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The fluorescence and phosphorescence of glycine -- a first step in the determination of glycine carbamate

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THE FLUORESCENCE AND PHOSPHORESCENCE OF GLYCINE -
A FIRST STEP IN THE DETERMINATION OF GLYCINE CARBAMATE

by

Henry George Fein

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Senior Thesis Submitted in
Partial Fulfillment of the Requirements of Graduation

DEPARTMENT OF CHEMISTRY
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This Thesis

Submitted by

Henry George Zein

to the

Department of Chemistry of Union College

in partial fulfillment of the requirements of the degree of

Bachelor of Science with a Major in Chemistry

is approved by

Robert W Schaefer

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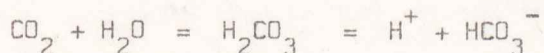
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HISTORICAL INTRODUCTION TO THE CARBAMATE REACTION

Cassidy (1) fully outlines the historical progress of research to 1968 about the carbamate reaction; this paper will simply summarize that effort.

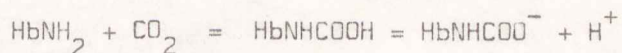
It is presently believed that carbon dioxide is transported by the blood in three ways: 1) dissolved CO_2 and carbonic acid, 2) bicarbonate ions, and 3) carbamate.

The first two forms, bound and free CO_2 , are dependent on the hydration of CO_2 . This hydration is the first step in the uptake of CO_2 by the blood, and occurs in the physiological pH range 7.0 - 8.0 (2,3,4):



It should be noted that this reaction is too slow to be of any physiological importance unless catalyzed by carbonic anhydrase. Meldrum and Roughton (5) isolated this substance from the red cells of the blood in 1933.

The carbamate reaction was first suggested by Henriques (6) in 1928, but it was not until 1934 that any experimental data could confirm this belief in a direct reversible reaction of CO_2 and hemoglobin. Ferguson and Roughton (7) found a small but fast uptake of CO_2 in blood where the carbonic anhydrase system had been inhibited. The following reaction was postulated (Hb = hemoglobin):



Staide and O'Brien (8) showed that reduced hemoglobin combines with more CO_2 as carbamate than does oxyhemoglobin. This data, of course, fits the physiological conditions proposed by Ferguson and Roughton.

In order to elucidate the reaction mechanism, experimentation has been

done with aqueous solutions of glycine, since it is chemically much simpler than hemoglobin, and also models the globin endings of the hemoglobin molecule. The hemoglobin- CO_2 reaction is believed to occur at the alpha-amino groups of alpha-amino side chains on hemoglobin. (1)

The quantitative assessment of the role of the carbamate reaction in CO_2 physiology is dependent on chemical analysis. Ferguson and Roughton (7) developed an analysis which depends on the fact that the barium salts of carbamates are soluble and reasonably stable at 0°C . and at very high pH, while under these conditions CO_2 , H_2CO_3 , and HCO_3^- are all converted to CO_3^{--} and precipitated as BaCO_3 . (9) By knowing the initial pCO_2 , it is possible to calculate what portion of the total CO_2 has combined with the alpha amino bearing substrate.

Roughton and Rossi-Bernardi (10) have proposed the use of a glycyl-glycine and CO_2 model for the carbamate reaction. Instead of the barium precipitate method, they have used a pH and pCO_2 electrode system to measure the uptake of CO_2 . Since the dissolved CO_2 and HCO_3^- can be calculated from the pCO_2 and pH measurements using the Henderson-Hasselbalch equation, the original CO_2 concentration only need be known to calculate the amount of carbamate formed.

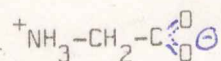
PROPOSED GOAL AND METHODOLOGY

All the carbamate literature is based on inferential evidence.

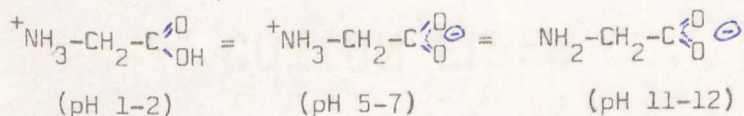
Neither the barium carbonate nor the more recent $p\text{CO}_2$ electrode methods are direct means of analysis. Moreover, both methods have inherent difficulties. The classical barium carbonate method is not suitable for the relatively unstable reaction product (1).

The reaction of CO_2 with amines was first investigated by Siegfried (11). Faurholt (12) has also studied the physical chemical aspects of this reaction. They both found that CO_2 reacts rapidly without the aid of any catalyst with the amino group, $-\text{NH}_2$, but never with the $-\text{NH}_3^+$ group. The reaction involves only the non-ionized alpha-amino group of the conjugated base species. The amount of carbamate formed at a given $p\text{CO}_2$, according to this work, should increase with increases in solution pH. This reaction occurs directly with free CO_2 , but not with H_2CO_3 , HCO_3^- , or CO_3^{2-} .

If the above is correct, then the model of the carbamate reaction using glycine must be carefully re-examined. Working independently, Adams (13) and Bjerrum (14) first demonstrated that glycine in a neutral medium (such as aqueous solutions in the physiological pH range) exists as a dipolar ion, called the zwitter-ion. It has the structure



The following pH dependent equilibrium exists:



Thus it is imperative to devise an analytical scheme that is not in itself pH dependent and does not alter the pH of the species being analyzed.

Spectroscopy is an obvious possibility for this problem. Cassidy (1) tried ultraviolet absorption spectroscopy with only very limited success. This paper will be concerned with efforts to devise and perform an analytical scheme for the determination of glycine carbamate making use of the Hitachi-Perkin-Elmer MPF-2A Fluorescence Spectrophotometer.

The fluorescence and phosphorescence of glycine is extensively studied as the first step in the analytical framework. The Hitachi instrument, as situated in these experiments, measures emissions from species of all half lives. Therefore, for the remainder of this paper, the phenomenon measured by the instrument will be termed phosphofluorescence unless otherwise stated.

Once the phosphofluorescence of glycine is established at different concentrations and pH, the same could be done with the disodium salt of glycine carbamate under the same conditions. Following this, the percentage of CO_2 uptake can be easily determined. CO_2 can be bubbled through a solution of known concentration of glycine, and its phosphofluorescence spectrum recorded after adjusting the pH. By the simple means of simultaneous equations, the percentage of glycine that has reacted to form glycine carbamate can be determined.

AN OUTLINE OF THE THEORETICAL CONSIDERATIONS OF PHOSPHORESCENCE AND FLUORESCENCE (15)

This section of the paper will deal briefly with some of the theoretical bases for the phenomena of fluorescence and phosphorescence in general. The following section will then concentrate more specifically on the energy levels of the electrons of the carbonyl group.

Molecules in the ground state have all possible electrons in pairs. The absorption of quanta of light results in the promotion of a single electron to a higher-energy orbital. In this excited state, all the electrons of the molecule remain in pairs with the exception of those two whose pair relationship has been broken by the energy absorbed from the light quanta. The spins of this latter pair (now in different orbitals) may be paired (the singlet state) or unpaired (the triplet state). The singlet state is quantum mechanically permitted, while the triplet state is forbidden. To each excited singlet state reached by absorption there corresponds a triplet state. This triplet state always has lower energy than the corresponding singlet.

As Figure 1 below indicates, fluorescence occurs when electrons drop back to the ground state from the lowest vibrational level of the first excited singlet state. According to current theory, all other deactivation shown (from the second excited state or within the first excited state) is radiationless. It should be noted that fluorescence is a rapid process; half-life values for the excited singlet states are of the order of 10^{-9} sec.

Phosphorescence occurs when the deactivation occurs through the excited triplet state. As noted above, the triplet state has lower energy than the corresponding singlet state, and therefore the phosphorescence occurs at longer wavelengths than the corresponding fluorescence.

The transitions between singlet and triplet states, as shown in Figure 2

Figure 1: Fluorescence Energy Level Diagram

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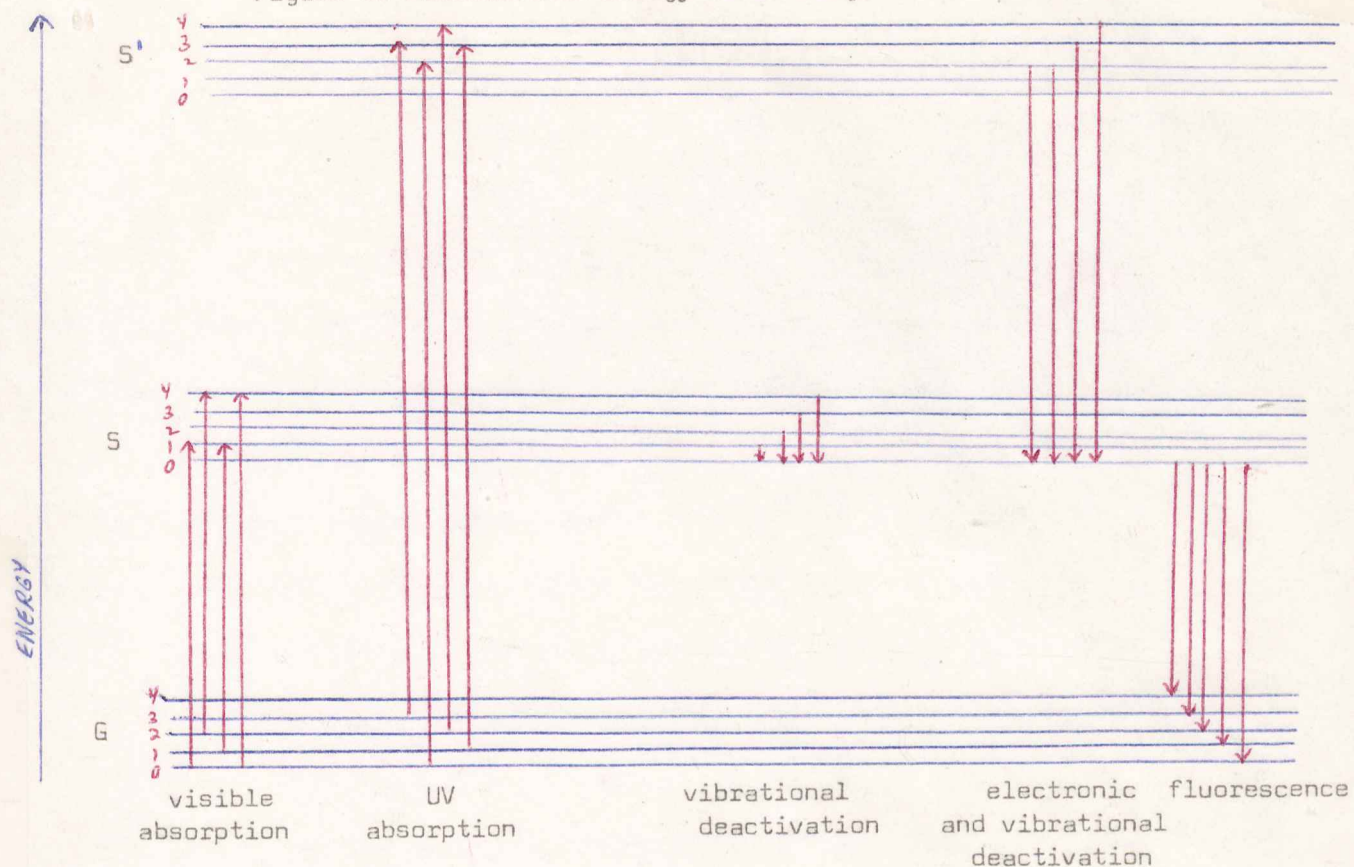
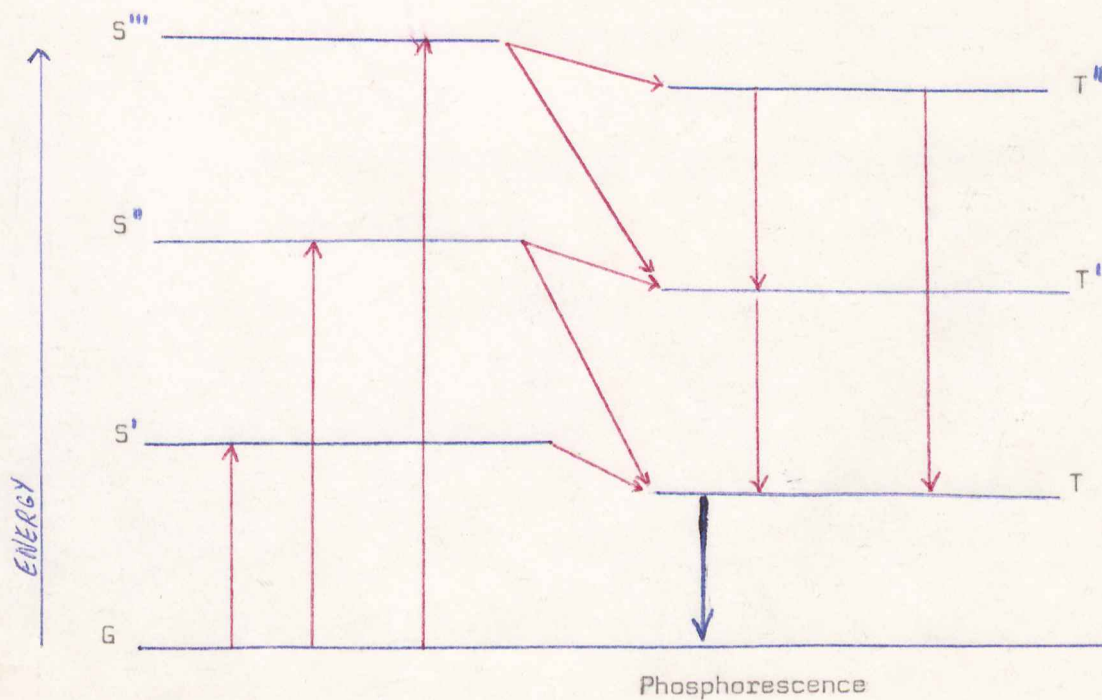


Figure 2: Phosphorescence Energy Level Diagram



below, are multiplicitly forbidden, and occur only rarely.

In the normal process of phosphorescence, the electrons are excited by the absorption of radiation to an excited singlet state. The electron then drops to the lowest excited triplet state by a single or a series of radiationless transitions, the nature of which is not well understood. Once the electron has arrived in the lowest excited triplet state, it still possesses excess energy. This energy is emitted as phosphorescent radiation. The major difference between the singlet and triplet states is that of stability; while fluorescence occurs with half-life values for the excited state of 10^{-9} sec., phosphorescence occurs with half-life values ranging from 10^{-8} sec. to hours.

The electron may return to the lowest excited singlet state from another excited state (singlet or triplet) if a collision occurs; this re-excitation results in a phenomenon called slow fluorescence.

When the transitions from the higher triplet states to the lowest triplet state are allowed, no phosphorescence from these states is observed. However, no such phosphorescence is observed even if the transitions are forbidden. This is because the decay period for even a forbidden transition is much shorter than for the multiplicity-forbidden transition from the lowest triplet back to the ground state.

THE PHOSPHORESCENCE OF THE CARBONYL GROUP

In the ground state, the carbonyl group electrons have the following configuration: $s^2 \pi^2 \gamma_0^2$. Aside from the sigma-bonding electrons, there are two electrons in the C-O π bond, and two sets of lone pairs: one s pair and one p_y pair, or some hybridized orbitals.

The excitation of least energy, as Figure 3 indicates, results in the following transition: $s^2 \pi^2 \gamma_0^2 \rightarrow s^2 \pi^2 \gamma_0 \pi^*$. This is the $n \rightarrow \pi^*$ transition, which is symmetry-forbidden. According to Murrell (16), "if we make a transition from an orbital which is localized over the whole molecule, then the transition density is still localized on the atom, since it is confined to regions of space in which both orbitals have non-zero values. It follows that the intensity of an $n \rightarrow \pi^*$ band depends only on the local symmetry of the non-bonding orbital." The π and π^* orbitals are perpendicular to the molecular axis, z , whereas n and σ orbitals are parallel to this axis. Because this transition is of least energy, it occurs at the longest wavelength ($E = h\nu$).

The $\pi \rightarrow \pi^*$ transition occurs far downfield from the $n \rightarrow \pi^*$ transition. Since this is a symmetry-allowed transition, its intensity is much greater, with $a_m = 7000 - 10,000$. This absorption usually occurs at 180-190 nm.

Recent work by this student and others requires a re-examination of the absorption spectrum for the $n \rightarrow \pi^*$ transition in molecules containing the carbonyl group. The work summarized by Jaffe and reproduced as Chart I below seems somewhat in error. Work done in this laboratory, summarized by Chart II, indicates that the $n \rightarrow \pi^*$ transition absorbs only in the 280-290 nm region (17). Because of the symmetry-forbidden nature

Figure 3: Energy Levels of the Carbonyl Group

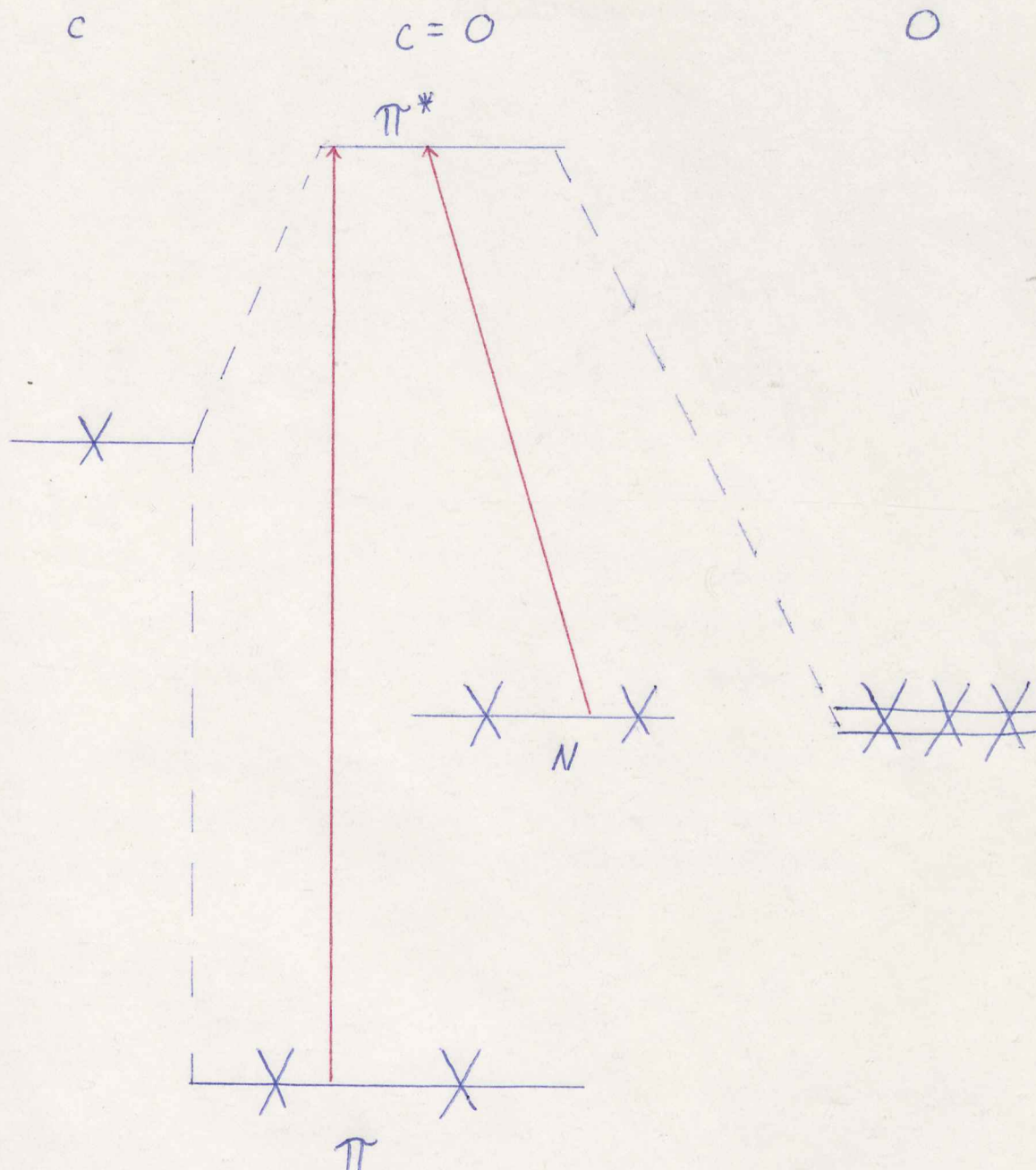


Chart I: The $n \rightarrow \pi^*$ Transition in Simple Carbonyl Compounds (Jaffe)

Compound:	max (nm):	a_m max:	Solvent:
$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{H}$	293.4	11.8	Hexane
$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{OH}$	204	41	Alcohol
$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{O}-\text{C}_2\text{H}_5$	204	60	Water
$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{NH}_2$	214	--	Water
$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{Cl}$	235	53	Hexane
$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{CH}_3$	279	14.8	Hexane

Chart II: The $n \rightarrow \pi^*$ Transition in Simple Carbonyl Compounds (Schaefer and Gray)

Please note: The Hitachi Instrument is a Single-Beam Instrument and Does Not Permit a_m Measurements. **All samples are Neat.**

Compound:	max (nm):
$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{OH}$	283
$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{O}-\text{C}_2\text{H}_5$	285
$\text{CH}_3-\overset{\text{O}}{\parallel}{\text{C}}-\text{NH}_2$	276
$\text{C}_2\text{H}_5-\text{O}-\overset{\text{O}}{\parallel}{\text{C}}-\text{NH}_2$	280
$\text{Na}^+ \text{O}^- - \overset{\text{O}}{\parallel}{\text{C}} - \text{NH} - \text{CH}_2 - \overset{\text{O}}{\parallel}{\text{C}} - \text{O}^- \text{Na}^+$	280 and 325

of this transition, the a_m values are quite small. In addition, this absorption, if viewed with an absorption spectrophotometer, appears as only a shoulder on the quite broad absorption band produced by the $\pi \rightarrow \pi^*$ transition (See Figure 4 below, which was prepared with a Cary 14 spectrophotometer).

Work done in this laboratory differs from previous attempts in a number of ways. Perhaps most importantly is the means by which the absorption is studied. Instead of using a double-beam absorption instrument, this laboratory used the Hitachi instrument to observe the absorption spectrum that produces the maximum phosphorescence. Since the $\pi \rightarrow \pi^*$ transition is not directly involved in the phosphorescence excitation, it does not form a large absorption band that masks the $n \rightarrow \pi^*$ transition band.

Figure 4: UV Absorption Spectrum of Glycine

15 % Glycine (2 M)

5 cm Beckmann Cells

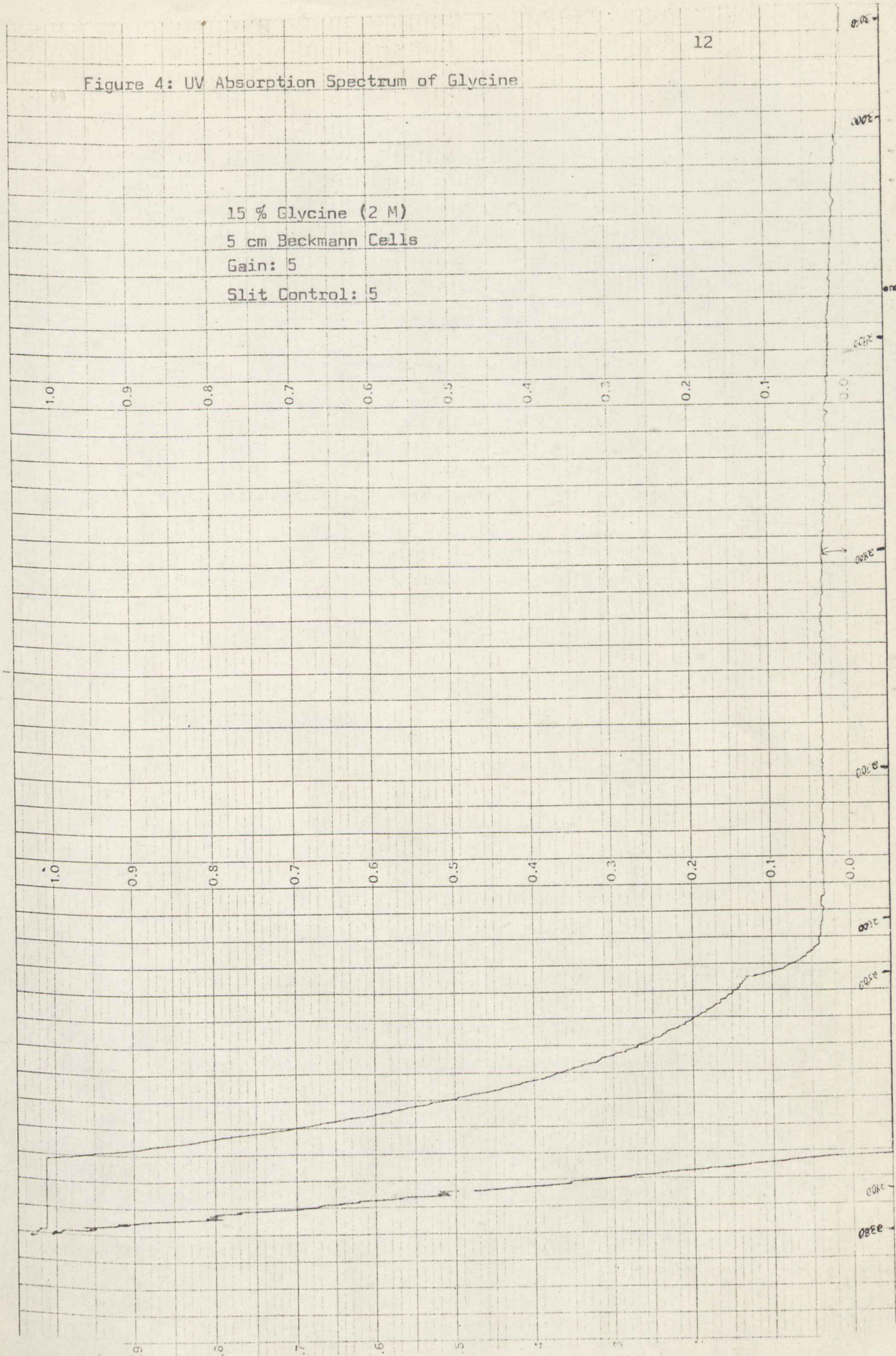
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EXPERIMENTAL RESULTS

As the above discussion indicates, the $\pi \rightarrow \pi^*$ transition absorption for glycine is at 280 nm. Therefore, all excitation of glycine in this study is at 280 nm. Slit width for both excitation and emission monochromators is 6 nm. The Reference Sensitivity is 1 and the Sample Sensitivity is 5. In all data below, the Hitachi instrument was set up as shown in Figure 5. There is continuous excitation radiation from A which irradiates the sample cuvette B. This cuvette is made of silica and is optically matched on all four sides. The emitted radiation that is studied passes through slit C to the monochromators and detector units.

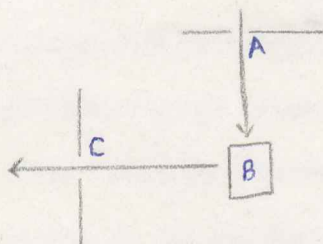


Figure 5: Hitachi Phosphofluorescence Set-Up

It should be noted here that this set-up analyzes emitted radiation from both fluorescence and phosphorescence. Since the excitation is continuous, there is no time factor involved in this process, and light emitted from species with any half-life will be recorded.

Readings were taken of glycine solutions of concentrations of 15 %, 10 %, 7.5 %, and 5 % and at pH of 6.8, 7.2, and 8.0. The aqueous glycine solutions were approximately pH 5.8 when first prepared. The pH was raised by the addition of 1-2 ml of 1 M NaOH to approximately 200 ml of glycine solution. The pH was continuously monitored by a single-electrode pHmeter.

All water used in these studies was carefully prepared to reduce interfering fluorescence. Distilled water was boiled for more than one hour to denature any proteins contained in bacteria in the water. The water was stored in a CO_2 -free bottle. No water more than ten days old was used in these studies.

In addition, all data reported in this study was achieved by subtracting off the phosphofluorescence of the water used as a solvent. Before each day's experiments, a sample of deaired water was analyzed on the Hitachi instrument, and the values achieved were subtracted from all those recorded for glycine solutions. This is to insure that what is reported is due solely to the phosphofluorescence of glycine itself.

Using this method, a phosphofluorescence peak for glycine was found at 420-440 nm. In all the studies below, the phosphofluorescence reported is that at 420 nm. This finding confirms the early work of Bertrand (18) who found the peak to lie at 467 nm when exciting with a mercury vapor lamp.

The initial results, as shown in Graph 1 below, were not good. The level of phosphofluorescence is low, and there is no indication of any appreciable pH or concentration dependence. This early data was prepared with precautions taken to ensure no CO_2 contamination, but no steps were taken with this set of experiments to remove any gases already in solution.

During a pause in this research because of a school vacation, other investigation in this laboratory resulted in significant findings that radically altered the experimental methods used by this researcher.

Using the Hitachi instrument and another attachment, and working at liquid nitrogen temperatures, a fellow experimenter showed that the

phenomenon being observed was phosphorescence, and not fluorescence as had been originally postulated. Although half-life times were not extensively studied, values of the order of 10^{-6} sec. were recorded. This work confirmed the absorption for the phenomenon to lie in the 280-290 nm region. The instrument response at liquid nitrogen temperatures was significantly higher than that at higher temperatures. This is to be expected, especially if one assumes that the $S \rightarrow T$ transition, which is radiationless in the UV and visible regions, occurs in the far IR (thermal). Lower temperature would increase the likelihood for such a transition to occur.

Furthermore, this work found the phosphorescence peak for carbonyl-containing species to lie in the 440-460 range. This is a slight shift to longer wavelengths from the phosphofluorescence spectra recorded with glycine at room temperatures.

Since the phenomenon this experimenter was observing was also at least partly phosphorescence, it was necessary to deairate all samples before taking measurements. This is necessary to remove all oxygen that might be dissolved in the water, and was accomplished by bubbling Nitrogen through all samples for a quarter hour. Oxygen molecules act as a quenching agent for phosphorescence.

Graph 2 indicates the great improvement the deairation affected. This is further proof that the phenomenon observed is phosphorescence. When the concentration of glycine exceeds 7.5 % (1 M), the increase in phosphofluorescence with increasing concentration begins to level off. This is due to self-quenching. When there are too many species in solution, this phenomenon occurs.

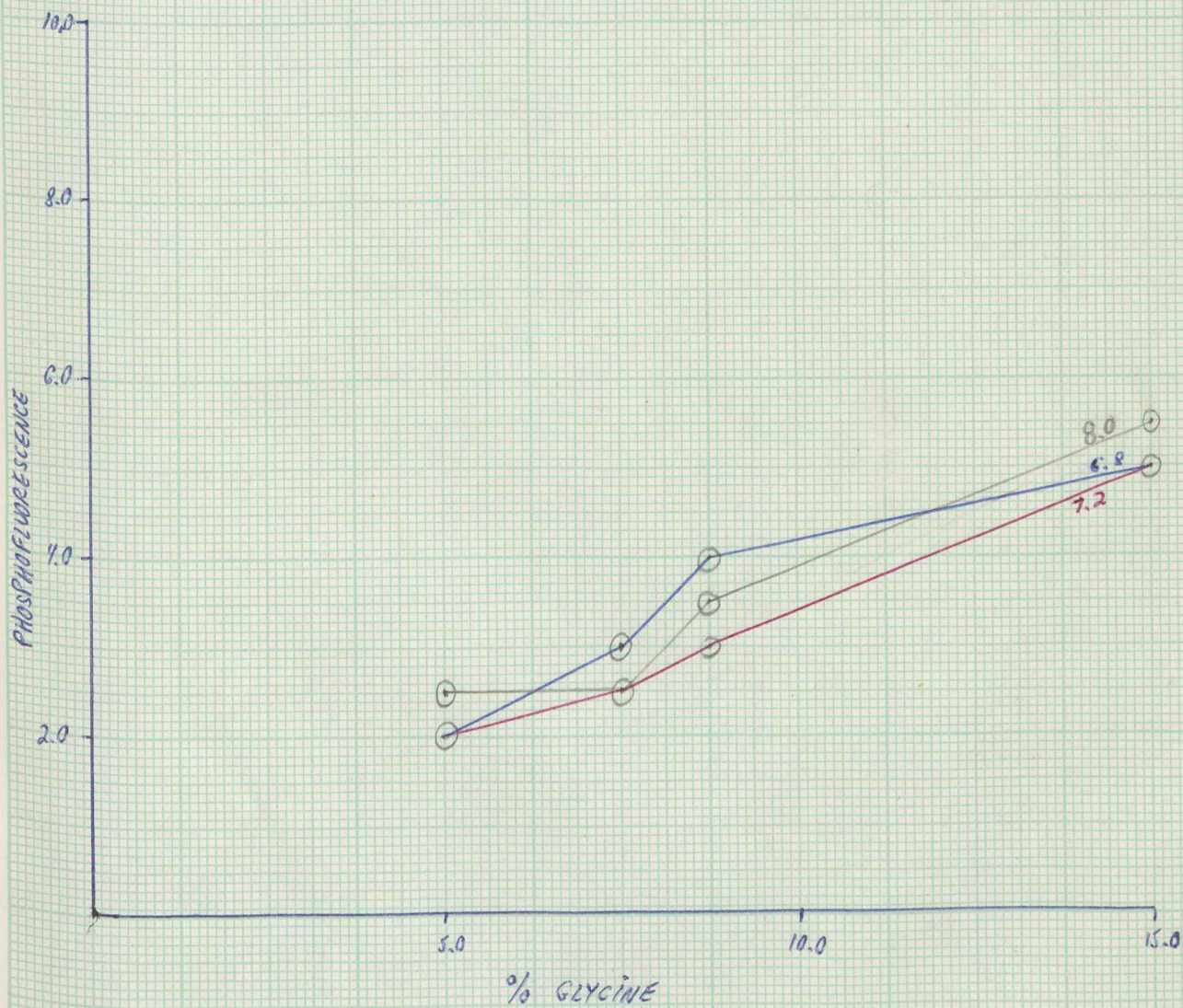
The next task was to try to show that the phosphofluorescence of glycine follows a straight-line plot at low concentrations. This experimenter tried

to show that at low concentrations (less than 1 M), the phosphofluorescence is directly related to the concentration of glycine.

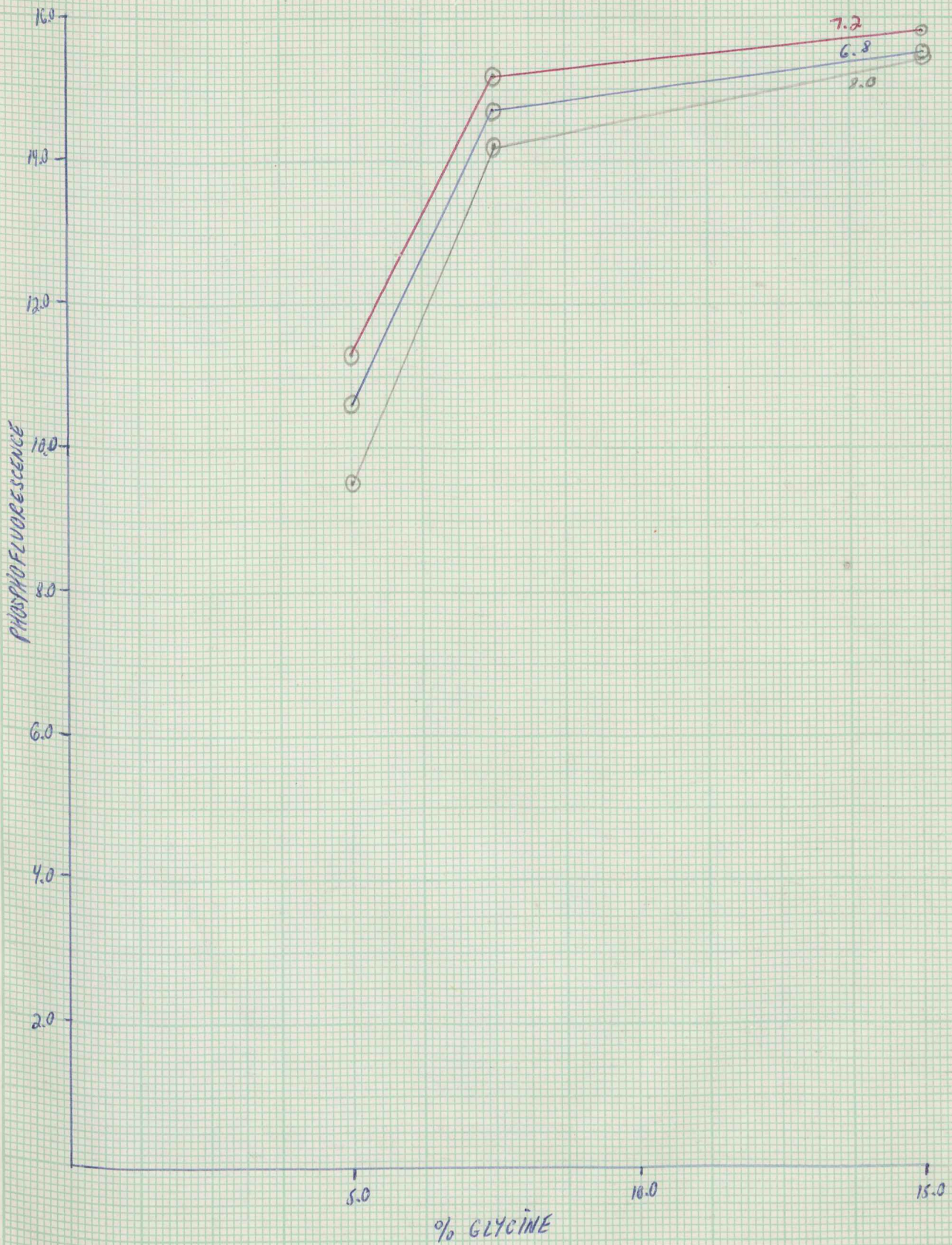
Graph 3 shows the results of this study, which was quite successful.

Graphs 2 and 3 likewise show the phosphofluorescence to be pH dependent. In all cases, the highest phosphofluorescence is shown at pH 7.2. This confirms earlier work done by others in this laboratory, and seems to indicate that the phosphofluorescence of the zwitter-ion is greater than that of either the conjugate acid or the conjugate base.

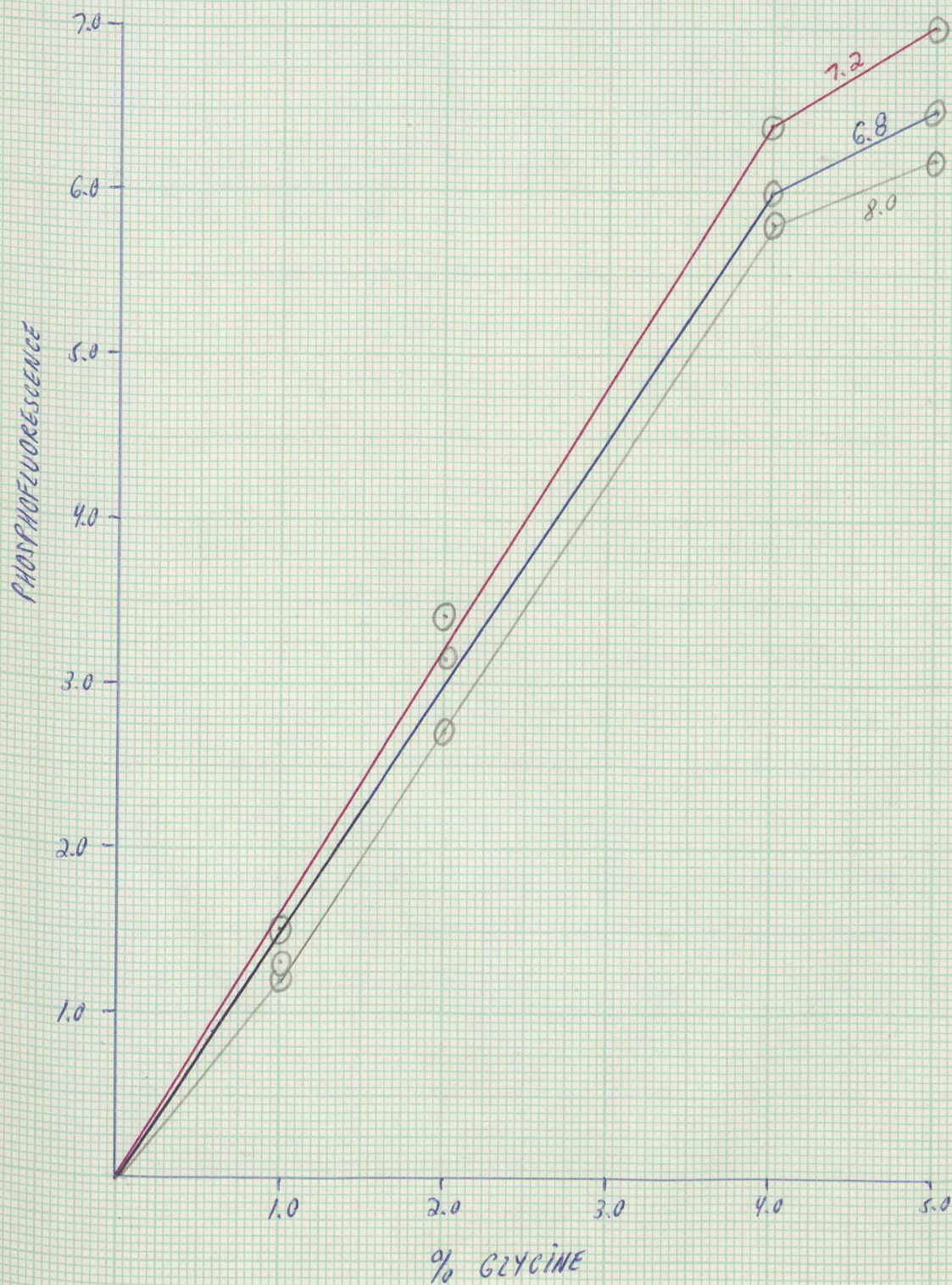
Graph 1: Early Studies of the Phosphofluorescence of Glycine



Graph 2: The Phosphofluorescence of Glycine: High Concentrations



Graph 3: The Phosphofluorescence of Glycine: Low Concentrations



FUTURE WORK ON THIS PROJECT

While a major hurdle in the completion of the scheme for the determination of glycine carbamate has been overcome, there still remains a substantial amount of work to be done before this project will be completed.

Preliminary work by this experimenter has shown that the disodium salt of glycine carbamate shows considerable phosphofluorescence in aqueous solution, with values approximately 100 times stronger than those recorded for glycine at the same concentration and pH. However, considerable problems remain before this compound can be fully characterized. The salt used in this laboratory appears to be contaminated with a strongly fluorescent material, possibly a phenolic, that shows a peak at approximately 380-400 nm. Recrystallization in ethanol is helpful in removing this impurity, but the yield is low and the starting material is expensive.

Other preliminary work bubbling CO_2 through glycine solutions shows a 10-15 % increase in phosphofluorescence. While this is encouraging, it is much smaller than expected if the carbamate reaction is occurring here. One possible problem that must be investigated is the work of Siegfried and Faurholt, which seems to indicate that the carbamate reaction occurs only at high pH. Whether their work is correct, and whether this is a phenomenon specific to glycine has yet to be determined.

SUMMARY

As a first step in the performance of an analytical scheme to determine glycine carbamate by spectrophotometric means, the phosphofluorescence of aqueous glycine solutions is studied.

Results show that phosphorescence plays a major role in the observed phenomenon, and that deaeration to remove oxygen from solution significantly improves the experimental data.

A family of curves is obtained for solutions with pH 6.8, 7.2, and 8.0 at concentrations of 15 % (2 M) glycine and less. For solutions of concentrations of less than 7.5 % (1 M) glycine, straight-line plots versus concentration are obtained.

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