NUCLEAR MAGNETIC RESONANCE STUDIES OF ENZYME-SUBSTRATE INTERACTION

by

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In partial fulfillment of the requirements for Ch 80

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ABSTRACT

The complex formed by the interaction of N-trifluoroacetyl-D,L-tryptophan with the enzyme alpha-chymotrypsin was studied, using nuclear magnetic resonance spectroscopy. The effect of the complex formation on the fluorine spectra of the D-isomer of the inhibitor is quite distinct, while no change is apparent with the L-isomer. The D-isomer appeared to be bound much more tightly than was the L-isomer. These results were obtained through studies of the change in line width of the resonance peak, and also studies of the downfield shift of the D-isomer resonance signal. It was shown that the D-isomer was sufficiently strongly bound to shorten the relaxation time by a factor of ten, and to increase the relative chemical shift of the D-isomer signal by 87 Hertz. The maximum effect occurs at pH 6.4.

Introduction

Recently, the mechanisms of enzyme action have received a great deal of attention. A number of experimental methods have been applied to study these enzyme-inhibitor systems. A popular, and very effective, technique has been nuclear magnetic resonance studies of either proton or fluorine nuclei on these compounds. This technique, combined with x-ray crystalography, has yielded a great deal of information about the nature of the active site of an enzyme. One enzyme which has been very conducive to study is alpha-chymotrypsin, which is found in a number of animals. A large number of substrates have been studied, and the amino acid D,L-tryptophan has often been used. This substrate is very sensitive to the conformation of the active site of the enzyme, and it is also one of the essential amino acids. Nuclear magnetic resonance studies yield parameters which characterize the interaction; for example, changes in magnetic environment (as measured by chemical shift), and changes in relaxation times (as measured by line widths). These two parameters are both affected, and reflect different characteristics of the enzyme-inhibitor interaction. Thus, both are valuable parameters for study.

Background

The nuclear magnetic resonance phenomenon depends

on the fact that protons, and certain other nuclei, have quantized spin states. These states are characterized by the magnetic quantum number M_s, and each state has a different energy in a magnetic field of magnitude H_o. These energies are given by:

$$1. \qquad E = M_s \cdot H_o \cdot \gamma$$

where γ is the magneto-gyric ratio for the particular nuclei. Transitions can occur between the energy states, accompanied by absorbtion of energy. Since these states are quantized, transition can only occur at certain energies, corresponding to the difference between the energies of the two states. This absorbtion of energy gives rise to the nuclear magnetic resonance signal. After a certain length of time, the excited state drops back into its original, energetically more stable state. This is done by transferring the added energy to other nuclei, or to the environment. This time constant which characterizes the decay of the excited state is the relaxation time of the resonance signal, and is very sensitive to the magnetic environment of the excited nuclei. The relation between the relaxation time and the lifetime of the excited state is shown in the Bloch formulation of the resonance phenomenon:

$$2. \qquad V = -M_o \frac{\gamma \cdot H \cdot T_2}{1 + T_2^2 [\omega - \omega]^2 + \gamma^2 H^2 \cdot T_1 \cdot T_2}$$

T_1 is the "spin-lattice" or longitudinal relaxation time,

while T_2 is the "spin-spin" or transverse relaxation time. T_1 is the measure of how long it takes the excited state to give up energy to the environment as translational, rotational, or vibrational energy. T_2 measures the length of time to exchange the energy between the excited nucleus and another nucleus, or any other local magnetic field, through dipole-dipole interactions. In solution, these two times are nearly identical, on the order of 0.1 seconds, for hydrogen.

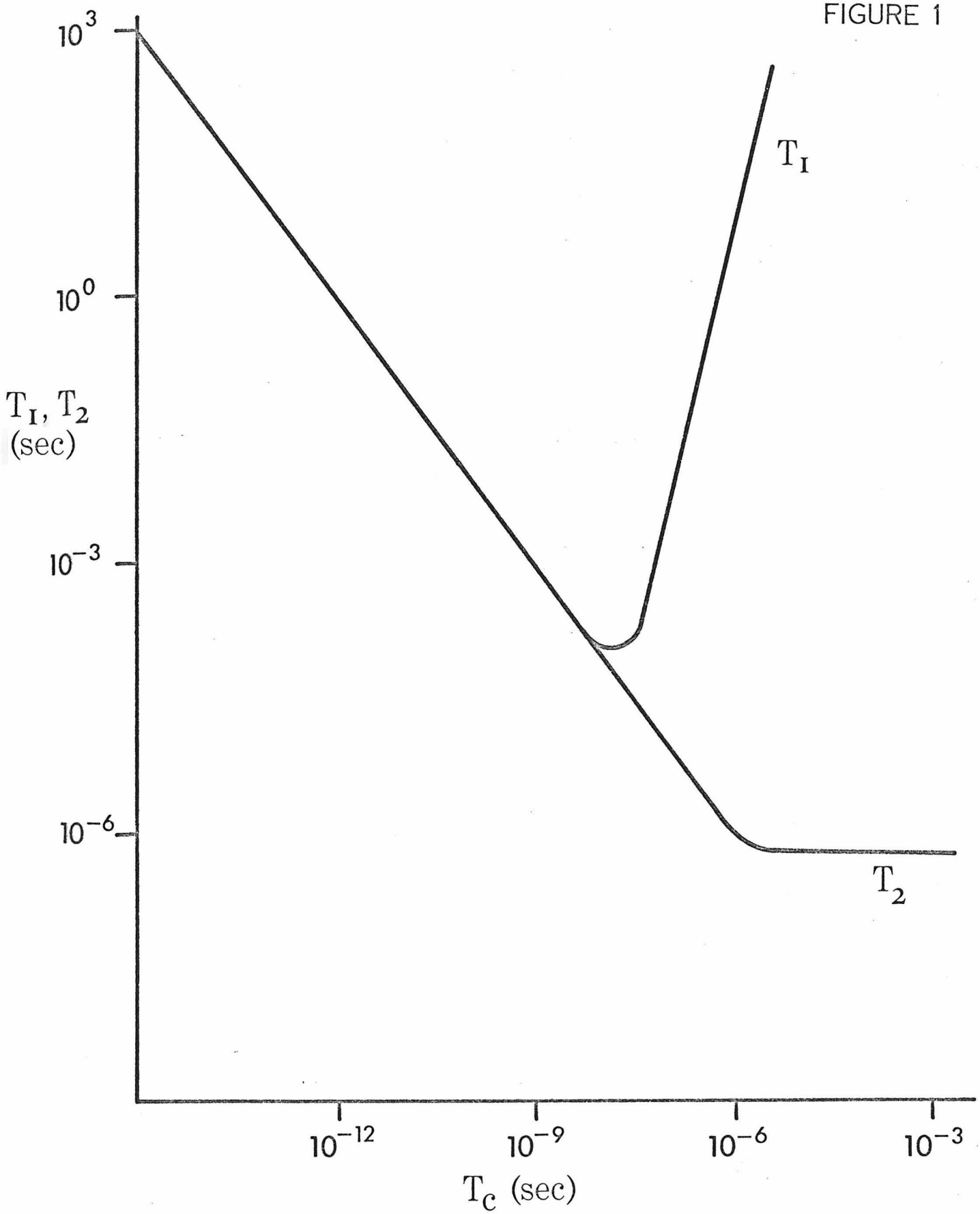
From the Bloch formulation, it can be shown that the relaxation time and the line shape of the signal are directly related. If we assume a Lorentzian line shape, the relaxation time is inversely proportional to the width of the peak at half the maximum height.

$$3 \qquad 1/T_2 = \pi \cdot \delta W$$

where δW is the width at half-height. The relaxation time is also related to the correlation time of a molecule. The correlation time is a measure of how rapidly the molecule turns over in solution; i.e. how long it takes for a molecule to "lose the memory" of its previous orientation in solution. Figure #1 shows the relation between correlation time and the relaxation times for hydrogen. (For a more complete description of the NMR phenomenon, see Appendix 1.)

The NMR phenomenon can be applied to many systems, including those of biological importance. One such system is the enzyme-substrate, or enzyme-inhibitor system.

FIGURE 1



Variation of Relaxation Times with Correlation Time

This interaction may be characterized by the equilibria

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \rightleftharpoons EP \rightleftharpoons E + P$$

or

$$E + I \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} EI$$

Since the magnetic environment is different at the active site of the enzyme, relative to solution, NMR is a powerful technique. Generally, certain nuclei of the substrate or inhibitor are studied, and the interaction is measured by observing the change in magnetic environment. When a nucleus has two possible magnetic environments, H_A and H_B, the equilibrium may be considered as

$$H_A \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} H_B$$

where the rate constants k_1 and k_{-1} are the same as in the enzyme-inhibitor interaction. This is because H_A is the magnetic environment of the molecule in solution, and H_B is the magnetic environment of the molecule, when bound to the active site of the enzyme. However, one must consider whether or not the exchange rate is sufficiently rapid. The exchange rate, also known as the "on-off" rate, is the measure of how rapidly a given molecule exchanges between the solution and the active site. If the exchange rate is rapid, then there is an averaging of the two environments, and the spectrum consists of one peak, whose position depends on the differences in the magnetic environments, and on the equilibrium constant. If, on the other hand, the

exchange rate is slow, there is no averaging effect between the two environments, and there are two separate peaks.

A measure of the exchange rate is the mean lifetime of the ES complex. If the mean lifetime, τ, of the complex is

$$\tau > \frac{\sqrt{2}}{\pi \cdot \Delta}$$

where Δ is the difference between the location of the resonance signals characteristic of the two environments, then the exchange rate is slow, and there are two separate peaks. However, as the mean lifetime decreases until

$$\tau < \frac{\sqrt{2}}{\pi \cdot \Delta}$$

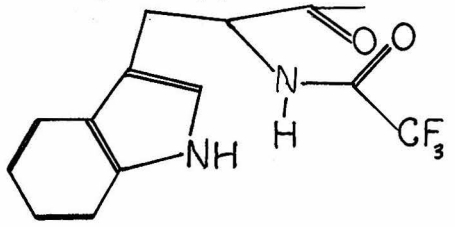
then the exchange rate is sufficiently fast that there is a single absorbtion which is the average of the two environments, appropriately weighted for relative populations. When the exchange rate is rapid, the observed spectrum represents an averaging of both magnetic environments, i.e. the positions of absorbtion, and also of the relaxation times, and hence of the line widths.

Two parameters measure the effect of enzyme-inhibitor interactions in NMR spectroscopy. The first parameter is δ. This value represents the actual change brought about by a specific amount of enzyme on a specific amount of substrate. This parameter is dependent on the concentrations of both components, the pH, and the strength of interaction. Δ is the second parameter

characterizing the interaction. This quantity, also known as the slope, is the value which δ would take if all of the substrate present were complexed; i.e. this parameter characterizes the magnetic environment H_B. The pH dependence of Δ provides an excellent means of studying the interaction, and its dependence on pH. Both relative chemical shift, and line broadening can be investigated using these two parameters.

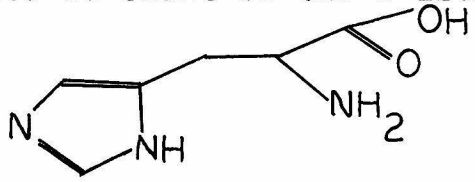
There are several possible sources of the binding interaction between enzyme and substrate. Hydrogen bonding between parts of the active site of the enzyme and certain parts of the substrate is a major interaction. A second factor is electrostatic attraction between the active site and the substrate. Also, dipole-dipole interactions may have some role in the binding.

These considerations may be applied to many specific systems, one of which is the interaction of the enzyme alpha-chymotrypsin and N-tfa-D,L-tryptophan.



This particular inhibitor has several advantages for study. Gerig and co-workers[1] have demonstrated that the amino acid D,L-tryptophan interacts strongly with the enzyme alpha-chymotrypsin, so we would expect the trifluoroacetyl derivative to do so also. Secondly,

the substance exists as two isomers, D and L-tryptophan. Previous studies with D and L isomers[2] show that, in general, the D-isomer binds significantly more strongly to the enzyme than does the L-isomer. Thus, the L-isomer can serve as an internal standard in the systems, for measurements of both the shift of the resonance signal, and the broadening of this signal. Interaction between the D-isomer and the active site of the enzyme causes deshielding of the trifluoroacetyl group of the D-isomer, and causes a downfield shift of the resonance signal. Histidine 57 is one of the amino acids at the active site of alpha-chymotrypsin. The ring currents of the imidazole group of this amino acid, in conjunction with hydrogen bonding, may be a large factor accounting for the deshielding. Other factors, including the bulk diamagnetic susceptibility of the enzyme may also contribute to shift of the D-isomer.



The position of the L-isomer is taken as the position of zero shift; also, the line width of this signal is taken as the standard against which the change in the line width of the D-isomer signal is measured. By doing this, any overall change in conditions which affect both isomers equally will be eliminated, and only effects arising from the interaction will be considered.

Effects which might affect both isomers equally would be a change in viscosity of the solution, due to the addition of the enzyme. Thus, the study of the enzyme-substrate system can be reduced to the study of the splitting of the D and L-isomer signals, and the change in the line width of the D-isomer signal relative to the signal of the L-isomer.

The trifluoroacetyl derivative is used, so that fluorine resonances may be studied rather than the proton resonances. The advantage of this is twofold. First, the fluorine nuclei are much more sensitive to the small changes in magnetic environments than are protons, so that the effect on the D,L splitting is greater and more easily measureable. This added sensitivity is essentially a result of the fact that the fluorine atoms have occupied p-orbitals, which extend further, are more diffuse, and so are more sensitive to environmental perturbations than the 1s orbitals, which lie close to the nucleus.

Secondly, in N-tfa-D,L-tryptophan, there are many hydrogen absorbtions, for the many hydrogen nuclei in the molecule. However, there is just one fluorine resonance, for the three fluorine nuclei are magnetically equivalent. Also, there is negligible spin-spin coupling to the fluorine atoms, whereas such coupling would greatly complicate the absorbtion of hydrogen nuclei elsewhere in the molecule.

There is just one overall factor which affects the change in line width of a signal; namely the degree of freedom of the fluorine nuclei. The magnitude of the parameter δW is dependent on how tightly bound the molecule is, for the binding makes transfer of energy to the environment much easier.

The interaction may be studied quantitatively, by using the parameters δ and Δ which were introduced earlier. If we take E_o and S_o as the initial concentrations of the enzyme and the substrate, and ES as the amount of enzyme-substrate complex, there is the relationship

$$4. \qquad ES = \frac{\delta}{\Delta} S_o$$

This can be justified if we refer back to page 6, where δ was given as the average of the two magnetic environments, appropriately weighted for relative populations, and Δ was the value of δ if all the substrate present were in the form of enzyme-substrate complex. From this relationship, expressions for E and S can be found, which are the concentrations of free enzyme and free substrate at equilibrium:

$$5A. \qquad E = E_o - ES = E_o - \frac{\delta}{\Delta} S_o$$

$$5B. \qquad S = S_o - ES = S_o - \frac{\delta}{\Delta} S_o$$

There is also the expression for the dissociation constant for the system:

$$6. \qquad K_s = \frac{E \cdot S}{ES}$$

Combining the above relationships, a quadratic equation can be derived for ES, as a function of E_o, S_o, and K_s, all of which are measureable quantities:

$$7. \quad [ES]^2 - ES \cdot [E_o + S_o + K_s] + E_o S_o = 0$$

This equation may be solved, using the general solution for a quadratic equation. For each data point, consisting of E_o, S_o, and δ, we can now plot ES/S_o versus δ. From equation 4, we see that the slope of this plot, using a least squares fit, is Δ. Also associated with this value is a least squares error. However, all of the above calculations assume a value for K_s.

There are several methods which may be used in assuming a value of K_s. One technique is to use a computer program which calculates Δ and the least square error in Δ, using the above method, for each value of K_s, from $K_s = 10^{-9}$ Molar, to $K_s = 10^{1}$ Molar. The value of K_s which yields the smallest least square error between the observed values of δ and the values of δ calculated from equation 4 is taken to be the value of K_s at this pH.

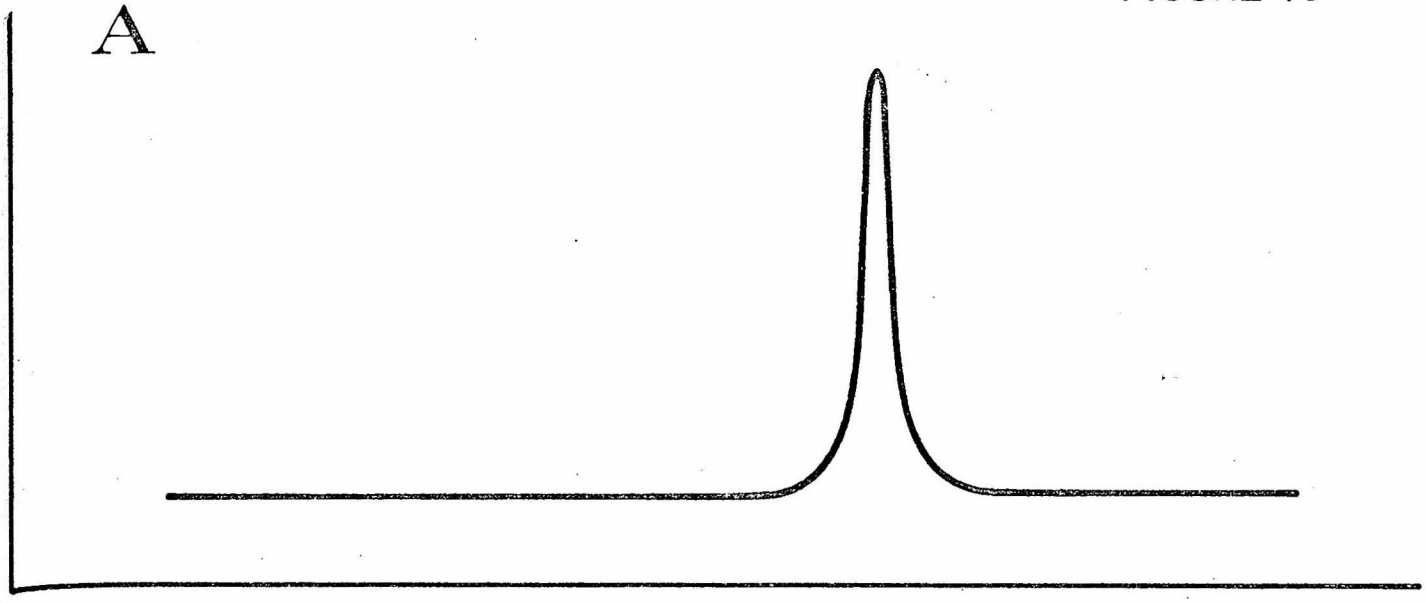
Another technique is to assume a very simple dependence of K_s on pH. In one case, a constant value of K_s was used throughout. An equilibrium dialysis was done by Johnson and Knowles[3], who arrived at the value of $2 \pm 1 \times 10^{-3}$ M., for the interaction of D,L-tryptophan with alpha-chymotrypsin. Assuming that the trifluoroacetyl derivative of this compound is not too dif-

ferent, a reasonable value for K_s is 3.16×10^{-3} M. Both methods of assuming K_s may be used, and from K_s, a value of Δ can be found.

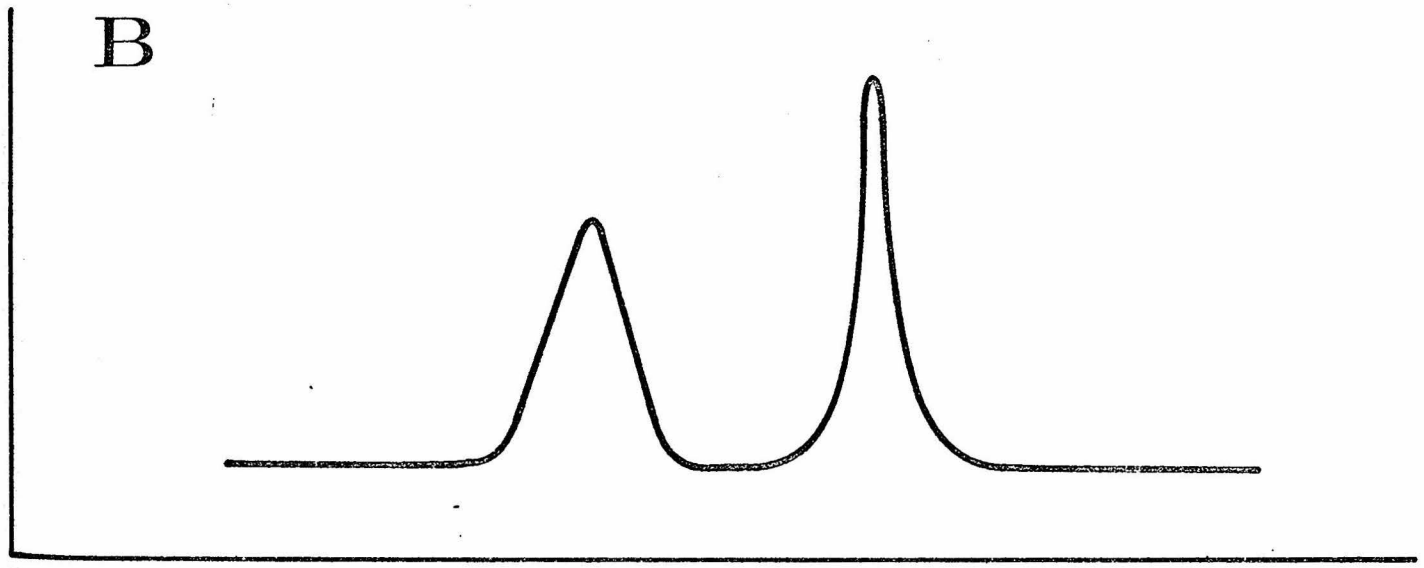
Results

The most qualitative results are demonstrated by the comparison of the spectrum of the substrate alone, with the spectra of the enzyme-substrate complex (see Figure #10). The spectrum of the substrate alone was one sharp peak, with a line width of slightly more than one Hertz. The addition of the enzyme had definite, readily observable effects. The peak corresponding to the fluorine resonance of the L-isomer did not shift its position at all. Also, the line width of this signal did not increase significantly. However, the peak corresponding to the D-isomer shifted downfield by a substantial amount; when S_o = 0.0050 moles/liter, the shift was 13.5 Hertz. At high substrate concentrations, S_o = 0.0200 m/l, the splitting was 4.0 Hz. Also, the line widths of the D-isomer signal increased as the substrate concentration decreased, in the presence of the enzyme. At S_o = 0.0050 m/l, the difference between the line widths of the D and L-isomer signals was about 2.0 Hz. This represents a change of from 100% to 200% of the line width of the compound in solution. Table #1, in the appendix, gives all of the individual data points—

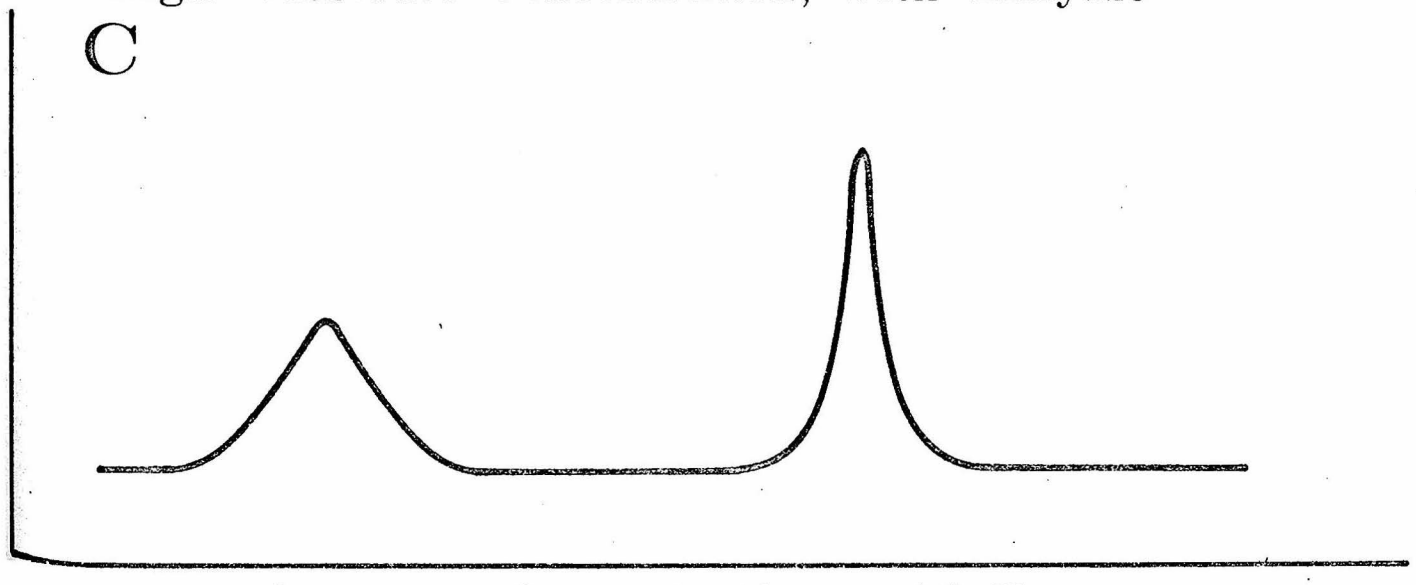
FIGURE 10



Zero Enzyme Concentration



High Substrate Concentration, with Enzyme



Low Substrate Concentration, with Enzyme

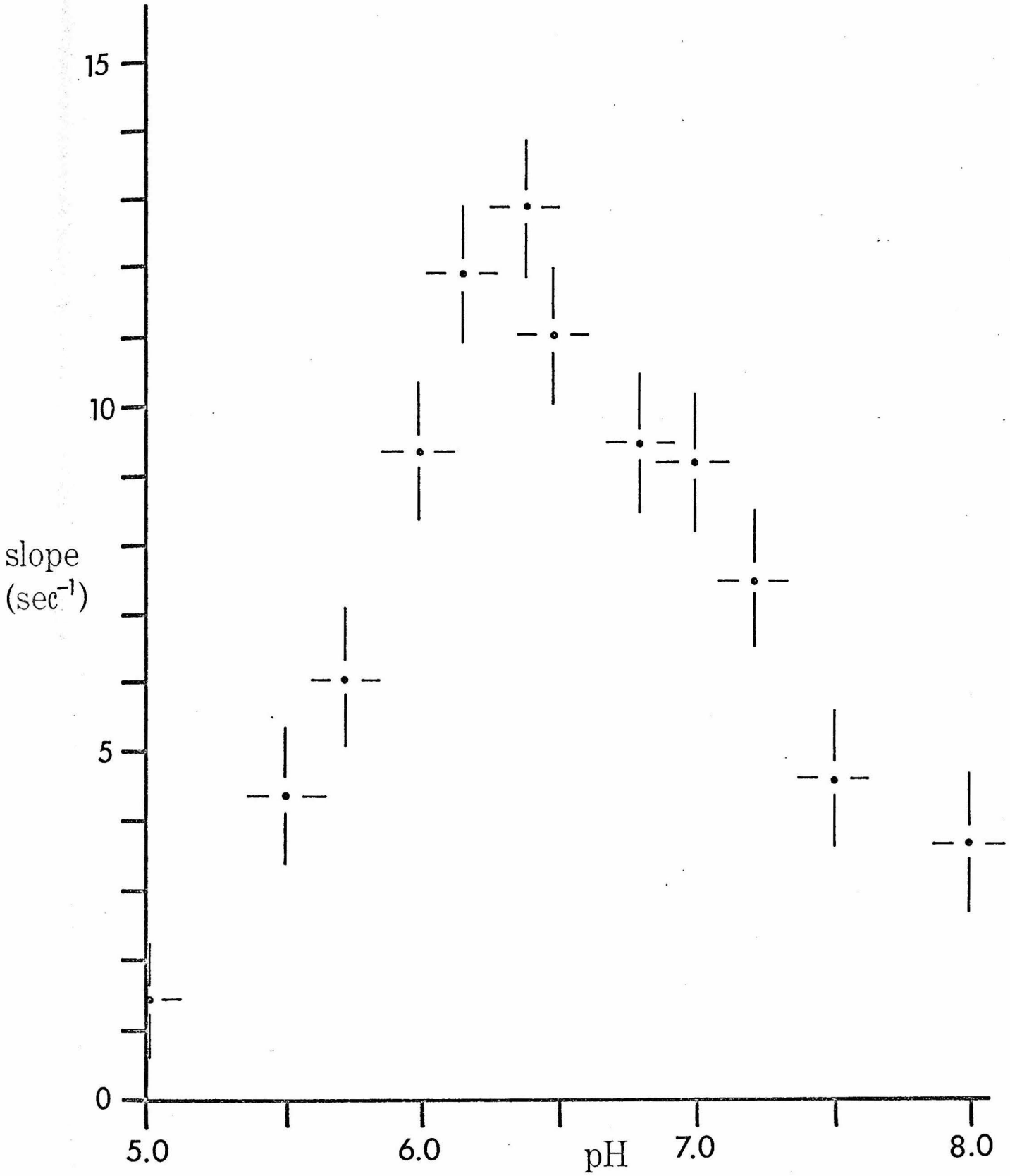
E_o, S_o, δW, and d,l splitting—for all binding curves.

The quantitative study was divided into two sections; namely, the relaxation time results and the D,L splitting results. In the pH range from 5.0 to 8.0, about 20 binding curves were run; at some pH values, several binding curves were run. Throughout the runs, the enzyme concentration remained constant. The uncorrected concentration of the enzyme was 0.0025 moles/liter. However, to obtain this value, we assume that the enzyme is one hundred per cent active, which was not true. Titrations were run to find the actual activity of the enzyme. The results were constant, at about 70% activity. When we took this into consideration, the corrected concentration of the enzyme, E_o, became 0.00175 m/l.

In a binding curve, at a fixed pH, from 5 to 7 data points were taken, each with a different substrate concentration. The substrate concentrations, or actually the concentrations of the D-isomer, ranged from 0.0050 to 0.0200 m/l.

The value of the overall parameter Δ for the line width broadening was calculated at each of the pH values, and where applicable, an average of Δ for several binding curves was taken. If we assume the equilibrium constant to be $K_s = 3.16 \times 10^{-3}$ M., then there is a smooth curve in plotting Δ against pH (see Figure #2). The maximum value of the slope was 12.9 $sec.^{-1}$, at pH

FIGURE 2



$1/T_2$ Slope Versus pH, at Constant K_S

6.4. The shape of the plot was a very sharp rise, to the maximum, and then a sharp decline; rising from a slope of 1.3 Hz. at pH 5.0, and dropping from the maximum to 3.7 Hz at pH 8.0.

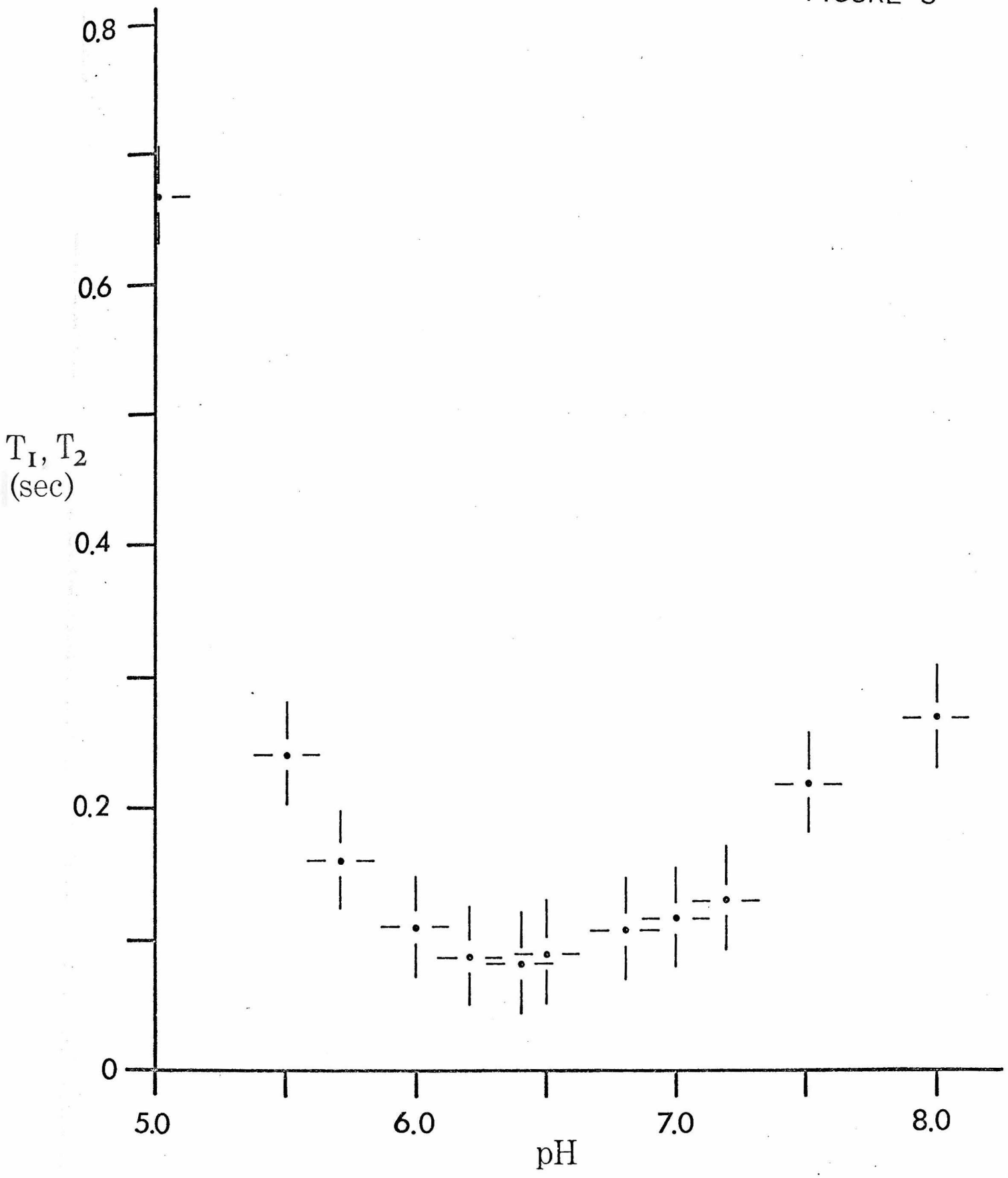
The parameter Δ has a direct relationship to the relaxation time T_2. This relationship is (see Eqn. 3)

$$1/T_2 = \pi \cdot \Delta$$

From this, T_2 can be plotted versus pH, or $\pi \cdot T_2$ versus pH. This figure, #3, shows a sharp mimimum at pH 6.4, and at this point the relaxation time is approximately 0.03 seconds. All of these results for T_2, however, were calculated assuming that $K_s = 3.16 \times 10^{-3}$ M.

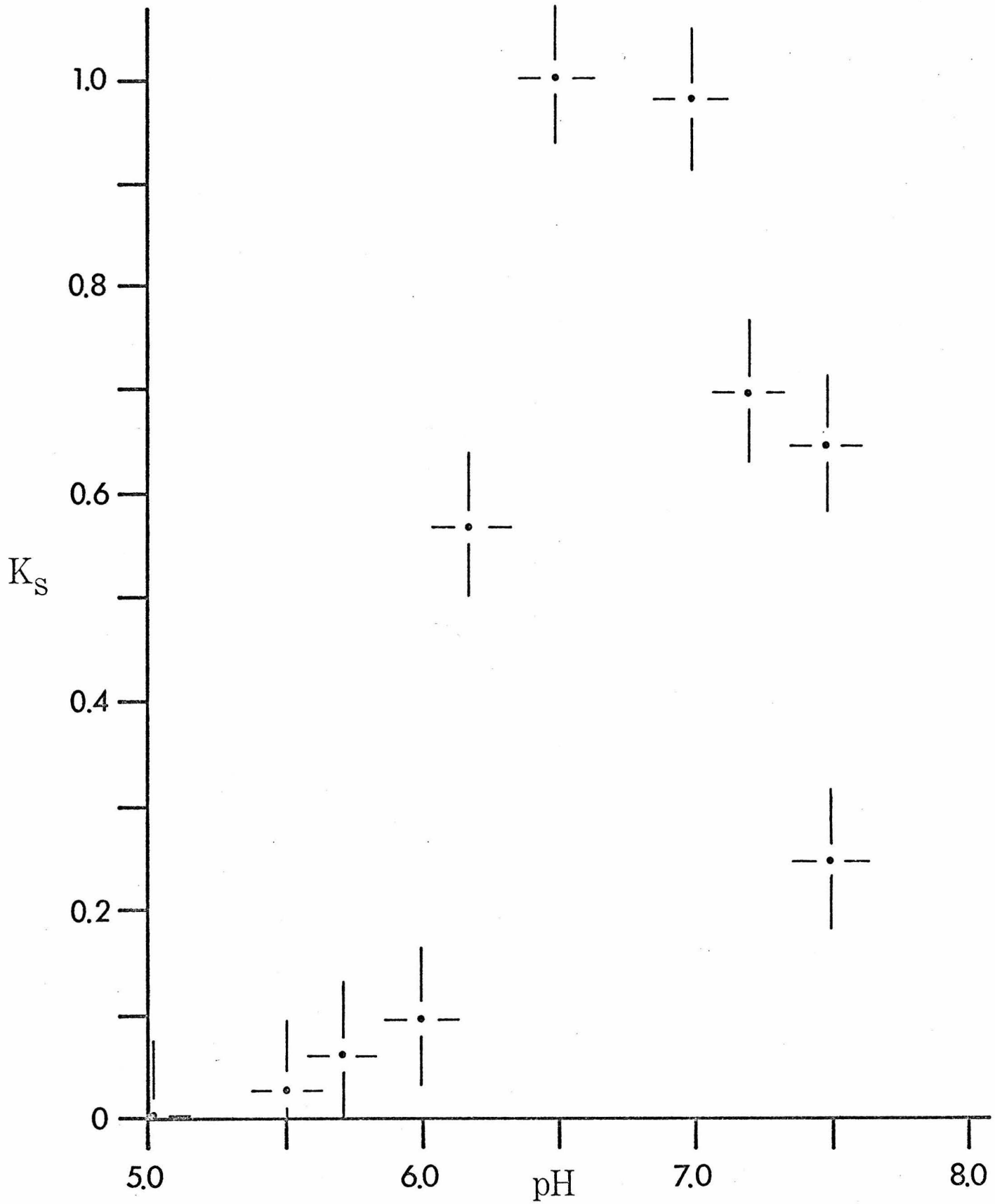
The computer arrived at different values for the value of K_s and of the slope. In the search for the K_s which yielded the minimum error in the slope, the computer found K_s to be very pH dependent. This dependence, shown in Figure #4, is very much like the plot of slope versus pH at constant K_s. As a result, the plot of slope versus pH, using the computer values of K_s, had a shape very similar to the plot at constant K_s, but the magnitude of the values was very different. The maximum value of the slope was found as 20,000 Hz, corresponding to a K_s of 1.0 M. This slope yields a relaxation time of 1.7×10^{-5} seconds. From the order of magnitude of these values, we suggest that the computer did not find the right values for K_s, and, as a result, found extremely high values for the slope Δ.

FIGURE 3



Relaxation Time Versus pH

FIGURE 4



Computer Values of K_S Versus pH

One reason that these values are not reasonable, is that a relaxation time T_2 of 10^{-5} seconds corresponds to a correlation time on the order of 10^{-5} seconds. However, the correlation time for the enzyme in solution has been determined to be much faster than this, on the order of 10^{-8} seconds, and it is impossible for the substrate to have a correlation time much slower than the enzyme to which it is bound.

There is an explanation regarding the computer's trouble in finding the correct value of K_s. The computer sought the value of K_s which gave the smallest least square error in the slope. For the D,L splitting, this minimum was sharp and distinct. However, for the relaxation time results, there was no sharp minimum at any K_s. Rather, there was at best, just a slight dip in the plot of least square error versus Log K_s. Since there was no distinct minimum, the computer could not find a consistent K_s. In figure #11, both of the error versus Log K_s are shown, and from this figure, the differences are clear.

However, the shape of the computer plots of slope versus Log K_s tells a great deal. By comparing these plots at a number of pH values, we see that the shapes of these curves are nearly identical throughout, and that the only difference is the baseline—the value of the slope at $K_s = 10^{-9}$ M. One of these plots is shown in Figure #5. If the baseline is plotted against pH, (see Figure #6), the shape of the plot is just that of

FIGURE 11

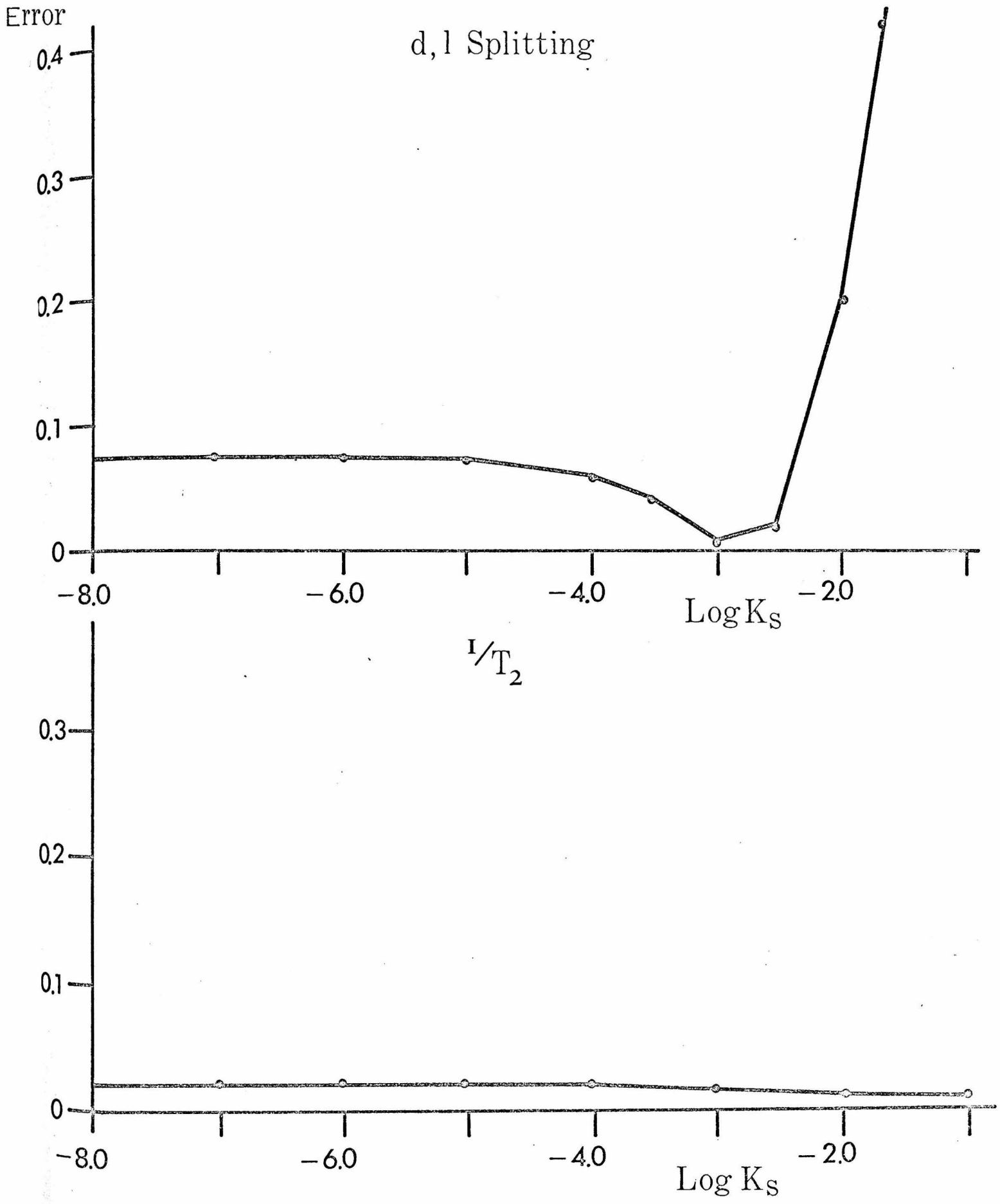
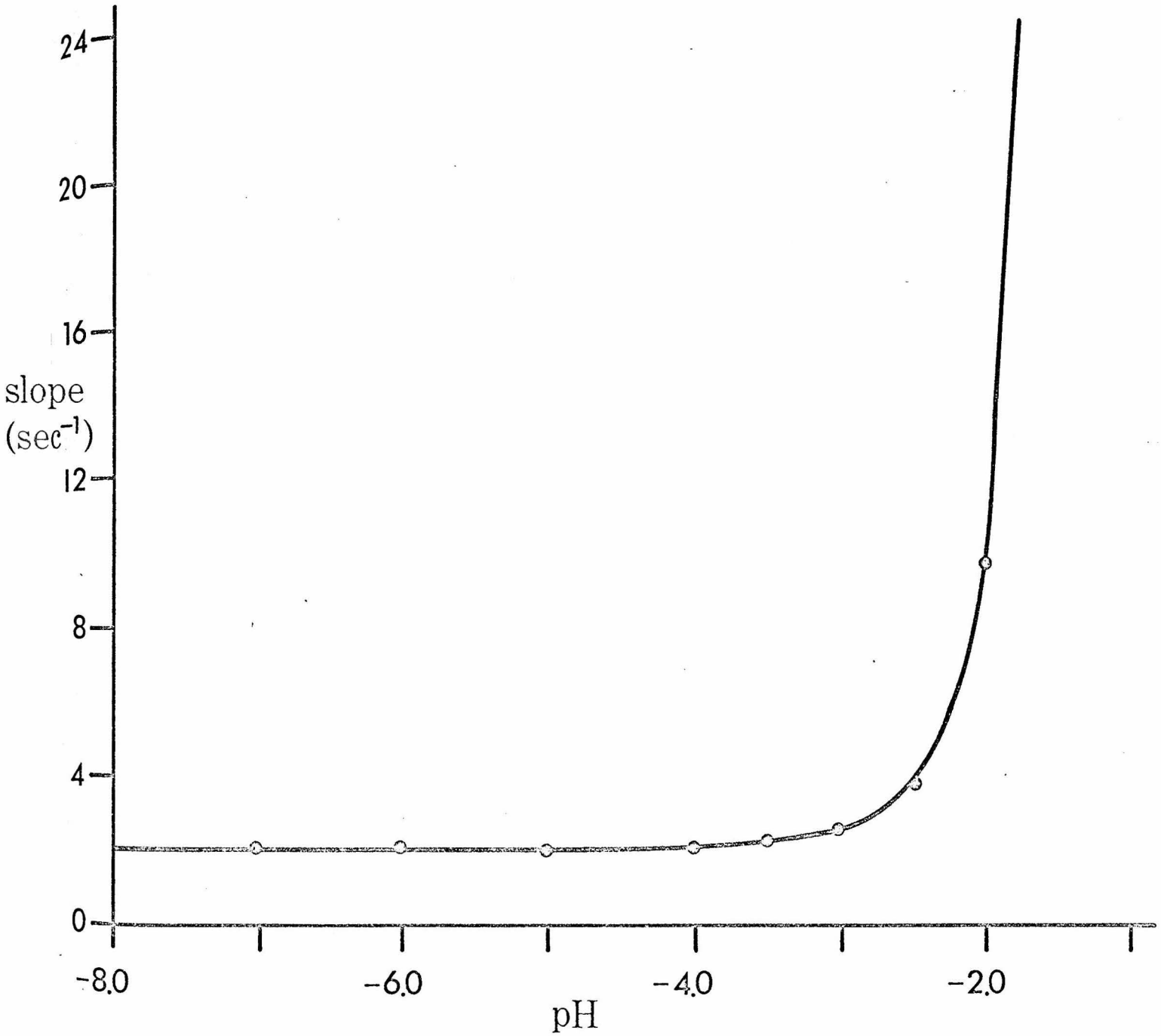
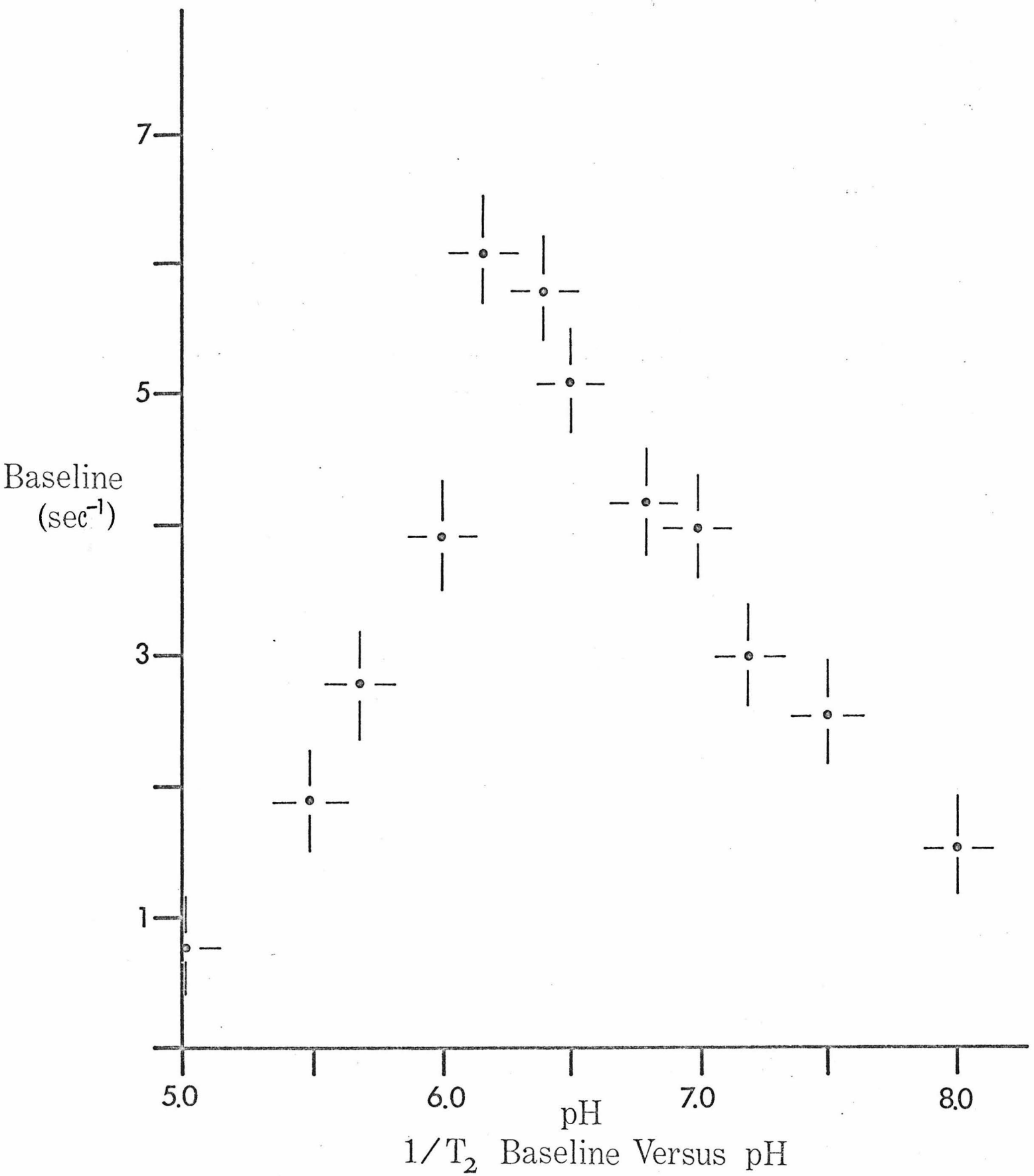

Error
d, l Splitting
0.4
0.3
0.2
0.1
0
−8.0
−6.0
−4.0
−2.0
Log K_S
$1/T_2$
0.3
0.2
0.1
0
−8.0
−6.0
−4.0
−2.0
Log K_S

FIGURE 5



Sample Computer Plot of Slope Versus LogK_S at pH 5

FIGURE 6



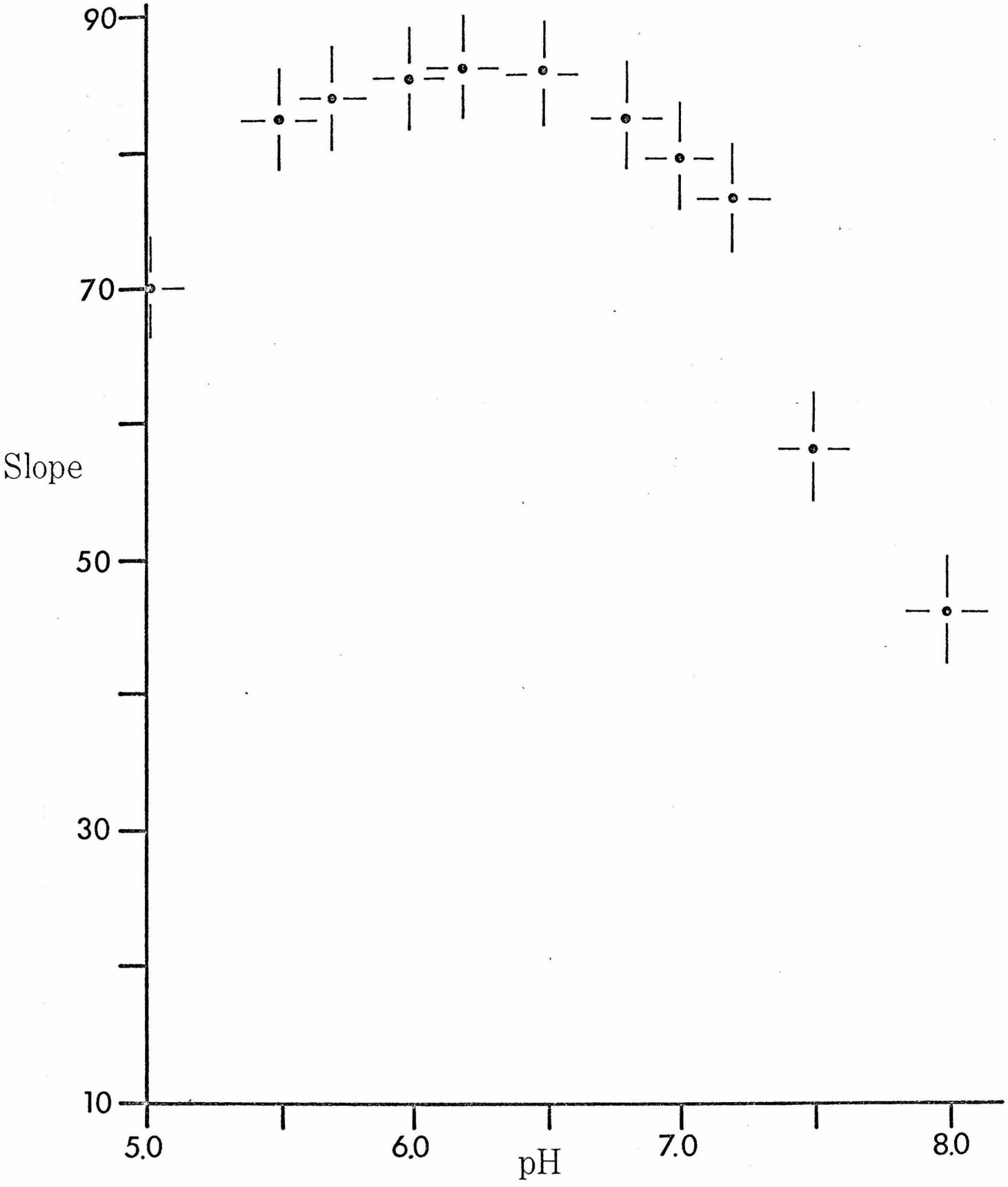
$1/T_2$ Baseline Versus pH

the slope versus pH plots. The magnitude of the baseline is lower than the slope at each pH, but the shape is identical, which suggests that the baseline is also a valid quantity for qualitative examination. So, the shapes of all three plots—slope (computer K_s) vs pH, slope (constant K_s) vs pH, and slope ($K_s = 10^{-9}$ M.) vs pH—are the same. Since, from equilibrium dialysis, K_s appears to be approximately 3×10^{-3} M., the assumption that $K_s = 3.16 \times 10^{-3}$ should introduce no qualitative error into the results, and only a very small percentage error in the quantitative results.

The calculations were also made using the D,L splitting data and Δ parameter. The plot of slope versus pH, assuming a constant K_s of 3.16×10^{-3} M., is similar to that found from relaxation time calculations. There are two minor differences. The first is that the entire curve is shifted upward. The maximum is still found at pH 6.4, but is now about 87 Hz. The second difference is the sharpness of the curve in the pH range 5.0 to 6.4. For the $1/T_2$ versus pH plot, there is a very sharp increase over this pH range. However, for the D,L splitting, there is just a slight increase over this pH range (see Figure #7). The values for D,L splittings run from about 70 Hz at pH 5.0 to the maximum of 87 Hz at pH 6.4. The drop in the slope in the pH range 6.5 to 8.0 is quite sharp, analogous to the results from the relaxation time data.

This D,L splitting data was analyzed through the

FIGURE 7

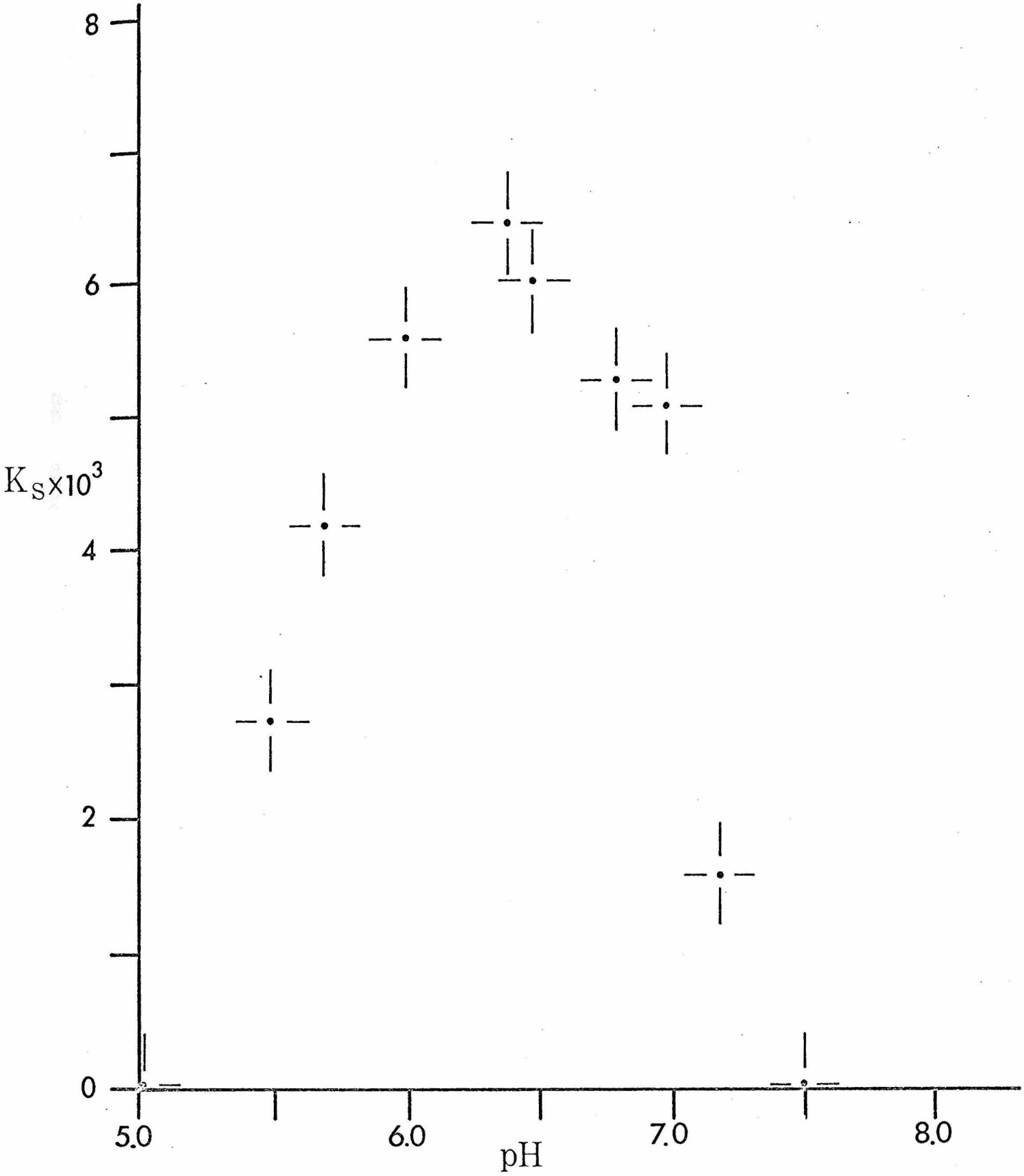


d,l Splitting Slope Versus pH

computer program, with similar results. Again, the computer-selected values for K_s were dependent on pH, although the values selected were near the equilibrium dialysis value. The computer range for K_s was from 1.0×10^{-3} M., to 7.0×10^{-3} M. (see figure #8). The assumed value of 3.16×10^{-3} M falls in the middle of this range. Since the selected values were close to the assumed value of K_s, the computer values for the slope were also close. These computer values are shown in Figure #9. It appears that the shape of the plot is the same as the shape at constant K_s, and that there is a small shift upward of the entire curve. The maximum now is about 1.4 ppm, or about 130 Hz, and still occurs at pH 6.4. The computer plots of the D,L splitting slope versus K_s showed the same shape throughout, and only differed by the value of the baseline, $K_s = 10^{-9}$ M. These values for the baseline, when plotted against pH, showed the same shape as the other D,L splitting slope plots. These three plots suggest that the shape of the curve is correct, and that the magnitude is approximately that at $K_s = 3.16 \times 10^{-3}$ M.

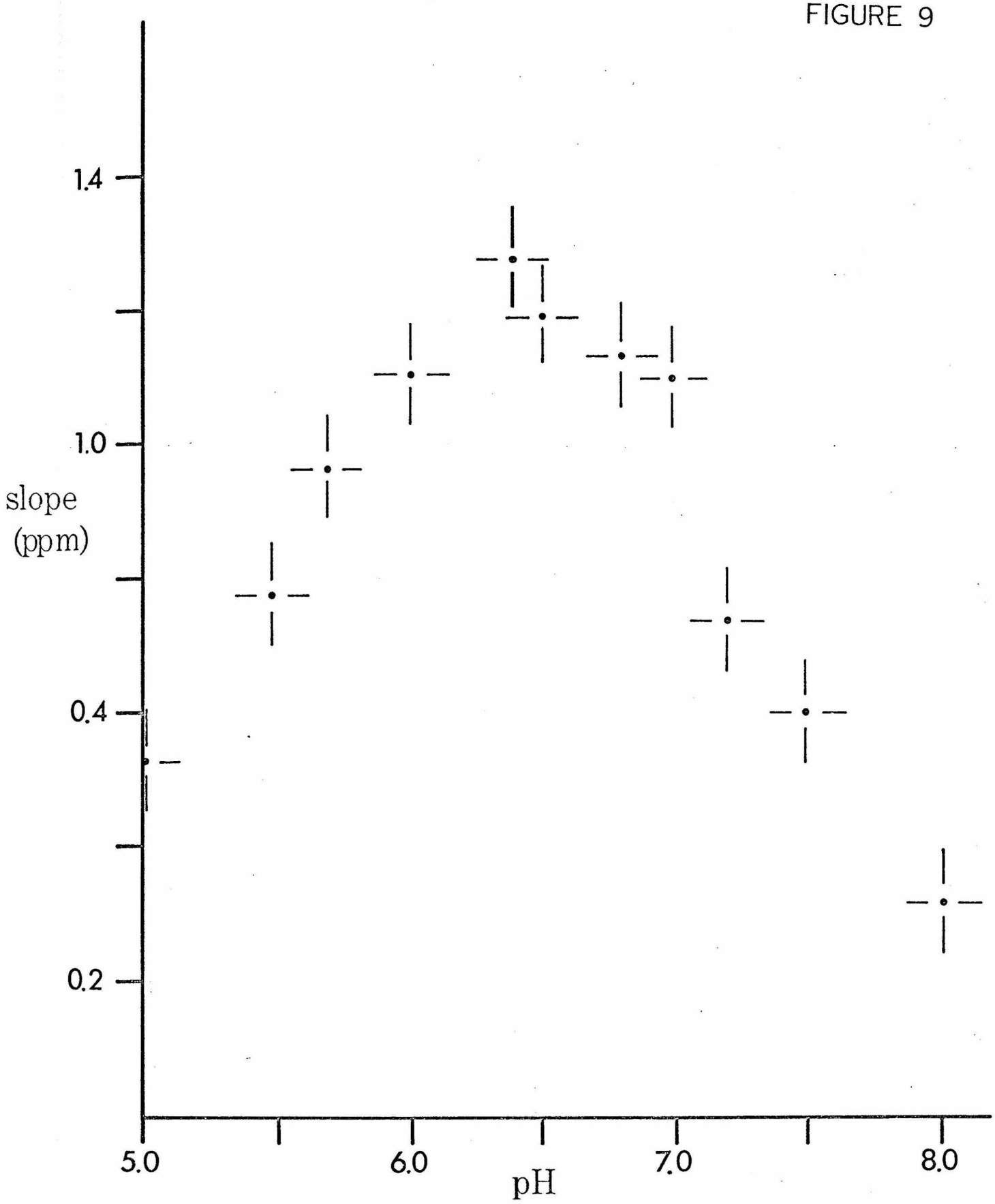
The value of the intercept in the computer plot of ES/S_o versus δ is another quantity which needs consideration. This plot is linear with a slope equal to Δ. We know that this plot should include the origin, for at zero enzyme concentration, and a non-zero substrate concentration, the effect on either the line broadening or the D,L splitting is zero. So, the intercept is a

FIGURE 8



Computer-Selected K_S Versus pH, d,l Splitting Parameter

FIGURE 9



Computer-Selected Values of d,l Splitting Slope Versus pH

measure of how close the actual line came to the origin, and is a measure of the error in the experiment. Throughout the experiment, a certain experimental error has been assigned to each value of the slope. The intercept should be within this experimental range of the origin. The intercepts at $K_s = 3.16 \times 10^{-3}$ M are the values studied. From tables #2 and #3, we can see that the intercepts are within the experimental error value which was assigned to the slopes. The assigned error for the line width was taken as ± 0.5 Hz., while for the D,L splitting slope, the error was taken as ± 3 Hz.

Another approach may be applied to this problem. This is to include the origin as one of the data points when the computer is analyzing the data, and finding the value of the slope. This procedure can be justified in that it is known that this point should fall on the line, and should be considered. Also, it is argued that the inclusion of this point would make selection of K_s more difficult, and the more accurate K_s would be selected. In herent in this method is the fact that the intercept would be smaller than when the origin is not included. The argument against this technique is that the intercept provides an excellent means of observing the error in the experimental values, which provides a check on the experiment. This measure of the error is lost if the origin is included as a data point.

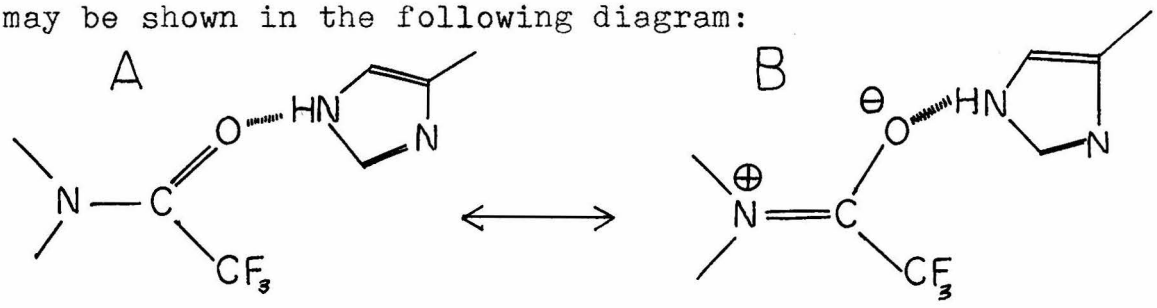
Nevertheless, if the intercept is within the ex-

perimentally assigned error, the differences in the slopes in these two methods should not be large. Five binding curves were analyzed again, this time including the origin. The result was a consistent shift of the values for the slope downward, by some 10%. This shift was not entirely uniform, but the general shape of the pH plots were maintained. An interesting point is that the K_s values selected by the computer for the D,L splitting, under these circumstances, were not improved.

Discussion

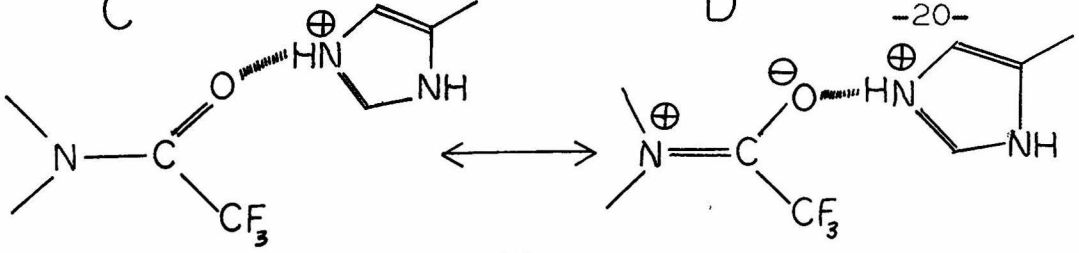
Several factors affect the interaction of the enzyme and the substrate, some of which affect both the relaxation time and the D,L splittings, while other factors affect only the relaxation times. If hydrogen bonding is a major source of interaction between the enzyme and the substrate, then the results can be explained in the following manner:

The imidazole group of Histidine 57 interacts with the trifluoroacetyl group of the substrate through hydrogen bonding. The imidazole group can exist in two forms, neutral and protonated. The overall interaction may be shown in the following diagram:



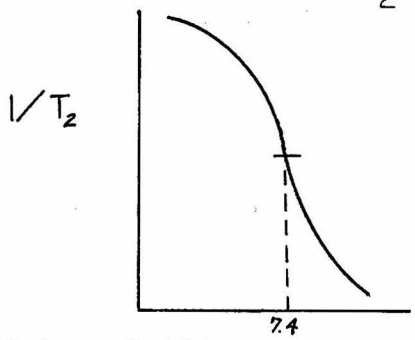
I

C D



II

At high pH values, complex I is prevalent, while at lower pH values, complex II dominates. Canonical form D makes a greater contribution to complex II than form A does to complex I. As a result, there is more double-bond character to the carbon-nitrogen bond in complex II than in complex I. This double bond character hinders rotation about the carbon-nitrogen double bond, increasing the rigidity of the molecule, and shortening the relaxation time. If this were the only factor affecting the interaction, we would have a $1/T_2$ versus pH curve of the type:



The half-height of this curve would occur near pH 7.4, for this pH value is the pK_a of the imidazole group. At this p^H, equal amounts of complex I and complex II are present.

However, in the pH range below 6.5, other factors work to lower the $1/T_2$ slope, and increase the relaxation times. One factor may be conformational changes in the active site of the enzyme in the pH range 5.0 to 6.5. If this were the case, there would not be as good a fit of the substrate on the enzyme as there was

at higher pH values. Since there would be a less perfect fit, there would also be a weaker interaction, and with a weaker interaction is associated more freedom of movement, and longer relaxation times. This imperfect fit would seem to negate the increase of hydrogen bonding at low pH values, below 6.5, and lengthen the relaxation time significantly.

A similar analysis may be applied to the results for D,L splitting. We observed that the D-isomer signal was shifted downfield relative to the stationary L-isomer signal, when the D-isomer interacted with the enzyme. The downfield shift corresponds to a deshielding of the fluorine nuclei. One cause of this deshielding may be the ring current of the imidazole group of Histidine 57. A ring current will deshield when the fluorine nuclei are held in the same plane as the ring, and will shield when the nuclei are held perpendicular to this plane, i.e. above the ring. If the orientation is correct, the deshielding effect will increase as the fluorine nuclei are held more tightly, and more closely to the ring. Also, there will be a greater ring current in the protonated form of the imidazole group, than in the neutral form. This is because in the neutral form, charge separation is necessary to delocalize the pi electrons, and set up a ring current. However,in the protonated form, charge separation is not necessary, and as a result, the ring current will be greater, and the deshielding will be greater.

Another effect increasing the deshielding with lowering pH is that in complex II, there will be more electron withdrawal from the amide than in complex I. This will also contribute to a greater deshielding effect with complex II than with complex I. Both of these factors, ring current and electron withdrawal, may explain the behavior of the D,L splitting slope in the pH range 6.5 to 8.0.

An imperfect fit of the substrate in the enzyme at lower pH values would hold the substrate further away from the ring current, and would also possibly change the angle of orientation of the substrate relative to the ring, and the deshielding effect would decrease. This would account for the slight decrease in the slope over the pH range 5.0 to 6.5.

The results suggest that the effect of the imperfect fit of the substrate in the active site of the enzyme at lower pH values affects the relaxation times more than the D,L splittings. Other factors may also have some effect on the interaction of the enzyme and the substrate. These include electrostatic attraction, and dipole-dipole interactions, both of which are pH dependent factors.

Conclusions

Several conclusions may be drawn from the results of this experiment. Clearly, the enzyme has a signif-

icant effect on the D-isomer of N-tfa-tryptophan, which is demonstrated through changes in the line width, and also through the shift of the signal from the L-isomer signal. The L-isomer appears to bind much less than the D-isomer, and probably not to any measureable degree. Secondly, calculation of the absolute effect—either D,L splitting or relaxation time—shows clearly that these effects are pH dependent. The pH dependence, however, is somewhat different for the two variables considered. The maximum effect occurred at pH 6.4 for both variables; at this point the relaxation time was 0.03 seconds, and Δ for the D,L splitting was 87 Hertz.

Several mechanisms may be postulated to explain these results. One mechanism is that the increasing dominance of the imidazolium ion at decreasing pH values increases hydrogen bonding to the substrate at the active site of the enzyme. This holds the molecule more rigidly, decreasing the relaxation time. Also, this hold the fluorine nuclei close to the ring current of the imidazolium ion, which deshields these nuclei, and causes the downfield shift. Conformational changes may be a factor which lowers the slopes in the pH range 5.0 to 6.5.

There still remains a great deal to be done in this area, with different substrates, and with modified enzymes of chymotrypsin, and also with entirely different enzymes. Also, temperature dependent studies

will be important, for they will reveal whether the exchange rate between the enzyme and the substrate is a factor in the line broadening. These are just a few of the remaining areas to be investigated, and studies here and elsewhere will reveal a great deal about the interactions of enzymes and substrates.

Experimental

Samples of the N-tfa-D,L-tryptophan and the alpha-chymotrypsin are prepared gravimetrically, taking 25,000 as the molecular weight of the enzyme. The substrate is first weighed out in a 1 milliliter volumetric test tube, and dissolved in about 0.7 ml of 0.1 Molar pH 7.0 citrate buffer. The enzyme is then weighed out, and added to the solution in the test tube. The enzyme was most readily dissolved by centrifuging. The pH of the solution was then adjusted to the desired value by the addition of 0.1 M citrate buffers of different pH values. The pH was measured with a glass electrode and pH meter. The volume was then adjusted to exactly 1.0 ml by the addition of citrate buffer of the correct pH. The enzyme was stored, when not in use, vacuum-dessicated in a freezer.

Spectra were taken on a Varian Associates HA 100 nuclear magnetic resonance spectrometer. A computer time-averaging device was available, but for this work was not used. The internal lock signal was obtained

by using a sealed capillary tube, containing trifluoroacetic acid,inserted into the NMR tube. The temperature was the ambient temperature of the probe. The resolution of the spectrometer was adjusted between each of the samples, and is reflected in the fact that the line width of the L-isomer was very constant on a given day, and also from day to day.

The first part of the work was done with a supply of the substrate synthesized by a member of the research group shortly before the work was done. However, after this work had begun, more substrate was necessary, and was synthesized. The procedure was:

D,L tryptophan (1.0 grams, 4.8 mMoles) was dissolved in trifluoroacetic acid (5.6 ml). To this was added anhydrous ethyl ether (6.5 ml). After the tryptophan had dissolved, the solution was cooled in an acetone-ice bath. When cool, trifluoroacetic anhydride (1.0 ml) was added dropwise over several minutes. This addition was accompanied by vigorous shaking. After all of the anhydride was added, the solution was allowed to stand in the temperature bath for 30 minutes. By this time, a large amount of yellowish precipitate had formed. This precipitate was filtered, and crudely dried for 15 minutes under water aspirator vacuum. The precipitate was then added to about 50 ml of distilled water. This mixture was stirred for 30 minutes, and the yellowish color of the precipitate disappeared, leaving a pure, white color. The solid was the filtered,

and the water removed by suction. The precipitate was then recrystallized from 100 ml of hot toluene, forming long, white needles. Yield: 0.63 gram, 44%. Melting point: 154-155°C, sharp, with no residue. Spectra with and without enzyme were identical to those of the substrate previously used.

D,L splitting and changes in line width were measured by the distances between peaks or line widths of the recorded spectra. The error in the D,L splitting is on the order of 0.1 Hz. At dilute concentrations, the line widths were not too distinct, and using a Lorentzian line fit, a reasonable approximation to the actual shape was made. This may introduce an error on the order of 0.3 to 0.4 Hz, in the line width change at broad line widths. Errors of this magnitude appear to have not affected the quantitative results to a significant degree, and have not affected the conclusions at all.

Acknowledgements

I would like to thank Dr. John Richards for his ideas, his patience, and his encouragement throughout the last year. I also appreciate the patience and understanding of Dr. Richards entire research group, helping me with my many blunders and questions in the lab. Without the help of these people, this work would never have been completed.

APPENDICES

and

BIBLIOGRAPHY

Appendix #1

RELAXATION PHENOMENA IN NUCLEAR MAGNETIC RESONANCE

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for

Chemistry 81

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RELAXATION PHENOMENA IN NUCLEAR MAGNETIC RESONANCE

Nuclear magnetic resonance phenomena (NMR) in many compounds is an important field which the chemist may apply to his work. This report on NMR has been done within the context of a course Ch 81 - topics in chemistry. The goal has been a working knowledge of NMR and a more thorough grounding in relaxation phenomena, and to study the means by which relaxation has been applied to research in enzymes and macromolecules.

The concept of NMR encompasses many principles, and means of applying the theory. NMR may be approached from the viewpoint of the chemical shift of the resonance peaks, of spin-spin interactions of the magnetic nuclei, or of relaxation phenomena. This paper takes the latter viewpoint. However, the overall theory of NMR is necessary as a foundation for the specific application of relaxation phenomena.

The basis for the theory of NMR is the concept of spin of nuclei. Because nuclei have spin, as well as charge, each nuclei then has a magnetic moment. This magnetic moment, set up by the nuclear spin is given by:

$$\mu = \gamma(I\hbar)$$

In this expression, I is the nuclear spin, and γ is the magnetogyric ratio for the nucleus. The quantity $I\hbar$ is the largest value of angular momentum which the nucleus is allowed to have. The angular momentum is allowed values from $+I$ to $-I$, so that the magnetic moment will have $2I+1$ distinct values or states. It is the tran-

sitions between these states which provide the basis for NMR. In a uniform magnetic field, these allowable values for the magnetic moment become a system of energy levels, given by:

$$E = \mu \cdot H_o$$

where H_o is the magnetic field in the +z direction. With $2I+1$ orientations of the magnetic moment, there are similarly $2I+1$ energy levels, with equal spacing of $\frac{\mu H_o}{I}$. For the simplest case, that of $I = \frac{1}{2}$, we have energy levels of μH_o and $-\mu H_o$. NMR measures this energy differences between levels, since we also know that $E = h\nu_o$. By varying either H_o or ν_o, we can determine the energy of the transitions between the possible states.

However, within a molecule, not all of the nuclei feel the same field. This is due to dipole interactions between the nuclei, which contributes to the total field, and screening effects of the surrounding electrons of each nucleus. Together, these factors create a slightly different effective field at each different nucleus, and due to this, the energy levels are just slightly different for different nuclei. The final result is that transitions for one nucleus do not occur at the same energies as do those of another nucleus. So, on an NMR spectrum of such a molecule, we will see more than one peak—~~one~~ a different peak for each different ~~energy of transition.~~ transition energy. The separation of these peaks on a spectrum is known as a chemical shift, and is one viewpoint from which NMR may be approached.

Nuclei within a molecule differ in another way. This

is known as spin-spin interaction:

$$I_{1,2} = I_1 \cdot I_2$$

for two nuclei, #1 and #2. These spins are coupled from nucleus to nucleus by the bonds which connect them, directly or indirectly. For example, a methyl group has three hydrogen nuclei, and has four possible different arrangements of spin, with total angular momentum of $3/2, \frac{1}{2}, -\frac{1}{2}, -\frac{3}{2}$ units of $\hbar$. A nearby nucleus will feel four different fields at various times, so that the expected peak is split into four peaks - slightly separated. There are three ways ~~to form~~ these three nuclei can produce a total angular momentum of $\frac{1}{2}$ or $-\frac{1}{2}$, while one way to form $\frac{3}{2}$ or $-\frac{3}{2}$. So, the peaks corresponding to $\pm\frac{1}{2}$ have three times the amplitude of those corresponding to the methyl group with angular momentum of $\pm\frac{3}{2}$. This is the nature of spin-spin interactions and the effect of spin-spin splitting of resonance peaks.

We should now look at the actual process by which the absorbtion of energy occurs at resonance. When a nucleus with magnetic moment is in a static magnetic field, the field causes the magnetic moment to precess about the axis of the magnetic field. The frequency of this precession, known as the Larmor angular frequency is:

$$\omega_0 = \gamma H_0$$

This frequency is very important for this is the frequency which is matched to cause resonance. Once this magnetic moment is precessing about the z axis, we apply a second

magnetic field, much smaller in magnitude. This is in the xy plane, and is a rotating field about the z axis. This secondary field creates a torque on the magnetic moment, attempting to "pull" it into another orientation, so as to flip the spin over. However, unless this field is rotating at the identical frequency as the precession of the magnetic moment (i.e. the Larmor frequency), the rotating field is going in and out of phase so rapidly that the torque on the magnetic moment is not significant. However, when the two frequencies just match, so that the rotating field is just at the Larmor frequency, the two are continually in phase and there is a large, constant torque on the magnetic moment. This causes it to oscillate rapidly with great amplitude. This process requires energy, which the rotating field provides, and this is the energy absorbtion at resonance. The energy absorbtion is recorded as the frequency of the rotating field is varied over a large range.

It is, however, difficult to apply a rotating field in the xy plane. Another way to cause the same field is to apply a magnetic field in a fixed direction, such as on the x axis, and vary this field sinusoidally. The sinusoidal field can be broken into two components, both of which are rotating field vectors, one clockwise and the other counterclockwise about the z axis. One, due to sign, has little affect on the resonance, for it is in phase only once every full rotation. This, for the most part, is a classical treatment of the absorbtion phenomena. If we use a quantum mechanical treat-

ment, we arrive at equivalent results for a spin $\frac{1}{2}$ nucleus. In this treatment, the probability of a transition is derived as a function of the Larmor frequency and the rotating field frequency. This probability is:

$$P(+\rightarrow -) = \frac{\theta^2}{\left(1-\frac{\omega_0}{\omega}\right)^2+\left(\frac{\omega_0}{\omega}\right)\theta^2} \sin^2\left[\frac{\omega T}{2}\left(\left(1+\frac{\omega_0}{\omega}\right)^2+\frac{\omega_0}{\omega}\theta^2\right)^{1/2}\right]$$

where θ is the angle the resultant field makes with the static field, and ω_0 is the Larmor frequency. This gives a resonance as ω approaches ω_0. In either case, we have resonance occuring when the rotating field in the xy plane is at actually the Larmor frequency, and in the same direction.

This absorbtion of energy will occur at the Larmor frequency, causing a resonance peak with a finite line width. This line width is determined by several factors, one of which is known as the spin-lattice relaxation time. This characteristic time is a measure of how rapidly other degrees of molecular freedom can induce transitions between the states. This affect, with others, causes the states to have a finite lifetime. The mean lifetime of a state is just the spin-lattice relaxation time. There are several mechanisms by which spin-lattice relaxation can occur; these will be discussed later. Other factors can also account for the line width, although the spin-lattice relaxation factor is always present. One factor is spontaneous emission of energy, causing a transition. Bloembergen estimates a lifetime from this affect of about 10^{19} years! This factor is negligible compared with the

other factors determining lifetime. A third factor is another type of relaxation—spin-spin relaxation. This results from interactions of magnetic moments from various nuclei, so that there is a transfer of spin between two nuclei. However, in this process, there is no net change in the energy of the system. This process also leads to a finite lifetime, but is not always important in comparison to spin-lattice relaxation, except in crystals, where positions are fixed. For nuclei with spin greater than $\frac{1}{2}$, there is also the affect of electric quadrupole interactions which can cause transitions between states. The line width, then, is a measure of the lifetime of a state involved in the absorbtion of energy and resonance, and is determined by several factors, the most important of which is generally spin-lattice relaxation.

A set of equations which applied to the resonance phenomena was derived by Bloch. These, known as the Bloch formulation, concentrate on the rate of change of the total magnetic moment, and its component along each of the coordinate axes.

$$\frac{d(M)}{dt} = -\gamma(M \times H)$$

This equation is broken into components, and then the contributions from the rotating field are added, so that we get the complete equations, in terms of the relaxation times, the magnetic moments, and the magnetic fields. These complete equations are:

$$\frac{dM_x}{dt} = \gamma(M_y H_0 + M_z H_1 \sin \omega t) - \frac{M_x}{T_2}$$

$$\frac{dM_y}{dt} = \gamma(M_z H_1 \cos \omega t - M_x H_0) - \frac{M_y}{T_2}$$

$$\frac{dM_z}{dt} = \gamma(-M_x \sin\omega t - M_y \cos\omega t) H_1 - \frac{M_z - M_0}{T_1}$$

Where T_1 is the spin-lattice relaxation time and T_2 is the spin-spin relaxation time. These equations, however, are easier to solve is we make a change of variable, and set:

$$M_x = u \cos\omega t - v \sin\omega t$$

$$M_y = -u \sin\omega t - v \cos\omega t$$

We can then solve for u, v and M_z in a steady state case, which sets all of the time derivatives to zero, so that the equations are readily solved. The solutions are:

$$u = M_0 \cdot \frac{\gamma H_1 T_2^2 (\omega - \omega_0)}{1 + T_2^2(\omega_0 - \omega)^2 + \gamma^2 H_1^2 T_1 T_2}$$

$$v = -M_0 \cdot \frac{\gamma H_1 T_2}{1 + T_2^2(\omega_0 - \omega)^2 + \gamma^2 H_1^2 T_1 T_2}$$

These solutions to the Bloch equations can give us the shapes of the resonance peaks for NMR. There are actually two such peaks, the absorbtion mode from the variable v and the dispersion mode from the variable u. From these solutions, with some manipulation, we can see the effect of each of the relaxation times on the line-shapes of the peaks. The size of T_1, the spin-lattice relaxation time, is generally larger than T_2, so that its affect is much greater. By studying the spin-lattice relaxation time in more detail, we can get additional information from spectra about the nature of the samples.

The relaxation time is a measure of the lifetime of a state—how rapidly it can interact with other molecules to cause a transition from that state. At this point, we will confine ourselves to a nucleus of spin $\frac{1}{2}$, for this has only a nuclear magnetic moment associated with

its spin. Because the nucleus has only a magnetic moment, it can interact only with magnetic fields from external sources, including other nuclei. These may be either within the same molecule, or nuclei in other molecules. This interaction with other magnetic moments has the final affect of creating local magnetic fields near the magnetic moment in question. These local fields, regardless of source, vary greatly due to thermal motion and other degrees of molecular freedom. As the local field varies, the total field at that nucleus will likewise vary, which in turn varies the Larmor frequency. As this frequency varies, it will eventually match the applied, rotating field, and will cause a transition from the original state. By varying the local field through interactions with nearby nuclei, transitions can be caused back down to the lower state, so that relaxation can affect or determine the lifetime of the upper state.

There are several mechanisms by which a magnetic moment may interact with a nearby nucleus. The first is magnetic dipole interaction. By this mechanism, the local fields are caused by other magnetic moments, from two sources. The first source are magnetic moments located within the same molecule. These nuclei are at a fixed distance, but there are many possible orientations. The relaxation time can be determined in terms of the rotational motion of the molecule. This, in turn, can be found by using the model of the molecule as a sphere in a viscous liquid. This model is not perfect, but works

quite well within experimental measurements. We find for this contribution to the relaxation time,

$$\left(\frac{1}{T_1}\right)_{INTRA} = \frac{2\pi\eta a^3 \hbar^2 \gamma^4}{6^6 KT}$$

where η is the viscosity of the solution in poise, b is the fixed distance of nuclear separation, and a is the radius of the sphere in our model. Approximating the values, we get a value for $\frac{1}{T_1}$ of $0.26\,sec^{-1}$.

Similarly, the affect from nuclei associated with other molecules can be found. In this case, the distances are not fixed. This case is treated as a diffusion process by several authors, including Bloembergen, Purcell and Pound. This contribution to the relaxation time can be found in terms of the diffusion constant of the liquid, and then in terms of the viscosity, just as for the intramolecular contribution. This intermolecular contribution is:

$$\left(\frac{1}{T_1}\right)_{INTER} = \frac{3\pi^2\gamma^4\hbar^2\eta N_0}{KT}$$

All of the above applies to molecules with two applicable nuclei, such as water, but this can be applied to larger, more complex systems. The results are:

$$\left(\frac{1}{T_{1i}}\right)_{INTRA} = \frac{2\pi\hbar^2\gamma_i^2\eta a^3}{3KT}\left[3\gamma_i^2\sum_j' r_{ij}^{-6} + 2\sum_f \gamma_f^2 r_{if}^{-6}\right]$$

$$\left(\frac{1}{T_{1i}}\right)_{INTER} = \frac{\pi^2\hbar^2\gamma_i^2\eta N_0 a^3}{KT}\left[3\gamma_i^2\sum_j' \frac{1}{r_{ij}} + 2\sum \frac{1}{r_{if}}\right]$$

For a nucleus "i", the sum over "j" are those nuclei of the same species, while the sum over "f" are those nuclei not of the same species.

These relations have been shown to hold quite well experimentally, in work by Bloembergen, Purcell and Pound.

They plotted the spin-lattice relaxation time T_1 vs. the quantity η/T, where η is the viscosity and T is the temperature. This was done for ethyl alcohol under varying conditions, and the plot was found to be linear as expected. This plot is in the appendix to this report, and confirms the affect of dipole interactions in relaxation phenomena.

There is another source of relaxation in some solutions—paramagnetic ions. The paramagnetic substance has at least one unpaired electron, which can contribute a magnetic field to the local field since there is not a cancelling field from the opposite-spin paired electron. This magnetic field fluctuates, and can cause transitions similar to dipole interactions. The major difference is a mean square nuclear moment in the expression for T_1, yielding the following expression for the contribution to the relaxation time from paramagnetism:

$$\frac{1}{T_1} = \frac{4\pi^2\gamma^2\eta N_p \mu_{eff}^2}{KT}$$

This contribution can also be observed experimentally, which was done by the above authors. They varied the concentration of paramagnetic ions in solution and recorded the relaxation time for each run. In a plot of these quantities, the result is quite linear, verifying the above expression. This plot is also in the appendix.

There is a final contribution to spin-lattice relaxation time in spin $\frac{1}{2}$ nuclei. This results from anisotropic shielding of the magnetic field by the electrons.

The electrons surrounding a nucleus will shield it to some degree from the spplied magnetic fields. If this shielding is not uniform, then electrons will form an electron current, and from these currents, magnetic fields. These fields can then cause transitions in a manner similar to dipole-dipole interactions. The major difference is that this contribution is dependent on the external magnetic field, while the other contributions are not. The actual relation has been derived, and is known as the McConnell-Holm formula:

$$\frac{1}{T_1} = \frac{2}{15} \gamma^2 H_0^2 (\Delta\sigma)^2 \tau_c$$

where $(\Delta\sigma)$ is related to the differences in shielding in directions perpendicular and parallel to the axis of symmetry, and τ_c is the correlation time just as with inter and intramolecular relaxation. Thus, this contribution is proportional to the inverse of the square of the applied magnetic field.

For nuclei with spin greater than $\frac{1}{2}$, we also have relaxation from electric quadrupole interactions. In these cases, electric quadrupoles of nuclei interact to cause transitions analogous to the magnetic dipole interactions.

These are the major causes of transitions between states due to spin-lattice relaxation. These include magnetic dipole interactions, secondary magnetic fields from unpaired electrons in paramagnetic substances, and anisotropic electron shielding of the applied magnetic

field. The local magnetic fields from each source vary rapidly in frequency and amplitude, and can cause transitions between states. These transitions contribute to the finite lifetime of a state involved in the resonance phenomena, and to the line width of the absorbtion peak due to the resonance.

The theory of relaxation phenomena can be applied in many cases to provide information about molecules and reactions, often in biochemistry. We know that the resonance peak or curve is directly related to the relaxation time through the width of the peak. If there are no interfering ions, such as paramagnetic ions, then a comparison of the line width under different conditions will give a good indication of factors such as binding sites on enzymes. For example, if an enzyme has three parts which may be considered binding sites, an NMR spectrum may be taken. After a substrate is added to the enzyme, by retaking the spectrum, we may be able to see a definite change. The resonance peak which is associated with the binding site will have its relaxation time shortened so that the peak will be broadened. This occurs since the substrate can preferentially affect the relaxation at the binding site while not affecting nearly as much the rest of the molecule. By observing the change in line width of the peaks on a spectrum, we can determine which portion of the enzyme is binding the substrate. One example is the complex of L-Epinephrine and ATP. The molecule of epinephrine has two

major parts, a ring and a side-chain. The NMR spectrum of this compound was taken. After adding 0.1 Molar ATP, a second spectrum was taken. It was noticed that the peaks involved with the ring were almost unchanged, while those peaks associated with the side-chain were broadened considerably. This tells us that the substrate is most likely binding to a part of the side-chain. This process may be extended to be even more specific and yield more information about the binding between an enzyme and substrate. These spectra are reproduced in the appendix.

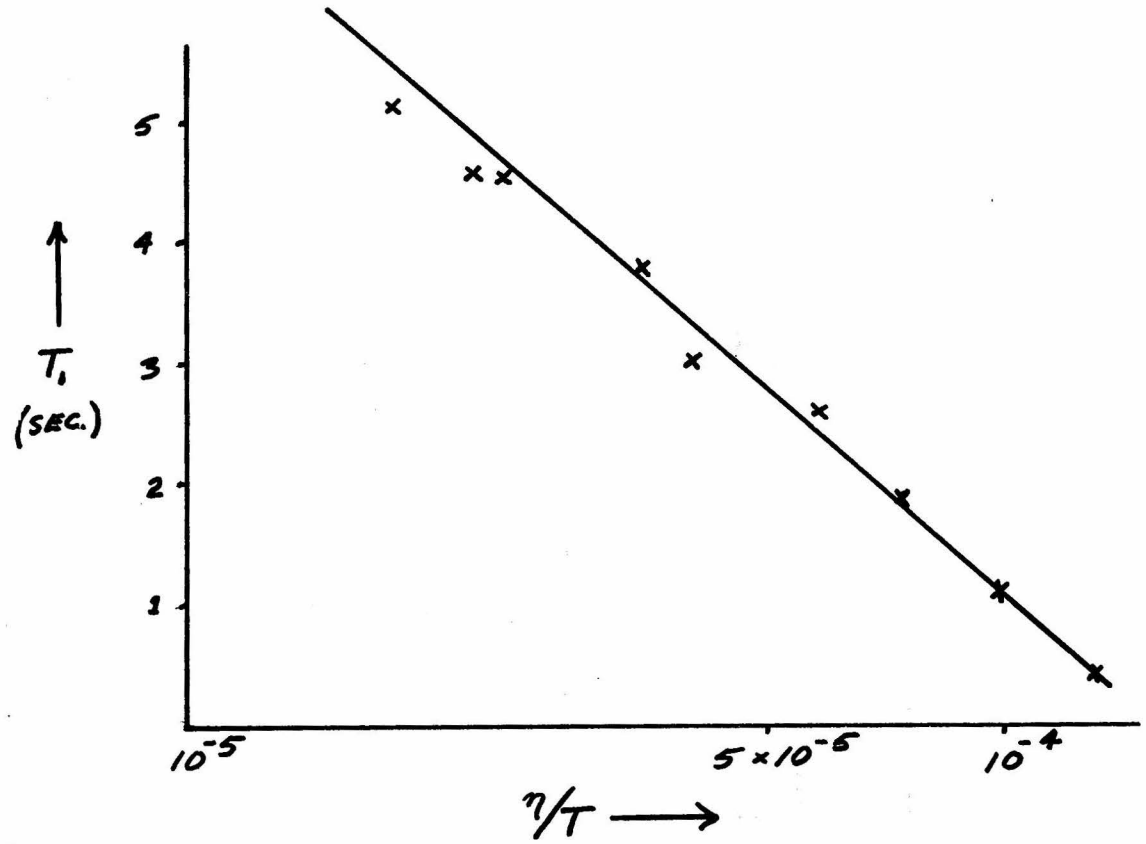
The relation of spin-lattice relaxation time to the amount of substrate present is very specific. Oleg Jardetsky studied the affect of albumin, the substrate, on penicillin. He varied the concentration of albumin from 0 to 5%, and found the relaxation time T_1. A plot of T_1 vs. concentration of substrate proved linear for several different runs. This plot can also be found in the appendix.

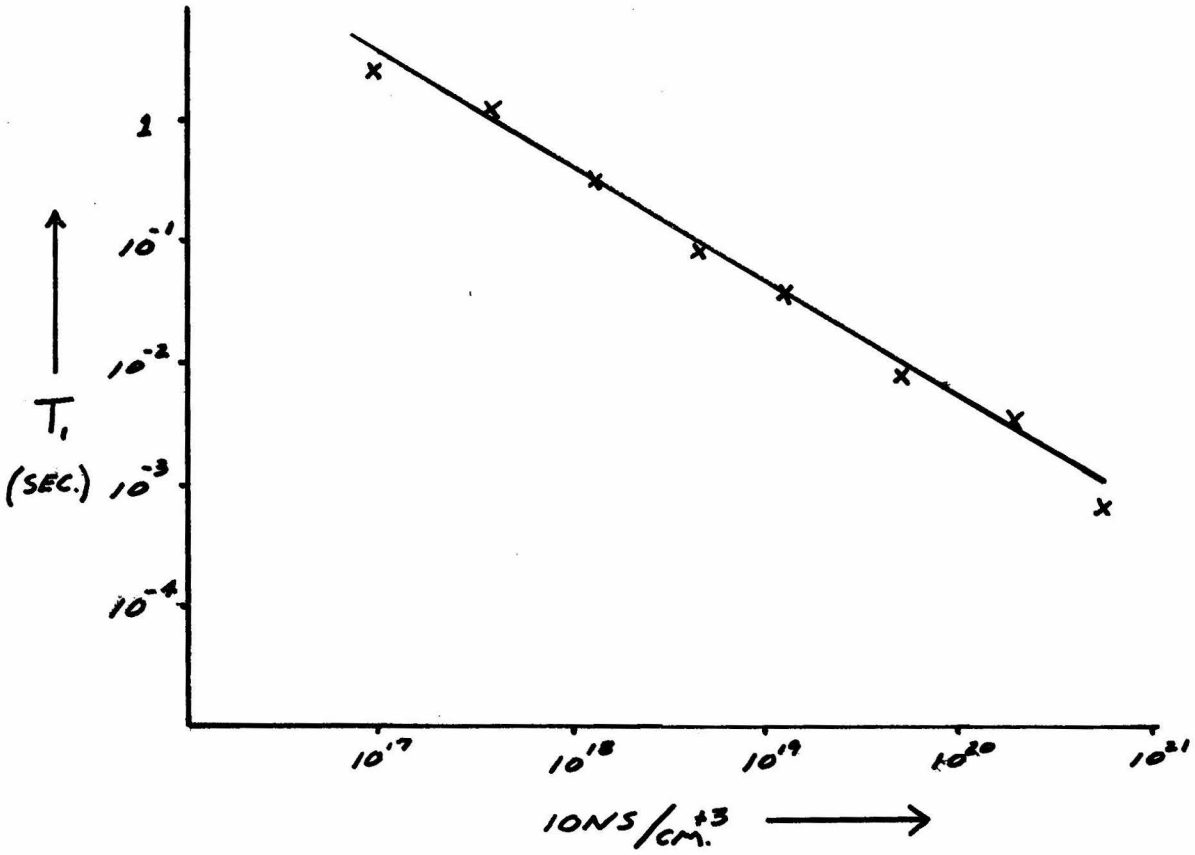
This paper has given only the introduction to the theory of Nuclear Magnetic Resonance, and much remains to be studied in this area. The applications are becoming more and more useful, and the area of relaxation phenomena will prove extremely valuable in the future. Through continued application of NMR, as well as a study of the theoretical aspects, one can obtain a more complete understanding of the NMR phenomena.

APPENDIX

and

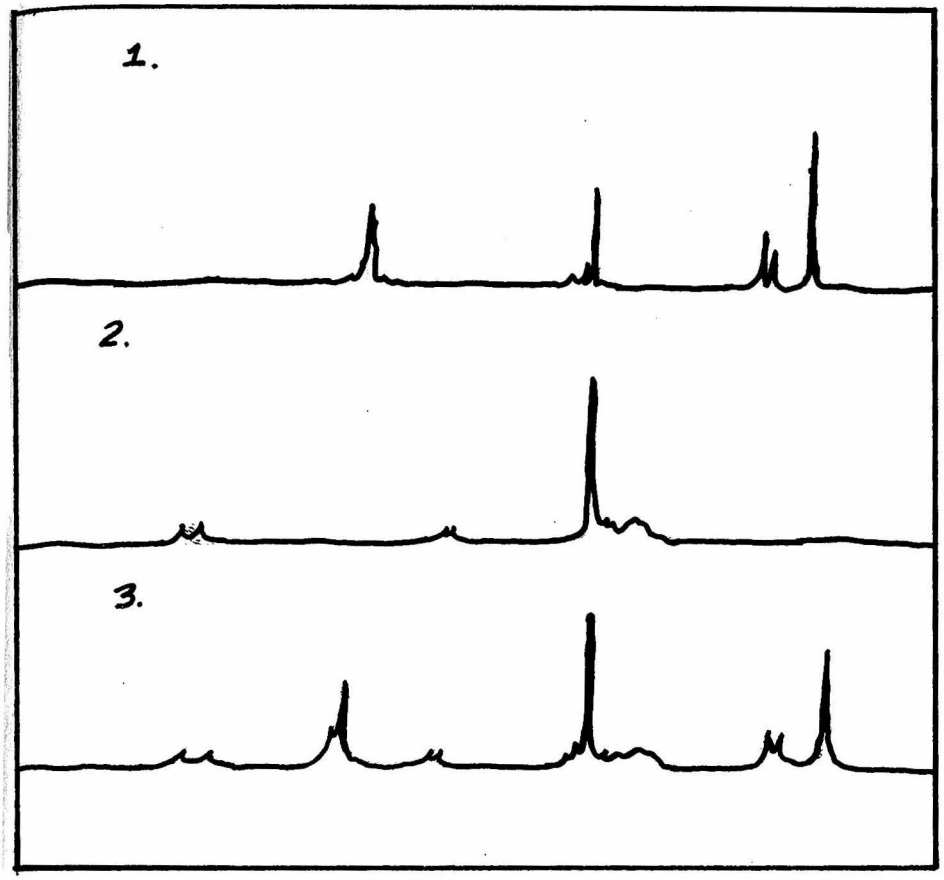
Bibliography





Pople, Schneider & Bernstein High Resolution Nuclear Magnetic Resonance P. 204, 208

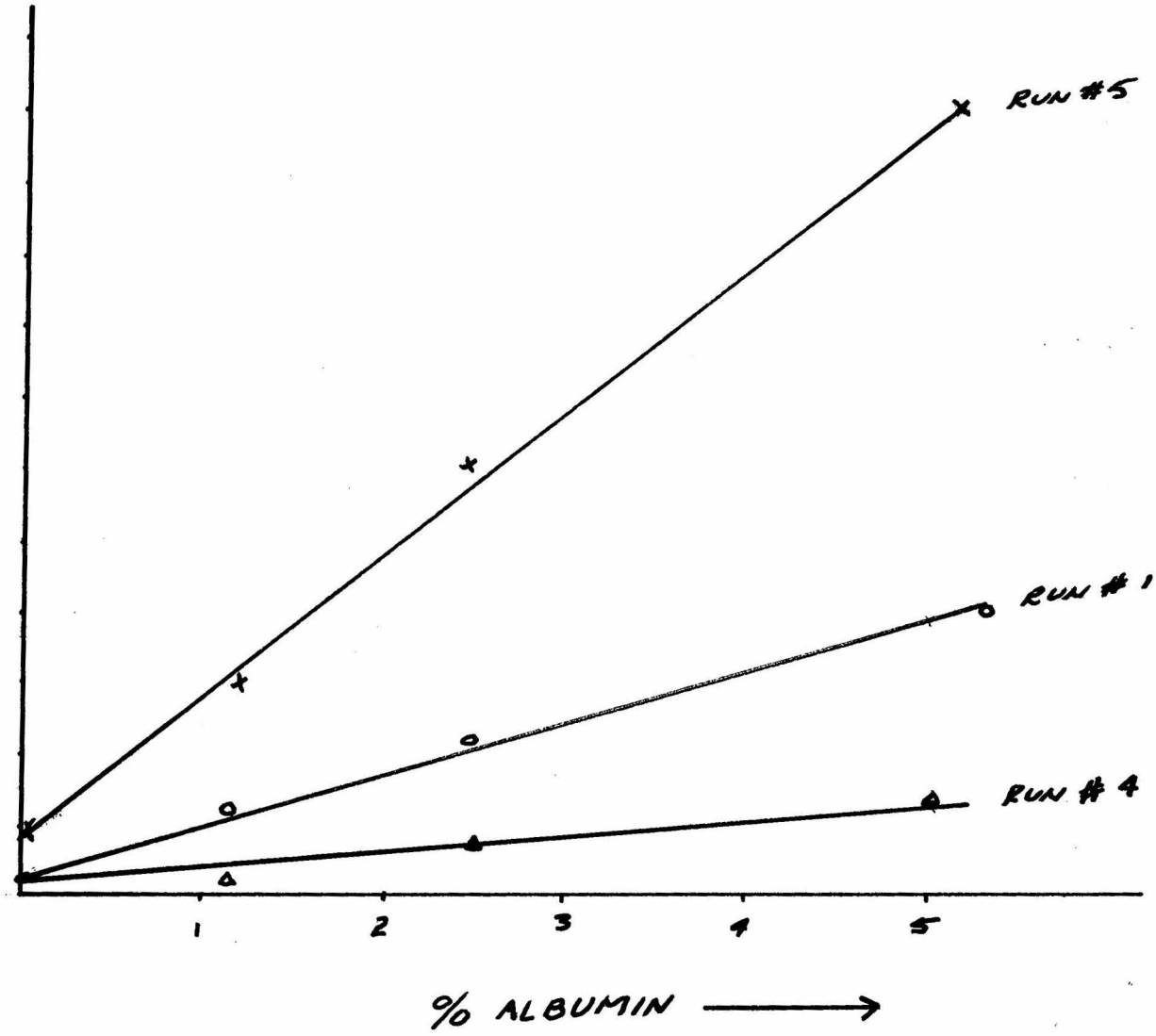
Proton Magnetic Resonance Spectra of Epinephrine Complexes



1. 0.5 M L-Epinephrine at pH = 1.5
2. 0.1 M Adenosinetriphosphate (ATP) at pH = 1.5
3. 0.3 M L-Epinephrine & 0.1 M ATP at pH = 1.5

Advances in Chemical Physics, Vol. VII p. 519

Effect of Albumin on 0.2 % Penicillin peaks



Advances in Chemical Physics, Vol. VII pg. 525

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DATA TABLE #1

Binding Curve #7 - pH = 5.0

E_o	S_o	δw	D,L split
0.00175 M	0.0056 M	0.80 Hz	12.00 Hz
0.00175	0.0086	0.60	7.60
0.00175	0.0125	0.70	6.10
0.00175	0.0158	0.60	4.85
0.00175	0.0208	0.60	3.90

Binding Curve #6 - pH = 5.5

E_o	S_o	δw	D,L split
0.00175	0.0050	1.00	14.10
0.00175	0.0071	1.20	10.60
0.00175	0.0101	0.90	7.85
0.00175	0.0140	0.60	5.55
0.00175	0.0208	0.60	4.45

Binding Curve #18 - pH = 5.7

E_o	S_o	δw	D,L split
0.00175	0.0055	1.40	13.55
0.00175	0.0086	1.50	9.65
0.00175	0.0120	0.80	7.45
0.00175	0.0151	1.10	5.90
0.00175	0.0201	0.75	4.55

Binding Curve #3 - pH 6.0

E_o	S_o	δw	D,L split
0.00175	0.0035	1.50	17.57
0.00175	0.0075	1.30	10.30
0.00175	0.0108	1.25	6.70
0.00175	0.0121	0.30	6.60
0.00175	0.0131	0.50	5.82
0.00175	0.0210	0.10	3.66
0.00175	0.0226	0.00	3.35

Binding Curve #20 - pH = 6.0

E_o	S_o	δw	D,L split
0.00175	0.0058	1.40	12.60
0.00175	0.0123	0.80	6.80
0.00175	0.0155	0.55	5.10
0.00175	0.0198	0.45	4.00

Binding Curve # 23 - pH = 6.2

E_o	S_o	δw	D,L split
0.00175	0.0060	-	13.35
0.00175	0.0088	1.70	9.35
0.00175	0.0123	1.30	7.40
0.00175	0.0156	1.10	6.35
0.00175	0.0185	1.05	4.85

Binding Curve #15 - pH = 6.5

E_o	S_o	δw	D,L split
0.00175	0.0058	2.25	15.05
0.00175	0.0090	1.75	10.50
0.00175	0.0115	1.95	8.30
0.00175	0.0153	1.45	6.40
0.00175	0.0193	1.10	4.80

Binding Curve #25 - pH = 6.5

E_o	S_o	δw	D,L split
0.00175	0.0058	2.00	13.25
0.00175	0.0086	2.00	10.00
0.00175	0.0116	1.85	8.30
0.00175	0.0146	1.20	6.35
0.00175	0.0191	0.75	4.60

Binding Curve #9 - pH = 6.8

E_o	S_o	δw	D,L split
0.00175	0.0058	1.40	12.55
0.00175	0.0083	1.30	12.30
0.00175	0.0113	1.30	9.45
0.00175	0.0148	0.90	5.85
0.00175	0.0183	0.80	4.65

Binding Curve #13 - pH = 6.8

E_o	S_o	δw	D,L split
0.00175	0.0050	1.95	14.35
0.00175	0.0078	1.85	10.45
0.00175	0.0128	0.85	7.40
0.00175	0.0148	0.80	6.15
0.00175	0.0181	0.80	5.15

Binding Curve #24 - pH = 7.0

E_o	S_o	δw	D,L split
0.00175	0.0051	2.00	14.35
0.00175	0.0091	1.80	9.65
0.00175	0.0128	1.50	7.25
0.00175	0.0151	1.10	6.20
0.00175	0.0200	0.90	4.95

Binding Curve #4 - pH = 7.2

E_o	S_o	δw	D,L split
0.00175	0.0033	1.30	19.28
0.00175	0.0066	1.30	10.98
0.00175	0.0100	0.65	8.03
0.00175	0.0130	0.60	6.43
0.00175	0.0155	0.45	4.73

Binding Curve #12 - pH = 7.2

E_o	S_o	δw	D,$^{\perp}$ split
0.00175	0.0055	1.40	11.40
0.00175	0.0085	1.30	8.15
0.00175	0.0105	1.15	6.95
0.00175	0.0160	0.75	4.75
0.00175	0.0208	0.40	4.00

Binding Curve #10 - pH = 7.5

E_o	S_o	δw	D,$^{\perp}$ split
0.00175	0.0050	-	9.50
0.00175	0.0086	0.95	7.45
0.00175	0.0125	0.60	5.80
0.00175	0.0158	0.55	4.85
0.00175	0.0205	0.50	3.85

Binding Curve #19 - pH = 7.5

E_o	S_o	δw	D,L split
0.00175	0.0088	1.10	9.20
0.00175	0.0116	1.30	7.30
0.00175	0.0153	1.10	5.50
0.00175	0.0196	0.95	4.90

Binding Curve #11 - pH = 8.0

E_o	S_o	δW	D,L split
0.00175	0.0055	0.80	9.05
0.00175	0.0088	1.00	6.50
0.00175	0.0116	0.65	5.20
0.00175	0.0156	0.50	4.75
0.00175	0.0185	0.50	4.05

DATA TABLE #2

$1/T_2$ slope values

Binding Curve #	slope	intercept	pH
3	9.54 Hz	0.5 Hz	6.0
4	6.41	0.1	7.2
6	4.46	0.3	5.5
7	1.49	0.5	5.0
9	6.01	0.4	6.8
10	6.56	0.0	7.5
11	3.70	0.2	8.0
12	8.67	0.1	7.2
13	12.00	0.3	6.8
15	9.97	0.5	6.5
16	12.90	0.0	6.4
18	6.10	0.4	5.7
19	2.70	0.8	7.5
20	9.10	0.2	6.0
22	12.90	0.1	6.2
23	11.10	0.1	6.2
24	9.25	0.3	7.0
25	11.9	0.1	6.5

DATA TABLE #3

D,L splitting Parameter Values

Binding Curve #	slope	intercept	pH
3	87.6 Hz	3.1 Hz	6.0
4	88.7	4.5	7.2
5	45.9	0.0	8.0
6	82.0	2.2	5.5
7	70.2	1.5	5.0
8	78.0	2.0	6.5
9	85.0	1.6	6.8
10	45.9	0.7	7.5
11	46.3	0.2	8.0
12	64.4	0.8	7.2
13	79.3	1.3	6.8
15	97.8	2.8	6.5
18	83.7	1.9	5.7
19	69.1	0.6	7.5
20	82.3	2.3	6.0
23	82.4	1.6	6.2
24	78.9	1.1	7.0
25	82.3	1.6	6.5

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