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The developement [sic] of phosphorometric, fluorometric and absorption analyses for the measurement of carbamates and carbamic acids as an explanation of carbon dioxide transport in blood

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THE DEVELOPEMENT OF PHOSPHOROMETRIC, FLUOROMETRIC AND ABSORPTION ANALYSES FOR THE MEASUREMENT OF CARBAMATES AND CARBAMIC ACIDS AS AN EXPLANATION OF CARBON DIOXIDE TRANSPORT IN BLOOD

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is approved by

Robert W Schafer

LB

Dedicated to one

ROBERT A. KURTTER

-whose decision to study economics allowed me to find a deep interest in chemistry.

ACKNOWLEDGEMENTS

I would like to thank the faculty of the Department of Chemistry, Union College for all their interest and encouragement. Special thanks to Drs. Thomas Werner and Peter Frosh for the many very stimulating and interesting discussions.

Above all, my heartfelt thanks and appreciation to Prof. Robert W. Schaefer, my advisor. His understanding, timely criticism, and thoughtful advice on every topic will be sorely missed in the days to come; my many thanks.

E. T. Gray

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LIST OF SPECTRA, GRAPHS, DIAGRAMS AND TABLES

SPECTRA, GRAPHS, DEAGRAMS, AND TABLES (con't)

ABSTRACT

Phosphorometric, flourometric, and absorption analyses have been developed for the measurement of amino acids, carbamic acids, and carbamates. The absorption analysis may be done in any solvent while the other two methods are limited to non-re-emitting solvents. Water is not excluded in any of the analyses. A carbamate or carbamic acid can be detected even in the presence of amino acids as low as 0.5%, with an overall accuracy of ± 0.3%.

Tentative assignments have been made for the n,π^* direct triplet excitation bands for the carbonyls of glycine and its carbamic acid, acetyl chloride, ethyl acetate, and acetamide. The first two are based on absorption and phosphorescence data while the last four are based on phosphorescence data alone.

INTRODUCTION:

A SHORT HISTORY OF THE CARBAMATE REACTION AND ITS MEASUREMENT

Until 1934, it was believed that carbon dioxide was transported in the body in two principle forms; hydrated molecular CO_2 or carbonic acid, and bicarbonate ions.

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

It was realized, however, that the hydration of CO_2 in the physiological pH range (7.0-8.0) is relatively slow (1,2,3). It is, in fact, too slow to be physiologically important unless the reaction is catalyzed by a carbonic anhydrase. Meldrum and Roughton (4) isolated carbonic anhydrase from red blood cells. Then, in 1934, Ferguson and Foughton (5) inhibited the carbonic anhydrase system and still found a small but fast uptake of CO_2 by blood. At this point a direct and reversible reaction was postulated between CO_2 and hemoglobin, the new compound thus formed being called a carbamate ($HbNH_2$ stands for hemoglobin):

$$HbNH_2 + CO_2 \rightleftharpoons HbNHCOOH \rightleftharpoons HbNHCOO^- + H^+$$

The reaction as shown above is that of CO_2 and the α-amino ending of the amino acid groups of hemoglobin. This reaction is analygous to the reactions studied by Faurholt

(6). He found that CO_2 reacts rapidly and without the need of a catalyst with the $-NH_2$ functional group but not with the $-NH_3$ group. He also found that only molecular CO_2 and none of the other equilibrium products of CO_2 hydration would react with the amino group. This means that when simple α-amino acids are used as models of hemoglobin for studying the carbamate reaction, the reaction being studied will be between molecular CO_2, and either the conjugate base or the neutral form of the amino acid.

Using glycine as an example, the follwing equilibria are present in aqueous solution:

$$\underset{\text{conjugate acid}}{NH_3CH_2CO_2H} \overset{-H^+}{\underset{+H^+}{\rightleftharpoons}} \underset{\text{neutral form}}{NH_2CH_2CO_2H} \rightleftharpoons \underset{\text{zwitterion}}{NH_3CH_2CO_2^-} \overset{-H^+}{\underset{+H^+}{\rightleftharpoons}} \underset{\text{conjugate acid}}{NH_2CH_2CO_2^-}$$

The dipolar ion is commonly called the "Inner Salt" or "zwitterion". The pK_A between the conjugate acid and the zwitterion-neutral form equilibrium is 2.3, and the pK_A between the zwitterion-neutral form equilibrium and the conjugate base, is 9.6. This would indicate that amino acid ends of hemoglobin in physiological media may be in equilibrium between the zwitterion and neutral forms. Therefore, the carbamate formation reaction that takes place in the body probably involves molecular CO_2 and the neutral form of the substrate (7).

To understand the role of carbamates in the physiological process, it would be useful to be able to quantitatively analyze their presense. Ferguson and Roughton (5) developed a quantitative analysis in which a carbamate was measured by precipitating the uncombined CO_2 which remained after equilibrium had been established between the amino acid and its carbamic acid. This analysis was dependent on the facts that "Barium salts of carbamates are soluble and reasonably stable at 0^oC and at very alkaline pH, whereas under these same conditions, CO_2, H_2CO_3 and HCO_3^- are all converted to $CO_3^=$ and are precipitated as $BaCO_3$ (5)." By knowing the pCO_2, they claimed that the amount of uncombined CO_2 could be measured.

Roughton and Rossi-Bernardi (8) used a pH and pCO_2 electrode system to measure the uptake of CO_2 by an amino acid. The basis for this system was that the CO_2 and HCO_3^- concentrations can be calculated from the pCO_2 and pH measurements using the Henderson-Hasselbalch equation:

$$pH = pk' \log\frac{(\text{bound } CO_2)}{(\text{free } CO_2)}$$

To calculate the amount of carbamate formed, one supposedly only needed to know the initial pCO_2.

In 1968, Bruce Cassidy (9) attempted to develope an ultraviolet spectroscopic method of quantitative determin-

ation of a carbamate, and a year later G. John Tiberio (10) attempted to develope a fluorescent method of determination. The material in this paper is a development from these two initial efforts towards a direct measurement of carbamates.

PROPOSED GOALS AND METHODOLOGY

The method of Ferguson and Roughton (15) assumes a maximum yield of carbamate at pH's of 12 or higher which would favor the equilibrium concentration of the conjugate base. However, Giustina and Temelcou (11) state that the amount of carbamate bound to glycylglycine (diglycine) at pH 12 is zero. They find a maximum yield of carbamate at pH's between 8 and 9 which would suggest a reaction of CO_2 and the neutral form of the zwitterion-neutral form equilibrium. When barium hydroxide is added to the reaction equilibrium to precipitate the $CO_3^=$, the rise in pH would be enough to destroy any carbamate present. Also, this method would perturb physiological reaction conditions which would be a hindrence in application of direct physiological studies, irrespective of its quantitative exactness.

The pH-pCO_2 method also has some drawbacks. The electrode system has an inherent "lag" time which forces the use of upwards of 100ml. of solution for each measurement. This amount of solution may be acceptable for a glycine or diglycine system but not so acceptable if a blood carbamate analysis was desired.

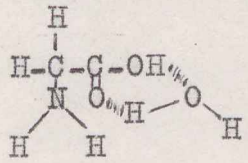
Seemingly, the most desireable method of measurement of this system would be one where the reaction solution

could be measured quickly, directly, and accurately. A spectroscopic approach would fit these criterion if some form of spectroscopy could be shown applicable to the problem.

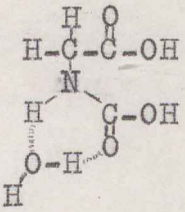
Cassidy (9) hoped to apply ultraviolet absorption to measure a carbamate. He observed an increased absorbance of diglycine over that of glycine and attributed this difference to an n,π^* transition of the $-\overset{O}{\overset{\|}{C}}-NH-$ group which glycine itself does not have. This same group is also present on a carbamate but when he tried to observe a change in the spectra of glycine or diglycine when the solutions are bubbled with CO_2, no definitive absorption difference could be obtained. He did, however, firmly establish a pH dependency of the absorption of glycine and diglycine.

Didier Betrand (12) reported that glycine and similar compounds exhibited fluorescent spectra in the 400-700nm. region. Instrumentally, this is a much better working region than the 200-250nm. range which Cassidy worked with using absorption. Tiberio (10) hoped to make use of this advantage by trying to observe a difference of fluorescence intensity between an amino acid and its carbamic acid in aqueous systems. He found that the observable difference between glycine and a glycine solution bubbled with CO_2 was not negligable and that the reaction might be measured

quantitatively by this method. His proposal for the cause of fluorescence were ring structures such as:



and



ring structure for glycine

ring structure for glycine carbamate(10)

This research began with the following goals: 1) the direct measurement of a carbamate using fluorescence spectroscopy and 2) the determination of the causes of its fluorescence. Glycine will be used as a model instead of hemoglobin for measuring the addition of CO_2 in carbamate formation initially. Hopefully other amino acids could be studied once the procedure is established. A wide variety of compounds and solvents are employed in order to isolate and identify the causes of fluorescence in amino acids and carbamates.

EXPERIMENTAL APPARATUS

All emission and excitation spectra were taken on the Hitachi-Perkin Elmer MPF-2A Recording Spectrofluorimeter. This instrument is not equipped to record spectra which are totally corrected for non-linear responses of the light source, the monochrometer, the phototube, or the amplifier. No attempt was made for correction as it was not necessary for this work. The accessories used for this instrument were those specifically designed by Hitachi Ltd., Tokyo, Japan and sold commercially. Included were the standard fluorescence equipment (one cm. cells), a thermostated and controlled atmosphere sample holder, a solid sample holder, and phosphorescence equipment.

All absorption spectra were obtained on a Cary 14 Recording Spectrophotomer. In this particular instrument, the baseline was totally horizontal at wavelengths above 250nm. Therefore, no corrections were necessary as all interested absorption measurements were made at greater than 250nm.

The pH measurements were made with a Photovolt Model 110 Electronic Photovoltmeter using a single combination glass-internal reference electrode.

The nitrogen used to degas samples was Matheson re-

search grade dry nitrogen and the carbon dioxide was obtained from sublimation of dry ice. Both these gases were purified as explained in the text. The liquid nitrogen used for phosphorometric measurements was found to be spectrally pure and therefore no attempt was made at purification.

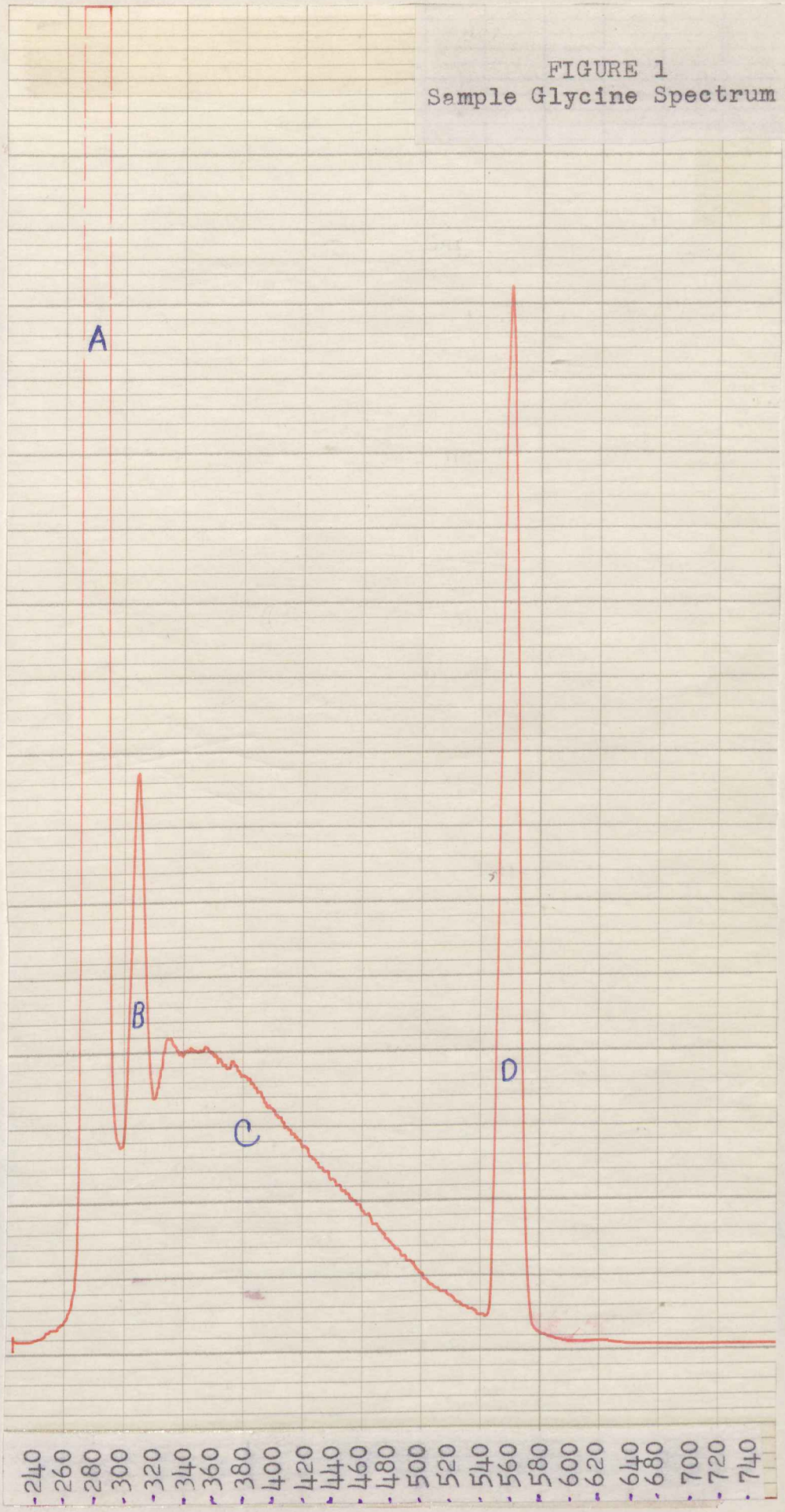
All amino acids and N-carboxyglycine disodium were obtained from Nutritional Biochemicals Corp. except for DL α-aminophenylacetic acid which was obtained from Aldrich Chemical Co., Inc. The ethyl carbamate (urethan) was obtained from Matheson Coleman and Bell. All chemicals were used without purification unless otherwise stated.

FLUORESCENCE: Experimental

To establish the fluorescence of glycine using the Hitachi instrument, a 1M aqueous solution of glycine was prepared using 'house' distilled water without attempting any purification and without degassing solutions. A similar spectrum was obtained to that shown in Figure 1, p.12. Region A is the excitation wavelength being scattered by the sample, region B is the water Raman line, region C is considered the glycine emission and region D is the excitation overtone. Regions A and D could have easily been filtered out but it was felt that any artificial tampering with the spectra might inhibit the developement of an analysis. Therefore, this spectrum and all following spectra were taken without filters or any other type of light impedence of perturbation.

Most distilled water is allowed to sit in a reserve tank for a length of time which allows it to dissolve some gases from the air. One of these gases is carbon dioxide. This is the primary reason why 'house' distilled water commonly has a pH well below 7. If glycine did not fluorsce and its carbamic acid did, Region C in Fig. 1. might actually be indicative of the carbamate formed when glycine was origionally put into solution. This would make a car-

FIGURE 1
Sample Glycine Spectrum



bamate analysis procedure quite simple (CO_2 is not a fluorescent compound in water (13)).

In order to make the glycine solutions with CO_2 free water and in a CO_2 free atmosphere, the following apparatus was devised:

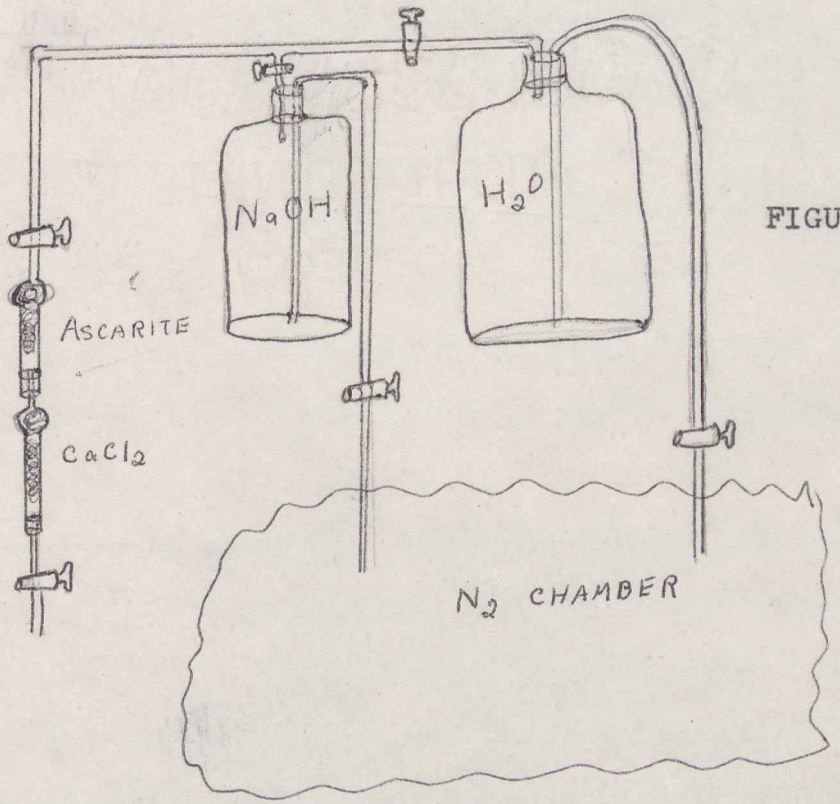


FIGURE 1D

To avoid all impurities in the apparatus, all tubes are glass with Tygon tubing as connecters only when necessary, and definately not where it might come in contact with liquid. All stopcocks are teflon (stopcock grease fluoresces, eliminating ground glass), and all other apparatus was rinsed extensively with distilled water after washing.

page 14 missing due to typographical error

For testing purposes, a 1M glycine solution was prepared and a fluorescence spectrum obtained as follows. The entire system was purged with N_2 and distilled water was boiled vigorously for 30 minutes to remove all dissolved gases. This water was pulled into its resevoir through tube A and was allowed to cool in the CO_2 free atmosphere. Its pH was monitered at 6,98. A 4M NaOH solution was then boiled similarly for 30 minutes and pulled into its receptacle through tube B. It was also allowed to cool in the CO_2 free atmosphere. One tenth mole of glycine was put into the glove bag which was again purged with N_2. Then 100ml. of a 1M solution of glycine was mixed in the nitrogen atmosphere and brought to pH 7.2.

Before it was possible to take a fluorescence spectra of the solution, it was necessary to be sure no appreceable amount of CO_2 would diffuse back into the solution as the spectrum was being run. To do this, some of the pH 6.98 water was brought back into the atmosphere and the pH was constantly monitered. In 30 minutes no measurable change could be seen.

The CO_2 free glycine solution was measured for fluorescence and a spectrum which was within experimentaly error of the first spectrum obtained was realized. However, the water R_{aman} and excitation harmonic were noticably lower in

intensity.

A Beer's Law concentration curve was then obtained in order to find the fluorescent working region for glycine. Four solutions of glycine and water were made at concentrations of 0.5, 1.0, 1.5, and 2.0M with no attempt to control pH. The 0.5M and 1.0M solutions showed a linear increase in peak height and were substantiated by solutions of 0.25M and 0.75M as linear up to 1.0M. The 1.5M and 2.0M solutions leveled off in height but increased greatly in width. (see Fig. 2, p.17)

As a precaution, solutions of 1M glycine at various pH's were measured by fluorescence from pH 5.5 (untampered glycine solution) to pH 11.0. The maximum response was found to be between pH's 7.1 and 7.4 (see Fig. 2, p.18).

Carbon dioxide addition studies were run with solutions again of 0.5, 1.0, 1.5, and 2.0M glycine. Their fluorescence intensities were measured at pH 7.2. After the spectra were taken, the samples were saturated with CO_2 by simply bubbling the gas through the solution for 15 minutes. The pH's of the CO_2 saturated solutions were monitered until they came to a minimum. This took about 10 minutes with vigorous bubbling. The fluorescence spectra of these solutions were then obtained after returning the solutions to pH 7.2. With CO_2 addition, region C (refer to Fig. 1)

Figure 2
Fluorescence Concentration Sketch of Glycine

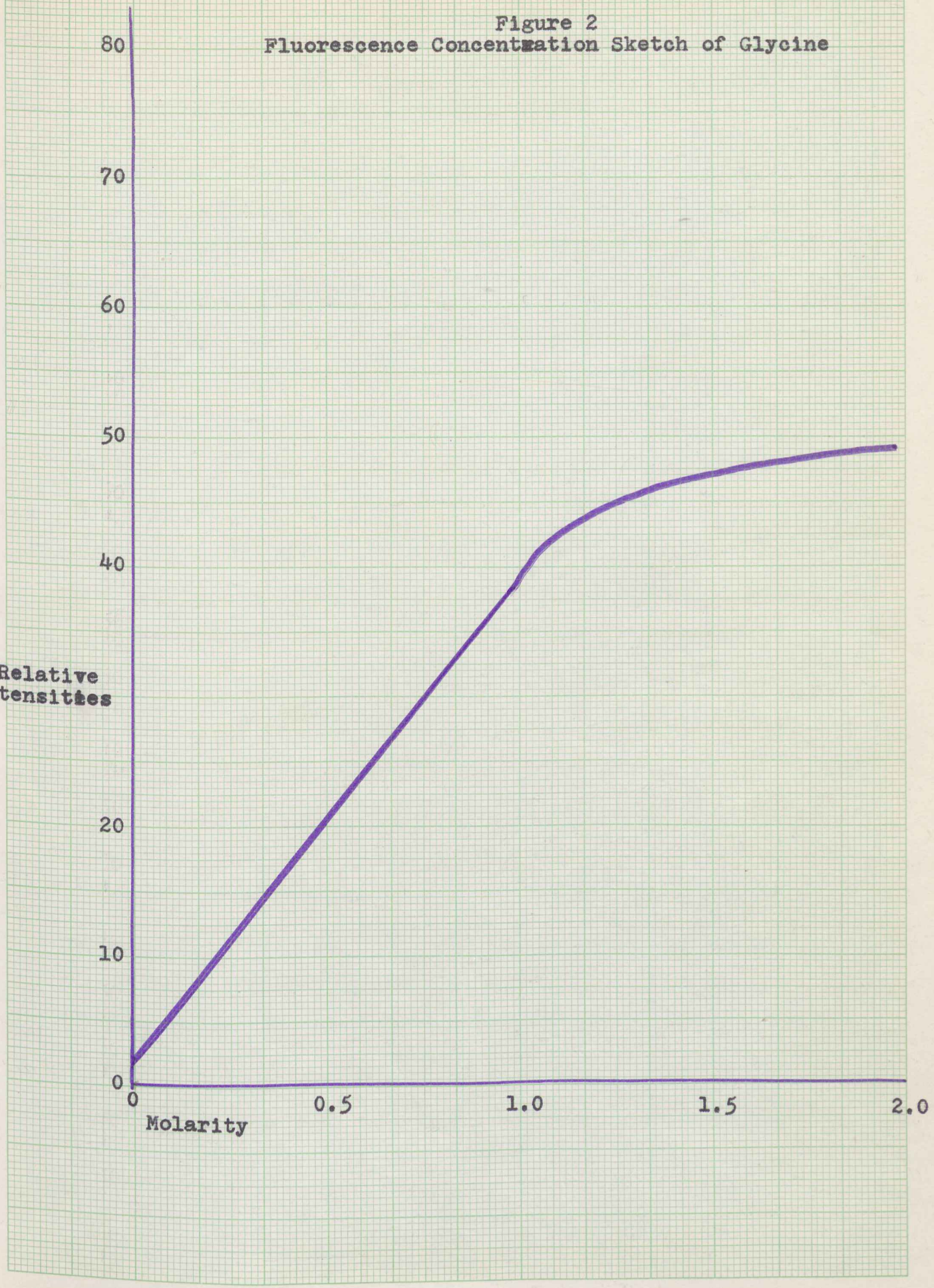
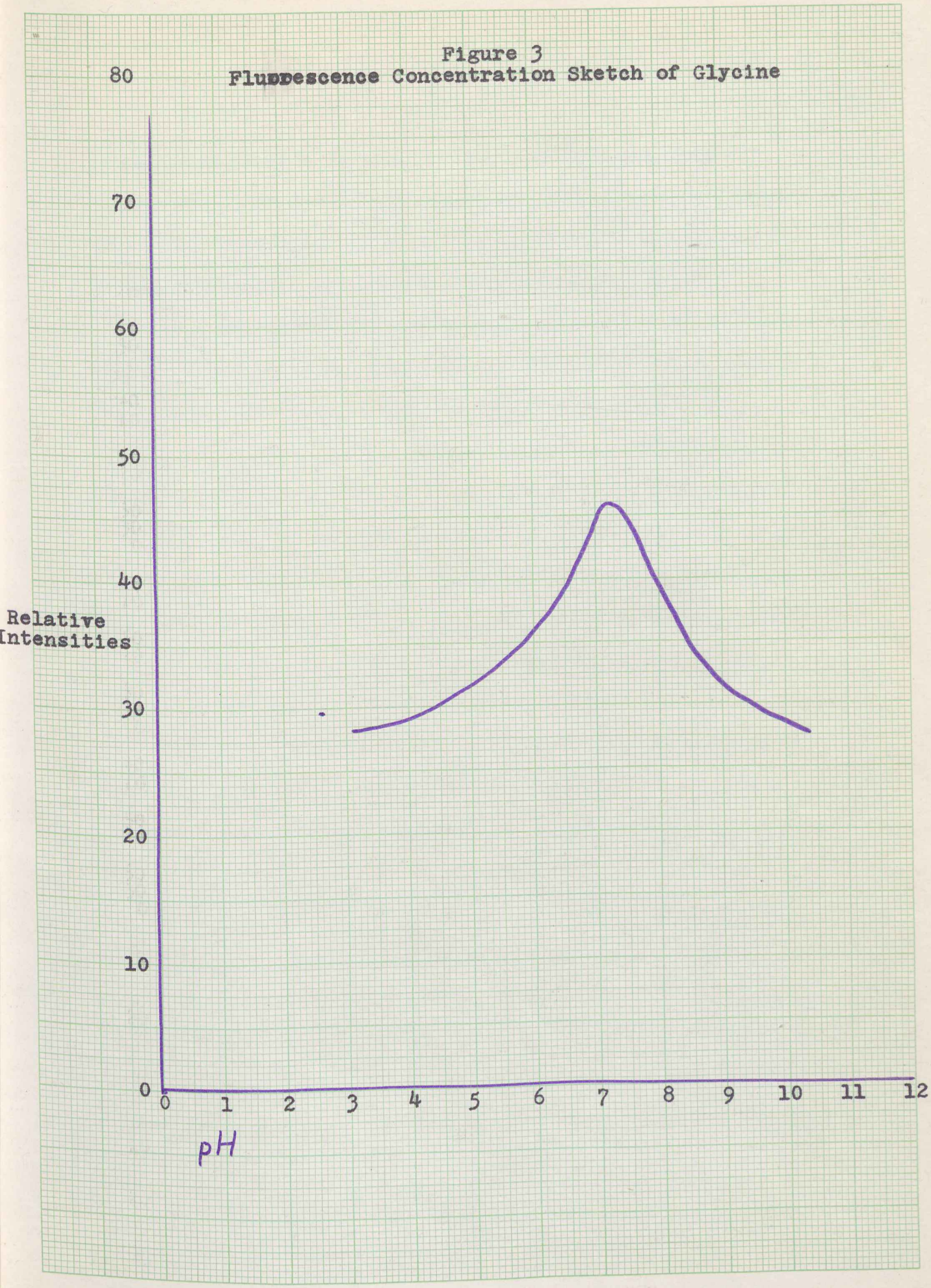


Figure 3
Fluorescence Concentration Sketch of Glycine



ares to the Inch

showed a definate increase. The increase was over the entire width of the region which therefore required differential plotting. These differences were, however, so erratic that they were not at all quantitative.

The compound N,N-dimethylglycine was used as a control for the CO_2 addition reaction. The N-substituted methyl groups should prohibit the addition of CO_2 completely. Solutions of 0.2, 0.1, 0.75 and 0.50M were used as this compound tended to fluoresce quite well, even after purification. Spectra were obtained for each solution after returning them to pH 7.2. Then CO_2 was added as before and the fluorescence intensities of these solutions were measured at pH 7.2. Absolutely no difference was seen after CO_2 addition.

Three amino acids with unsaturated six-membered rings were then analysed by fluorescence. The idea of adding CO_2 to α-phenylglycine, N-phenylglycine, and α-phenylalanine was dropped quickly when the N-phenylglycine began to polymerize and the other two were too insoluble in water to be quantitative.

The change of fluorescence and/or reaction of glycine and CO_2 with temperature was next investigated. The usual four solutions of 2.0, 1.5, 1.0 and 0.5M were made with their pH's at 7.2. Fluorescence spectra of these sol-

utions

utions at 10° intervals from 20° to 50°C were obtained. The same temperatures were used when fluorescence spectra of these solutions saturated with CO_2 were obtained. Both before and after CO_2 was added, the spectral intensity of the solutions decreased with increasing temperature. Ability to notice a difference in the spectra after CO_2 addition also diminished with increased temperature.

Finally, CO_2 was monitered fluorescently as it saturated CO_2 free water and as predicted by the literature, no change was seen in any part of the spectrum.

Discussion

The overwhelming problem in all of the previous experiments has been their inconsistancy. When it became apparent that these inconsistancies were not because of technique problems, other sources of error were questioned. Water which was freshly boiled was fluorescently much cleaner than water which had been held for two or three days. Saturated sulfuric acid-chromic acid cleaning solution was substituted for soap. The CO_2 was bubbled through concentrated nitric acid and then twice through water to insure its purity. However, even after every precaution had been taken, the intensity of glycine alone or that of a solution of glycine saturated with CO_2 was still slightly erratic. Quantitative work using this mode of measurement seems limited.

Information was gained, however, from these experiments. The equilibrium amount of CO_2 was found not to be prohibitive. All fluorescent intensities of glycine were shown to be linear with concentration and have a maximum in the pH range 7.1 to 7.5 which, conveniently, is physiological pH. There is definate CO_2 uptake and the carbamate formation is real as shown by the control reaction. The lowering of fluorescent intensity with increased temp-

erature is expected because of the increased rate of excitated state relaxation through internal conversion. The relative disappearance of the carbamate increase would be expected to take place with increased temperature if there was not a large increase in the amount of carbamate formed. This seems to be the trend which the results followed although the experiment is far from definitive.

THE CAUSES OF FLUORESCENCE:

Experimental

Ethanol, dioxane, acetone, methanol, tetrahydrofufuryl alcohol, n-methylpyrrolidone, 2,2,4-trimethylpentane, and water were purified. Ethanol-water mixtures were investigated as solvent possibilities in order to observe the fluorescent properties of glycine. The small amount of glycine which would dissolve in the mixtures of higher ethanol content prohibited the determination of any trend of fluorescence vs. the amount of water present. An attempt at dissolving enough glycine in any of the above solvents (except water), or any combination thereof, proved unsuccessful.

If water is necessary for glycine to fluoresce, and these interactions were removed completely, it should, therefore, not exhibit fluorescence. To test this, solid samples of glucine, DL asparagine monohydrate, DL aspartic acid, DLα-alanine, β-alanine, N,N-dimethyl glycine HCl and its recrystalized base, ethyl carbamate, DL glutamic acid, glycine ethyl ester, glycine butyl ester, glycine methyl ester, glyclglycine, DL leucine, DLα-phenylglycine, N-phenylglycine and DL phenylalanine were analysed by fluorescence. The last three solids showed a fluorescence

characteristic of their benzene structures. All of the other compounds gave no peaks except the excitation peak and its harmonic. Figures 4 and5 on pages 25 and 26 are examples of these spectra.

In order to remove some of the possible sights of hydrogen bonding, one molar solutions of the methyl, ethyl, and butyl esters of glycine were analysed. The spectra of these compounds were only slightly different from a glycine spectrum.

The pH dependency of fluorescence was investigated by experimenting with ethyl carbamate. Ethyl carbamate has a fluorescence maximum at 325nm. when excited at 280nm. The height of this peak was seen to be linear below three molar. A 0.5M solution was then fluorescently measured at pH's rangeing from 2.0 to 11.5. All of the resulting spectra were exactly the same, i.e. no pH dependency could be seen. A sample spectrum is shown on page 27.

Finally, the following compounds were compaired spectrally in hope of determining the common fluorescent structure in glycine;(p.28). All possible parts of glycine are represented along with their possible combinations. A close look at the above list would seem to indicate that only those compounds with a carbonyl present exhibit fluorescence.

Figure 4
Solid α-phenylglycine Spectrum
Sample Sensitivity-5
Excitation Wavelength-280nm.
Excitation Slit Band Width-1.25nm.
Emission Slit Band Width-1.25nm.
Emission Scan

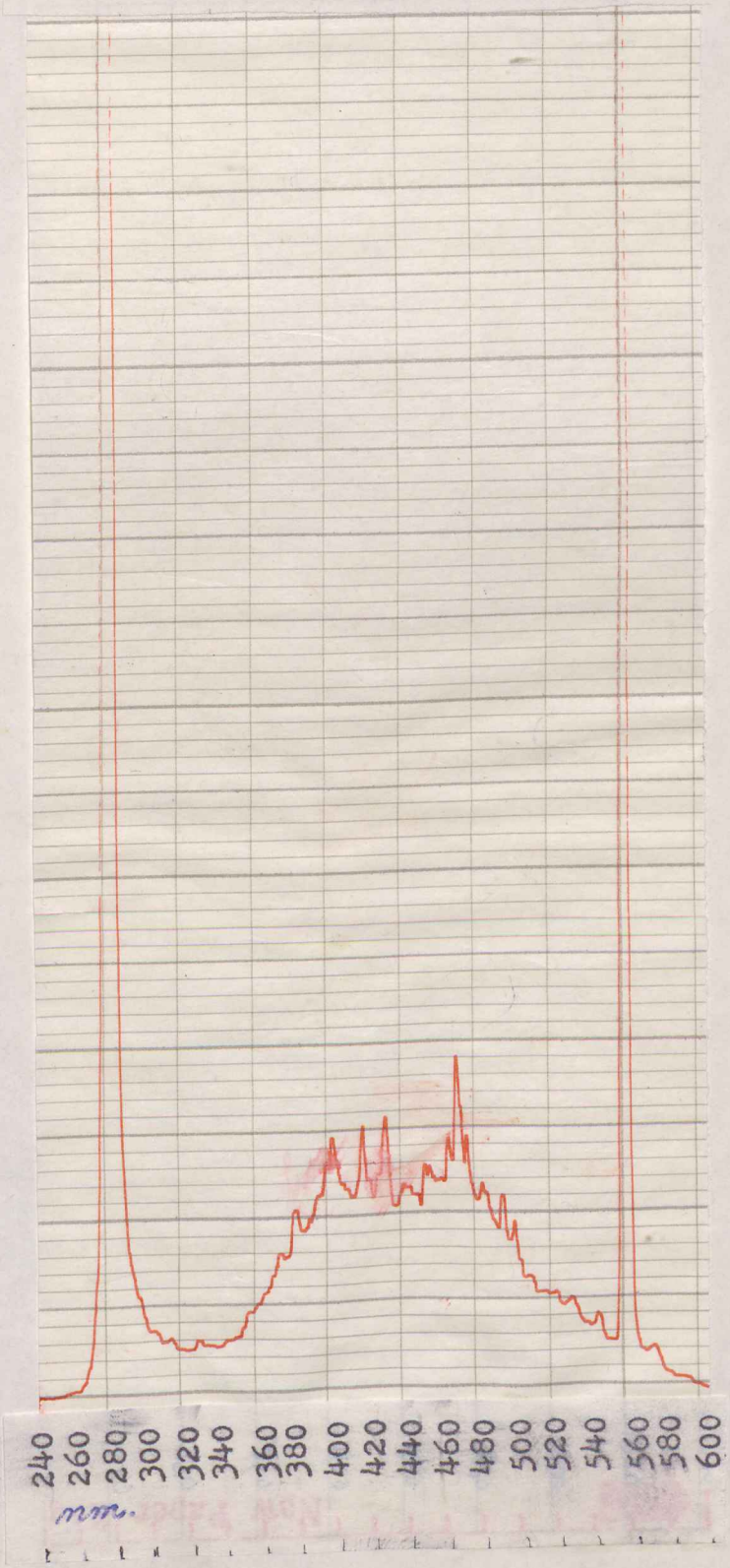


Figure 5
Solid Glycine Spectrum
Sample Sensitivity-6
Excitation Wavelength-280nm.
Excitation Slit Band Width-2.0nm.
Emission Slit Band Width-2.0nm.
Emission Scan

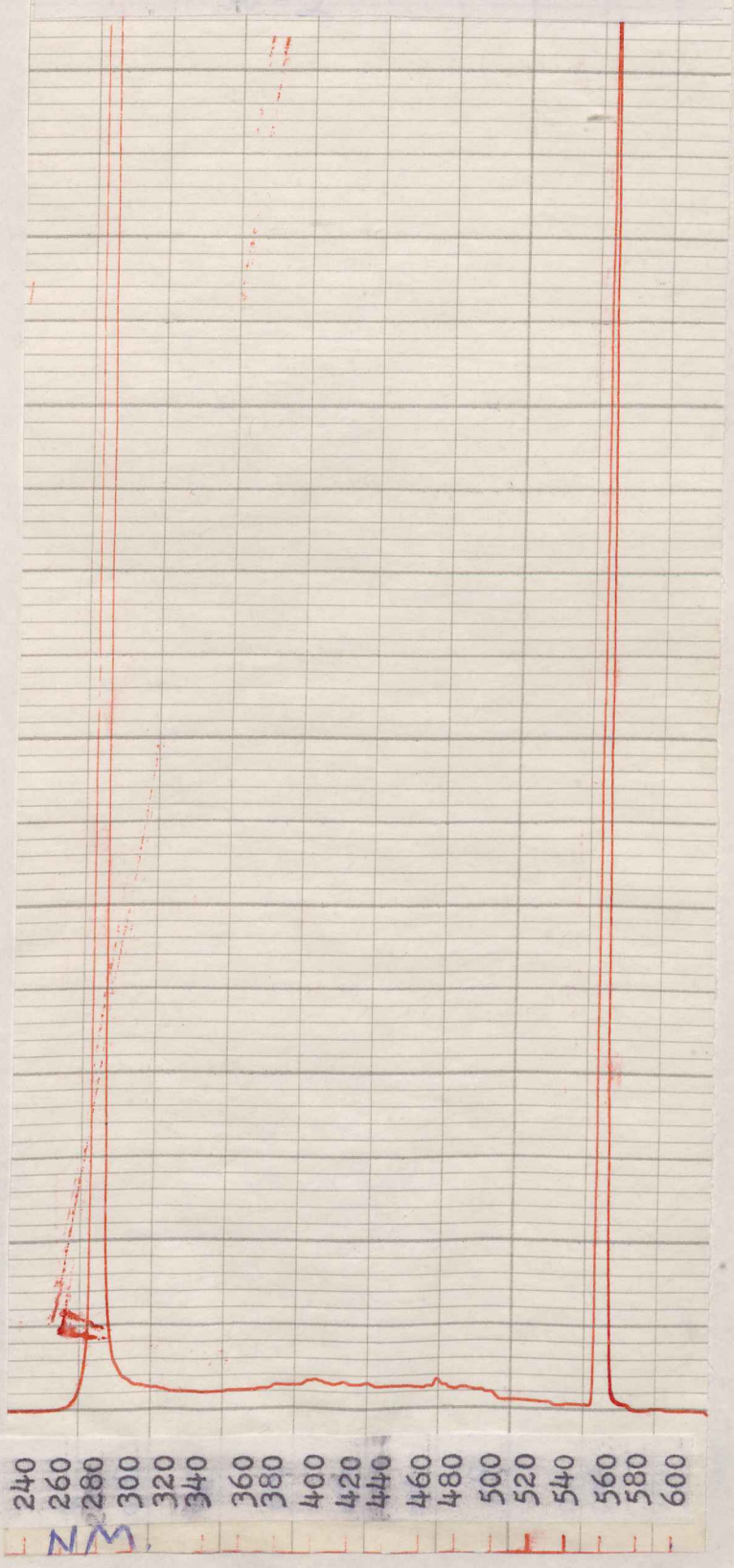


Figure 6
Sample Ethyl Carbamate Spectrum
Sample Sensitivity-5
Excitation Wavelength-280nm.
Excitation Slit Band Width-6.0nm.
Emission Slit Band Width-6.0nm.
Emission Scan
0.5 Molar

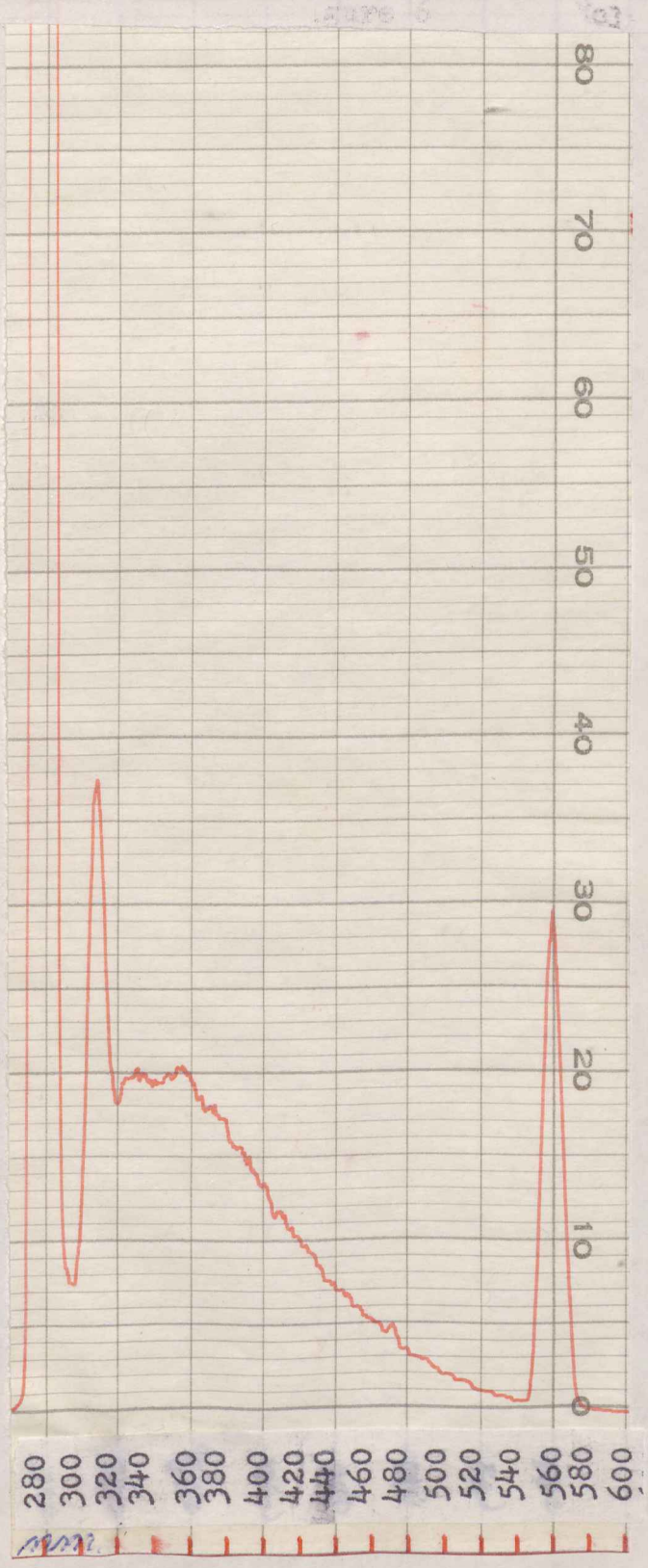
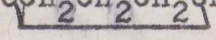
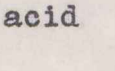
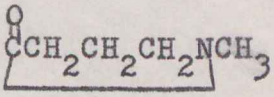
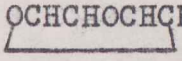


FIGURE 7

Compounds for Interpretation of Glycine Fluorescence

Compound	Structural Formula	Fluorescence
Methanol	CH_3OH	NO
Ethanol	CH_3CH_2OH	NO
Tetrahydrofufuryl Alc.		NO
Acetic acid		YES
Propionic acid		YES
Glycine		YES
N-methylpyrrolidone		YES
Acetone		YES
Dioxane		YES*
2,2,4-trimethylpentane	$CH_3C(CH_3)_2CH_2CH(CH_3)CH_3$	NO
2-aminopropionol	$CH_3CH(NH_2)CH_2OH$	NO
2-propylamine	$CH_3CH(NH_2)CH_3$	NO

*This fluorescence spectrum is not at all similar to glycine.

Discussion

As a help to the reader, a brief backround of the theory of the fluorescence phenomina will be presented.

Fluorescence is one of the pathways by which energy that has been absorbed by a molecule may be re-emitted. The following diagram should be helpful:

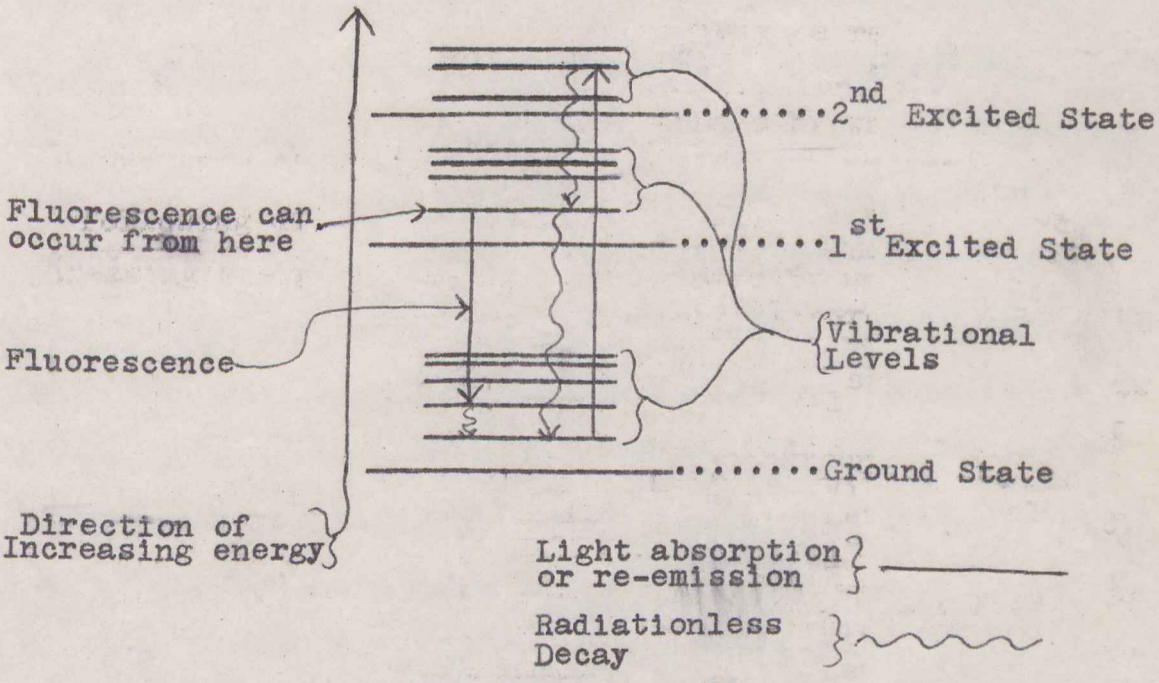


Figure 8. Fluorescence

Because of the energy differences involved, all but a very, very few molecules are in the lowest vibrational energy level of the ground state under normal circumstances. When a molecule is 'hit' with a certain quant-

ized amount of energy (only light energy will be used in this research although any energy source strong enough to induce an excitation may be used) an electron can be promoted to a higher energy state called an excited state. This electron may actually be promoted to any vibrational level of any excited electronic state.

The energy levels of the excited electronic states are usually close enough that low lying vibrational levels of two different electronic states can overlap in what is called coupling. This coupling allows for a very efficient non-radiative decay, called internal conversion, of the electron all the way down to the lowest vibrational level of the first excited state. At this point the coupling effect is usually less efficient because of the increased energy difference of the first excited and ground states, and flourescence therefore has a chance to compete with the radiationless decay processes.

Now, instead of all the energy being given off to solvent in the form of heat or kinetic energy, some of this energy is emitted as a quantum of light when the electron decays to the ground state. The re-emitted light will show a positive Stokes shift because the re-emmited light will be at lower energy. Two reasons for this effect

are 1)the non-radiative loss of energy when the molecule decays to the lowest excited state from a higher one and 2)the Frank-Congdon principle. Also, since the intensity of the fluorescence is (everything else being a constant) proportional to the absorption of the species, the larger the molar absorptivity of a transition, the more favorable will be the fluorescence. Therefore, most fluorescence occurs by the decay of a π,π^* transition and would indicate the presence of double bonds or an unsaturated ring system.

As shown in Fig. 1., the spectrum of glycine in water is quite complicated. Also, it was felt that glycine was possibly fluorescing only because water was hedrogen bonding to the molecule in solution. Being able to work with glycine spectra taken in solvents other than water would be a help to both problems. It was for this reason that non-aqueous solvents were investigated for spectroscopic use. Unfortunately, these solvents either had fluorescent structures themselves, or glycine was not appreciably soluble in them.

When no fluorescence was observed in the solid samples of the saturated amino acids, hydrogen bonded water seemed to be a possible answer for glycine's fluorescence. However, no change was seen between the glycine

spectra in water and those of the esters. Also, no pH dependency was observed for ethyl carbamate as was reported by Tiberio (10).

These anomalies led to comparing the flourescence of small saturated hydrocarbons with the functional groups of glycine attatched to them in all possible combinations in order to hopefully isolate the flourescing structure. The fact that only carbonyl compounds were flourescing in the same manner as glycine, coupled with the other results given above, prompted the possible use of phosphorescence as a tool in determining the flourescing chromophore.

PHOSPHORESCENCE OF CARBONYL COMPOUNDS:

Experimental

Initially, the phosphorescence cells (which will be refered to as p-cuvettes) and the low temperature Dewar fladk were shown to be clean of any phosphorescent material at 77°K which is the temperature at which all samples will be run. Also pure water was analysed by phosphorescence and revealed only the slightest amount of response at the most sensitive settings of the instrument.

A 1M solution of β-alanine was then prepared and was not degassed. The sample was analysed at room temperature and gave no response. The sample was then lowered to 77°K and excited with light at 280nm. producing a re-emission at 410nm. An excitation spectrum of the peak at 410nm. was taken and the maximum excitation was shown to be at 280nm. The maximum signal occured with a chopper speed of 6000RPM.

This peak at 410nm. was then monitered as the sample was allowed to warm up to room temperature. During this warm-up period, a phosphorescence 'flash' was observed. A solid sample of β-alanine was then excitated with light at 280nm. and a spectrum similar to the spectrum of the solution was obtained.

A 1M solution of glycine (all samples are degassed) and a solid sample of this compound were analysed by phosphorescence and both samples produced a spectrum which re-emitted at 430nm. when excited at 280nm. The disodium salt of glycine's carbamic acid was analysed and revealed an emission peak at 450nm. when excitated at 280nm. and a peak at 490nm. when excited at 325nm. Both excitation peaks were observed in the excitation spectrum of either emission peak.

For a negative test, 2,2,4-trimethylpentane was analysed for phosphorescence and a small response was obtained which decreased upon continued purification. Hexane was analysed following the same procedures and produced similar results.

Correlary data was obtained by analysing the compounds which Jaffe (14) gives in his chart of selected carbonyl compounds and the n,π^* absorptions. Phosphorescence and excitation spectra were obtained and are presented on pages 35-40. Also, a reproduction of Jaffe's table is presented on page 41 extended to include the above phosphorescence values.

Figure 9
Acetaldelyde
Solvent--Ethanol
Excitation Slit Band Width-10nm.
Emission Slit Band Width-10nm.
Chopper Speed-6,000 RPM
77°K
Sample Sensitivity-6

Emission Spectrum
Excitation Wavelength-303nm.
Emission Scan
Wavelength Maximum-(477±1)nm.

Excitation Spectrum
Emission Wavelength-477nm.
Excitation Scan
Wavelength Maximum-(304±1)nm.

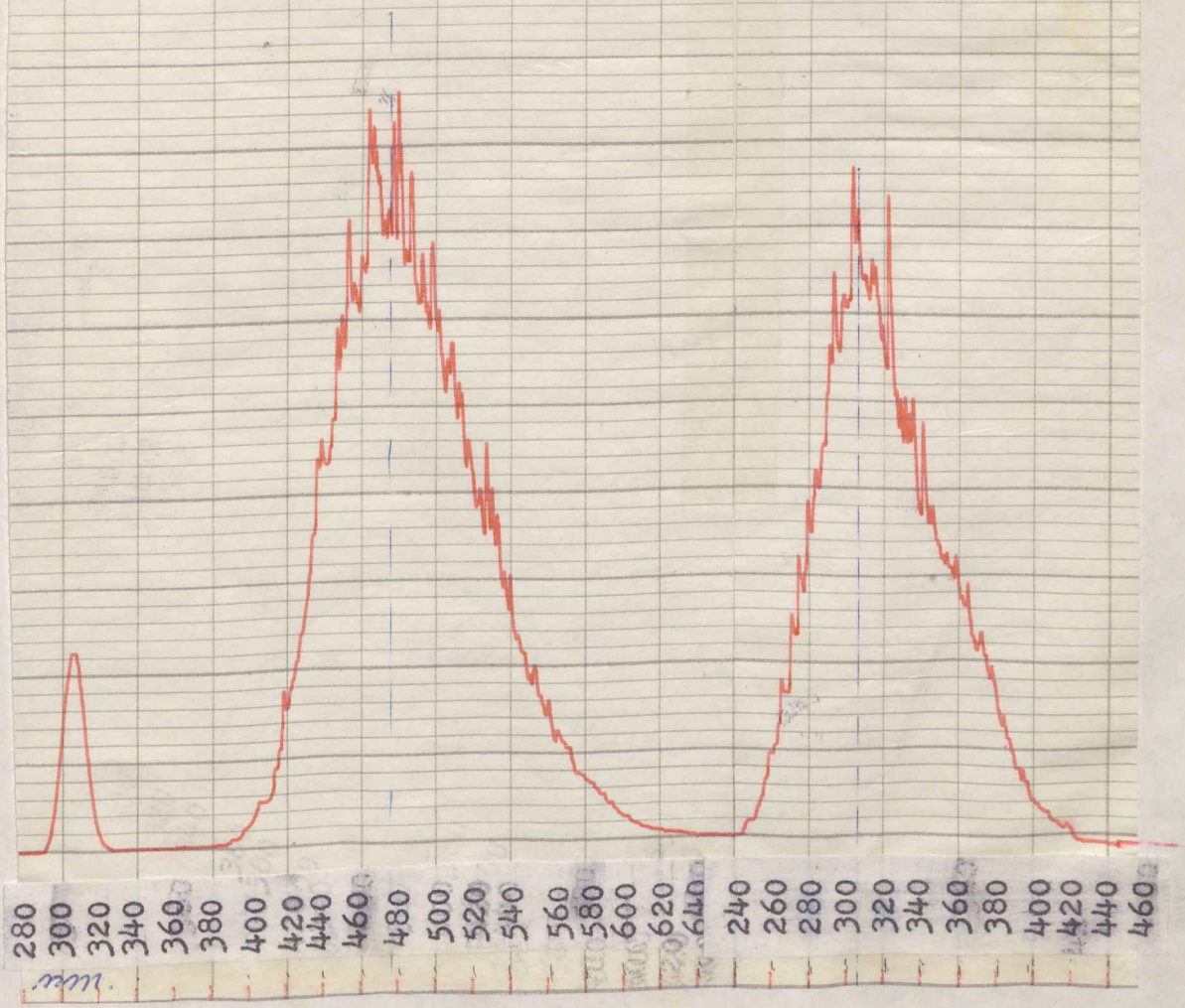


Figure 10
Acetic Acid
Solvent--Ethanol
Sample Sensitivity-6
Excitation Slit Band Width-10.0nm.
Emission Slit Band Width-10.0nm.
Chopper Speed-6,000 RPM
77°K

Emission Spectrum
Excitation Wavelength-280nm.
Emission Scan
Wavelength Maximum-(444±1)nm.

Excitation Spectrum
Emission Wavelength-444nm.
Excitation Scan
Wavelength Maximum-(287±1nm.

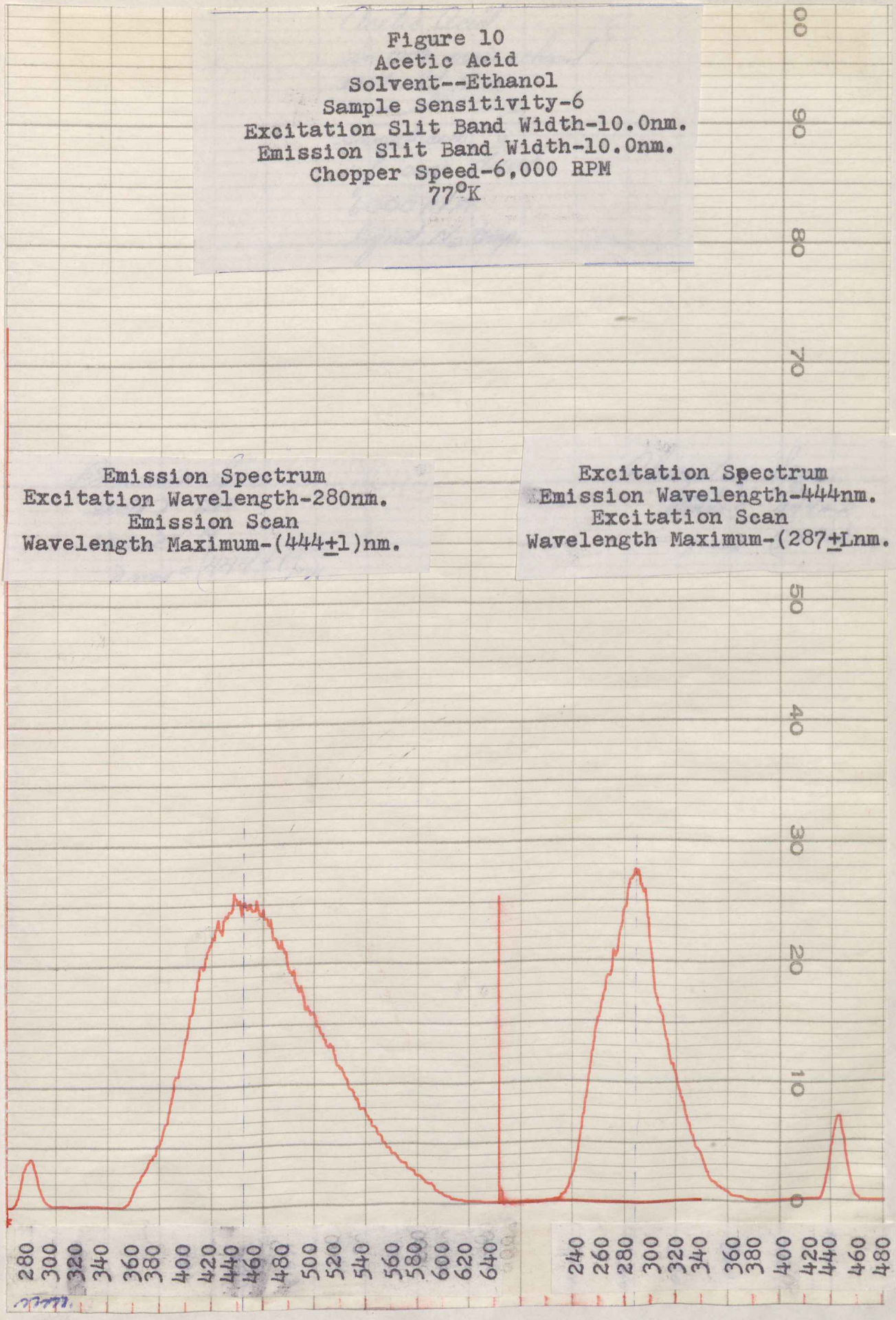


Figure 11
Acetamide
Solvent--Water
Sample Sensitivity-6
Excitation Slit Band Width-16.0nm.
Emission Slit Band Width-16.0nm.
Chopper Speed-6,000 RPM
77°K

Emission Spectrum
Excitation Wavelength-280nm.
Emission Scan
Wavelength Maximum-(437±1)nm.

Excitation Spectrum
Emission Wavelength-(437)nm.
Excitation Scan
Wavelength Maximum-(279±1)nm.

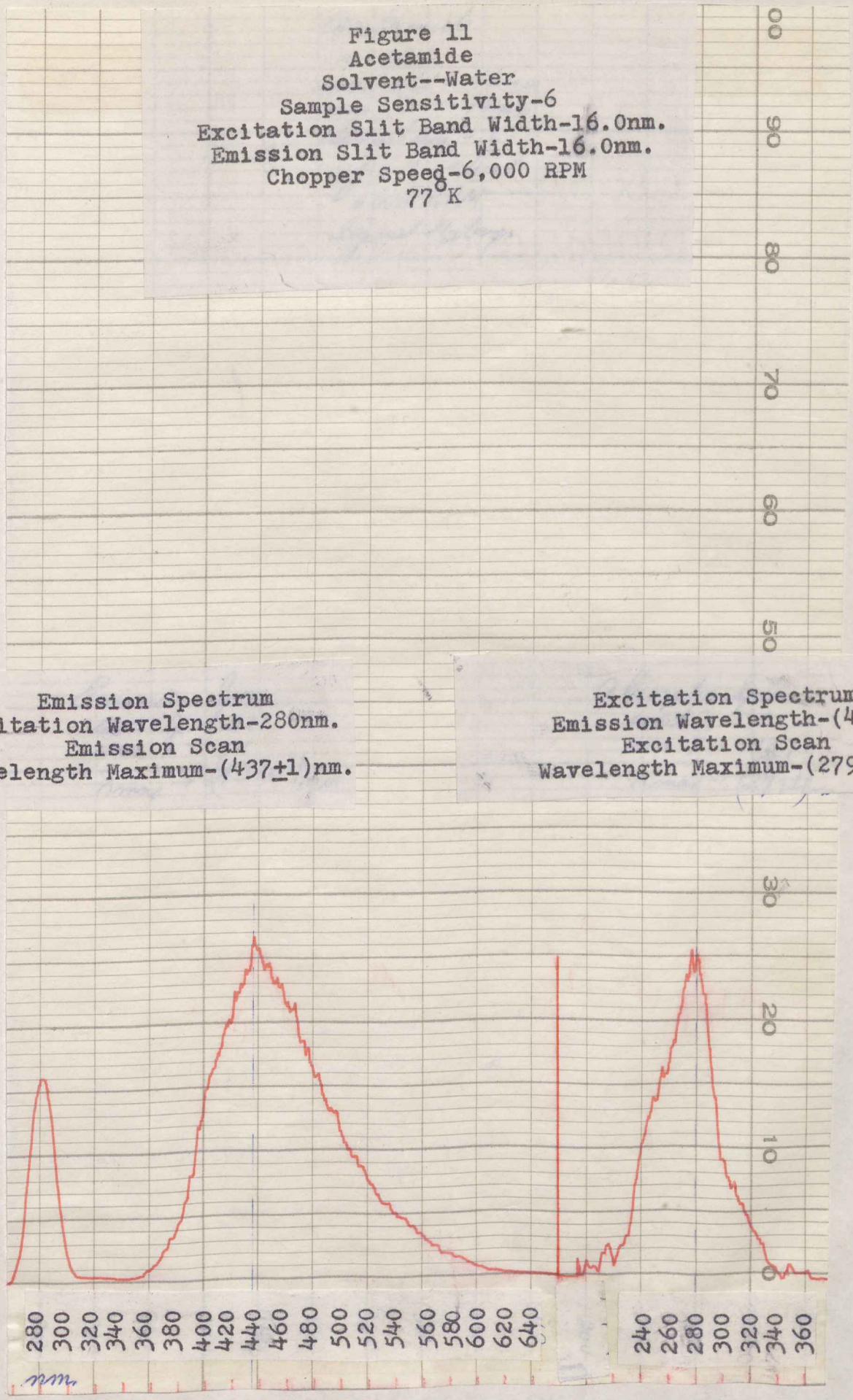


Figure 12
Ethyl Acetate
Neat
Sample Sensitivity-6
Excitation Slit Band Width-16.0nm.
Emission Slit Band Width-16.0nm.
Chopper Speed-6,000RPM
77°K

Emission Spectrum
Excitation Wavelength-291nm.
Emission Scan
Wavelength Maximum-(437±)nm.

Excitation Spectrum
Emission Wavelength-437nm.
Excitation Scan
Wavelength Maximum-(295±1)nm.

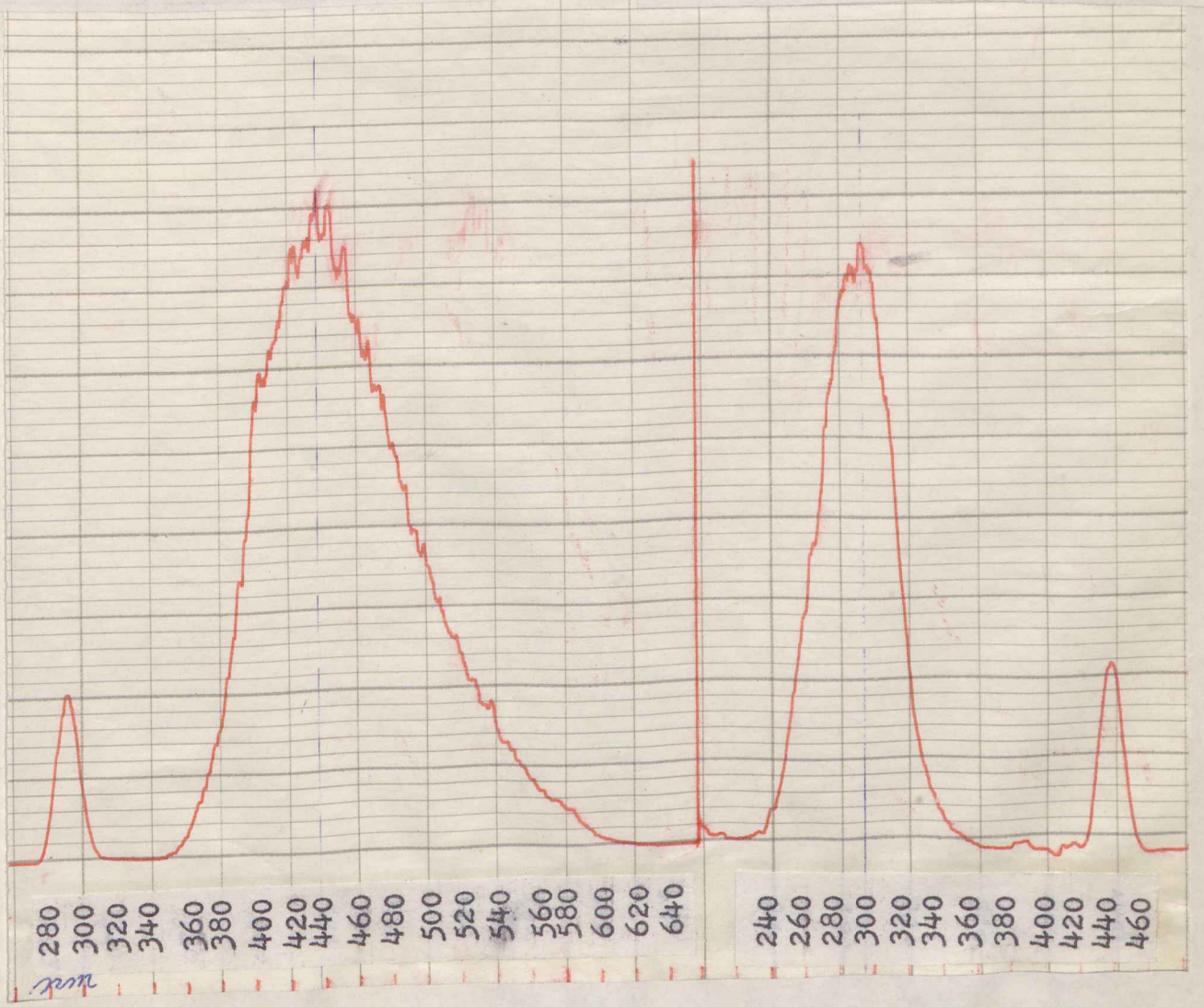
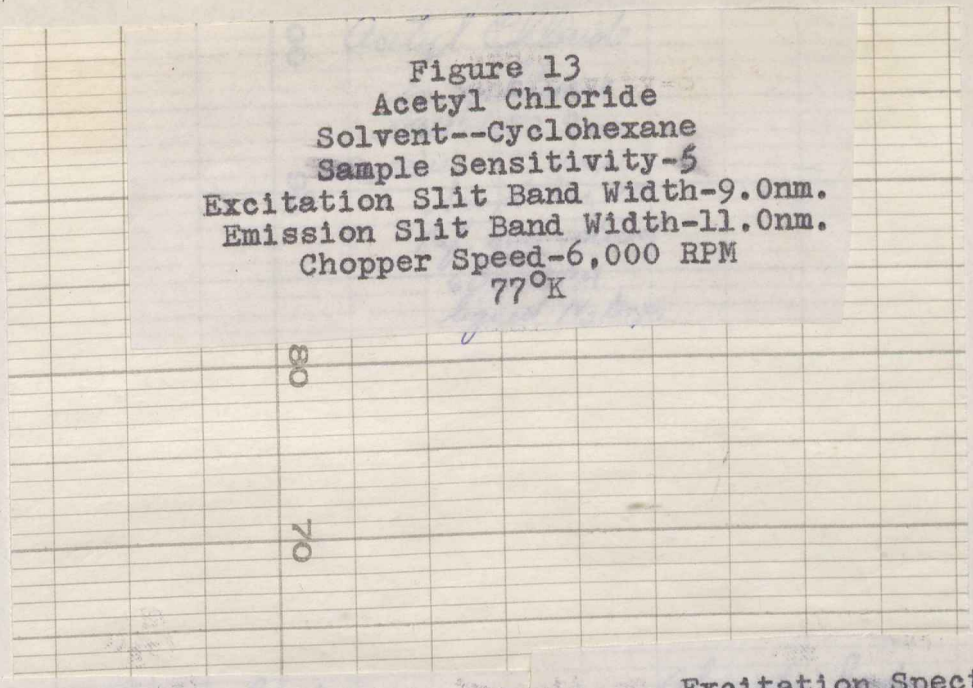


Figure 13
Acetyl Chloride
Solvent--Cyclohexane
Sample Sensitivity-5
Excitation Slit Band Width-9.0nm.
Emission Slit Band Width-11.0nm.
Chopper Speed-6,000 RPM
77°K



Emission Spectrum
Excitation Wavelength-294nm.
Emission Scan
Wavelength Maximum-(398±1)nm.

Excitation Spectrum
Emission Wavelength-398nm.
Excitation Scan
Wavelength Maximum-(291±1)nm.

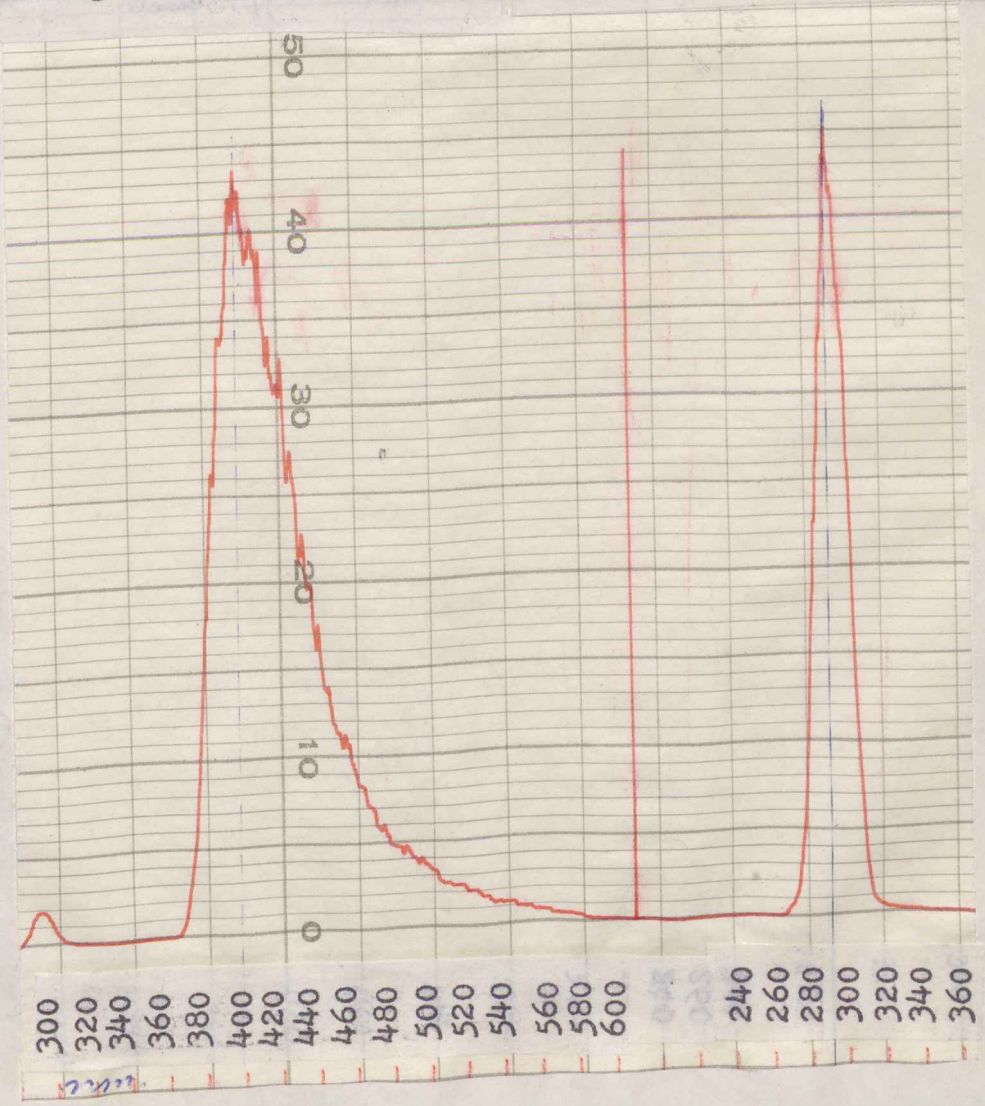


Figure 14
Acetone
Solvent--Cyclohexane
Sample Sensitivity-4
Excitation Slit Band Width-9.0nm.
Emission Slit Band Width-11.0nm.
Chopper Speed-6,000 RPM
77°K

Emission Spectrum
Excitation Wavelength-303nm.
Emission Scan
Wavelength Maximum-(441±1)nm.

Excitation Spectrum
Emission Wavelength-441nm.
Excitation Scan
Wavelength Maximum-(318±1)nm.

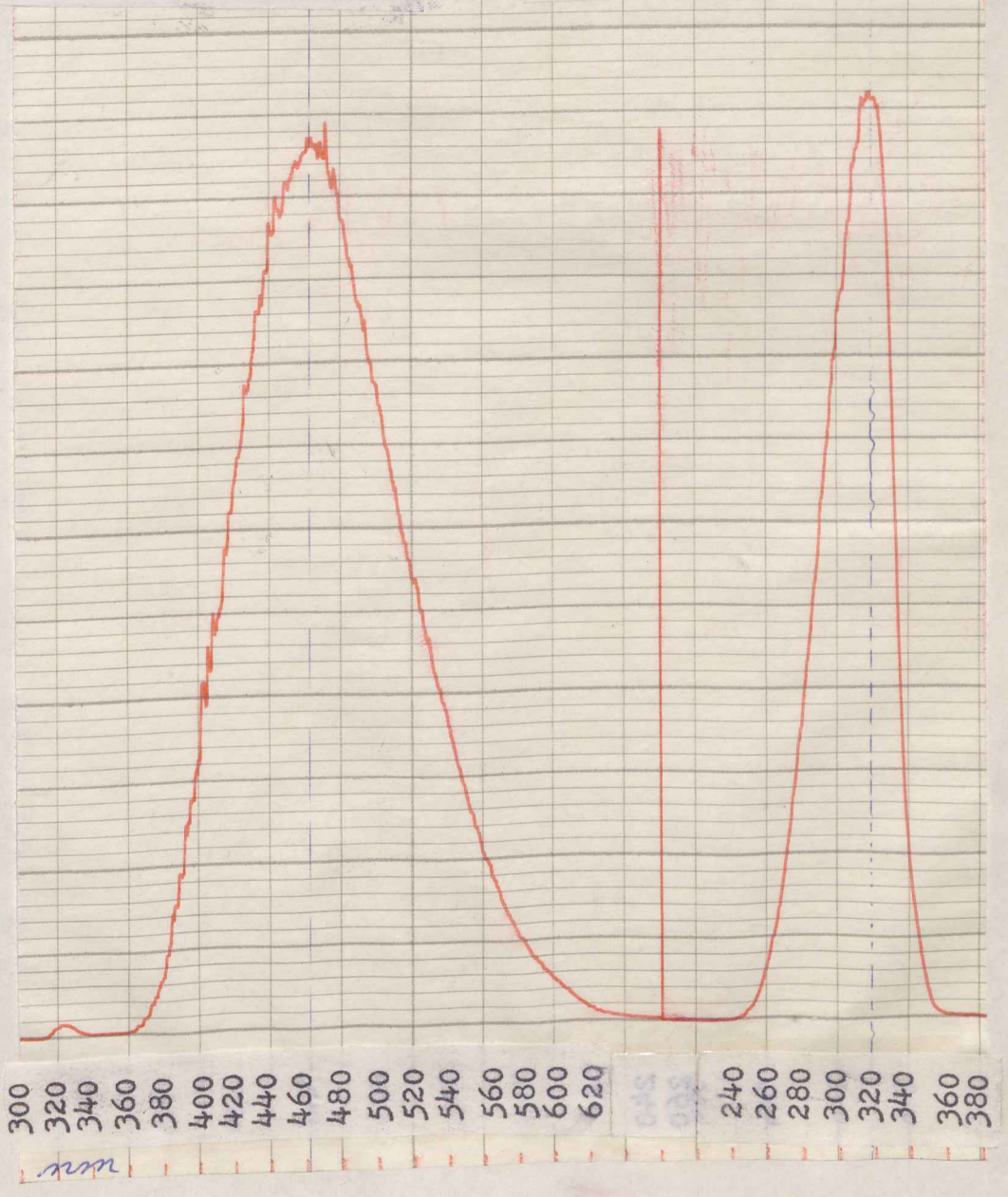


FIGURE 15
n,π^* Transition Information for some Carbonyls

Compound	Absorption λ_{max}(nm.)	ϵ_{max}	Phosphorescnece(nm.) Excitation	Emission
Acetaldehyde	293	11.8	304	477
Acetic Acid	204	41	287	444
Ethyl Acetate	204	60	295	437
Acetamide	214	—	279	437
Acetyl Chloride	235	53	291	398
Acetone	279	14.8	318	441

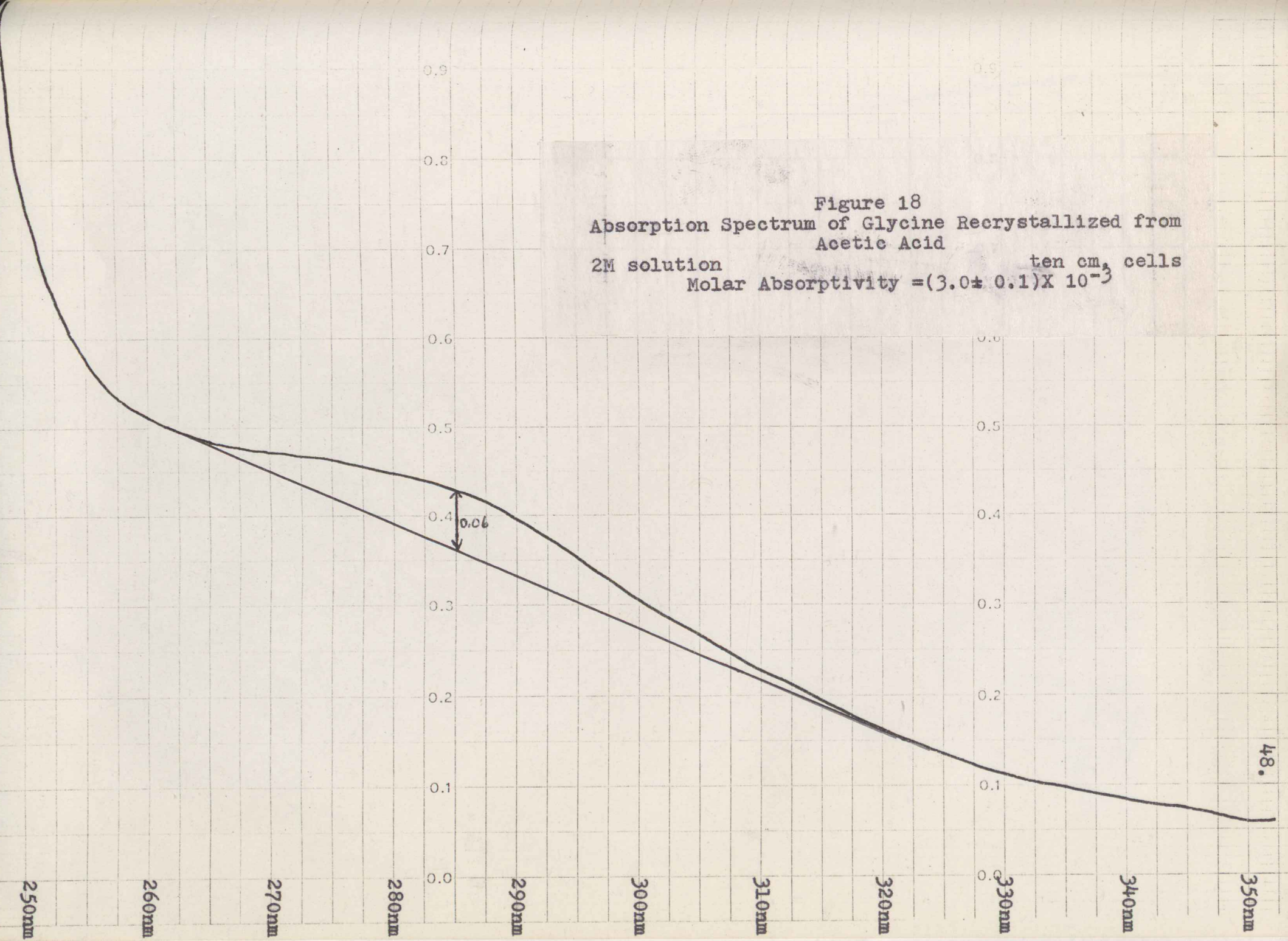


Figure 18
Absorption Spectrum of Glycine Recrystallized from Acetic Acid

2M solution ten cm, cells

Molar Absorptivity =(3.0± 0.1)X 10^{-3}

Figure 20

Absorption Spectrum of N-carboxyglycine disodium
ca. 0.5M solution one cm. cells.

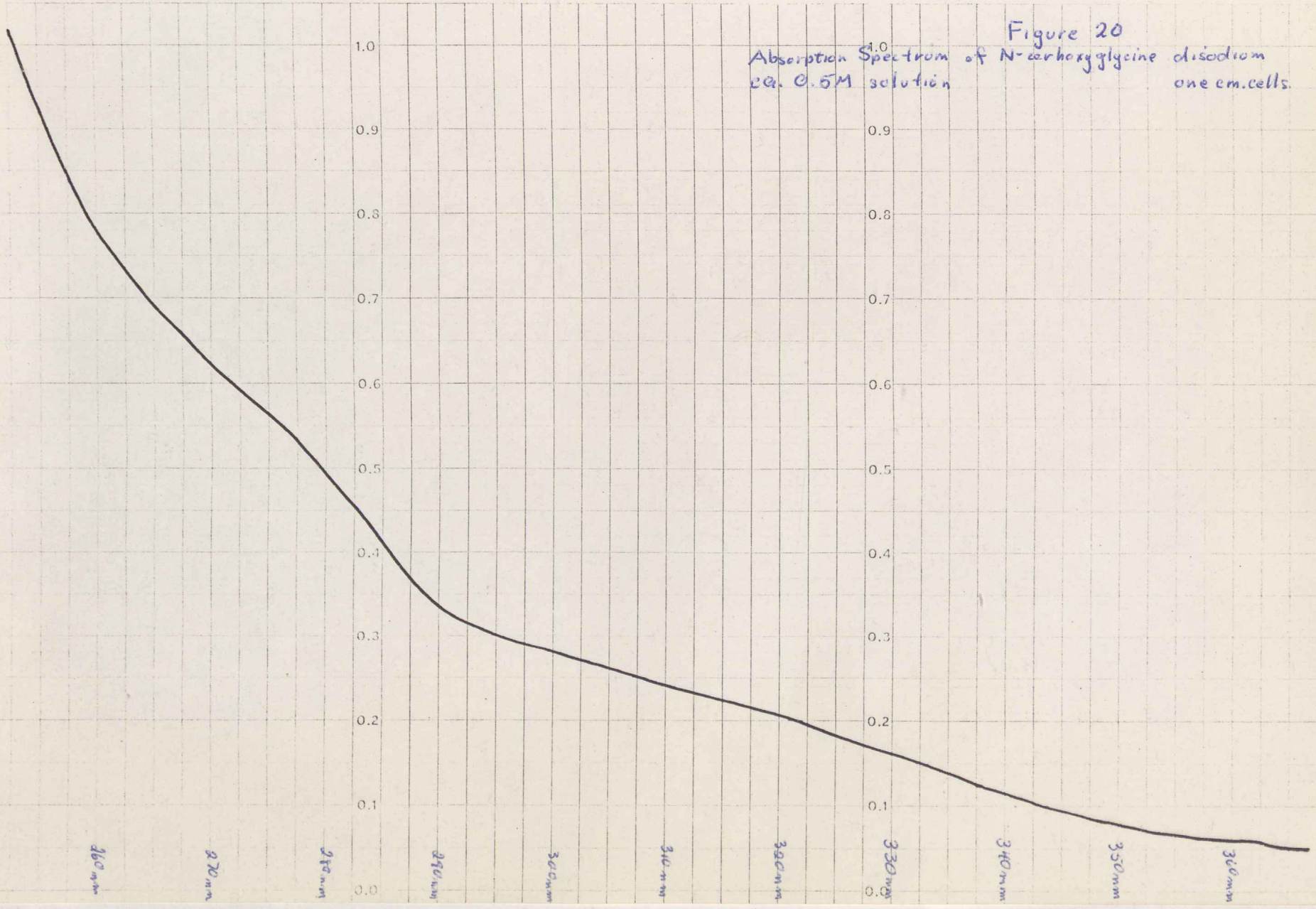
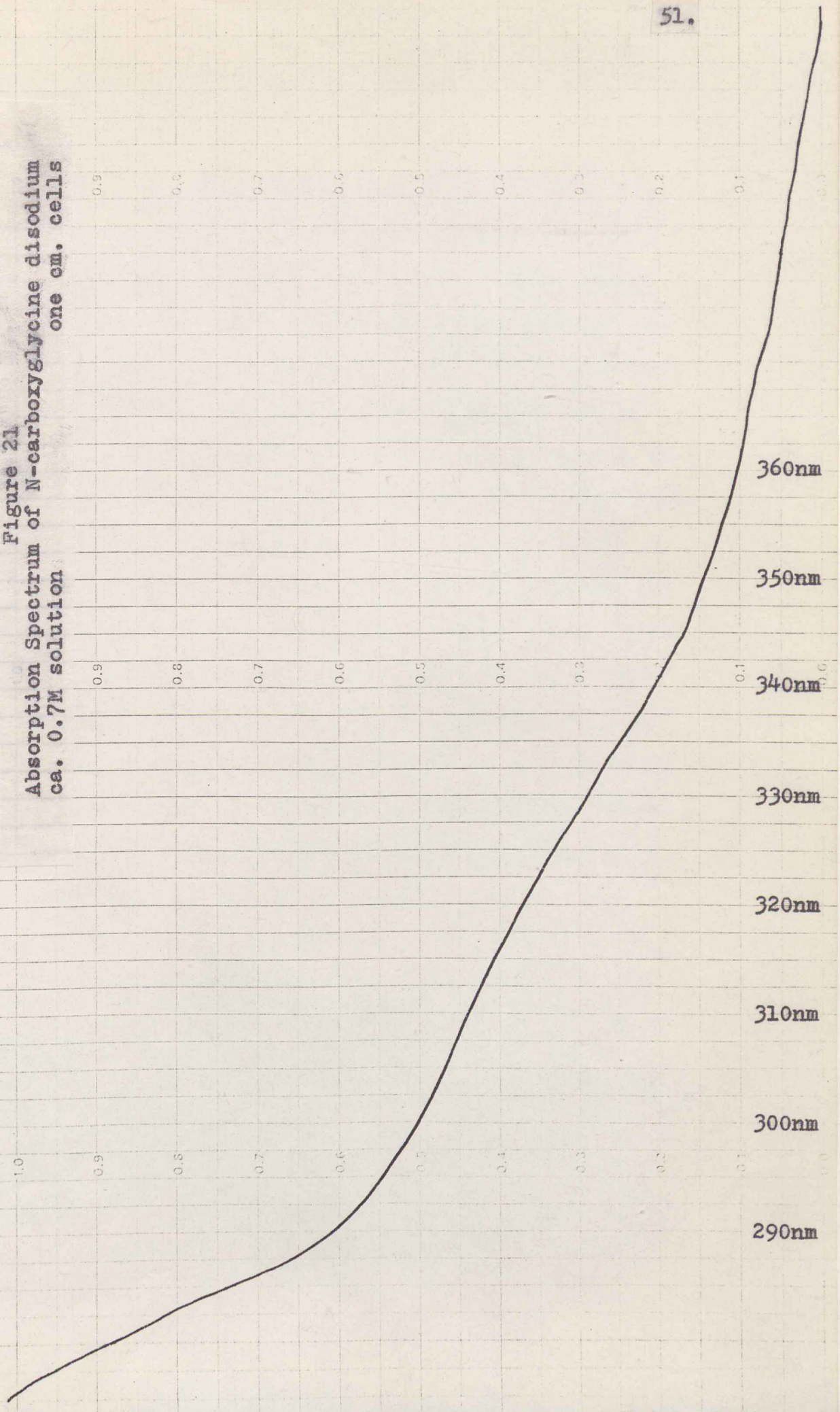


Figure 21
Absorption Spectrum of N-carboxyglycine disodium
ca. 0.7M solution
one cm. cells



Discussion

The phenomino of phosphorescence takes place when a molecule in an excited triplet state decays back to the ground state through a radiative process. This state may be acheived by direct excitation from the ground state (an exceedingly forbidden transition) or may be formed when vibrational levels of the excited singlet state couple with vibrational levels of the excited triplet state. This transition, termed 'inner system crossing', is similar to that of the non-radiative fluorescence decay, except that in this case the electron involved flips its spin. The following diagram is similar to the one shown on page 29 given for fluorescence and now extended by the inner system crossing pathway. Notice that the excited triplet state can be depopulated by a non-radiative decay just as the excited singlet state can.

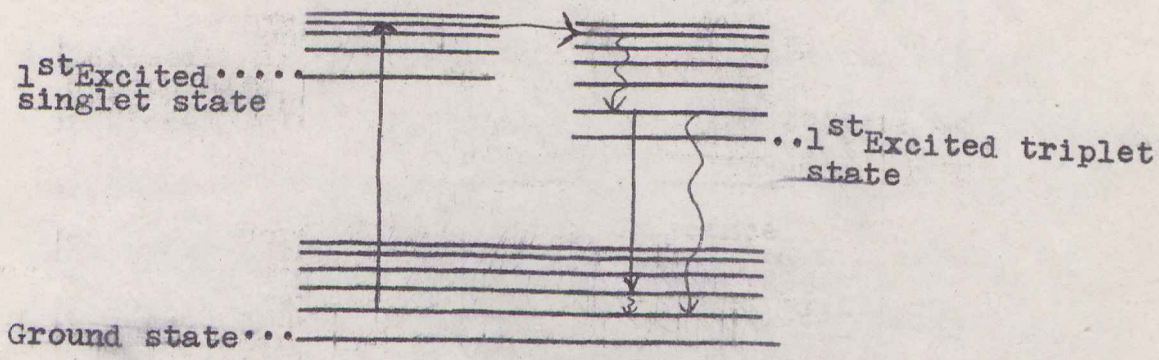


Figure 16. Phosphorescence

Instrumentally, only the sample compartment of the Hitachi must be modified. The sample is placed in a glass tube which has a 1mm. silica tip (this is the p-cuvette). This tube sits in a Dewar flask which is also equipped with silica tips (i.e. both walls of the Dewar) for the passage of ultraviolet light. The whole assembly is then placed in the sample compartment such that these tips are surrounded by a rotating chopper. The chopper speed may be varied as desired up to 10,000 RPM with an accuracy of ca. ±200 RPM from the dial setting alone. All samples are analysed at reduced temperature with liquid nitrogen being used exclusively in this case.

After the equipment and the liquid nitrogen were shown to be optically clear, the phosphorescence spectrum which was obtained from both the solid and the aqueous solution of β-alanine indicated that a hydrogen bonding solvent was not necessary for re-emission. The only functional group present which is able to absorb light is the carbonyl which is absorbing at 280nm. From this, it would follow that the two carbonlys of glycine's carbamic acid are causing the two excitation peaks seen for that compound.

The values Jaffe gives for the n,π^* singlet-singlet (S-S) absorptions do not match the excitation values obtained for the same compounds, except for acetaldehyde and

acetone which agree within about 10nm. Also, all the excitation values are shifted to longer wavelengths vs. the absorption values. The excitation values range from 279nm. to 304nm. where the absorption values range from 204nm. to 293nm.

In his book on electronic absorption spectra, Suzuki (15) states that formaldehyde shows an n,π^* direct triplet excitation (S-T) at about 390nm. Sandorfy (16) reports an n,π^* (S-T) for acetone at 400nm. with an absorption coefficient of ca. 10^{-3}. This could mean that the observed excitation for acetaldehyde and acetone are the (S-S) n,π^* transition, while the rest of the compounds are re-emitting from the triplet state which was excited directly. If the observed phosphorescence spectra have, in reality, resulted from the absorption and re-emission of the carbonyl as stated above, and not from an impurity as could be likely at the solution concentrations being used, this transition band should be measurable in an absorption spectra. Also, the molar absorptivity of peaks involved should not decrease with intensive purification and should have a value of ca. 10^{-3}. Time limitation factors dictated that only one compound could be chosen for intensive purification. Because glycine was to be the physiological model in the carbamate analysis, it was the logical choice.

ABSORPTION OF GLYCINE AND ITS CARBAMIC ACID:

Experimental

Glycine was recrystalized from 10% acetic acid (17) in the following manner. A 10% solution of acetic acid was saturated with glycine at room temperature by raising the solution's temperature and allowing the excess to precipitate. To 100ml. of this saturated solution ca. five grams of glycine was added and the solution was then brought to near boiling. When all the glycine was dissolved, the solution was placed in a Dewar flask and allowed to cool very slowly. Total descent to room temperature proceeded for approximately ten hours. This process allowed very little occlusion of liquid and moderate to large crystals. These crystals were then washed, dried, ground, and dried again by vacuum desiccation.

Because acetic acid is one of the carbonyl compounds in question, a pure water recrystallization was thought to be more suitable. Approximately 400ml. of water were saturated with glycine at room temperature as described above. Ten grams excess was added and the solution was again brought to near boiling. It cooled for approximately 18 hours in the Dewar flask and again good sized crystals were obtained with little or no occlusion of

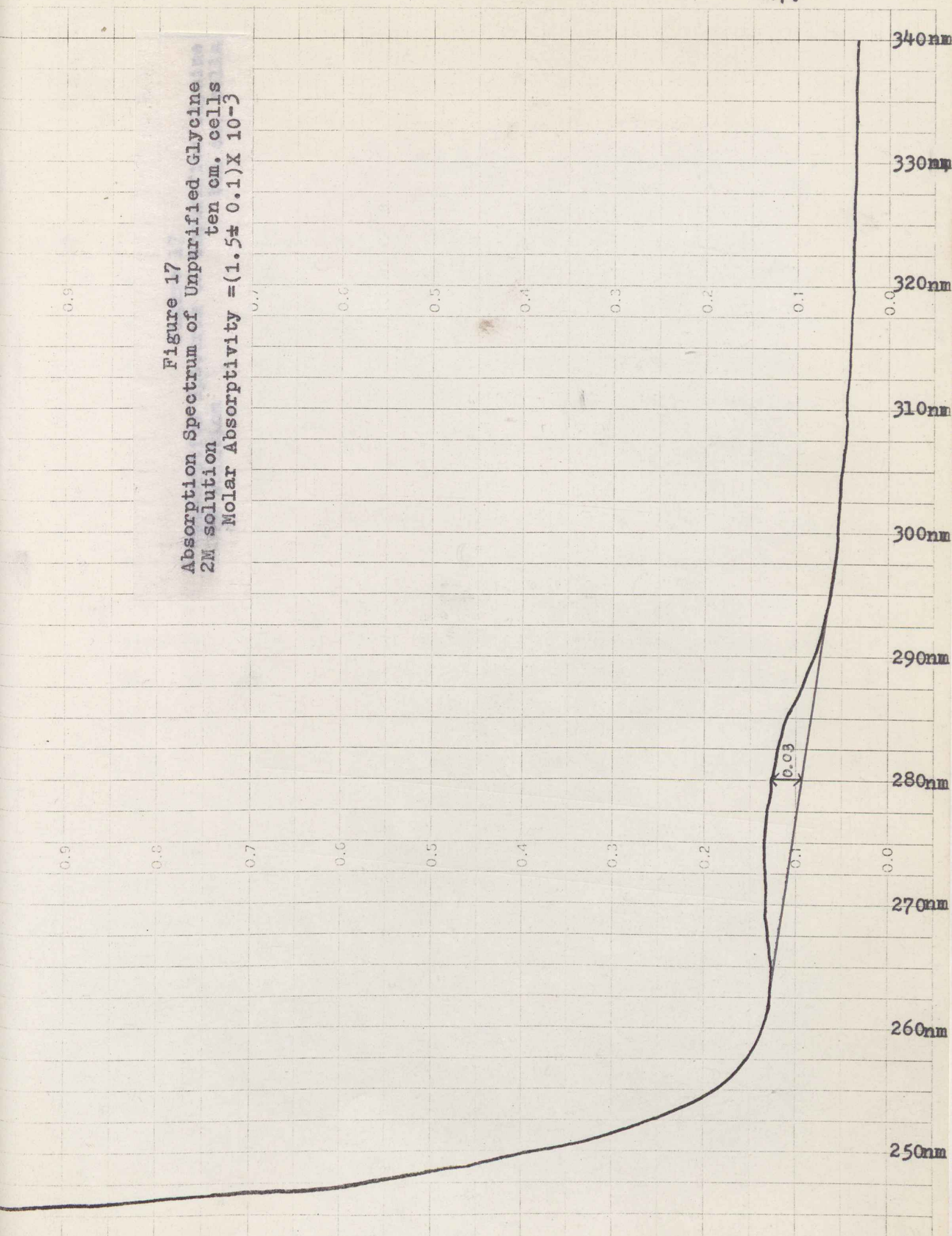
liquid. These crystals were then recrystalized in the same manner with about five grams being realized out of the initial 90 grams used.

Absorption spectra were obtained on a Cary 14 absorption instrument using 2M solutions and 10cm. cells. These extreme measures seemed necessary to be able to observe the theoretical 10^{-3} molar absorptivity. All of the spectra were taken with solutions at pH 7.5. Absorption spectra were obtained in this manner for unpurified glycine, glycine recrystallized from 10% acetic acid, and glycine from the second water recrystallization. These spectra are presented on pages 47-49.

As seen from the spectra, the n,π^* (S-T) band can be observed at 280nm. and the molar absorptivity of this remains basically unchanged throughout all recrystallization attempts in the region of $(2.0\pm0.6)X10^{-3}$. The higher value obtained for the recrystallization of acetic acid is probably due to some occluded liquid.

An unpurified sample of N-carboxy glycine disodium analysed by absorption. Using a much weaker solution and 1cm. cells, the spectra on page 50 was obtained. The band for the n,π^* (S-T) transition for the -N-C(=O)-OH group is seen better on page 51.

Figure 17
Absorption Spectrum of Unpurified Glycine
2M solution ten cm. cells
Molar Absorptivity =(1.5± 0.1)X 10-3



Discussion

The absorption band of glycine at 281±2nm. with a molar absorptivity of $(2.0\pm0.6)X10^{-3}$ has tentatively been assigned as an n,π^* direct triplet excitation. The consistancy of the molar absorptivity throughout all recrystallization attempts would seem to be proof of this assignment vs. the possibility of an impurity contamination causing the absorption.

The double absorption bands of glycine's carbamic acid, one at 281nm. and one at 325nm., bear out the excitation measurement that was done on the phosphorescence peak of this compound. A likely explanation of this phenomina is that the two different carbonyls are absorbing at different wavelengths. One of the carbonyls is attached to a carbon, i.e. as in glycine (λ_{max} 281), and the other is attached to a nitrogen, as in a carbamate (λ_{max} 325).

As seen from the absorption spectra, glycine has a negligable absorption at wavelengths greater than 300nm. when compaired to that of the carbamic acid. This is because the n,π^* (S-S) band has tailed out to almost nothing before it reaches 300nm. and the n,π^* (S-T) band has a total width of only 400nm. which means very little of this band tails beyond 300nm. However, in the case of the

carbamic acid, the whole n,π^* (S-T) band is at wavelengths greater than 300nm. and the n,π^* (S-S) of the nitrogen bonded carbonyl still has a large tail absorbing beyond 300nm. It would seem very likely that a good quantitative method for measuring a carbamate in aqueous solution could be easily developed keeping these ideas in mind. However, time limitation factors forced the decision to only experiment with the analytical technique which would seem to be the most sensitive and accurate, i.e. phosphorescence.

THE MEASUREMENT OF A CARBAMATE BY PHOSPHORESCENCE

Experimental

The pH dependency of glycine and its carbamic acid were analysed in order to find the effects of slight pH changes at physiological pH, and thereby hopefully find a pH range where the intensity of the response would not be greatly sensitive to pH changes.

Great care was taken in preparing samples and taking spectra in order to keep impurities to a minimum. All water was boiled vigorously for at least 30 minutes and was used immediately. All glassware was washed with acid cleaning solution and rinsed thouroughly with distilled water. The nitrogen used to degas samples was scrubbed through a chain of six bubblers, numbers one and three containing concentrated nitric acid and the rest containing distilled water. These bubblers were cleaned and refilled daily. The samples were dried under vacuum desiccation for two days after being finely powdered.

One molar solutions of glycine and the disodium salt of its carbamic acid were monitered for phosphorescence intensity as the pH was changed. The physical properties of the glycine solution seem to remain unchanged while the carbamate began to effervesce as the solution became

more and more acidic.

The graphs of concentration vs. pH presented on pages 56 and 57 were obtained. Notice that the pK_A which is 9.6 at room temperature for glycine has shifted to ca. 6.7 at 77°K as would be predicted by the Gibbs-Duhem relationship (18). Also notice the very flat portion of both curbes at pH's greater than 7.

These graphs show that glycine and its carbamic acid can be measured at pH's greater than 7 without changing the results. This is convenient in that the disodium salt of glycine's carbamate has a pH of roughly 9.0 when origionally dissolved. The phosphorescence spectra may be obtained at this pH, therefore, instead of having to add acid to raise the pH and lose CO_2 in the process. Glycine can still be brought to pH 7.5 with NaOH as before.

Concentration curves were then made for glycine and its carbamic salt. Their relative slopes are sketched on page 58. A sketching is all that is necessary in that the amount of impurities in the water being used can change the actual intensities of glycine and its carbamate. This requires that a consistant supply of water, totally unchanging in purity, must be used. Otherwise, all measurements must be made with the same sample of water.

Observing all the above-mentioned precautions, a pure

Figure 22
Phosphorescence Dependency of the pH of Glycine

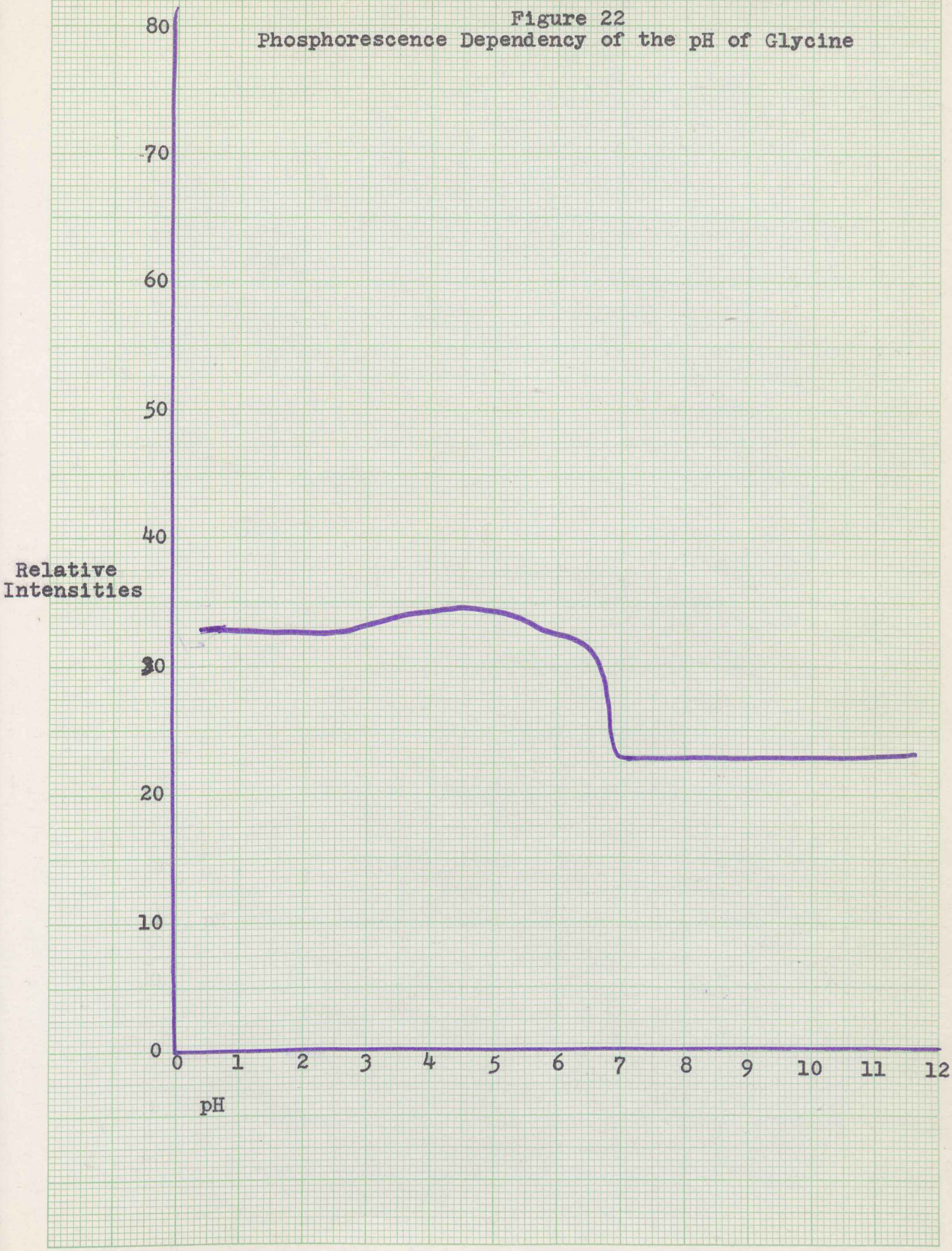


Figure 23
Phosphorescence pH dependency of
N-carboxyglycine disodium

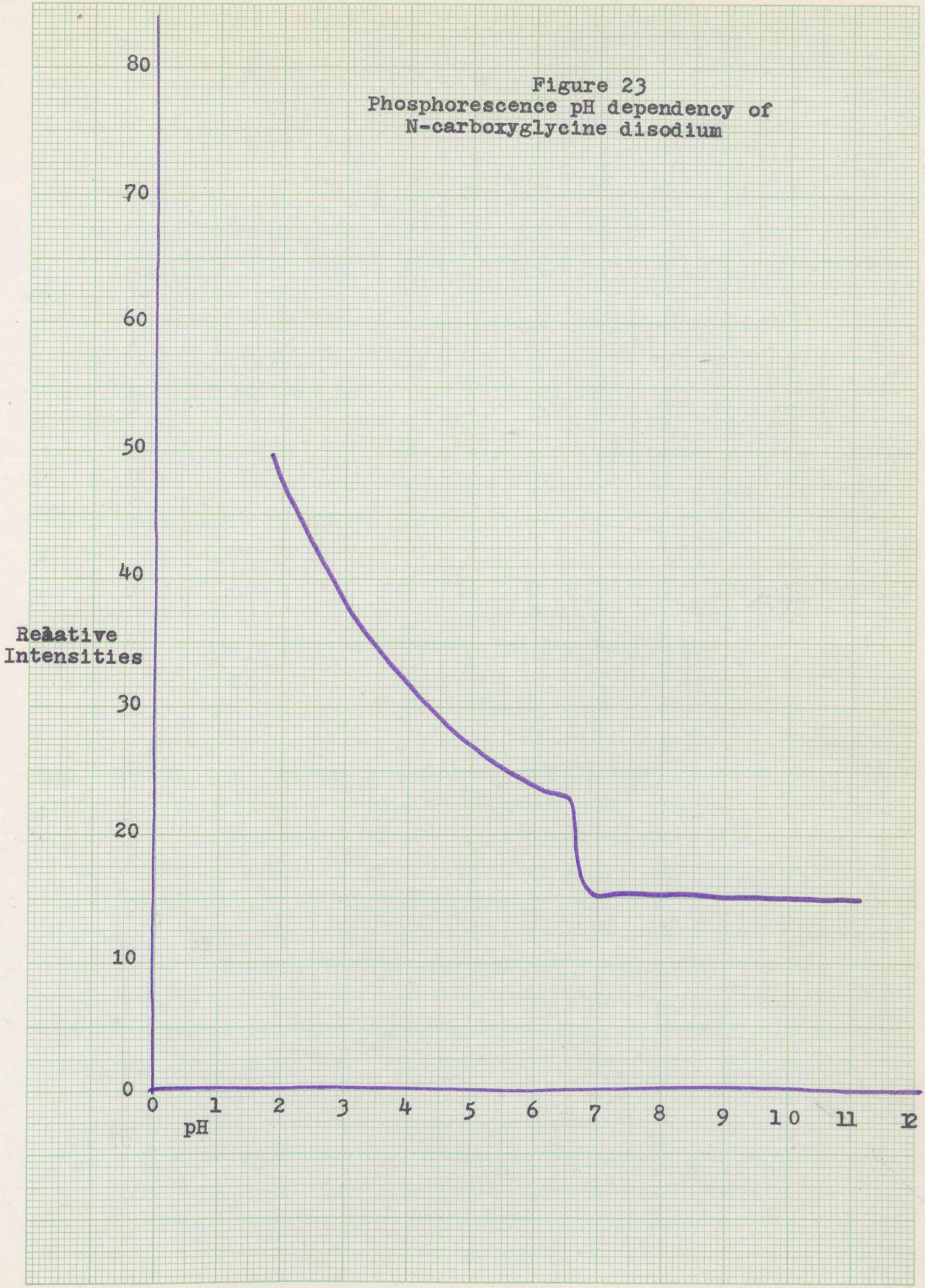
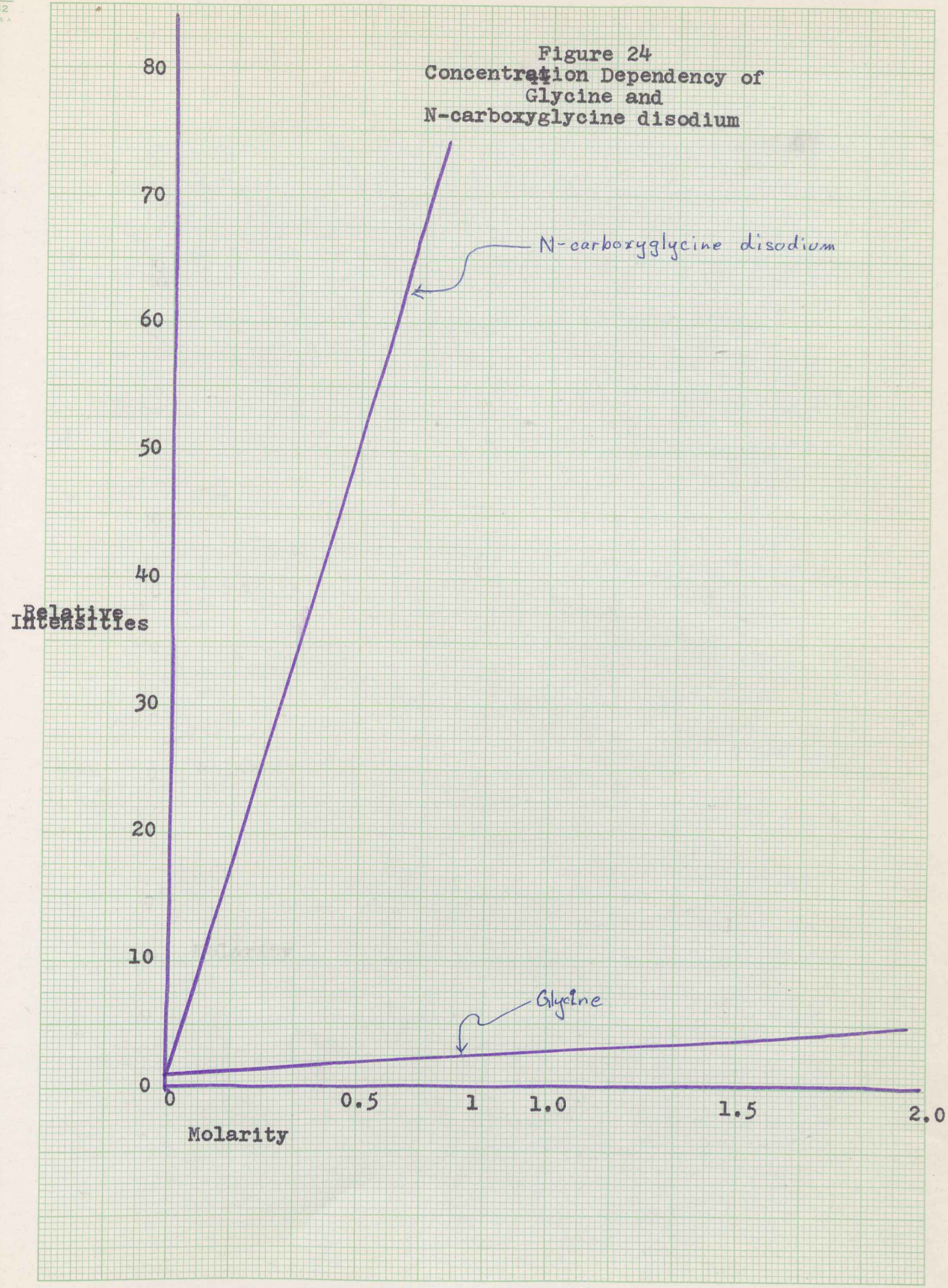


Figure 24
Concentration Dependency of
Glycine and
N-carboxyglycine disodium



water sample, a one molar solution of glycine, a saturated solution (aqueous) of CO_2, and a solution of glycine which had been bubbled with CO_2 for 15 minutes were analysed by phosphorescence with an excitation mavelength of 281nm. For maximum accuracy, all emission peaks were weighed and are, therefore, reported in grams. The values (1), (2), and (3) were taken directly from area measurements while (4) and (5) were calsulated from concentration curves such as the ones on page 58.

(1)-Emission peak of a 1M glycine solution minus the water backround.....0.015gm.=$M_{gly.}$

(2)-Emission peak of CO_2 saturated water.....0.007gm.

(3)-Emission peak of a 1M glycine solution bubbled with CO_2 minus #(2).....0.031gm.=M_{total}

(4)-Weight of emission peak of glycine per mole.....0.005gm.=$I_{gly.}$

(5)-Weight of emission peak of glycine carbamate per mole.....0.223gm.=$I_{carb.}$

It follows that the summation of the number of moles present of each type of molecule in solution, multiplied by their respective intensities per mole, equals the total intensity of the sample. If X is the number of moles of carbamate formed (in grams of area):

$$(I_{gly.})(M_{gly.}-X) + (I_{carb.})(X) = M_{total}$$

or,

$$(0.005)(0.014-X) + (0.223)(X) = 0.031$$

$$0.218(X) = 0.031$$

$$X = 0.031/0.218 = 0.143 \text{ or } 14.3\% \text{carbamate}$$

This preliminary value showing a 14.3%carbamate formation was measured at pH 7.5.

Discussion

A carbamic acid derivative of an amino acid can now be measured directly in aqueous solution at physiological pH. With a control of water impurities it is a quick and easy measurement. If the measurements were all made at an excitation wavelength of 325nm., the glycine and CO_2 backrounds could most likely be ignored as they would be very small.

With the procedure used in this preliminary trial, the carbamic acid could have easily been detected as low as 0.5% of the total mixture with an error of $\pm$0.3%. If some type of constant impurity could be introduced to increase the radiative decay of the triplet state vs. the non-radiative processes, the backround impurities would no longer be a variable while the response of the carbonyl would increase. This would make the method much more accurate and sensitive.

One problem which any researcher will encounter in this procedure is the short lifetime of the p-cuvettes. When their temperature is dropped from room temperature to 77°K in 20 seconds or less, they seem to withstand the shock well. However, when they are allowed to warm back up to room temperature in less than 10 minutes, they tend to

shatter. By simply allowing these tubes to remain in the atmosphere above liquid nitrogen for a few minutes while constantly but very slowly removing them, their rate of loss is small.

SUMMARY AND CONCLUSIONS

A carbamate or carbamic acid can be measured directly in aqueous solution and at any pH desired by phosphorescence, fluorescence or absorption methods. The phosphorescence method has actually been undertaken and a preliminary value of 14.3±0.3% has been obtained for the reaction of CO_2 and glycine forming a carbamic acid. The fluorescence method had previously been unsuccessful in producing consistant data but some of the interference problems have since been removed and this method may now be satisfactory although not as accurate as the phosphorescence method. The absorption method has not been attempted but the amino acid reactant and the carbamic acid product are vastly different in their absorption greater than 300nm. An analysis in the 300nm. to 400nm. range should prove fruitful in providing a very simple analysis with accuracy compairable to that of the phosphorescence at higher carbamate concentrations. The only question would be in the area of differing accuracies as the concentration of carbamate changes.

All of the techniques are quite inexpensive to maintain once in operation. Also, they are all quite easy and fast with the most accurate and sensitive technique (phosphorescence) being, relatively, the hardest and slow-

est technique.

These studies have also produced a tentative band assignment for the carbonyl of glycine. It shows an n,π^* direct triplet excitation at 281±2nm. with a molar absorptivity of $(2.0\pm0.6)X10^{-3}$. This band has remained constant throughout all purification procedures and its wavelength maximum has been correlated through phosphorexcence excitation spectra.

Other n,π^* direct triplet excitations have ostensibly been observed although they have not been verified as in the case of glycine. The absorptionand phosphorescence excitation peaks have been observed at 325nm. on the carbamic acid of glycine. Phosphorescence excitation spectra have also shown that ethyl acetate, acetamide, acetic acid, and acetyl chloride have possible n,π^* direct triplet transitions in the 280nm. to 295nm. range.

If what has been seen to date has been correctly interpreted, it would be a simple matter to extend these ananyses to that of any saturated carbonyl. The phosphorescence excitation might even be used to distinguish between different elements directly bonded to carbonyl groups.

SUGGESTIONS FOR FURTHER WORK

The knowlege that amino acids and carbamates can be measured in solution spectroscopically is only the beginning of what could extend into many years of research.

The three different types of carbamate measurement should be checked against themselves with regard to accuracy and sensitivity ranges. From here, dosed CO_2 studies could be carried out and then reactant temperature could be made a variable in all those studies, especially studies at body temperature. Then other amino acids could be used and their intensities compared with that of glycine and its carbamate; conceivably, mixtures and total carbamate formation which would model the body could eventually be attempted.

One improvement in the method itself would be the addition of a constant impurity such as bromide ion or possibly a short organic molecule such as ethanol or methanol in small quantities. This type of manipulation may increase the radiative response of glycine and its carbamic acid, making the procedure much more accurate and possibly much more stable.

The assignment of n,π^* direct triplet excitation of carbonyls is in its infancy. All saturated carbonyl compounds could theoretically be open to investigation and assignment

of direct triplet excitation bands. All of those compounds in Jaffe's table (17), urethan, and N-carboxy-glycine disodium would be a good start. These compounds would need to be intensely purified before absorption and correlated phosphorescence data could be obtained.

Those spectra which have commonly been called flourescence spectra of glycine and all other related compounds also have excitation maxima at or close to 280 nm. It would seem that this could only be achieved if the directly populated triplet state were very quickly losing its energy in partially radiative form. This is almost unheared of when the triplet state is formed from inner system crossing from the excited singlet state. However, no references could be found as to the rules governing the radiative decay of directly populated triplet states. A theoretical calculation (or an experimental one) of the rate constants involved, along with an in depth search into the geometry changes which these molecules may undergo during these transitions would be exceedingly beneficial to the total understanding of the system.

Glycine is known not to be optically active. However, its carbamic acid could be optically active because of the overall geometry. The application of spectropolarimetry, optical rotatory dispersion and circular dichroism to the

study of this compound would give interesting results, although it is doubtful whether these methods would be easier, more accurate, or more sensitive than the methods described earlier. These techniques could, however, give some added information as far as the theoretical aspects of the system are concerned.

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