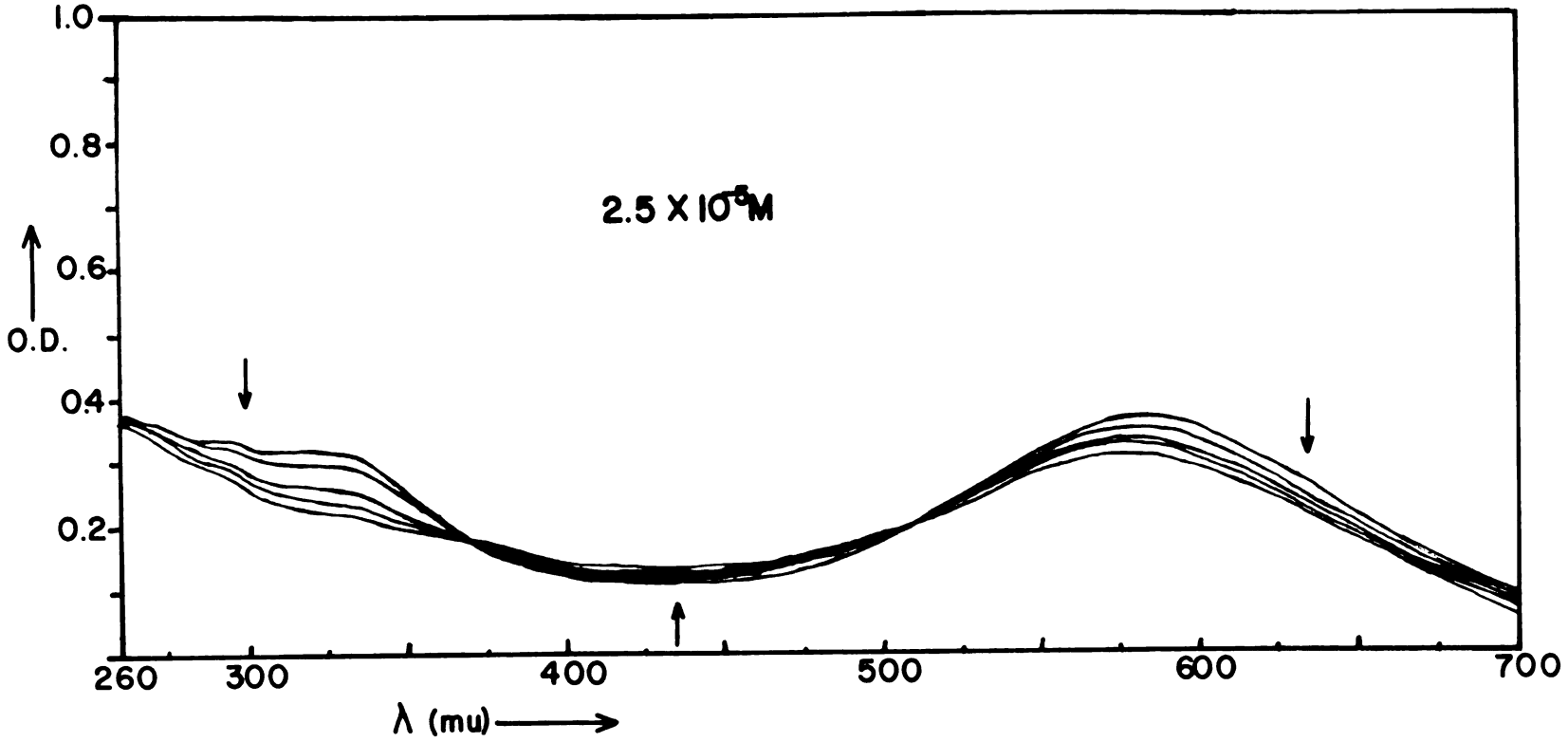


FIGURE 20. Absorption Spectra Showing Effect of Irradiation of a Protonated Solution of 9-Cis Retinal with Indole

Infra-red Spectra

The initial infra-red measurements were made on the retinal-pyrrole system. Samples were prepared by combining a small amount of crystalline retinal with a few drops of pyrrole and diluting with CCl_4. (Pyrrole is a liquid at room temperature.)

Figure 21 is a typical I.R. spectrum of all-trans retinal in CCl_4. The peaks evident at 2860, 2940 and 2960 cm^{-1} are typical for the all-trans configurations of conjugated systems. The strong band at approximately 1650 cm^{-1} represents the terminal aldehyde group. No spectrum was measured for pyrrole alone as such spectra are readily available. The appearance of an extremely strong band at approximately 3500 cm^{-1} in the spectrum of the unprotonated solution of retinal and pyrrole (Figure 22), a band not present in either the typical pyrrole or retinal I.R. Spectra, is possibly indicative of an OH group. The remaining bands are chiefly those associated with the basic structure of the retinal polyene system or of pyrrole. There was no way to ascertain the extent of reaction of the mixture, and the presence of a fairly strong 1650 cm^{-1} band probably indicates an unreacted or excess amount of retinal.

A more complete set of measurements were made using indole. Figure 23 shows the typical I.R. spectrum of indole. The doublet of strong bands apparent between 3400 and 3500 cm^{-1} is typical of the NH group in indole. Solutions for I.R. measurements of the unprotonated mixture were prepared by dissolving unmeasured amounts of retinal and indole crystals in a small amount of CCl_4. As a result, the molecular proportions of the constituents and the resulting extent of reaction

were unknown. The spectrum of this solution is, for the most part, merely a combination of the characteristic bands of the individual reactants (Figure 24). The marked change in the ratio of the doublet bands associated with indole (see above) seems to indicate that the band at approximately 3480 cm^{-1} is not merely representative of the indole structure. Extrapolating from the spectrum of the complex with pyrrole, it may be possible to ascribe at least part of the strength of this band to an OH group.

Figure 25 shows the I.R. spectrum of a KBr pellet of a protonated compound of 13-cis retinal and indole. The weak to moderate bands between 2490 and 2700 cm^{-1} can be representative of a C⁻N type structure. However, an exact structural assignment based on the appearance of these bands, cannot be definitely made. Of greater interest is the complete absence of the bands characteristic of NH group of indole (3420 and 3480 cm^{-1}) and the CH=O group of retinal (approximately 1650 cm^{-1}).

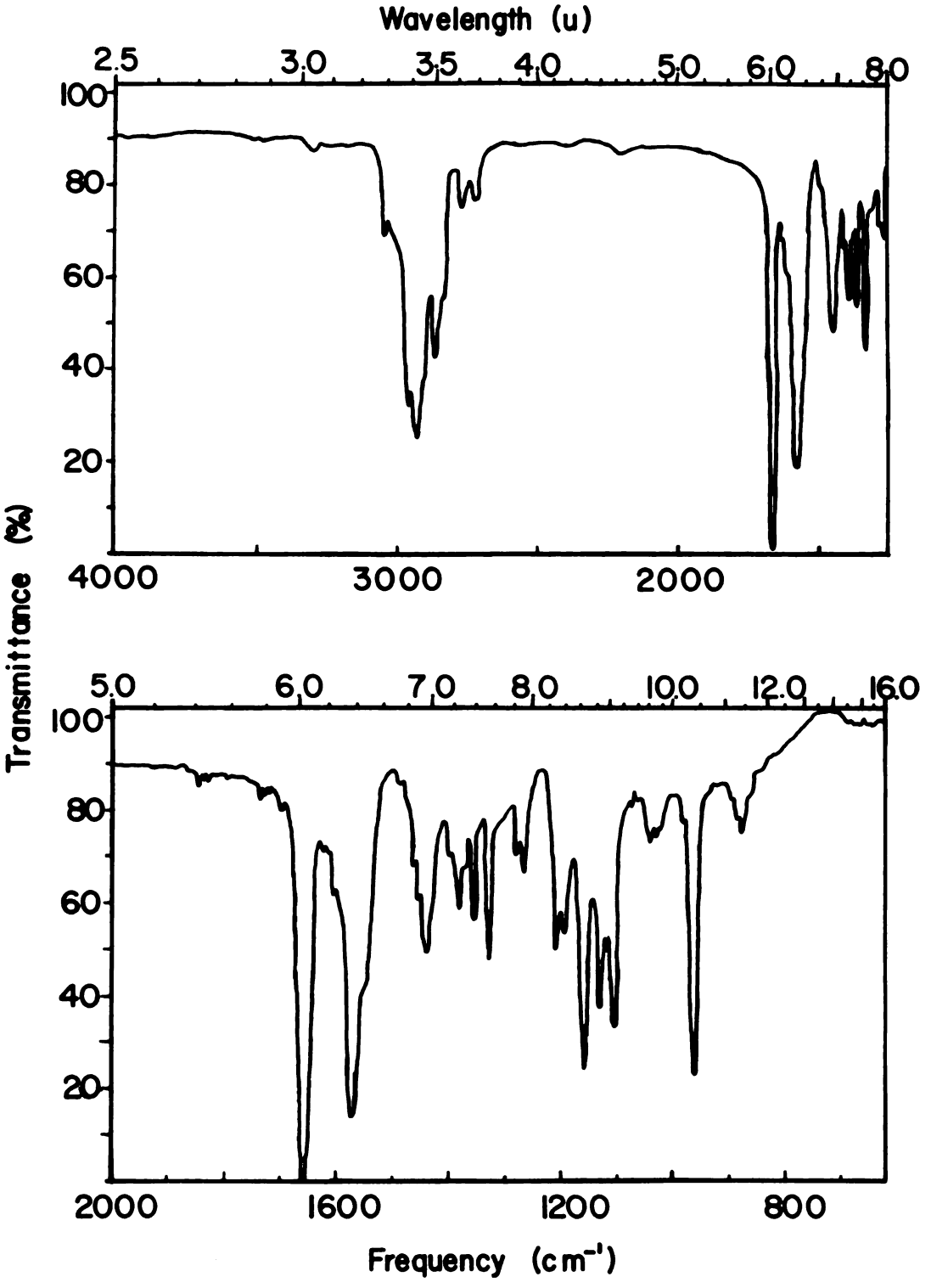


FIGURE 21. Infra-Red Spectrum of All-Trans Retinal in CCl_4

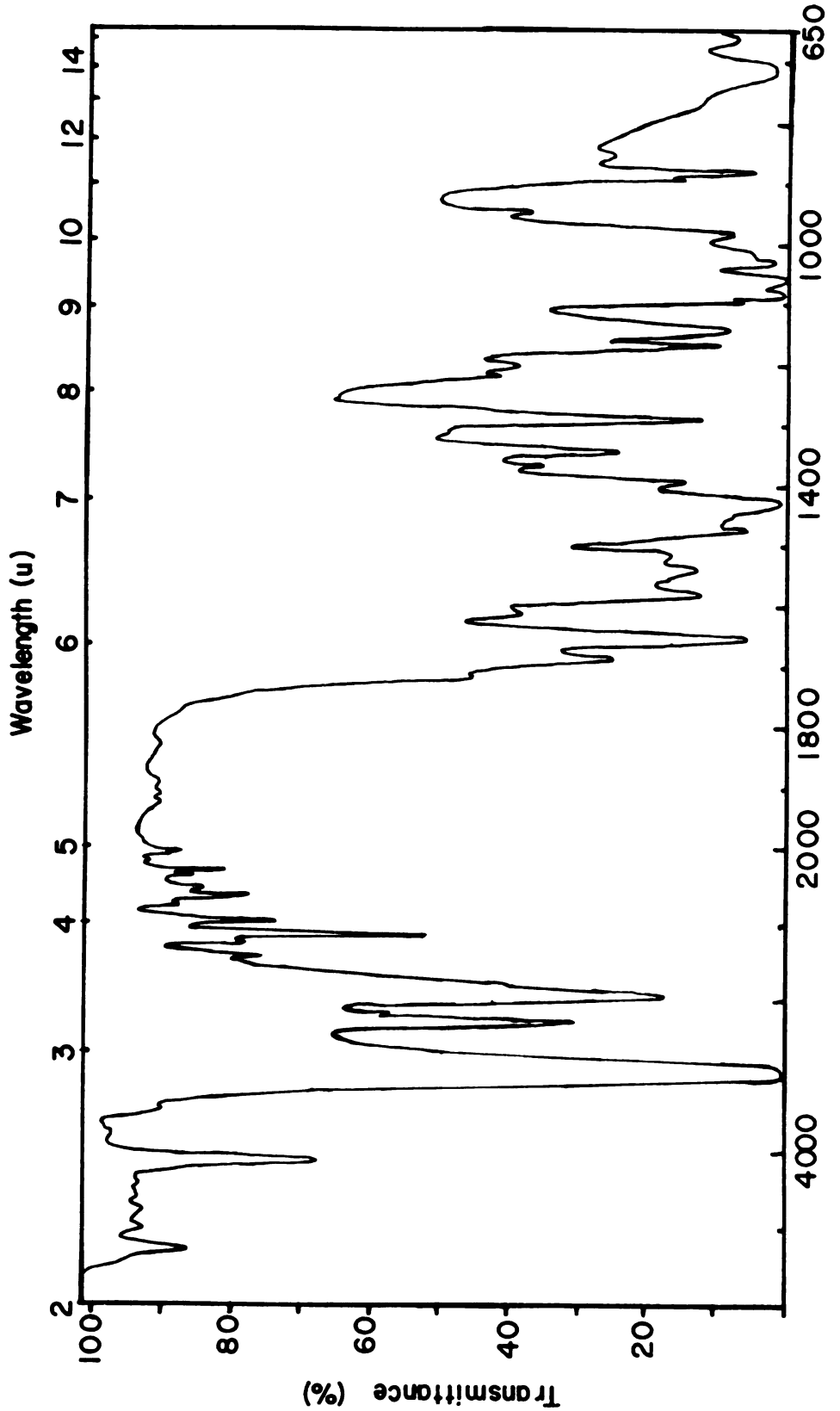


FIGURE 22. Infra-Red Spectrum of All-Trans Retinal with Pyrrole

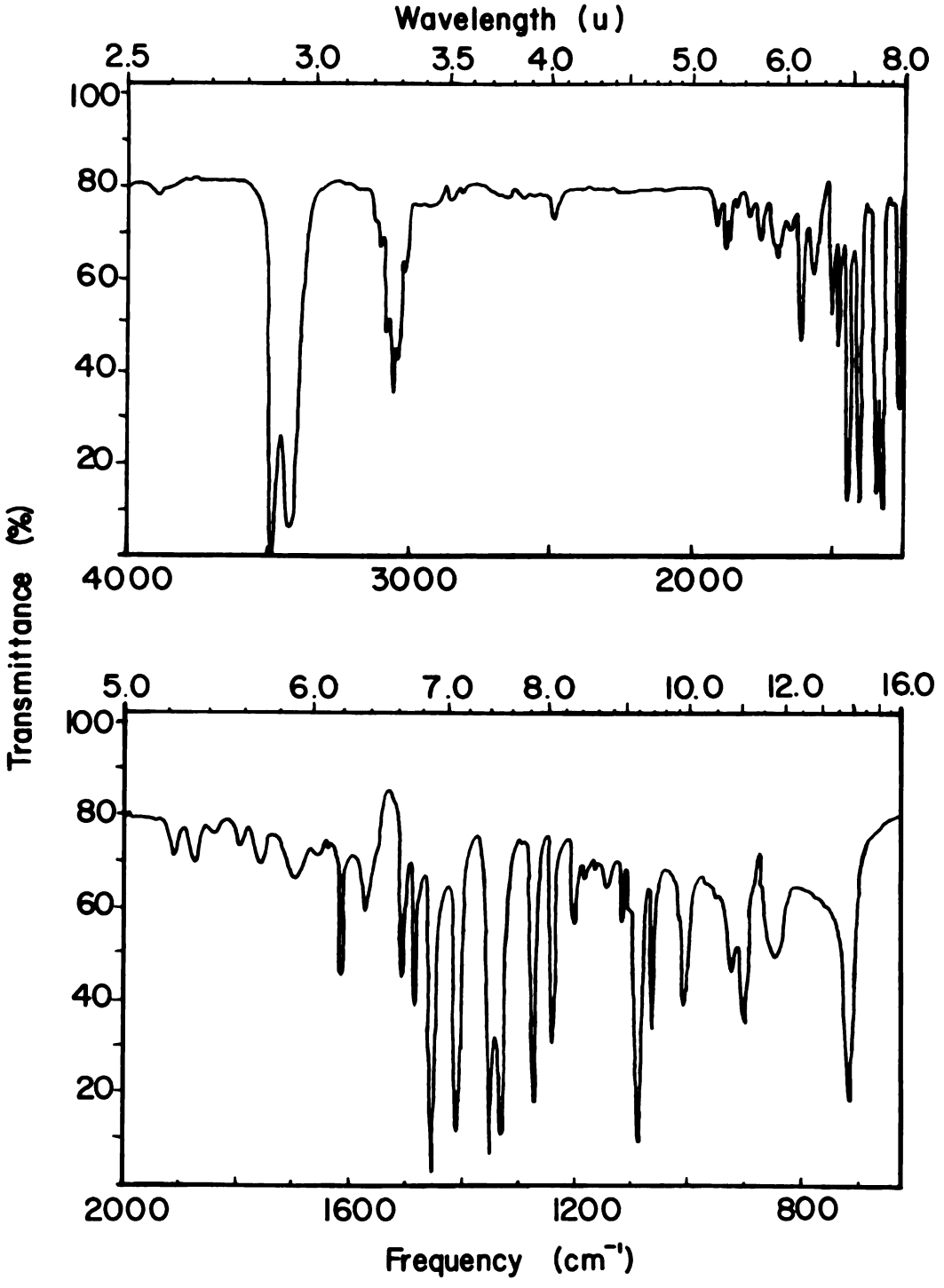


FIGURE 23. Infra-Red Spectrum of Indole in CCl_4

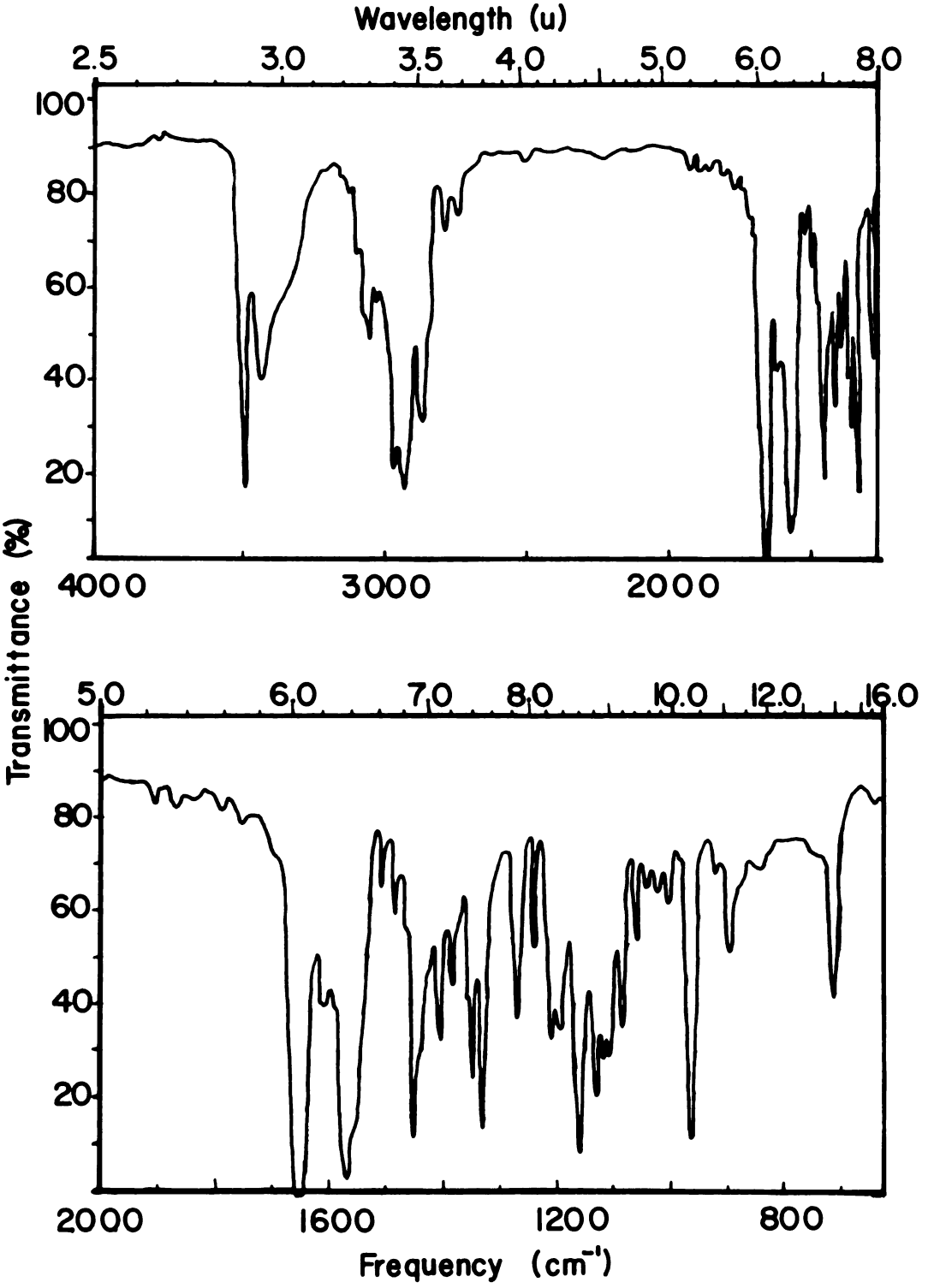


FIGURE 24. Infra-Red Spectrum of All-Trans Retinal with Indole

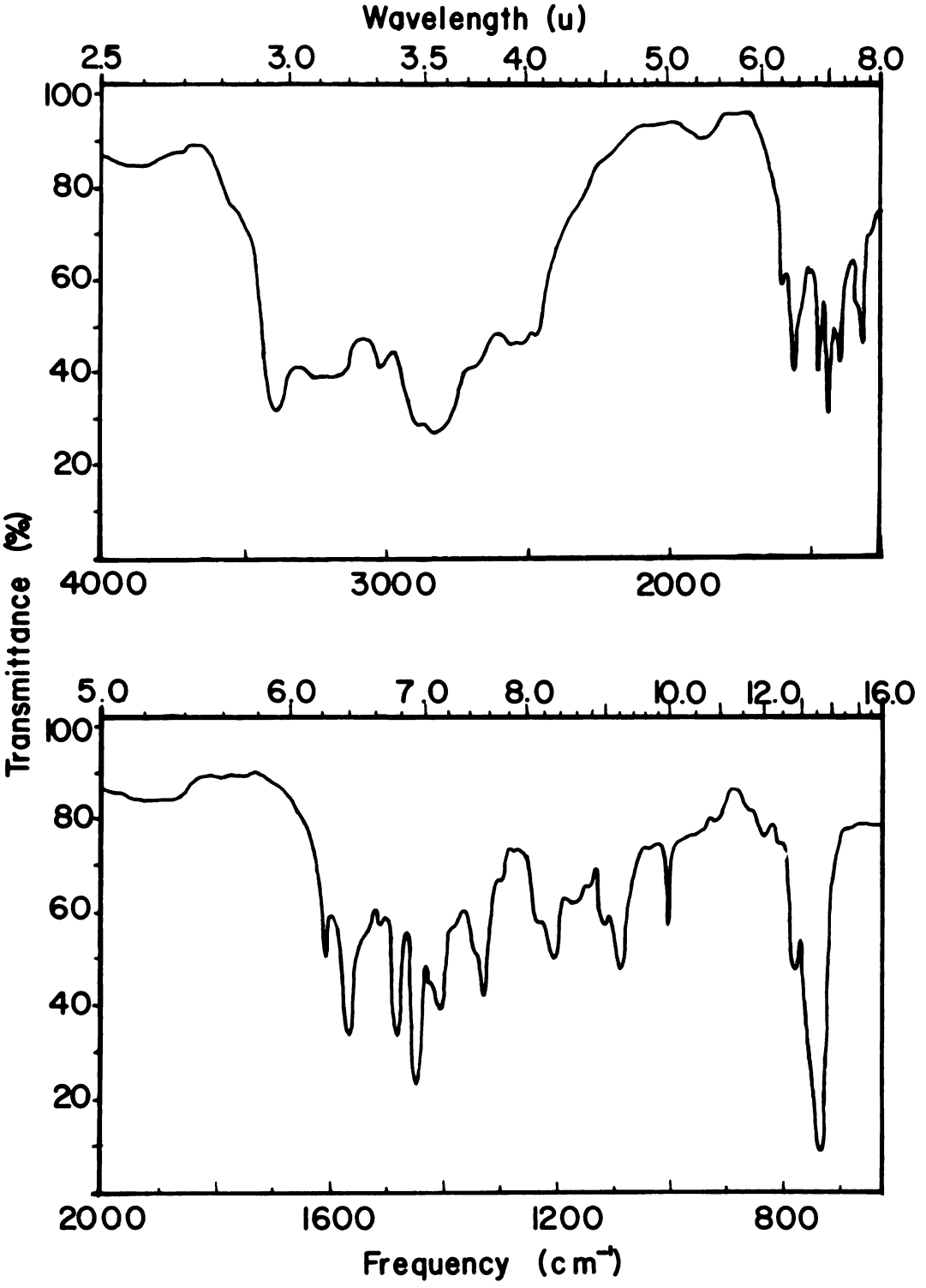


FIGURE 25. Infra-Red Spectrum of a KBr Pellet of 13-Cis Retinal with Indole and HCl

Microanalytical Results

The microanalytical results were inconclusive. Assuming a one-to-one reaction, the expected composition is: C-80.06%, H- 8.15%, N-3.33% and Cl-8.44%. The analyses produced the following percentages: C-72.31%, H-6.49%, N-6.08% and Cl-15.25%. If the reaction is assumed to involve one retinal to two indole molecules, the expected percentages still differ from the measured values in the same directions as the above. Since purification, to date, has been impossible, the discrepancies may result from the presence of unreacted constituents, adsorbed HCl or possibly the presence of solvent molecules. The extreme temperature dependence of the reaction and general instability of the compound may result in side reactions which would also interfere with accurate analysis.

Solvent Effects

Attempts to find a suitable solvent for the purification and recrystallization of the protonated compound proved futile. The compound is virtually insoluble in such non-polar solvents as carbon tetrachloride, cyclohexane and benzene. It readily dissolves in such solvents as ethanol, acetone, dioxane, chloroform, formamide and N,N-dimethylformamide at both room temperature and 0°C. However, it is extremely unstable in all of these solvents at both temperatures. The solution soon takes on the yellow color of retinal or becomes a pink to dirty orange.

Sulfolane, a polar solvent commonly used for organic salts, was investigated as a possible solvent. Since the compound is readily hydrolyzed, the sulfolane used was first vacuum distilled to remove

traces of water. However, the compound proved to be unstable in this solvent also, and spectral measurements of the solution proved identical, as far as placement of characteristic peaks, with Figure 11.

DISCUSSION

Proposed Reaction Scheme and Final Structure

In recent years much attention has been given to the structure, preparations and reactions of enamines. The term "enamine" is synonymous with α,β-unsaturated amine.[24] It was coined by Wittig and Blumenthal in 1927 to indicate a general structural feature in which a nitrogen atom replaces an oxygen atom in the familiar "enol." Typical enamine compounds can be formed by condensation of aldehydes and ketones with secondary amines in the presence of dehydrating agents.[25] In the case of derivatives of aromatic aldehydes, the formation of carbinolamines has been observed.[26] Investigating similar condensation reactions of imines and saturated heterocyclic amines with aldehydes, Kostyanovskii and Pan'shin, working at temperatures ranging from -30° to -70°C, prepared and isolated carbinol type compounds.[27] The structure of the products was confirmed by proton magnetic resonance spectra. By the treatment of an anhydrous alcoholic solution of formaldehyde with piperidine at -70°C they succeeded in preparing, in quantitative yield, white, crystalline N-piperidinocarbinol (Figure 26). It is unstable at room temperature, both in the crystalline state and in solution, but is stable at dry ice temperature. The low stability arises from its ease of dissociation, or reversibility. Immonio-carbon compounds of the type ($N^{+}=CH_2$) X^{-}, where the X is a more electronegative anion than OH, can be obtained by reaction with H (Halide).

Although the compounds resulting from condensations of aldehydes

and cyclic amines and imines differ in structure from the typical enamine, the resulting salt linkage, on treatment with H (halide), is consistent with the typical enamine salt structure.[25] Since the process of their formation is essentially identical to that of enamine salts---condensation of an aldehyde and a "disubstituted amine" followed by acidification---it does not seem unreasonable to classify the final structure as an "enamine salt."

From the above facts and the results reported here, it is proposed that the nature of the reaction of retinal with imines and secondary amines is similar to those identified by these Russian workers. The reversibility of the reaction between aldehydes and heterocyclic amines presents the possibility that such a reversibility is also present in the retinal systems. This could account for the failure of visible spectra of several of the unprotonated solutions (with diphenylamine, pyrrole, indole and carbazole) to evidence the presence of any new or shifted bands. In these cases, the reaction may strongly favor the dissociated form of the constituents, at least at the temperature at which the spectra were measured. The proposed formation of a positively charged nitrogen atom on reaction with a hydrogen halide, can explain the marked bathochromic shift evidenced on reaction of the unprotonated solutions with anhydrous HCl. The effects of introducing a positively charged nitrogen atom into a polyene chain still require investigation. From the work of Pitt, et.al.,[11] however, the introduction of this charged atom has been shown to result in a fairly extensive bathochromic shift.

The increase in extent of shift with increase in conjugation of

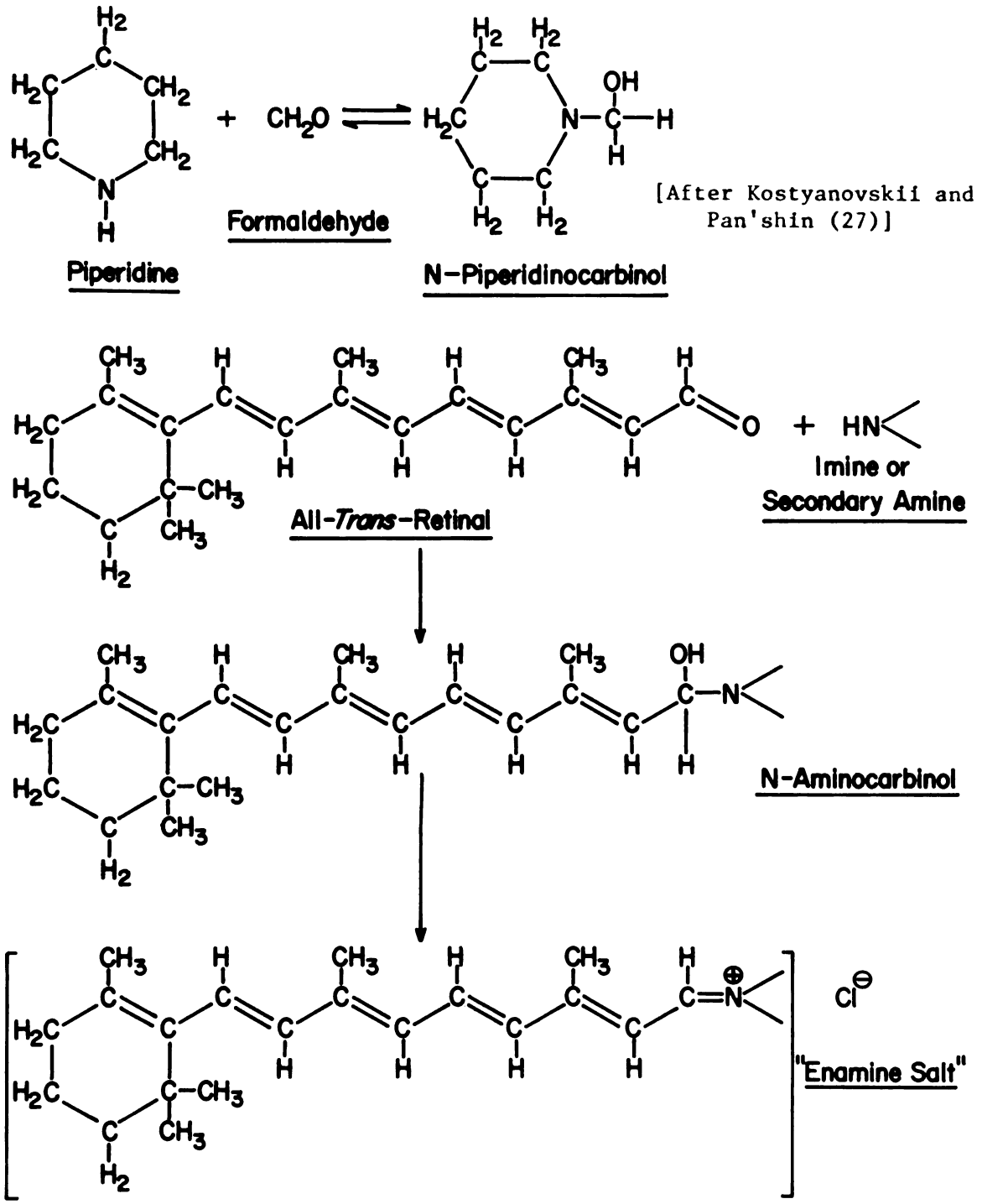


FIGURE 26. Reaction of Piperidine and Formaldehyde and Proposed Reaction of Retinal with Imines and Secondary Amines

the reacting compound (as evidenced in Figure 5) may be a combined effect of increase in conjugation of the system as a whole and an increase of the positive charge on the nitrogen from inductive effects. The extent of shift which could be expected from the addition of such conjugated compounds as used in the reported experiments is difficult to estimate, as all compounds used were cyclic in nature. Although much work has been done on the spectral effects of increasing conjugation in polyene systems,[28,29] linear conjugation chains of carbon atoms were mainly used for these studies. Spectral effects resulting from increased conjugation by the introduction of cyclic regions requires further investigation.

The hypothesis that a red shift in the λ_{max} can be attained by increasing the positive charge on nitrogen, by the inductive effect of substituents, was proposed by Rosenberg and Krigas[30] from their work with Schiff's bases of retinal. The λ_{max}'s of acidified Schiff's bases formed from retinal and various singly substituted meta- and para-anilines were compared. As the substituents become more "electron withdrawing," the λ_{max} of the resulting acidified compounds becomes increasingly red-shifted.

Absence of the I.R. bands representative of the NH and CH=O groups in spectra of the protonated compound, provides further evidence that the reaction does involve these portions of the reacting molecules, as postulated. The tentative assignment of the approximately 3500 cm^{-1} band of the unprotonated solutions is consistent with the proposed reaction scheme. Absence of an identifiable OH band in these spectra, however, would not discredit the hypothesized structure, as the

favored direction for the reversible initial reaction is unknown. Moreover, lack of evidence for a reaction from the visible spectra of unprotonated solutions of pyrrole and indole indicates the possibility that the dissociated constituents may be heavily favored in these cases.

The insolubility of the protonated compound in such non-polar solvents as cyclohexane, carbon tetrachloride and benzene is consistent with postulated structure---that of an "enamine salt." Also indicative of this type of structure is the extreme degree of adsorption of the protonated species on an ammonium chloride precipitate, as evidenced in the indicator studies of the compound.[31] More definite identification of the exact structure of the complexes will require nuclear magnetic resonance spectra.

Reasons for the failure of the conjugated compounds containing two nitrogen atoms to react with retinal are largely unknown. Work with imidazole, a compound belonging in this group, has evidenced a failure to react with nitrous acid, despite the presence of the imino group.[32] This seems to indicate that the imino group of this class of compounds has different reactive properties from the typical imino grouping, possibly as result of the rapid tautomeric shifts of hydrogen from one nitrogen to the other---a tautomerism which has been well established for such compounds. This may possibly explain why reaction of such compounds with retinal was not evidenced.

Comparison with Schiff's Bases as Model Systems

Naturally occurring visual pigments based on retinaldehyde show absorption maxima over the range 440-560 mμ.[12] The postulate of a conjugate acid form of the Schiff's base can satisfactorily account

for only a portion of the required spectral shift on union of retinaldehyde with an opsin. Moreover, it has been shown[33] that cattle metarhodopsin can exist in two forms---metarhodopsin I with λ_{max} at 478 mμ and metarhodopsin II with λ_{max} at 380 mμ. They can be interconverted by changing the pH---metarhodopsin II predominating at lower pH. The absorption maximum of metarhodopsin II is at too short a wavelength for it to be a conjugate acid of a Schiff's base. Moreover, the bathochromic shift of nearly 100 mμ on conversion to metarhodopsin I cannot be ascribed to protonation of a Schiff's base since it is favored by alkaline conditions and reversed by acid. These facts have lead to the argument that rhodopsin itself is unlikely to be a Schiff's base,[1] the indicated carbon-to-nitrogen link for rhodopsin still being maintained, nevertheless.

Results of reactions with imines and secondary amines indicate the possibility of the existence of a second type of carbon-to-nitrogen linkage with retinaldehyde. Table II includes the spectral ranges and chemical properties for protonated solutions of these systems along with those of protonated Schiff's bases. The extent of bathochromic shift attainable by the proper selection of suitable imines and/or secondary amines is more comparable to the shifts found for naturally occurring pigments than is that evidenced by protonated Schiff's bases. The evidence of "photosensitivity" for these compounds is probably inconsequential. The initial or photochemical step in the degradation of rhodopsin has been shown to involve a photoisomerization of the chromophore. Therefore, the possible failure of protonated Schiff's bases to evidence some type of photosensitivity

Table II: Comparison of "enamine salts" and protonated Schiff's bases

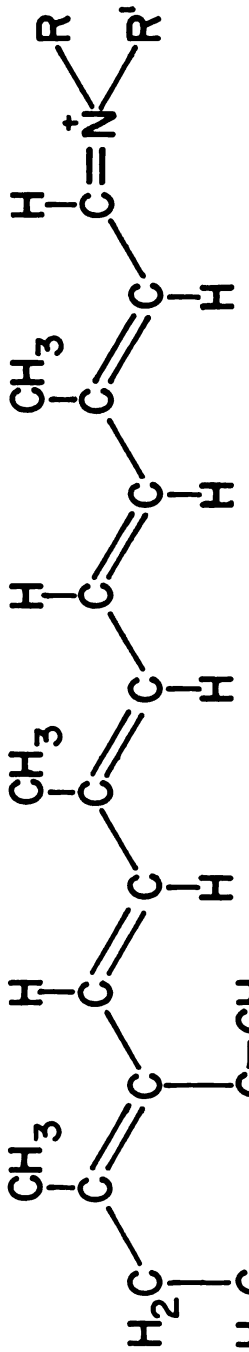
"Enamine salts"	Protonated Schiff's bases
λ_{max} - 450-650 mμ	λ_{max} - 420-460 mμ[11] -498 -574 mμ[30]
readily hydrolyzed	readily hydrolyzed
indicator-like properties	indicator-like properties[11]
"photosensitive"	no data available
stability extremely temperature dependent	no temperature dependence indicated

does not depreciate their value as a model system of the linkage involved. The temperature dependence of the imino and secondary amine systems, however, may be of relevance, as the stability of the intermediates of rhodopsin degradation, subsequent to the photochemical reaction, have been shown to be extremely temperature dependent, as indicated in Figure 1.

Relevance of the Postulated Structure to Visual Pigment Chemistry

Of possible relevance to the type of systems reported here, is the hypothesis of Morton and Pitt concerning the linkage between retinaldehyde and opsin in rhodopsin.[1] From all available evidence, they conclude that the C=N grouping determined for N-retinylideneopsin indicates the presence of this linkage in rhodopsin. Simple azomethine derivatives of this sort are readily decomposed by hydroxylamine, giving retinene oxime. However, rhodopsin and metarhodopsin are not affected by treatment with this compound. (Rhodopsin is also reported to be unreactive with borohydride until it has reached the metarhodopsin II stage of its degradation process.[7]) This seems to indicate that the C=N linkage must be shielded in some way. They propose a retinylene ammonium structure (Figure 27), where R represents the polypeptide chain involved in the N-retinylideneopsin structure and R' an unknown grouping (possibly on another polypeptide chain) joined by a link hydrolyzed when metarhodopsin is converted to N-retinylideneopsin.

These authors prepared retinylidene-ammonium iodide (R and R' both CH_3 in Figure 27) by reaction of the Schiff's base formed from retinal and methylamine with methyliodide.[12] Their proposed structure for the



[After Morton and Pitt (1)]

FIGURE 27. Retinylene Ammonium Structure

resulting compound is identical with structure of the "enamine salt" postulated above. They point out that their compound shows several similarities to metarhodopsin---its instability at room temperature, its decomposition on treatment with alkali and the position of its absorption maximum. The instability at room temperature of the systems reported here as well as the effects of "alkaline" conditions, has already been noted. Morton and Pitt point out that the comparison of the spectroscopic characteristics should not be pushed too far, as the nature of the substituents R and R' in their structure obviously influence the position of the λ_{max}.

They further point out that consideration of the chemical properties of rhodopsin, lumirhodopsin, metarhodopsin, N-retinylidenedopsin and retinaldehyde suggests that in rhodopsin there are at least three links joining retinal to the opsin. One of these is split in the conversion of rhodopsin to prelumirhodopsin; another is broken when metarhodopsin converts to N-retinylideneopsin (or metarhodopsin I converts to metarhodopsin II in the case of cattle rhodopsin); and finally the carbon-to-nitrogen link is hydrolyzed to give retinaldehyde. Although they themselves indicate that this interpretation is no more than a working hypothesis, it is interesting to speculate how the retinal linkage postulated here could fit into their scheme. Using C=N linkage as a starting point, it seems plausible that a side group in opsin might react with the nitrogen atom to form the $C{=}N^{+}\swarrow$ type of linkage postulated. The formation of such a linkage could reasonably account for the extent of bathochromic shift evident on reaction of the aldehyde with an opsin. The exact extent of the shift

would depend on the nature of the side group involved. This type of linkage would be unreactive with those compounds known to attack typical Schiff's base linkages---e.g., borohydride. If the side group of opsin participating in this linkage were to be split off at some stage of the degradation process, a result possibly of a change in the protein configuration, the linkage would convert to that of a typical Schiff's base and would be reactive with borohydride, as is the metarhodopsin II form of cattle rhodopsin. This scheme, however, is as hypothetical as the one proposed by Morton and Pitt, but does present a new possibility for the interpretation of the chemistry of visual pigments.

REFERENCES

1. Morton, R. A. and Pitt, G. A. J. "Visual Pigments," Fortschritte der Chemie Organischer Naturstoffe, 14, 244. (1957)

2. Hubbard, R. and Wald, G. "Cis_Trans Isomers of Vitamin A and Retinene in the Rhodopsin System," Journal of General Physiology, 36, 269. (1952)

3. Wald, G. "The Photoreceptor Process in Vision," American Journal of Ophthalmology, 40, Number 5, Part II, p. 18. (1955)

4. Wald, G. "The Visual Function of the Vitamins A," Vitamins and Hormones, 18, 417. (1960)

5. Pitt, G. A. J. "A Survey of the Chemistry of Visual Pigments," Experimental Eye Research, 3, 316. (1964)

6. Kropf, A. and Hubbard, R. "The Mechanism of Bleaching Rhodopsin," Annals New York Academy of Sciences, 74, 266. (1958)

7. Hubbard, R., Bownds, D. and Yoshizawa, T. "The Chemistry of Photoreception," Symposia on Quantitative Biology, 30, 301. (1965)

8. Hubbard, R. and Kropf, A. "Molecular Aspects of Visual Excitation," Annals New York Academy of Science, 81, 388. (1959)

9. Bridges, C. D. B. "Studies on the Flash Photolysis of Visual Pigments-III," Vision Research, 2, 201. (1962)

10. Ball, S., Collins, F. D., Dalvi, P. D. and Morton, R. A. "Studies in Vitamin A. 11. Reactions of retinene with Amino Compounds," Biochemical Journal, 45, 304. (1949)

11. Collins, F. D. and Morton, R. A. "Studies on Rhodopsin. 2. Indicator Yellow," Biochemical Journal, 47, 10. (1950)

12. Pitt, G. A. J., Collins, F. D., Morton, R. A. and Stok, P. "Studies on Rhodopsin. 8. Retinylidenemethylamine, an Indicator Yellow Analogue," Biochemical Journal, 59, 122. (1955)

13. Collins, F. D. "Rhodopsin and Indicator Yellow," Nature, 171, 469. (1953)

14. Dartnall, H. J. A. The Visual Pigments. New York: John Wiley, 1957. Pp. 122-123.

15. Wald, G. Light and Life. Baltimore: Johns Hopkins Press, 1961. Pp. 724-749.

16. Liebman, P. A. Discussion, "The Chemistry of Photoreception," Symposia on Quantitative Biology, 30, 315. (1965)

17. Rosenberg, B. "Photoconductivity and the Visual Receptor Process," Journal of the Optical Society of America, 48, 581. (1958)

18. Rosenberg, B. "A Physical Approach to the Visual Receptor Process," Advances in Radiation Biology, Volume 2. New York: Academic Press, 1966. Pp. 193-241.

19. Rosenberg, B. "Photoconduction and Cis-Trans Isomerism in β-Carotene," Journal of Chemical Physics, 31, 238. (1958)

20. Rosenberg, B. "Photoconduction in a Hindered cis-Isomer of β-Carotene and its Relation to a Theory of Visual Receptor Process," Journal of the Optical Society of America, 51, 238. (1961)

21. Rosenberg, B., Heck, R., and Aziz, K. "Color Responses in an Organic Photoconductive Cell," Journal of the Optical Society of America, 54, 1018. (1964)

22. Rosenberg, B., Heck, R. J. and Aziz, K. "A Physical Basis for the Chromatic S Poatentials in Colour Vision," Photochemistry and Photobiology, 4, 351. (1965)

23. Rosenberg, B., Orlando, R. A. and Orlando, J. M.,"Photoconduction and Semiconduction in Dried Receptors of Sheep Eyes," Archives of Biochemistry and Biophysics, 93, 395. (1961)

24. Szmuszkovicz, J. "Enamines," Advances in Organic Chemistry, Methods and Results, Volume 4. (Raphael, R. A., Taylor, E. C. and Wynberg, H., editors). New York: Interscience Publishers, 1963. Pp. 1-114.

25. Blaha, K. and Cervinka O. "Cyclic Enamines and Imines," Advances in Heterocyclic Chemistry, Volume 6. (Katritzky, A. R. and Boulton, A. J., editors) New York: Academic Press, 1966. Pp. 147-227.

26. Kostyanovskii, R. G. and Bystrov, V. F. "Aryl-N-Ethyleneiminocarbinols," Bulletin of the Academy of Sciences, USSR. Division of Chemical Science, 1, 151. (1963)

27. Kostyanovskii, P. G. and Pan'shin, O. A. "N-Piperidinocarbinol," Bulletin of the Academy of Sciences, USSR. Division of Chemical Science, 1, 164. (1963)

28. Jaffe, H. H. and Orchin, M. Theory and Applications of Ultraviolet Spectroscopy. New York: John Wiley and Sons, Inc., 1962. Pp. 220-241.

29. Murrell, J. N. The Theory of the Electronic Spectra of Organic Molecules. New York: John Wiley and Sons., Inc., 1963. Pp. 67-90.

30. Rosenberg, B. and Krigas, T. M. "Spectral Shifts in Retinal Schiff Base Complexes," Photochemistry and Photobiology (to be published).

31. Willard, H. H., Furman N. H. and Brecker, C. E. Elements of Quantitative Analysis. New York: D. Van Nostrand Company, Inc., 1956. Pp. 115-117.

32. Hofmann, K. Imidazole and Its Derivatives Part I. The Chemistry of Heterocyclic Compounds, Volume 6. New York: Interscience Publishers, Inc., 1953.

33. Matthews, R. G., Hubbard, R., Brown, P. K. and Wald, G. "Tautomeric Forms of Metarhodopsin," Journal of General Physiology, 47, 215. (1963)