A Thesis Entitled

"HIGH RESOLUTION NUCLEAR MAGNETIC RESONANCE STUDIES OF BIOLOGICALLY SIGNIFICANT MOLECULES"

submitted by

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ABSTRACT

Fluorine magnetic resonance has been used to study the interaction of N-trifluoroacetyl-X-phenyl alanines with the enzyme α-chymotrypsin with varying pH and buffer systems. The aromatic and aliphatic binding sites of the enzyme were studied simultaneously using N-trifluoroacetyl-fluoro phenyl alanines as the enzyme inhibitors, the aromatic fluorine spectra being simplified by heteronuclear decoupling. The specific nature of the interaction between α-chymotrypsin and the N-trifluoroacetyl-X-phenyl alanines was demonstrated using N-trans cinnamoyl imidazole as a competitive inhibitor. A new method for the estimation of α-chymotrypsin is suggested. The inherent limitations of n.m.r. for studying these types of interaction are discussed.

The n.m.r. technique has been used to study the rotation about the carbon-nitrogen bond of some N,N dimethyl-X-phenyl carbamates. The free energy of activation ($\Delta G^{\ddagger}$) for the rotation process has been shown to depend on the nature of the substituent in the aromatic ring. Linear correlations have been obtained between $\Delta G^{\ddagger}$ and the Hammett substituent constant, also between $\Delta G^{\ddagger}$ and the

pK_a of the parent phenol. The latter correlation has enabled the study to be extended to include some heterocyclic carbamates.

The carbamate study was then used as the basis for studying phenyl thiocarbamate, phenyl acetamide and benzamide derivatives. The differences between the series have been interpreted on the basis of the transmission of pi-electronic effects from the phenyl ring through the intermediate oxygen, sulphur and methylene groups to the carbon-nitrogen bond.

To My Wife,

Marianne.

Acknowledgments

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"The great pleasure in life is doing
what others say you cannot do."

Walter Bagehot

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PART I

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CHAPTER ONE

Introduction

The study of enzyme systems presents one of the greatest scientific challenges of our times. Despite the extensive studies of the last forty years, the mechanisms of reactions of even the simplest enzymes are not fully understood. Over the last decade the traditional physical techniques for studying enzyme systems, e.g. UV spectroscopy[1], and potentiometric titrimetry[2], have been augmented by newer techniques such as X-ray diffraction[3], electron spin resonance (e.s.r.)[4] and nuclear magnetic resonance (n.m.r.)[5].

The n.m.r. method offers several advantages over the traditional techniques, but is not without its disadvantages. The greatest advantages stem from the small energy required to cause the transition which gives rise to the resonance signal; this small energy requirement means that the macroscopic system under study is not perturbed by the experimental measurements. The parameters which are measured in a routine n.m.r. spectrum, namely chemical shift, line intensity, spin-spin coupling constant and

line widths, all give useful information for biological systems. These will be considered in more detail in section 1.2.

In part I of this thesis, the application of the n.m.r. technique to the study of enzyme systems will be considered in detail, together with the necessary theoretical background.

1.1 General Theory

Nuclear magnetic resonance can be used to study several different aspects of the enzyme catalytic process and to understand these fully, it is necessary to outline briefly more general aspects of traditional enzyme kinetics.

A. Kinetic Scheme for Simple Enzyme Reactions

The simplest possible kinetic scheme for an enzyme reaction was proposed by Michaelis and Menten[6]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + \text{products} \qquad \ldots\ldots 1$$

The substrate (S) combines with the enzyme (E) to form a 1 : 1 molecular complex (ES). This is an equilibrium interaction (probably involving several pre-equilibria), of which the equilibrium constant is <u>either</u>

$$K_S = \frac{[E]\,[S]}{[ES]} \qquad \ldots\ldots 2$$

where K_S is the dissociation constant of the enzyme substrate complex,

<u>or</u>

$$K_D = \frac{[ES]}{[E]\,[S]} \qquad \ldots\ldots 3$$

where K_D is the association constant of the complex.

The enzyme - substrate complex then undergoes a chemical change with velocity constant k_2 to form the "inhibited" enzyme (ES') (which may be isolated in favourable cases); this in turn breaks down with a velocity constant k_3 to regenerate the enzyme and give products.

This type of system is amenable to study by n.m.r. techniques only when the life-times of the intermediates are sufficiently long and the steady state concentration

is large enough for n.m.r. detection. For example, when $k_2 \approx k_3 \approx 0$, equation 1 reduces to

$$E + S \rightleftharpoons ES \qquad \ldots\ldots 4$$

Binding occurs between the enzyme and substrate with no net enzymic reaction. When k_2 is large and $k_3 = 0$, it is possible to isolate the irreversibly inhibited enzyme. Both of these extreme cases may be studied by the n.m.r. technique.

B. Quantitation of n.m.r. Chemical Shift and line-width changes

The two situations which can be readily studied by n.m.r. are:

(a) When $k_2 \approx k_3 \approx 0$ (i.e. equation 4), the substrate molecule is exchanging between free solution and the enzyme-substrate complex. This may lead to chemical shift changes and line-width variations in the substrate spectrum; these spectral changes may be put on to a quantitative basis.

(b) When k_2 is large, $k_3 \approx 0$ and the inhibited enzyme is isolable . The substrate molecule is now completely

bound to the enzyme, and this will produce marked changes in the n.m.r. spectra of both the substrate and the enzyme in favourable cases.

(i) Chemical Shift Changes

When a magnetic group is exchanging between two different non-equivalent structural sites, it experiences a different degree of magnetic shielding in each of these environments. The effect of the exchange process on the n.m.r. spectrum will depend on the exchange rate between the two sites[7]. If this exchange is slow (i.e. $\tau_A \gg \Delta^{-1} \ll \tau_B$ where τ_A, τ_B are the life-times of the group in sites A and B respectively and Δ is the difference in shielding between sites A and B in radians sec^{-1}), a separate resonance line will be observed for the magnetic group in each environment. When the exchange rate is fast (i.e. $\Delta^{-1} \gg \tau_A, \tau_B$) only one averaged resonance signal will be observed at a position intermediate between the two extremes.

The fast exchange case was put on a quantitative basis by Dahlquist and Raftery[7] for a system described by equation 4. The enzyme concentration [E] at any instant is

$$[E] = Eo - [ES] \qquad \ldots\ldots 5$$

where Eo is initial enzyme concentration.

The corresponding substrate concentration $[S]$ at any instant is

$$[S] = So - [ES] \qquad \ldots\ldots 6$$

where So is initial substrate concentration.

The chemical shift of the free inhibitor (with no enzyme present) is taken as zero; the chemical shift of the completely bound inhibitor is Δ and the experimentally observed chemical shift is δ (relative to free inhibitor = 0)

$$\delta = Pb\,\Delta \qquad \ldots\ldots 7$$

where Pb is the fraction of substrate present in the bound form

$$\delta = \frac{[ES]}{So}\,\Delta \qquad \ldots\ldots 8$$

Substitutions of equations 2, 5, 6 into 8 with subsequent rearrangement and the simplifying assumptions $\delta \ll \Delta$ and $So \approx K_S$ leads to

$$So = \frac{Eo\,\Delta}{\delta} - K_S - Eo \qquad \ldots\ldots 9$$

$$So/Eo = \frac{\Delta}{\delta} - K_S/Eo - 1 \qquad \ldots\ldots 10$$

It is therefore possible to plot either So against $1/\delta$ or So/Eo against $1/\delta$ to give straight line graphs from which K_S and Δ can be easily obtained. By measuring the change in K_S with temperature, the entropy and enthalpy changes may be obtained for the interaction, using the Arrhenius equation.

Another approach is the method of Spotswood, Evans and Richards[8] who assumed that $[S] \approx [So]$ and shifts are plotted according to

$$\frac{1}{\delta_{obs} - \delta_1} = \frac{1}{\Delta}\left(\frac{So}{Eo}\right) + \frac{K_D}{Eo} \qquad \ldots\ldots 11$$

where $\delta = \delta_{obs} - \delta_S$.

Sykes[9] obviated the necessity of these double reciprocal plot approaches by a modification of the computer method of Groves, Huck and Homer[10]. He derived an equation for the concentration of the enzyme substrate complex, ES, as

$$[ES] = \frac{(Eo + So + K_D) \pm \sqrt{(Eo + So + K_D)^2 - 4\,Eo\,So}}{2} \qquad \ldots\ldots 12$$

when $0 \leqslant \frac{[ES]}{Eo} \leqslant 1$

The value of K_D is chosen as the one giving the best linear plot of δ_{obs} against $^{ES}/_{So}$. The program yields values for K_D, Δ and δ_s, and has the considerable advantage that the shift of the group in free solution does not have to be measured.

(ii) Line-width Changes

The line-width ($\Delta\nu_{\frac{1}{2}}$) of an n.m.r. spectral line depends on the relaxation processes taking place in the system. The relaxation processes, in turn, depend upon the correlation time, τ_c, of the group giving rise to the spectral line. τ_c may be thought of as the time a molecule takes to turn through one radian or move through a distance comparable to its molecular dimensions[11]. The larger the molecule, the longer will be the correlation time; this means shorter relaxation times and a broader spectral line. When a small molecule with a magnetic group is spending part of the time complexed to a larger molecule, it will have a correlation time characteristic of the macromolecule for the duration of its binding. The correlation time is given by

$$\tau_c = \frac{4\pi\eta a^3}{3kT} \qquad \ldots\ldots 13^{(12)}$$

where η = viscosity of medium

a = radius of molecule.

This is related to the line-width by the explicit relationship

$$\Delta\nu_{\frac{1}{2}} = \frac{3k}{4}\gamma^4\hbar^2 I(I+1)\sum_j \overline{|Yoj|^2}\,\tau_{cj} \quad \ldots\ldots 14$$

where k = Boltzmann constant

γ = magnetogyric ratio

$\hbar$ = Planck's constant

I = nuclear spin number

τ_{cj} = Correlation time

Yoj is given by

$$Yoj = (1 - 3\cos^2\theta ij)r_{ij}^{-3} \quad \ldots\ldots 15$$

where

r_{ij} = distance between nucleus i and neighbour j

θij = the angle between r_{ij} and Ho

Bloembergen, Purcell and Pound[13] have computed the dependence of the spin-spin lattice relaxation time (T_1) and the spin-lattice relaxation time (T_2) upon the correlation time τ_c (figure 1).

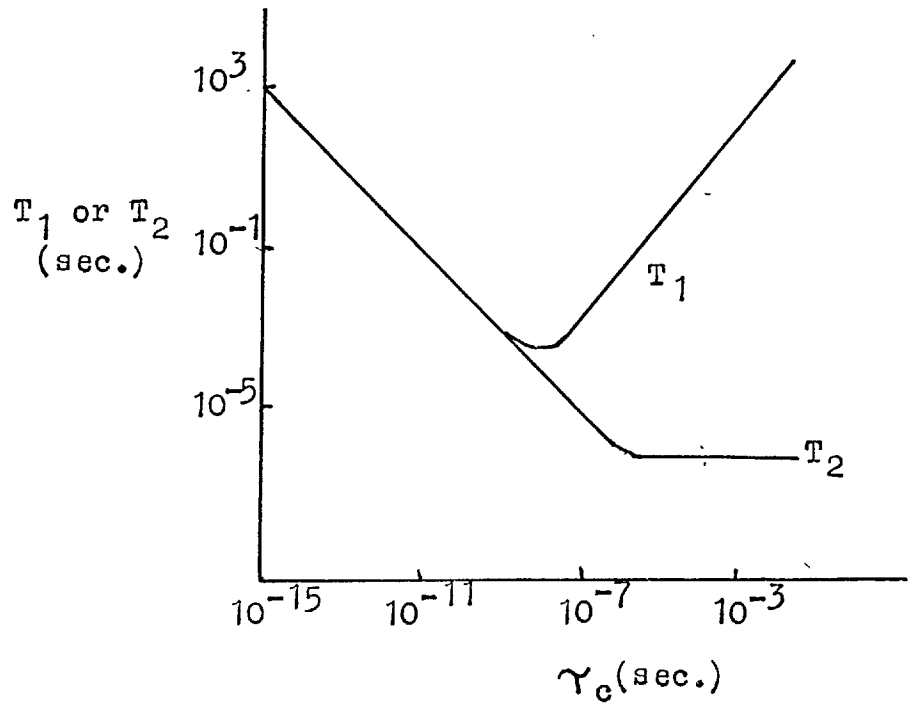


Figure 1.1: Relationship between relaxation times T_1 and T_2 and the correlation time τ_c

If the magnetic group on the substrate molecule is undergoing rapid exchange between the two extreme environments (such that $(\frac{1}{T_2})_{free} < (\frac{1}{T_2})_{bound} < (\frac{1}{T})$) the spectral line will appear broadened, and the amount of broadening can be related mathematically to the equilibrium constant for the exchange process[14] with similar reasoning to that used for chemical shift changes:

$$(\frac{1}{T_1})_{obs} = \alpha (\frac{1}{T_1})_{bound} + (1-\alpha)(\frac{1}{T_1})_{free} \quad \ldots\ldots 16$$

where α is the fraction of S bound.

This may be related to the observed line-width when $(\frac{1}{T_1}) = (\frac{1}{T_2})$ by

$$(\frac{1}{T_2}) = \pi \Delta \nu_{\frac{1}{2}} \text{ obs} \qquad \ldots\ldots 17$$

The relaxation times may be measured directly (and more accurately) by using spin echo techniques[15]. The use of line-width measurements for determination of T_1 and T_2 is only possible when the spectral lines are clearly visible ($\Delta \nu_{\frac{1}{2}} \leq 5$ Hz). When the lines are very broad, the dilute solutions used cause them to be indistinguishable from the base-line. This will clearly be the case when the irreversibly inhibited enzyme is isolated; the substrate molecule has a correlation time characteristic of the macromolecular protein and its high resolution n.m.r. spectrum is not detectable. In cases like this, the spin echo technique proves invaluable.

1.2 Use of n.m.r. to study the binding of small molecules to enzymes

Although n.m.r. has been widely used to study biological systems, only applications which involve complex formation between two molecules will be considered here, e.g. when a small substrate (or pseudo-substrate) molecule interacts with an enzyme. In principle, it is possible to study changes in the substrate spectrum in the presence of enzyme, or alternatively it is possible to study changes in the enzyme spectrum in the presence of substrate.

Changes in the n.m.r. spectrum of enzymes in the presence of small molecules have been observed and interpreted for simple enzymes with molecular weights less than 15,000 (e.g. lysozyme[16] and ribonuclease[17]). This approach, however, is of limited value with larger enzymes, as the long correlation times lead to very broad spectral lines. For example, an α-chymotrypsin molecule (monomeric molecular weight 25,000) has the overall dimensions $45 \times 35 \times 38$ A^{o} [18], with an average diameter in solution of 39 A^{o}. The calculated correlation time for such a molecule in aqueous solution is 4×10^{-8} secs, which corresponds to a line-width $> 1{,}000$ Hz for the enzyme spectrum, and obviously makes unambiguous assignments difficult.

The changes in "substrate" spectra which occur in the presence of protein have been widely studied and reported. The substrate may be either the true substrate for the enzyme or, more commonly, it may be a simple pseudo-substrate of a similar chemical type to the natural substrate. For example, in nature lysozyme catalyses the hydrolysis of complex polysaccharides, such as chitin, at α-glycosidic linkages while laboratory experiments have used simple glycosides of N-acetyl-glucosamine[19] as pseudo-substrates.

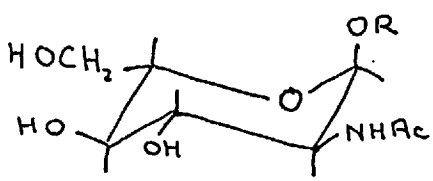
It is evident from section 1.1 that the n.m.r. experiments enable certain parameters to be evaluated, namely the dissociation constant K_S, the chemical shift Δ of the bound species, the relaxation times of the bound species and hence the bound correlation time τ_c. These parameters give information about the magnetic environment of the bound substrate which is potentially of importance. However, when the observed changes are interpreted, it is important to realise the experimental limitations of the technique, so that changes which are comparable with the inherent inaccuracies are not interpreted on a physical basis. The experiments should present evidence that the observed changes are due to an interaction of the substrate with the protein and if possible identify the particular part of the protein, and the specific amino acid residues

involved. A large proportion of the published work to date can be criticised for either misinterpretation or over-interpretation or the experimental results obtained.

Several different proteins and enzyme systems have been examined cursorily by n.m.r., such as lysozyme[20], ribonuclease[17], yeast alcohol dehydrogenase[21], α-chymotrypsin[22], horse serum cholinesterase[23], carboxypeptidase[24], and aspartate transcarbamylase[25]. Many of these systems have been used to demonstrate different aspects of the n.m.r. method, but a truly rigorous study has not been achieved.

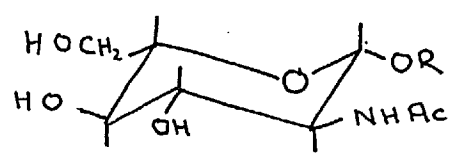
1.2.1 Chemical Shift Changes

The earliest demonstration of chemical shift changes occurring in substrate spectra in the presence of enzyme was for interaction of carbohydrates with lysozyme.



α anomer

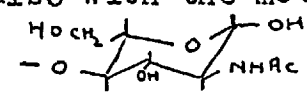
I



β anomer

II

Figure 1.2: Anomeric forms of D glucosamine derivatives

Thomas[20, 26] showed that the acetamido methyl resonance of the α-N-acetyl glucosamine (I, R = H) shifted 3.9 Hz upfield in the presence of lysozyme; furthermore, after ~60 minutes, mutarotation occurs (I ⇌ II) and two acetamido methyl resonances of almost equal intensity, identified as the α and β anomers, were observed (one shifted 1.4 Hz upfield, the other shifted 3.9 Hz upfield). Thus the α and β forms of N-acetyl-glucosamine were apparently interacting in different ways with the enzyme. Without any further evidence, the upfield shifts were said to be caused by the shielding of an aromatic ring and attributed to the shielding influence of the aromatic residue of tryptophan 108. The results were assumed to arise from specific binding of lysozyme to the α and β N-acetyl glucosamine, but no attempt was made to verify this. Similar n.m.r. changes were observed with the methyl glycosides of α (I R = CH_3) and β (II R = CH_3) N-acetyl glucosamine, and also with the methyl glycosides of N,N diacetyl chitobiose (R = ); these were interpreted in the same qualitative manner.

Raftery and his co-workers have also studied the lysozyme system using both chemical shift changes and line-width changes to obtain results. Initially, they studied the lysozyme - N-acetyl glucosamine interaction[7, 27],

together with the effects of temperature and pH on their measurements[28]. The values of K_S obtained ($\sim 10^{-2}$M) parallel those obtained using other spectroscopic methods, e.g. UV, suggesting that the n.m.r. method gives accurate and valid results or, at least, that n.m.r. is monitoring the same interaction as other techniques. The analysis also suggests that the simple binding model used ($E + S \rightleftharpoons ES$) is adequate to describe the system. The variation of pK_S with the pH of the enzyme solution was investigated according to the method of Dixon[29], and this led to the identification of a group whose pK_a was changed from 6.1 in the free enzyme to 6.6 in the enzyme - inhibitor complex. From a consideration of known X-ray structure of the enzyme this was assigned to glutamic acid 35. A plot of Δ with pH showed discontinuities at pH 4.7 $\pm$ 0.1 and pH 7.0 $\pm$ 0.5; the lower value was attributed to a group which did not appear to affect the binding constant, K_S, and other physical data led to the assignment of this to aspartic acid 103.

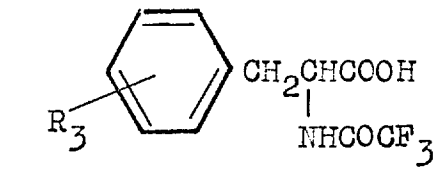
The same workers[30] studied the binding of disaccharides, trisaccharides and tetrasaccharides in an attempt to gain information about the three contiguous binding subsites which have been proposed on the basis of X-ray studies. They also investigated the nature of the functional groups required on the substrate[31] for binding

to occur, by using such pseudo-substrates as N-acetyl cyclohexylamine to test for necessity for the amide grouping and the D and L diasteriomers of 2-trans-acetamido cyclohexanol to demonstrate the need for both amide and hydroxyl functions. This series of papers represents the first rigorous demonstration of the power of n.m.r. for the study of this type of system. The results rely on the tacit assumption that the conformation of the enzyme as shown in solid X-ray structure still exists in solution. Even so, no direct experimental evidence was given to show that these effects were genuinely caused by binding to the "active centre" of the enzyme. One complicating factor, which was not investigated, was the use of buffered deuterium oxide solutions for the proton spectra of the carbohydrates in the presence of lysozyme. The use of deuterium oxide may lead to isotope exchange effects resulting in deuteration of different parts of the enzyme and also to kinetic isotope effects[32].

Another enzyme which has been studied by observing proton magnetic resonance chemical shift changes in pseudo-substrates is α-chymotrypsin. This is another relatively simple enzyme and is the object of work described elsewhere in this thesis. J.T. Gerig, for example, has studied the interaction of tryptophan derivatives[33] (figure 1.3,

Compound Type VIII) and trans cinnamic acid[34] (figure 1.3, Compound X) with ∝ -chymotrypsin. The latter paper suggests that the aromatic and vinyl protons of trans cinnamic acid are affected differently by the enzyme binding, which is interpreted as being due to the proximity of the phenyl ring of the trans cinnamic acid to an aromatic residue in the enzyme. The effects disappear completely when the enzyme is chemically modified (for example when Serine 195 is phosphorylated with diisopropyl fluorophosphate (figure 1.3, Compound XII), methionine 192 is alkylated with ∝-bromo-acetanilide (figure 1.3, Compound IX x = H), or histidine 57 is alkylated with L-1-tosylamido-2-phenyl ethyl chloromethyl ketone (figure 1.3, Compound XIII)). This is rather an ambiguous test for specific binding, because there is no direct evidence to suggest that the chemically modified enzymes have the same conformation as the native enzyme.

Many workers have attempted enzyme n.m.r. experiments using other nuclei as probes, and there have been several ^{19}F studies involving ∝ -chymotrypsin. They have mainly concentrated on tyrosine (figure 1.3, Compound VII), phenyl alanine (figure 1.3, Compound VI R_3 = H) and tryptophan (figure 1.3, Compound VIII) derivatives. These are considered in more detail in section 4.1.



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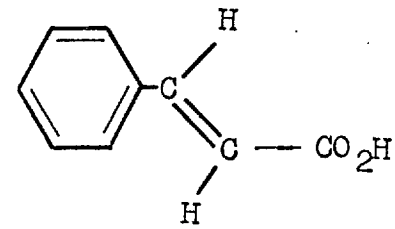

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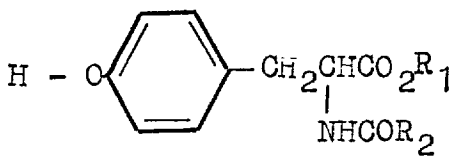

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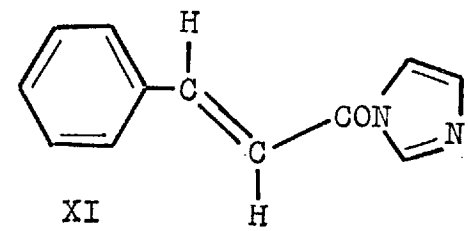

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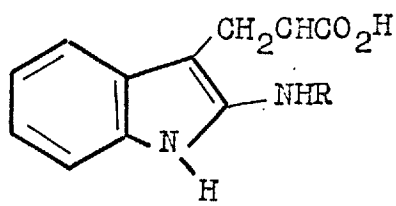

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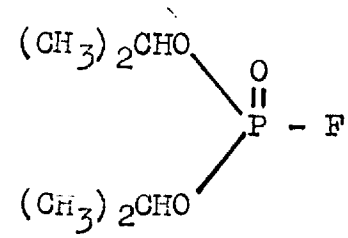

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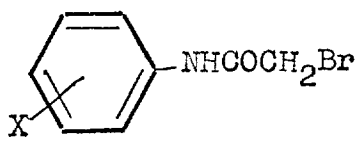

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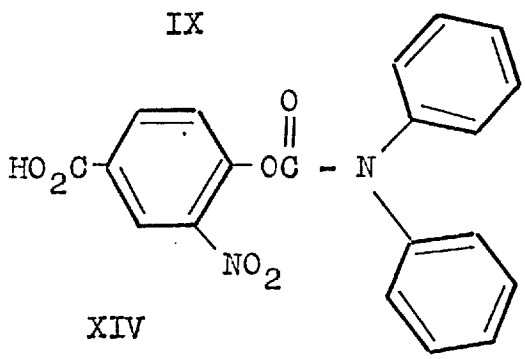

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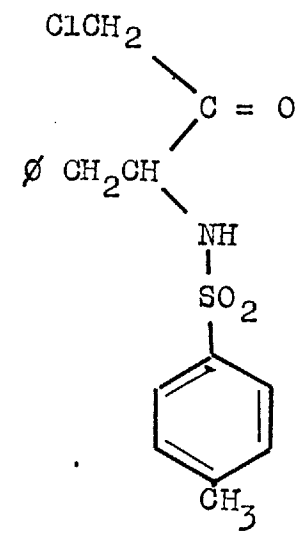


XIII

Figure 1.3: Pseudo-substrates used in experiments with the enzyme α-chymotrypsin

There have also been recent reports of the use of ^{19}F-resonance with carbohydrate derivatives interacting with lysozyme[38, 39].

1.2.2 Relaxation Measurements

Relaxation measurements have been used to study the interaction of small molecules with large molecules. In section 1.1 B, the background to studying relaxation time changes with binding was considered. The relaxation times (T_1 and T_2) can be measured in a variety of ways: spectral line widths[14]; progressive saturation techniques[40], and transient n.m.r. methods[9] may be used. Each of these different methods has been used with biological systems. The easiest, but least accurate method, is to observe changes in the spectral line-widths which occur on binding and relate these to relaxation times by the simple expression

$$\pi \Delta\nu_{\frac{1}{2}} = \frac{1}{T_2} \qquad \ldots\ldots 17$$

where $\Delta\nu_{\frac{1}{2}}$ = line-width at half peak height.

Jardetsky and co-workers have produced several excellent papers using line-width changes to estimate T_2 and the progressive saturation technique to measure T_1.

The systems studied by Jardetsky include the binding of penicillins and sulphonamides to bovine serum albumin[14,42] and a review of the general theory and early work has been published[41].

These workers developed various criteria to show that specific interactions were occurring, and were the first to show that the observed n.m.r. spectral changes were due to an interaction between the substrate molecule and a particular site on the protein, and not due to viscosity changes, substrate concentration changes or bulk magnetic susceptibility changes.

Viscosity or concentration changes will produce similar changes in relaxation times of different groups in the same molecule, e.g. sulphacetamide aromatic proton relaxation times change from 0.25 sec^{-1} to 0.5 sec^{-1} as the concentration increases from 0.1 M to 1 M and the methyl group relaxation times change from 0.45 sec^{-1} to 0.9 sec^{-1} over the same concentration range, both by a factor of 2. The relaxation times of the same groups were measured in the presence of varying concentrations of γ-globulin (in order to increase viscosity with no binding) and the increase in relaxation rates parallels the increase in viscosity for both peaks.

The viscosity of the most concentrated protein solution used was 1.25 times the viscosity of pure deuterium oxide,

whereas the increases in relaxation rates measured for the sulphacetamide system were

aromatic peaks	23	x	$(\frac{1}{T_2})$	D_2O solution	$\frac{1}{T_2}$
methyl	8	x	$(\frac{1}{T_2})$	D_2O	"

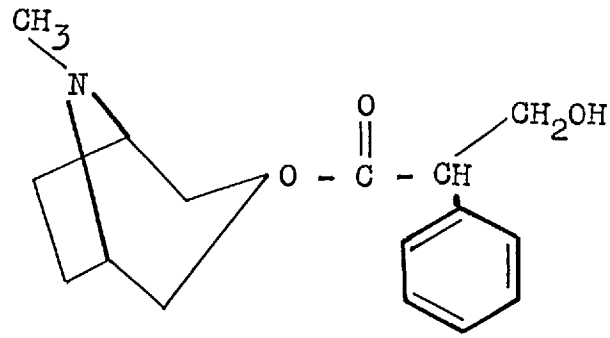
The relaxation rate depends upon the substrate to protein ratio, and decreases with increasing substrate concentration for a given protein concentration.

Protein systems which have been studied so far using line-width changes include yeast alcohol dehydrogenase[21], horse serum cholinesterase[23], acetyl cholinesterase[43], ribonuclease[17], antibody-hapten interactions[44], α-chymotrypsin[8] and lysozyme[20].

Kato has used line-width changes to study the interactions of various substrates and pseudo-substrates with acetyl cholinesterase and closely related enzymes. In the first paper of the series, n.m.r. integration was used to follow the appearance of acetic acid from the hydrolysis of acetyl choline by horse serum cholinesterase[23]. The line-widths of the acetyl choline resonance signals were monitored as the enzymic hydrolysis proceeded[23] (the sweep width was 10 Hz/cm, sweep time was 2 Hz/sec.) and were converted to relaxation times using equation 17. The line-

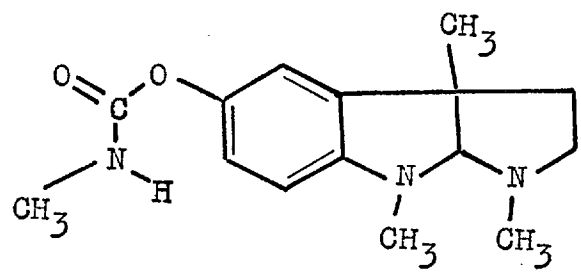
width of the quaternary ammonium resonance signal was observed to reach a maximum more rapidly than did the acetyl resonance signal, and this was interpreted as showing the binding of the positively charged trimethyl ammonium group to the anionic site of the enzyme as the first step in which the substrate molecule was anchored to the enzyme active centre, thus delivering the acetyl group to the esteratic site. The time taken for the trimethyl ammonium resonance to reach the maximum, however, (several minutes), rather suggests that this interpretation is incorrect. The same workers studied the interaction of eserine (figure 1.4, Compound XVI) and atropine (figure 1.4, Compound XV) with acetyl cholinesterase[43]. The line-widths at half-height were quoted to within 0.01 Hz but the experimental conditions (sweep width 5 Hz/cm, sweep time 0.5 Hz/sec.) imply that line-widths can be measured to 0.02 mm, which seems unreasonable. They found that both eserine and atropine bind to the enzyme to a similar extent, but after incubation with an irreversible inhibitor such as di-isopropyl fluorophosphate, the eserine binding is diminished, while the atropine still binds to the same extent. As atropine is a poorer anticholinesterase agent ($I_{50} \sim 10^{-1}$) than eserine ($I_{50} \sim 10^{-7}$) it was suggested that

the two molecules bind to different structural sites on the enzyme. This result could, however, be explained by simple competitive inhibition.



XV

ATROPINE



XVI

ESERINE

Figure 1.4: Pseudo-substrates used with Acetyl Cholinesterase

Transient Methods

Pulsed or transient methods for measuring relaxation times were pioneered by Bloembergen, Purcell and Pound[13], and Torrey[45], while Hahn[46] introduced the concept of spin echoes.

Essentially the experimental method is to allow the observed nuclei to come to magnetic equilibrium in the static magnetic field and an excitation pulse is then applied. The excitation pulse is a short pulse of radiofrequency energy at the resonant frequency, the duration and intensity of which are selected to nutate the nuclear polarisation by the desired angle (usually 180° for T_1 and 90° for T_2). The return to equilibrium is then monitored as a function of time.

This type of approach has been largely confined to biological systems involving paramagnetic probes, e.g. metallo enzymes, and an excellent review of this aspect of the topic by Mildvan and Cohn has recently appeared[47].

Sykes was the first person to demonstrate the potential application of this type of approach to enzyme systems using diamagnetic probes. He used the rotating frame method[9] which requires little modification to conventional high resolution n.m.r. spectrometers[48]. This experiment yields the function $T_{1\rho}$ which has been

shown to have the form[55]

$$\frac{1}{T_{1\rho}(H_1)} = \langle A \rangle^2 \frac{\tau}{1 + (\omega_1 \tau)^2} + \left(\frac{1}{T_1}\right)_{other} \quad \ldots\ldots 18$$

where $\left(\frac{1}{T_1}\right)_{other}$ is the contribution from relaxation processes which are not slow,
and $\langle A \rangle^2$ contains the physical constants of the slow relaxation mechanism.

In comparison T_2 has the form

$$\frac{1}{T_2} = \langle A \rangle^2 \tau + \left(\frac{1}{T_1}\right)_{other} \quad \ldots\ldots 19$$

$$\omega_1 = \gamma H_1 \quad \ldots\ldots 20$$

T_2 can therefore be obtained by measuring $T_{1\rho}$ as a function of H_1 and extrapolating to $H_1 = 0$. Sykes then derived the following equation which links $T_{1\rho}$ with K_D, Δ and k_{-1}.

$$\frac{1}{T_{1\rho}(0)} - \frac{1}{T_1} = \frac{Eo}{K_D + 1^0} \left(\frac{1}{k_{-1}}\right) \Delta^2 \quad \ldots\ldots 21$$

For a system with an observed chemical shift it is possible to obtain K_D and Δ as shown previously (equation 12, page 17).

From the combination of the transient and chemical shift approaches, it is possible to obtain K_D, k_{-1}, k_1 and Δ. Sykes has used this technique on several systems, e.g. lysozyme[49]; α-chymotrypsin[9]; aspartate transcarbamylase[25] and human serum albumin[50].

1.3 Biochemical background for α-chymotrypsin

This is a relatively simple proteolytic enzyme, with a monomeric molecular weight of approximately 25,000, found in the pancreas, whose main function in nature is the hydrolysis of peptides and proteins at (L) aromatic aminoacid residues. This enzyme has been shown to follow the simple kinetic scheme outlined in section 1.1.

Figure 1.4 gives the aminoacid sequence of the enzyme[65] and figure 1.5 shows a schematic drawing depicting the conformation of the enzyme (as suggested by X-ray)[66]. The residues which are thought to be close to the active centre of the enzyme are Serine 195, Histidine 57, Tyrosine 171, Tyrosine 146 and Methionine 192, as enzyme activity is lost if any of these residues are chemically modified. However, the lack of enzymic activity after chemical modification is rather an ambiguous test for the proximity of a particular group to the active centre as modification could either change the actual conformation of the enzyme or simply block the access to the active centre without necessarily blocking the active centre itself.

Degradation studies suggest that Serine 195 is the residue which is important during the hydrolysis of esters and amides.

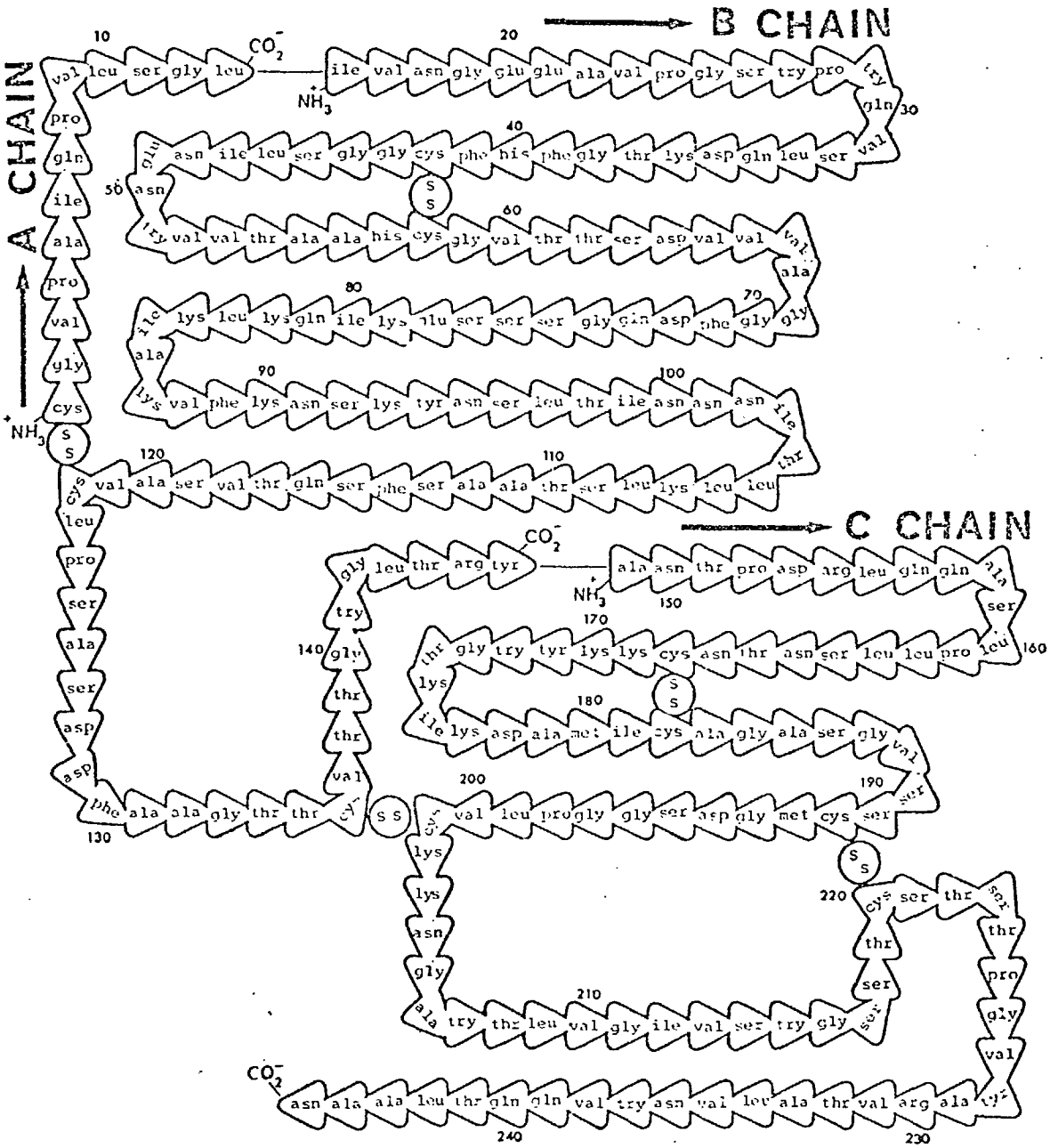


Figure 1.4: Amino acid sequence of α-chymotrypsin(65)

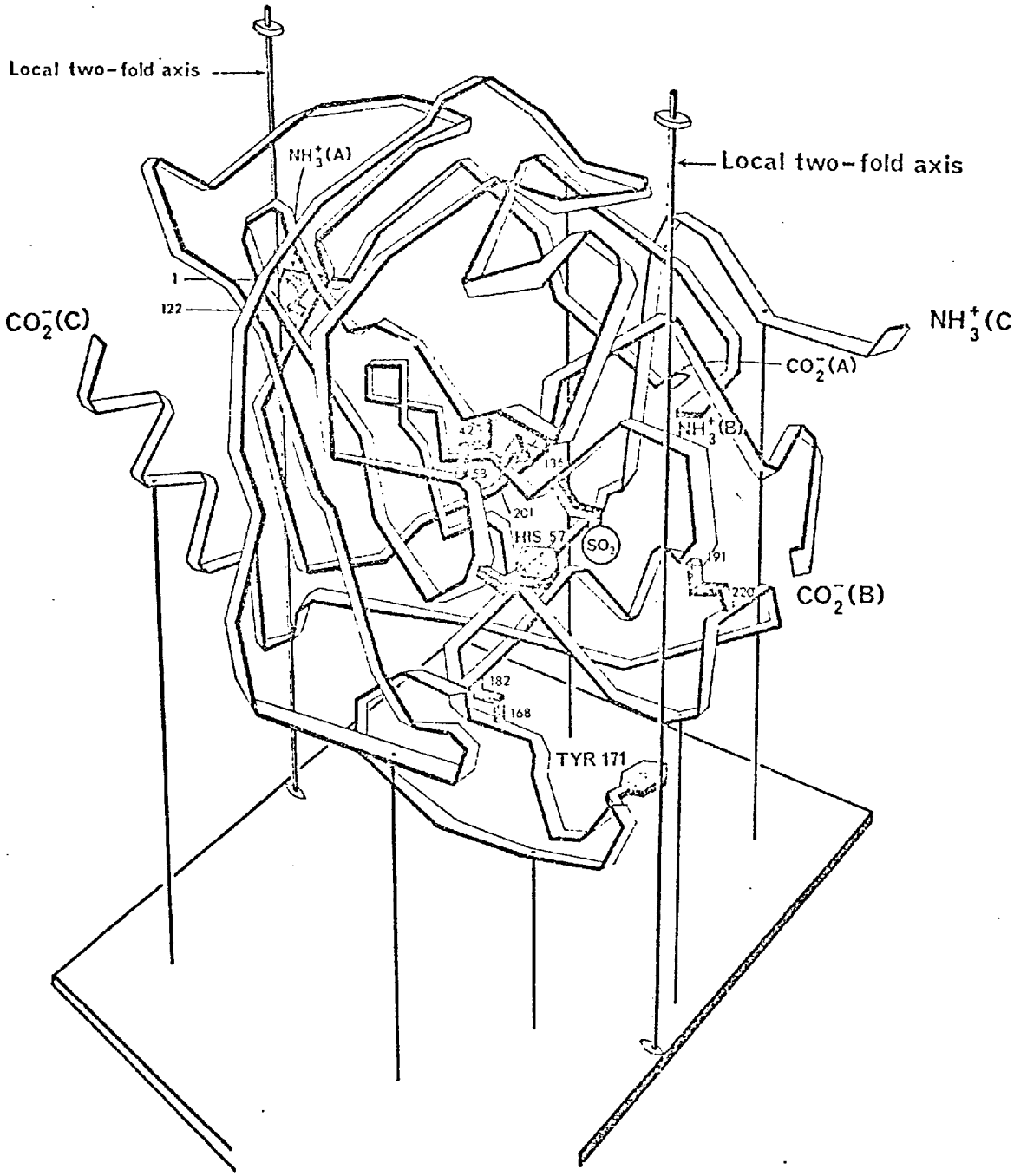


Figure 1.5: Schematic representation of the conformation of α-chymotrypsin

CHAPTER TWO

Experimental.

2.1 Nuclear Magnetic Resonance Measurements

The nuclear magnetic resonance measurements were made using a Varian HA100 spectrometer operating at 100 MHz and 94.1 MHz for proton and fluorine resonance respectively. The spectrometer was operated in the frequency sweep "locked" mode.

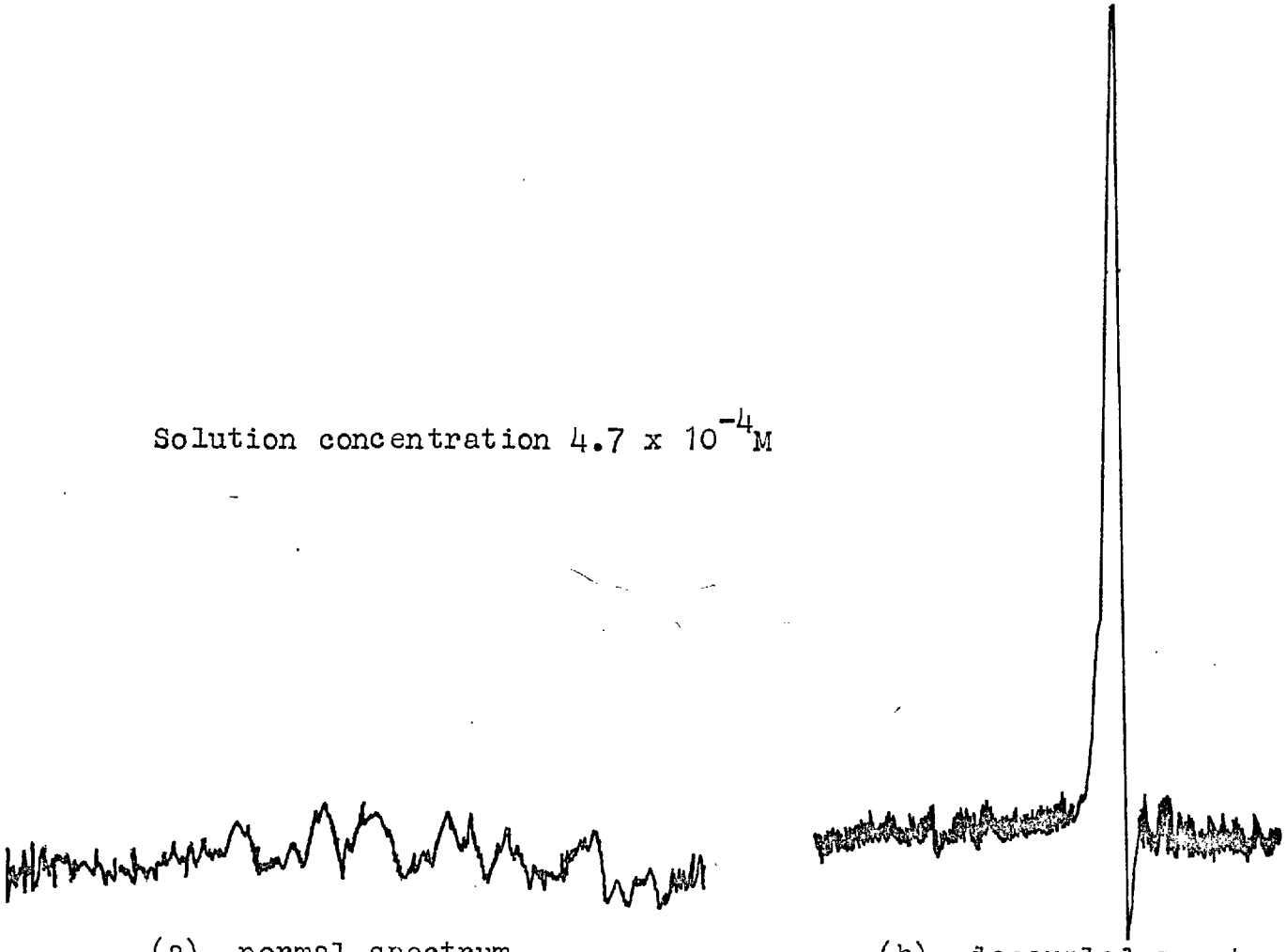
The internal field/frequency lock system has been modified to allow wide sweep (25 KHz) with automatic phase correction[51]. The instrument has also been fitted with a heteronuclear decoupler which enables fluorine resonance spectra to be decoupled from proton spectra (and vice versa)[52].

All spectra were recorded on pre-calibrated charts. Tetramethyl silane (TMS) and trifluoroacetic acid (TFA) were used as reference compounds and lock signals for proton and fluorine respectively. The chemical shift of the resonance signals relative to the internal reference was determined in the frequency sweep mode by measuring the frequency difference between the manual oscillator (lock signal) and the sweep oscillator. Frequencies were measured using either a Varian V-4315 (Beckmann Model 6000-4) frequency counter accurate to $\pm$ 0.1 Hz or a Racal type 801R frequency counter.

Spectra of dilute solutions were obtained using repetitive scanning with a time averaging computer (Varian CAT 1024).

The aromatic fluorine resonance spectra of derivatives of ortho fluorophenyl alanine, meta fluorophenyl alanine, para fluorophenyl alanine, were simplified by heteronuclear decoupling. The increase in sensitivity obtained by the Overhauser effect is illustrated in figure 2.1 for N-trifluoroacetyl meta fluorophenyl alanine (4.7×10^{-4}M). This increase in sensitivity is also obtained with the ortho fluorophenyl alanine and para fluorophenyl alanine derivatives on decoupling. This type of decoupler caused severe overheating of the spectrometer probe which was overcome by using the variable temperature accessory of the instrument to stabilise the temperature.

Solution concentration 4.7×10^{-4}M



(a) normal spectrum

(b) decoupled spectrum

Figure 2.1: The effect of heteronuclear decoupling on the ^{19}F resonance spectrum of N-trifluoroacetyl-DL-meta fluorophenyl alanine

2.2 Preparation of Buffer solutions

The following buffer solutions were prepared according to literature methods.

Table 2.1

Buffer Systems Used

Buffer System	pH Range	Ref.
Tris HCl	7.0 - 9.0	53
Phosphate	5.8 - 8.0	53
Citrate	5.8	54

After preparation, the buffer solutions were deoxygenated by bubbling oxygen-free nitrogen through the solution for two hours.

2.3 Enzyme Experiments

A typical enzyme experiment is described: α-chymotrypsin (sigma lot no. 500-2550)(50.0 mgs.) was dissolved in phosphate buffer pH 6.5 (2 ml) (Solution A). N-trifluoroacetyl-DL-ortho fluorophenyl alanine (19.6 mgs) was dissolved in phosphate buffer (2 ml) pH 6.5, (Solution B). The following solutions were then prepared from these stock solutions.

Table 2.2

Dilution of Substrate Solution

Solution	Vol. of Substrate solution	Vol. of Buffer
C	0.5 ml B	0.5 ml
D	0.5 ml C	0.5 ml
E	0.5 ml D	0.5 ml
F	0.5 ml E	0.5 ml
G	0.5 ml F	0.5 ml

Table 2.3

Preparation of enzyme/substrate solutions

Solution	Volume of enzyme solution	Volume of substrate solution
1	0.2 ml A	0.2 ml B
2	0.2 ml A	0.2 ml C
3	0.2 ml A	0.2 ml D
4	0.2 ml A	0.2 ml E
5	0.2 ml A	0.2 ml F
6	0.2 ml A	0.2 ml G

The fluorine-19 nuclear magnetic resonance spectra of solutions B to G and 1 to 6 were then obtained using an external capillary of trifluoroacetic acid as lock signal. The enzyme concentration was kept constant to minimise bulk magnetic susceptibility changes from solution to solution. The enzyme concentration was measured either using a U.V. method[55] or using the n.m.r. method

Results were calculated from equation

or using the method of Groves, Huck and Homer[10].

A typical graph is shown in figure 2.2.

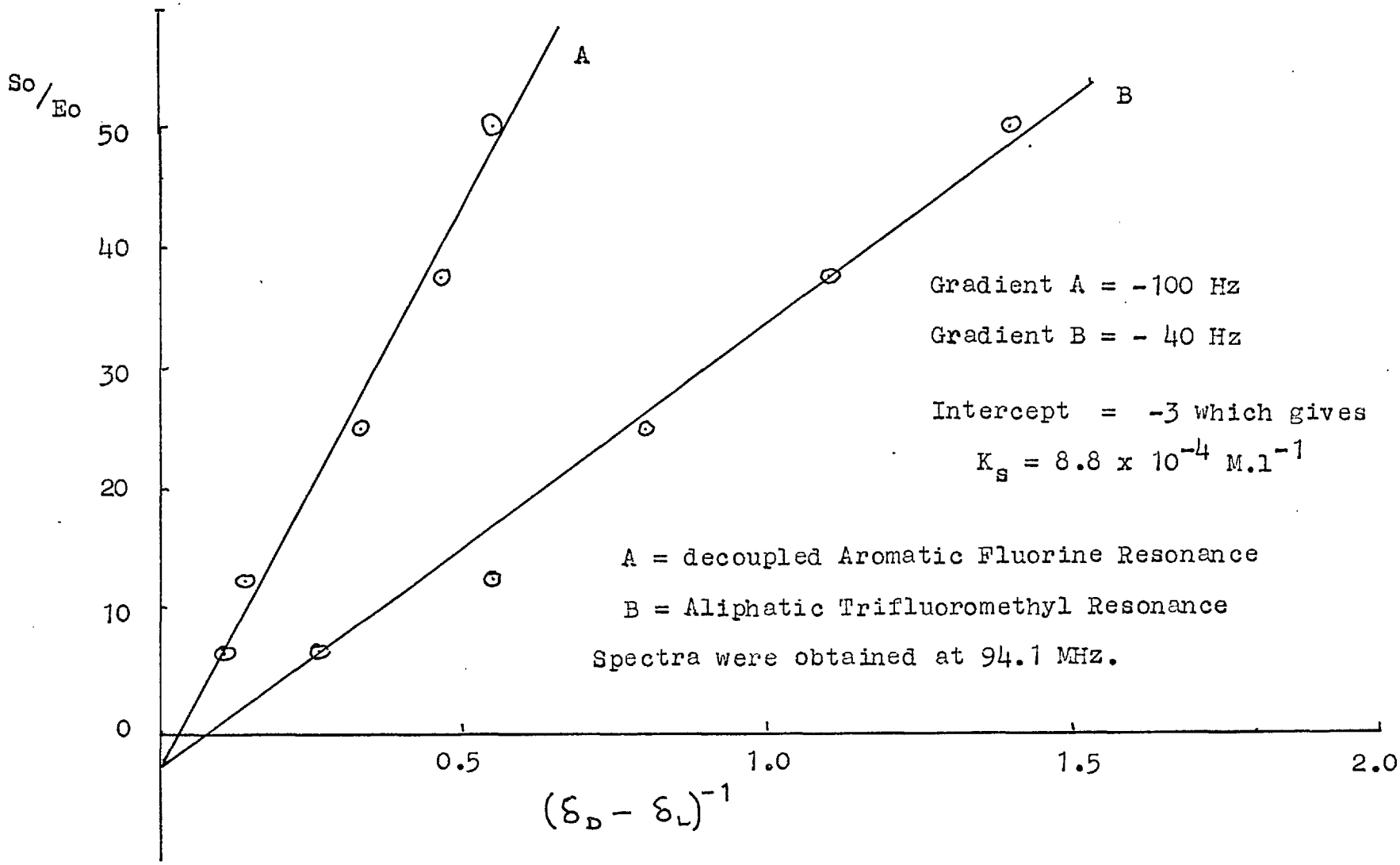


Figure 2.2: Typical Graph for determination of K_s and Δ . This example is N-trifluoroacetyl meta fluorophenyl alanine with α -chymotrypsin at pH 6.5 and 31°C.

2.4 Preparation of Compounds

The structures of all compounds described were verified by spectroscopic techniques. N.m.r. measurements were made using the following instruments: Varian HA100, Varian HA100D, Varian A60, Jeol MH60, Jeol C60HL. I.R. measurements were made using a Unicam SP200, Perkin Elmer Infracord, Perkin Elmer 437. U.V. measurements were made using a Unicam SP800. Microanalytical analyses were performed by either Analytical Chemistry Division, Shell Research Ltd., Sittingbourne, or Microanalytical Laboratory, Chemistry Department, Imperial College.

Purifications of the synthesized compounds were carried out by preparative thin-layer chromatography (T.L.C.), column chromatography or conventional techniques such as recrystallisation and distillation. Purity of compounds was tested by T.L.C. Grade III alumina was used for column chromatography unless indicated otherwise.

Notes on Syntheses Used

Substituted Phenyl Alanine Derivatives

These compounds were prepared according to the synthetic scheme outlined in figure 2.3. This is a slight variation of the literature method[56]. The carbonyl

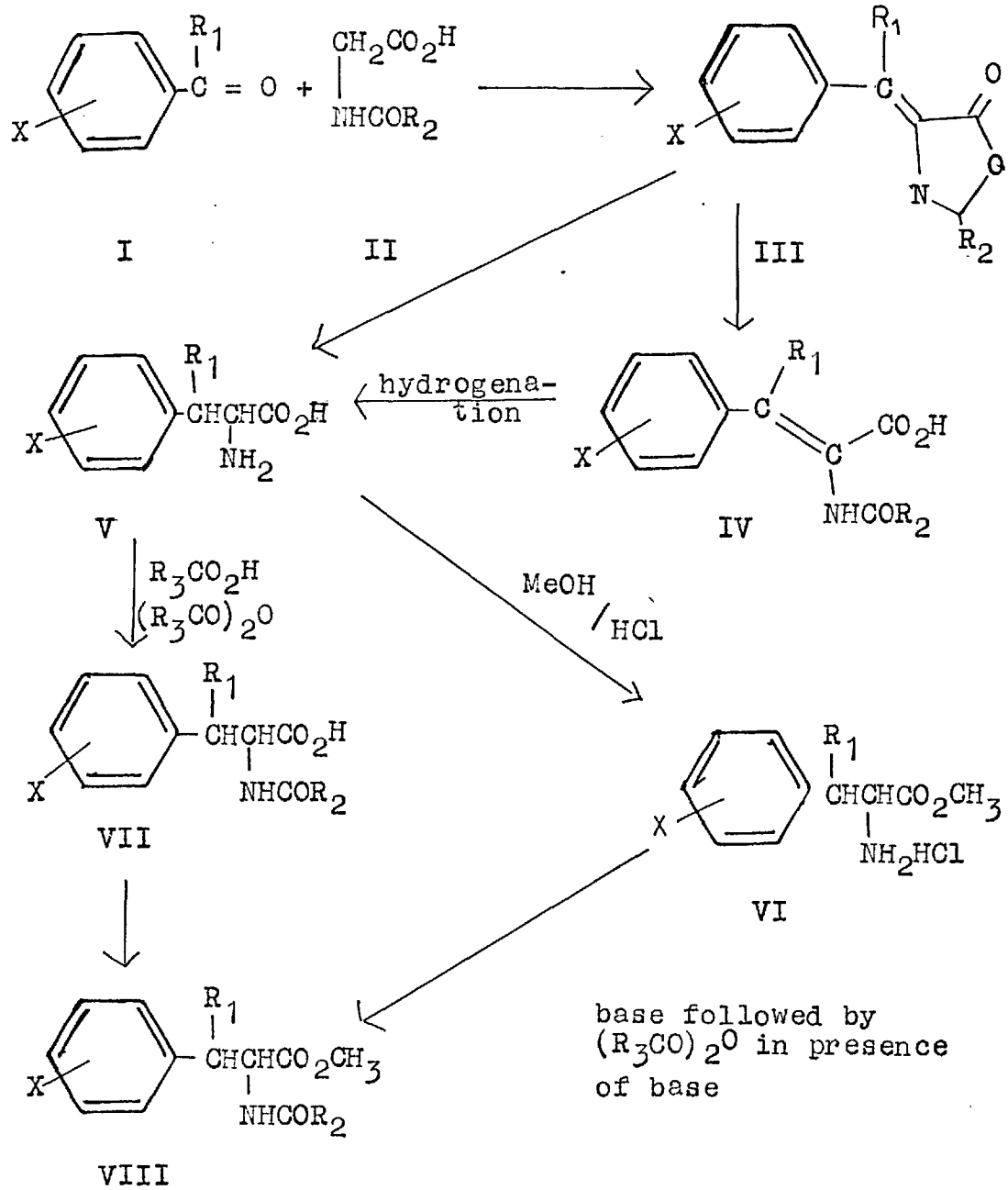
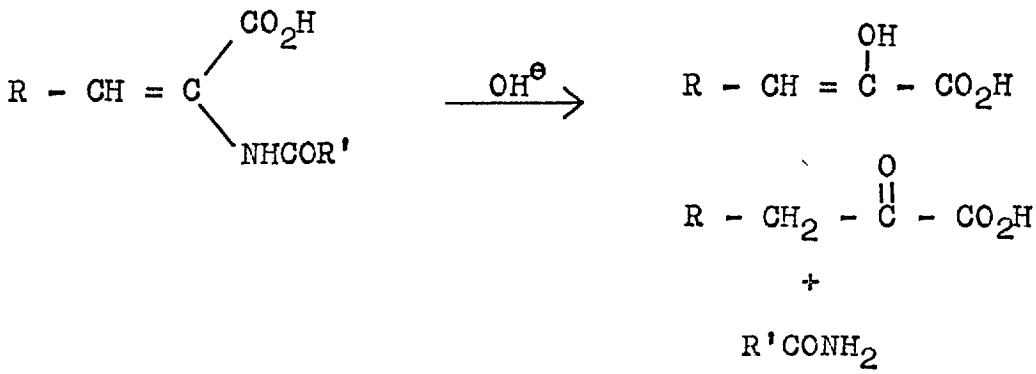


Figure 2.3: Reaction Scheme used for preparation of substituted propionic acid derivatives.

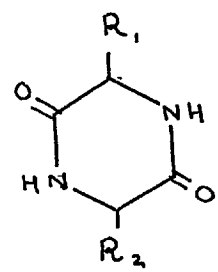
compound (I) is condensed with the glycine derivative (II) to form the oxazolone (III) (azlactone). The oxazolone is easily hydrolysed by, for example, aqueous acetone to form the acylamino cinnamic acid (IV). It is essential to keep the base concentration as low as possible, otherwise the following reaction occurs(57)



The stereochemistry of this reaction has been shown to give the cis derivative (i.e. aromatic ring cis to carboxyl group). The acylamino cinnamic acid is then hydrogenated either by phosphorus/hydriodic acid or by catalytic hydrogenation. The former method of hydrogenation gives low yields of the amino acid ($\sim$ 40 - 55%). It also hydrolyses the acyl amino group. The next stage of the reaction involves re-acylation of the amino group. The introduction of the desired acyl group was attempted in the first stage of the reaction by using suitably substituted glycine derivatives. It was hoped that the resulting series of

substituted oxazolones and acylamino cinnamic acids would prove interesting substrates for α -chymotrypsin. The introduction of a trifluoromethyl group or a monofluoromethyl group at this stage of the reaction proved impractical as the resulting oxazolone was too unstable to form.

It was also attempted to cut down the number of synthetic steps from the phenylalanine derivative (V) to the N-acyl phenyl alanine ester (VIII) by forming the common intermediate ester (VI) and then acylating this intermediate. Unfortunately, attempts to remove the hydrogen chloride to form the free amine resulted in the loss of methanol and dimerisation to form diketo piperazine derivatives of the type[57]



This was therefore abandoned.

Table 2.4

Compounds Prepared

Compound	Name
I	2-acetamido-propionic acid
II	2-benzamido-propionic acid
III	4-*cis*-benzylidene-2-phenyl-oxazolin-5-one
IV	4-*cis*-benzylidene-2-methyl-oxazolin-5-one
V	2-amino-DL-3-phenyl-propionic acid (phenyl alanine)
VI	2-amino-3-phenyl propionic acid methyl ester hydrochloride
VII	Trifluoroacetic anhydride
VIII	2-trifluoroacetamide-DL-3-phenyl propionic acid (N-trifluoroacetyl-DL-phenyl alanine)
IX	2-trifluoroacetamido-DL-3-phenyl-propionic acid methyl ester
X	2-trifluoroacetamido-L-3-phenyl-propionic acid (N-trifluoroacetyl-L-phenyl alanine)
XI	2-trifluoroacetamido-L-3-phenyl-propionic acid methyl ester
XII	4-*cis*(-2-fluorobenzylidene)-2-methyl-oxazolin-5-one

/ contd. .....

XIII	2-amino-DL-3-(2-fluorophenyl) propionic acid (ortho fluorophenyl alanine)
XIV	2-trifluoroacetamido-DL-3-(2-fluorophenyl) propionic acid (N-trifluoroacetyl-ortho fluorophenyl alanine)
XV	2-trifluoroacetamido-DL-3-(2-fluorophenyl)-propionic acid methyl ester
XVI	4-cis(-3-fluorobenzylidene)-2- methyl-oxazolin-5-one
XVII	2-amino-DL-3-fluorophenyl propionic acid (DL-metafluorophenyl alanine)
XVIII	2-trifluoroacetamido-DL-3-(3-fluorophenyl) propionic acid (N-trifluoroacetyl-DL-meta fluorophenyl alanine)
XIX	2-trifluoroacetamido-DL-3-(3-fluorophenyl) propionic acid methyl ester
XX	4-cis-(4-fluorobenzylidene)-2-methyl-oxazolin-5-one
XXI	2-amine-DL-3-(4-fluorophenyl)-propionic acid (DL-parafluorophenyl alanine)
XXII	2-trifluoroacetamido-DL-3-(4-fluorophenyl) propionic acid (N-trifluoroacetyl-DL-para fluoro phenyl alanine)

/contd. .....

XXIII	2-trifluoroacetamido-DL-3-(4-fluorophenyl) propionic acid methyl ester
XXIV	4-cis-(2-fluorobenzylidene)-2-phenyl-oxazolin-5-one
XXV	4-cis-(3-fluorobenzylidene)-2-phenyl-oxazolin-5-one
XXVI	4-cis-(4-fluorobenzylidene)-2-phenyl-oxazolin-5-one
XXVII	trans cinnamoyl chloride
XXVIII	N-trans cinnamoyl imidazole
XXIX	4-cis-(4-nitrobenzylidene)-oxazolin-5-one
XXX	2-amino-3-(4-nitrophenyl) propionic acid Attempted preparation
XXXI	2-amino-3-(4-nitrophenyl) propionic acid (DL-para nitrophenyl alanine)
XXXII	N-trifluoroacetamido-cyclohexane
XXXIII	N-monofluoroacetamido-cyclohexane
XXXIV	4-nitrophenyl acetate
XXXV	Resolution of (±) 1-phenyl ethylamine
XXXVI	2-trifluoroacetamido-DL-3-(4-nitrophenyl) propionic acid (N-trifluoroacetyl para-nitro-phenyl alanine)
XXXVII	2-trifluoroacetamido-DL-3-(4-hydroxyphenyl) propionic acid (N-trifluoroacetyl-DL-tyrosine)
XXXIX	2-trifluoroacetamido-L-3-(4-hydroxyphenyl) propionic acid (N-trifluoroacetyl-L-tyrosine)

I Preparation of Acetyl Glycine (Aceturic Acid)

Glycine (7.5 g, 0.1 m) was dissolved in a mixture of glacial acetic acid (20 ml) and freshly distilled acetic anhydride (10 ml). The mixture was stirred at room temperature overnight. The mixture was poured on to ice (100 g), the crystalline product was filtered off and dried under vacuo. The crude product (10.6 g ≡ 91% of theoretical yield) had m.p. 203 - 4°C (Lit.[56] 207 - 208°) it was therefore used without further purification.

II Preparation of benzoyl glycine (hippuric acid)

Glycine (7.5 g, 0.1 m) was dissolved in sodium hydroxide solution (75 ml, 0.1 m) in a conical flask. Benzoyl chloride (14.0 ml) was added portionwise (~3 ml), the flask was stoppered and shaken vigorously after each addition. The solution was poured on to crushed ice (10 g), concentrated hydrochloric acid was added dropwise until the solution was just acid to congo-red paper. The resulting crystalline mass was filtered off. The filtrate was washed with cold water (5 x 5 ml aliquots), dried under vacuo. The crude product was recrystallised from boiling water to yield white crystals (8.5 g ≡ 47.4% of theoretical) m.p. = 185 - 186°C (Lit.[56] 187°C).

III Preparation of 4-benzylidene-2-phenyl-oxazolin-5-one

Redistilled benzaldehyde (2.7 g, .025 m) was mixed with hippuric acid (4.5 g, .025 m), acetic anhydride (7.7 g, .075 m) and anhydrous sodium acetate (2.1 g, .025 m). The resulting mixture was heated in an oil bath until it completely liquefied. The flask and contents were then heated on a boiling water bath (2 hours). At the end of this time, the flask was removed from the water bath, ethanol (10 ml) was added cautiously and the mixture was allowed to stand overnight. The crystalline product was filtered off at the pump, washed with ice cold alcohol (2 x 2.5 ml), washed with boiling water, dried at 100°C. The crude product was recrystallised from benzene to yield pale yellow crystals, m.p. 163 - 164°C (Lit.[56] 167 - 168°C), yield 5.02 g (81% of theoretical).

IV Preparation of 4-cis-benzylidene-2-methyl-oxazolin-5-one

Redistilled benzaldehyde (4.0 g, .03 m) was mixed with acetyl glycine (2.9 g, .025 m), anhydrous sodium acetate (1.5 g, .01 m) and acetic anhydride (6.7 g, .065 m). The resulting mixture was heated on a water bath until solution was complete (20 minutes). It was boiled for one hour, cooled, stored at 0°C overnight. The pale yellow crystals were stirred with cold water (6.0 ml), and filtered.

The crude product was washed with cold ether until the odour of benzaldehyde was no longer present. The product was dried under vacuum. It was recrystallised from carbon tetrachloride to yield pale yellow crystals, m.p. 150°C (Lit.[56] m.p. 150°C), yield 3.6 g (64% of theoretical).

V Preparation of 2-amino-3-phenyl-propionic acid

2-phenyl-4-benzylidene-5-oxazolone (2.5 g, .01 m) was mixed with red phosphorous (2.0 g, .06 m) and acetic anhydride (12.5 ml) with vigorous stirring. Hydriodic acid (specific gravity 1.56; 12.5 ml) was added dropwise with stirring (one hour). The resulting mixture was refluxed (3 hours) with constant stirring, cooled, and filtered under suction. The unreacted phosphorous on the filter was washed with glacial acetic acid (2 x 1 ml protions). The filtrate and washings were evaporated to dryness under reduced pressure. Distilled water (10 ml) was added to the residue and it was evaporated to dryness again. The residue was then shaken with ether (15 ml)/water (15 ml) until solution was complete. The layers were separated. The aqueous layer was extracted with ether (3 x 10 ml portions). The combined ether extracts were discarded. Decolourising carbon (0.2 g) with sodium sulphite (10 mg) were added to

the aqueous portion, this solution was heated on a water bath until all of the ether had been removed. The solution was filtered, heated to boiling, neutralised with concentrated ammonia solution (until basic to congo-red paper). the solution was cooled and the colourless phenyl alanine which crystallised was filtered off. The crude product was washed with cold water (2 x 3 ml), alcohol (3 ml), it was then dried at 100°C. Yield = 1.1 g (60% of theoretical) m.p. 280°C (with decomposition) (Lit.[56] m.p. 284 - 288°C with decomposition.

This reaction was also attempted using 2-methyl-4-benzylidene-5-oxazolone instead of 2-phenyl-4-benzylidene-5-oxazolone with a very similar yield.

VI Preparation of 2-amino-3-phenyl propionic acid methyl ester hydrochloride[58]

Thionyl chloride (1.19 g, 0.01 m) was added slowly with cooling to dry methanol (25 ml). Phenyl alanine (1.65 g, 0.01 m) was added to the solution which was refluxed for four hours. The solvent was evaporated. The crude product was triturated with ether at 0°C until excess methyl sulphite was removed (about four hours). The solid was filtered off, dried under hivac. Yield of

hydrochloride 1.91 g (91% of theoretical). The high resolution n.m.r. spectrum in deuterium oxide solution was compatible with the proposed structure.

VII Preparation of trifluoroacetic anhydride

Because of its susceptibility to hydrolysis, the trifluoroacetic anhydride used for preparations described in this thesis was freshly prepared by the following procedure: Trifluoroacetic acid (5 ml) was mixed with phosphorous pentoxide (2 g), the resulting mixture was refluxed for twenty minutes. The trifluoroacetic anhydride was then distilled from the reaction mixture, b.p. 46°C. The product was redistilled before use.

VIII Preparation of 2-trifluoroacetamido-DL-3-phenyl propionic acid (N-trifluoroacetyl-DL-β-phenyl alanine)

Phenyl alanine (1.65 g, .01 m) was dissolved in trifluoroacetic acid (5 ml) with gentle warming. Trifluoroacetic anhydride (5 ml) was added dropwise with stirring. The mixture was left at room temperature overnight. The excess trifluoroacetic acid and trifluoroacetic anhydride were removed using hivac. The resulting product was

recrystallised from benzene/hexane to yield white needles (2.3 g) (98% of theoretical) m.p. = 128°C (Lit. m.p.[60] = 127.5°C).

IX Preparation of 2-trifluoroacetamido-DL-3-phenyl propionic acid methyl ester

2-trifluoroacetamido-3-phenyl-propionic acid (23.7 mg, .00001 m) was suspended in dry ether (2 ml). An ethereal solution of diazomethane was added dropwise, with shaking, until a permanent yellow coloration remained. The excess ether and diazomethane were removed under vacuum to yield 24.5 mg of crude product. This was then purified by T.L.C. to yield 20.0 mg chromatographically pure product which was characterised by n.m.r.

X Preparation of 2-trifluoroacetamido-L-3-phenyl propionic acid

2-amino-L-3-phenyl propionic acid (2.5 g, .015 m) was dissolved in dry benzene (25 ml). Freshly prepared trifluoroacetic anhydride (2.5 ml) was added dropwise with vigorous stirring. The solution went solid. It was heated slowly to 70°C, and cooled to room temperature. White

needles which crystallised were filtered off and recrystallised from water to yield 2.3 g (75% of theoretical) pure product, m.p. = 116 - 117°C (Lit.[59] = 119 - 20°C).

XI Preparation of 2-trifluoroacetamido-L-3-phenyl propionic acid methyl ester

2-trifluoroacetamido-3-phenyl propionic acid (24.0 mg, 10 m) was dissolved in dry ether (2 ml). An ethereal solution of diazomethane was added dropwise with stirring until a permanent yellow coloration remained. The excess ether and diazomethane were removed under vacuum. The crude product (25.0 mg) was purified by T.L.C. to yield 19.7 mg purified product which was characterised by n.m.r.

XII Preparation of 4-cis(-2-fluorobenzylidene)-2-methyl-oxazolin-5-one

2-fluorobenzaldehyde (250 mg, 0.002 m) was mixed with acetyl glycine (206 mg, 0.002 m) and anhydrous sodium acetate . Acetic anhydride (500 mg) was added. The mixture was heated on a steam bath (three hours). It was cooled, dry ethanol (2 ml) was added. The crude product was filtered off, dried to yield 386.8 mg of crude product. This was recrystallised from dry benzene to yield 375.0 mg (91.0% of theoretical) m.p. = 144 - 5°C.

XIII Preparation of 2-amino-3-(2-fluorophenyl) propionic acid (ortho fluorophenyl alanine)

4-cis(-2-fluorobenzylidene)-2-methyl-oxazolin-5-one (2.05 g, .01 m) was mixed with red phosphorous (2.0 g, .06 m) and acetic anhydride (12.5 ml) with vigorous stirring. Hydriodic acid (specific gravity 1.56 , 12.5 ml) was added dropwise with stirring over one hour. The resulting mixture was refluxed for three hours with constant stirring, cooled, and filtered under suction. The unreacted phosphorus on the filter was washed with glacial acetic acid (2 x 1 ml portion). The filtrate and washings were evaporated to dryness under reduced pressure. Distilled water (10 ml) was added to the residue and it was evaporated to dryness again. The residue was then shaken with ether (15 ml)/ water (15 ml) until solution was complete. The layers were separated and the aqueous layer was extracted with ether (3 x 10 ml). The combined ethereal extracts were discarded. Decolourising carbon (0.2 g) with sodium sulphite (10 mgs) were added to the aqueous portion, and this solution was heated on a water bath until all of the ether had been removed. The solution was filtered, heated to boiling, and neutralised with concentrated ammonia solution (until basic to congo red paper). The solution was cooled and the colourless ortho fluorophenyl alanine

which crystallised was filtered off. The crude product was washed with cold water (2 x 3 ml), alcohol (3 ml), it was then dried at 100°C. Yield = 1.05 g (57% of theoretical) m.p. ~ 258°C (with decomposition).

XIV Preparation of 2-trifluoroacetamido-DL-3-(2-fluorophenyl)-propionic acid, (N-trifluoroacetyl-ortho fluorophenyl alanine

Ortho fluorophenyl alanine (1.8 g, .01 m) was dissolved in trifluoroacetic acid (10.0 ml) with gentle warming. Freshly prepared trifluoroacetic anhydride (10.0 ml) was added dropwise with stirring. The resulting mixture was left at room temperature overnight. The excess trifluoroacetic acid and trifluoroacetic anhydride were removed using hivac. The resulting product was recrystallised from benzene/hexane to yield white needles, 2.0 g (95% of theoretical) m.p. = 130°C.

Microanalysis results for Molecular Formula = $C_{11}H_9F_4NO_3$

	C	H	N	F
Anticipated	47.31	3.22	5.01	27.24
Experimental	47.46	3.38	5.11	26.93

XV Preparation of 2-trifluoroacetamido-DL-3-(2-fluorophenyl) propionic acid methyl ester

2-amino-3-(2-fluorophenyl) propionic acid (20.9 mg, .00001 m) was suspended in dry ether (2 ml). An ethereal solution of diazomethane was added dropwise with stirring until permanent yellow colouration remained. The excess ether and diazomethane were removed under vacuum. The crude product (22.3 mg) was purified by T.L.C. to yield 18.0 mg purified product which was characterised by n.m.r.

XVI Preparation of 4 cis(-3-fluorobenzylidene)-2-methyl-oxazolin-5-one

3-fluorobenzaldehyde (1.24 g, .01 m) was mixed with acetyl glycine (1.03 g, .01 m) and anhydrous sodium acetate (0.82 g). Acetic anhydride (3 g) was added. The mixture was heated on a steam bath (three hours). It was cooled, dry ethanol (10 ml) was added. The crude product was filtered off, dried to yield 1.96 g of crude product. This was recrystallised from dry benzene to give 1.80 (87% of theoretical) m.p. = 126 - 127°C.

XVII Preparation of 2-amino-3-(3-fluorophenyl) propionic acid (meta fluorophenyl alanine

4-cis-(3-fluorobenzylidene)-2-methyl-oxazolin-5-one (1.02 g, .005 m) was mixed with red phosphorus (1.0 g, .03 m) and acetic anhydride (6.8g) with vigorous stirring. Hydriodic acid (6.25 ml, S.G. 1.56) was added dropwise with stirring over one hour. The resulting mixture was refluxed for three hours with constant stirring, cooled, and filtered under suction. The unreacted phosphorus on the filter was washed with glacial acetic acid (2 x 3 ml). The filtrate and washings were evaporated to dryness under reduced pressure. Distilled water (5.0 ml) was added to the residue and it was evaporated to dryness again. The residue was then shaken with ether (7.5 ml)/water (7.5 ml) until solution was complete. The layers were separated, and the aqueous layer was extracted with ether (3 x 5.0 ml). The combined ethereal extracts were discarded. Decolourising carbon (.1 g) with sodium sulphite (10 mg) were added to the aqueous portion, and this solution was heated on a water bath until all of the ether had been removed. The solution was filtered, heated to boiling and neutralised with concentrated ammonia solution (until basic to congo red paper). The solution was cooled and the colourless meta fluorophenyl

alanine which crystallised was filtered off. The crude product was washed with cold water (10 ml), alcohol (10 ml), it was then dried at 100°C. Yield = 1.1 g (60% of theoretical) m.p. = 262°C

XVIII Preparation of 2-trifluoroacetamido-DL-3-(3-fluorophenyl) propionic acid, (N-trifluoroacetyl-meta fluorophenyl alanine)

Meta fluorophenyl alanine (1.8 g, .01 m) was dissolved in trifluoroacetic acid (10.0 ml) with gentle warming. Freshly prepared trifluoroacetic anhydride (10.0 ml) was added dropwise with stirring. The resulting mixture was left at room temperature overnight. The excess trifluoroacetic acid and trifluoroacetic anhydride were removed using high vacuum. The resulting product was recrystallised from benzene/hexane to yield white needles 2.1 g (100% of theoretical) m.p. = 115 - 116°C.

Microanalysis results - Molecular Formula = $C_{11}H_9NO_3F_4$

	C	H	N	F
Anticipated	47.31	3.22	5.01	27.24
Experimental	47.5	3.29	5.05	27.00

XIX Preparation of 2-trifluoroacetamido-DL-3-(3-fluorophenyl)-propionic acid methyl ester

2-amino-3-(3-fluorophenyl) propionic acid (20.9 mg, .00001 m) was suspended in dry ether (2.0 ml). An ethereal solution of diazomethane was added dropwise with stirring until permanent yellow coloration remained. The excess ether and diazomethane were removed under vacuum. The crude product (23.0 mg) was purified by T.L.C. to yield the purified product (19.6 mg) which was characterised by n.m.r.

XX Preparation of 4-cis(-4-fluorobenzylidene) 2-methyl oxazolin-5-one

4-fluorobenzaldehyde (2.48 g, .02 m) was mixed with acetyl glycine (2.06 g, .02 m) and anhydrous sodium acetate (1.64 g). Acetic anhydride (6g) was added. The mixture was heated on a steam bath (three hours). It was cooled, and dry ethanol (20 ml) was added. The crude product was filtered off, and dried to yield 4.0 g of crude product. This was recrystallised from dry benzene to give 3.7 g (90% of theoretical) m.p. = 154°C (Lit.[61] 153.5°C).

XXI Preparation of 2-amino-3-(4-fluorophenyl) propionic acid (para fluorophenyl alanine)

4-cis-(4-fluorobenzylidene)-2-methyl-oxazolin-5-one (2.05 g, .01 m) was mixed with red phosphorus (2.0 g, .06 m) and acetic anhydride (12.5 ml) with vigorous stirring. Hydriodic acid (S.G. 1.56, 12.5 ml) was added dropwise with stirring over one hour. The resulting mixture was refluxed for three hours with constant stirring, cooled, and filtered under suction. The unreacted phosphorous on the filter was washed with glacial acetic acid (2 x 1 ml portions). The filtrate and washings were evaporated to dryness under reduced pressure. Distilled water (10.0 ml) was added to the residue and it was evaporated to dryness again. The residue was then shaken with ether (15 ml)/water (15 ml) until solution was complete. The layers were separated, and the aqueous layer extracted with ether (3 x 10 ml). The combined ethereal extracts were discarded. Decolourising carbon (0.2 g) with sodium sulphite (10 mg) were added to the aqueous portion. This solution was heated on a water bath until all of the ether had been removed. The solution was then filtered, heated to boiling, neutralised with concentrated ammonia solution (until basic to congo red paper). The solution was cooled and the colourless

para fluorophenyl alanine which crystallised was filtered off. The crude product was washed with cold water (2 x 3 ml), and alcohol, (3 ml), it was then dried at 100°C. Yield = 0.9 g (49% of theoretical), m.p. = 263°C (with decomposition).

XXII Preparation of 2-trifluoroacetamido-DL-3-(4-fluorophenyl) propionic acid, (N-trifluoroacetyl para fluorophenyl alanine)

Para fluorophenyl alanine (1.83 g, .01 m) was dissolved in trifluoroacetic acid (5 ml) with gentle warming. Freshly prepared trifluoroacetic anhydride (5 ml) was added dropwise with stirring. The resulting mixture was left at room temperature overnight. The excess trifluoroacetic acid and trifluoroacetic anhydride were removed using high vacuum. The resulting product was recrystallised from benzene/hexane to yield white crystals (2.5 g) (90% of theoretical), m.p. = 139 - 140°C.

Microanalysis results - Molecular formula = $C_{11}H_9NO_3F_4$

	C	H	N	F
Anticipated	47.31	3.22	5.01	27.24
Experimental	47.46	3.33	5.02	26.96

XXIII Preparation of 2-trifluoroacetamido-DL-3-(4-fluorophenyl) propionic acid methyl ester

2-amino-3-(4-fluorophenyl) propionic acid (20.9 mg, .1 m) was suspended in dry ether (3.0 ml). An ethereal solution of diazomethane was added dropwise with stirring until permanent yellow coloration persisted. The excess ether and diazomethane were distilled off under reduced pressure. The crude product (21.5 mg) was purified by T.L.C. to yield the purified product, 19.7 mg, which was characterised by n.m.r.

XXIV Preparation of 4-cis-(2-fluorobenzylidene)-2-phenyl-oxazolin-5-one

2-fluorobenzaldehyde (1.24 g, .01 m) was mixed with hippuric acid (1.79 g, .01 m), anhydrous powdered sodium acetate (0.82 g) and acetic anhydride (3 g). The solution was heated with continual shaking until it liquefied. The mixture was then heated on a steam bath for two hours. Ethanol (8 ml) was added with cooling. The mixture was allowed to stand overnight. The crystalline solid was filtered off, washed with ice cold ethanol (2 x 4 ml) and boiling water (2 x 2 ml). The filtrate was dried under

vacuum, and recrystallised from benzene to form 1.98 g pale yellow crystals (81% of theoretical), m.p. = 163 - 4°C (Lit.[62], 165.5°C corrected).

XXV Preparation of 4-cis-(3-fluorobenzylidene)-2-phenyl-oxazolin-5-one

3-fluorobenzaldehyde (1.24 g, .01 m) was mixed with hippuric acid (1.79 g, .01 m), anhydrous powdered sodium acetate (0.82 g) and acetic anhydride (3 g). The solution was heated with continual shaking until it liquefied. The mixture was then heated on a steam bath for two hours. Ethanol (8 ml) was added with cooling. The mixture was allowed to stand overnight. The crystalline solid was filtered off, washed with ice cold ethanol (2 x 4 ml) and boiling water (2 x 2 ml). The filtrate was dried under vacuum, and recrystallised from benzene to form pale yellow crystals, 2.13 g (87.5% of theoretical), m.p. = 155 - 6°C.

XXVI Preparation of 4-cis-(4-fluorobenzylidene)-2-phenyl-oxazolin-5-one

4-fluorobenzaldehyde (1.24 g, .01 m) was mixed with hippuric acid (1.79 g, .01 m), anhydrous powdered sodium acetate (0.82 g) and acetic anhydride (3 g). The solution was heated with continual shaking until it liquefied. The

mixture was then heated on a steam bath for two hours. Ethanol (8 ml) was added with cooling. The mixture was allowed to stand overnight. The crystalline solid was filtered off, washed with ice cold ethanol (2 x 4 ml) and boiling water (2 x 2 ml). The filtrate was dried under vacuum, and recrystallised from benzene to yield 1.93 g purified product (80% of theoretical), m.p. = 180 - 181°C (Lit.[61] 184 185.5°C corrected).

XXVII Preparation of trans cinnamoyl chloride

Trans cinnamic acid (1.48 g, .01 m) was refluxed with purified thionyl chloride (10 ml) until the evolution of hydrogen chloride and sulphur dioxide ceased (about 1½ hours). The excess thionyl chloride was distilled off. Dry benzene (2 x 5 ml portions) was added and distilled off, to azeotrope off the last traces of thionyl chloride. This crude acid chloride was then dissolved in dry benzene (20 ml) and used for preparation XXVIII.

XXVIII Preparation of N-trans-cinnamoyl imidazole

Cinnamoyl chloride (1.70 g) in dry benzene (20 ml) (preparation XXVII) was introduced dropwise over ten minutes

to imidazole (1.4 g, .02 m) in dry benzene (80 ml), maintaining the temperature below 10°C. The reaction mixture was vigorously stirred. After the addition of the acid chloride solution, the mixture was stirred at 10°C (five minutes), the temperature was then allowed to rise to 25°C. Dry benzene (30 ml) was added and the solution was stirred for twenty minutes. The imidazolium chloride was filtered off. The benzene solution was evaporated to leave 1.82 g crude product. This product was recrystallised from hexane to yield 1.76 g (89% of theoretical) purified product, m.p. = 133 - 135°C (Lit.[63] m.p. = 133 - 133.5°C).

XXIX Preparation of 4-cis-(4-nitrobenzylidene)-oxazolin-5-one

4-nitrobenzaldehyde (7.5 g, .05 m) was mixed with acetyl glycine (5.8 g, .05 m) anhydrous sodium acetate (2.0 g). Acetic anhydride (15 ml) was added. The mixture was heated on a steam bath (one hour). It was cooled, dry ethanol (20 ml) was added. The crude product was filtered off to yield 7.60 g crude product. This was recrystallised from carbon tetrachloride/chloroform to yield pale yellow crystals, m.p. 185 - 186°C.

XXX Attempted preparation of 2-amino-3-(4-nitrophenyl) propionic acid

4-cis-(4-nitrobenzylidene)-oxazolin-5-one (2.3 g, .01 m) was mixed with red phosphorus (2.0 g) and acetic anhydride (15 ml). Hydriodic acid (13.0 ml, S.G. 1.56) was added dropwise with stirring over one hour. This solution was then refluxed for four hours. The solution was cooled, filtered, and the unreacted phosphorus washed with glacial acetic acid (2 x 3 ml portions). Combined filtrate and washings were evaporated to dryness under reduced pressure. The residue was dissolved in water (15 ml)/ether (15 ml). The layers were separated and the aqueous layer extracted with ether (3 x 10 ml). The layers were separated and the combined ether layers were discarded. Carbon (0.2 g) and a trace of sodium sulphite were added. The solution was heated on a boiling water bath until the dissolved ether had all evaporated. The solution was filtered, heated to boiling, neutralised with concentrated ammonia (until alkaline to congo red paper). No product crystallised. The failure of this reaction was attributed to the opposing effects of the two amino groups and the carboxyl function.

XXXI Preparation of 2-amino-3-(4-nitrophenyl) propionic acid

2-amino-3-phenyl propionic acid (5.0 g, .03 m) was dissolved in nitric acid (50 ml of 8 M) and heated on a boiling water bath for eight hours. The solution was cooled to room temperature, neutralised with potassium hydroxide solution (alkaline to litmus paper). Stored at $0^{o}C$ overnight. The solid product was filtered at the pump. The crude product was washed with warm water (to remove any inorganic salt), acetone, dried under vacuum to yield 2.30 g product, m.p. = $235^{o}C$ with decomposition.

XXXII Preparation of N-trifluoroacetyl cyclohexylamine

Freshly prepared trifluoroacetic anhydride (5 ml) was added dropwise to a cooled solution of cyclohexylamine (1.0 g, .01 m) in dry benzene (5.0 ml) with vigorous stirring. The solvent was removed under vacuum to leave 1.95 g crude product. This was recrystallised from benzene/hexane to yield 1.71 g (88% of theoretical) purified product, white needles, m.p. $75^{o}C$.

XXXIII Preparation of N-monofluoroacetamido cyclohexylamine

Freshly prepared monofluoroacetyl chloride was added dropwise with vigorous stirring and cooling to a solution of cyclohexylamine (1.0 g, .01 m) in dry benzene. The solvent was evaporated under reduced pressure to yield 1.96 g crude product + cyclohexylamine hydrochloride. This was suspended in distilled water (10.0 ml), filtered, and dried under vacuum. The crude product was recrystallised from benzene/hexane to yield 1.45 g (91% of theoretical) white needles, m.p. 92°C.

XXXIV Preparation of 4-nitrophenyl acetate

4-nitrophenol (1.39 g, .01 m) was dissolved in sodium hydroxide (10 ml, 2.5 m). Crushed ice (50 g) was added. Acetic anhydride (12.5 g) was added and the mixture was shaken vigorously for ten minutes. The solution was extracted with ether (4 x 20 mls), and the layers were separated. The ether layer was washed with sodium hydroxide solution (3 x 10 ml, 0.1 m). It was then dried over magnesium sulphate, filtered and evaporated to yield of crude product which was recrystallised from benzene/ hexane to yield 1.7 g (90% of theoretical), m.p. = 83°C, (Lit.[64] = 83°C).

XXXV Resolution of (±) 1-phenyl ethylamine

(+) Tartaric acid (12.5 g) was dissolved in methanol (180 ml). The solution was refluxed on a steam bath and 1-phenyl ethylamine (10.3 g; .085 m) was added. The flask was removed from the steam bath and the side of the flask was scratched with a glass rod until a few crystals appeared. The solution was then stood at room temperature for 24 hours. The crystalline mass was filtered off, washed with methanol (10 ml) dried in vacuo to yield the (-)-amine-(+)-hydrogen tartrate. This was suspended in water (25 ml) and a solution of sodium hydroxide (13 g) in water (25 ml) was added. The solution was extracted with ether (3 x 20 ml) and the combined extracts were dried over magnesium sulphate and evaporated under reduced pressure to give the crude (-) amine. The crude (-) amine was distilled under reduced pressure, b.p. 72°C/10 mm, yield 5.0 g.

XXXVI Preparation of 2-trifluoroacetamido-DL-3-(4-nitrophenyl)-propionic acid.

2-amino-DL-3-(4-nitrophenyl)-propionic acid (2.1 g, .01 m) was dissolved in trifluoroacetic acid (5.0 ml).

Trifluoroacetic anhydride (5.0 ml) was added dropwise. The solution was stood at room temperature (24 hours). The excess trifluoroacetic acid and trifluoroacetic anhydride were removed under vacuum. The oily product was triturated with petroleum ether (60 - 80). The crystalline product was recrystallised from benzene/hexane to yield 2.0 g (97.0% of theoretical), m.p. = 162 - 3°C)

XXXVII Preparation of 2-trifluoroacetamido-DL-3-(4-hydroxyphenyl) propionic acid

2-amino-DL-3-(4-hydroxyphenyl) propionic acid (1.81 g, .01 m) was dissolved in trifluoroacetic acid (5.0 ml). Trifluoroacetic anhydride (5.0 ml) was added dropwise. The solution was stood at room temperature for twenty four hours. The excess trifluoroacetic acid and trifluoroacetic anhydride were removed under vacuum. The oily product was triturated with petroleum ether (60 - 80). The crystalline product was recrystallised from benzene/hexane to yield 2.60g (94% of theoretical), m.p. = 184 - 5°C.

XXXIX Preparation of 3-trifluoroacetamido-L-3-(4-hydroxyphenyl) propionic acid

2-amino-L-(4-hydroxyphenyl) propionic acid (1.81 g, .01 m) was dissolved in trifluoroacetic acid (5.0 ml).

Trifluoroacetic anhydride (5.0 ml) was added dropwise. The solution was stood at room temperature for twenty four hours. The excess trifluoroacetic acid and trifluoroacetic anhydride were removed under vacuum. The oily product was triturated with petroleum ether (60 - 80). The crystalline product was recrystallised from benzene/hexane to yield 2.58 g (93% of theoretical), m.p. = 191°C.

CHAPTER THREE

RESULTS

Table 3.1

Variation of Chemical Shift of the trifluoromethyl resonance signal of N-trifluoroacetyl meta fluorophenyl alanine with n.m.r. tube relative to the same external capillary of trifluoroacetic acid

Solution	1	2	3	4	5
Shift of CF_3(Hz)	-305.2	-304.4	-304.4	-303.8	-304.8

The same solution of N-trifluoroacetyl meta fluorophenyl alanine was used for all measurements - concentration 1.7×10^{-2}m.

Measurements were made 94.1 MHz.

Table 3.2

Attempt to find suitable internal reference for fluorine-19 resonance studies of -chymotrypsin-substrate systems

Compound	Effect on Enzyme Solution
Trifluoroacetic acid pH re-adjusted to 7.0	denatured
Sodium trifluoroacetate	denatured
1,1,1 trifluoroacetone	denatured

Table 3.3

Effect of addition of (L) N-trifluoroacetyl phenyl alanine to (DL) N-trifluoroacetyl phenyl alanine in presence of α-chymotrypsin at pH 6.0, 31°C

Solution	Moles of (DL) enantiomer	Moles of enzyme	Moles of (L) enantiomer added	Observed Chemical Shift (Hz) of CF_3	
				L	D
1	4.0×10^{-3} M	1.07×10^{-4} M	-	-305.0	-305.3
2	4.0×10^{-3} M	"	2.0×10^{-3} M	-305.1	-305.3
3	2.0×10^{-3} M	"	-	-305.2	-306.5
4	2.0×10^{-3} M	"	2.0×10^{-3} M	-305.2	-306.5
5	1.0×10^{-3} M	"	-	-305.1	-307.1

/contd. .....

Table 3.3 (contd.)

6	1.0×10^{-3} M	1.07×10^{-4} M	2.0×10^{-3} M	-305.0	-307.1
7	5.0×10^{-4} M	"	-	-305.3	-308.7
8	5.0×10^{-4} M	"	2.0×10^{-3} M	-305.1	-308.7

Shifts were measured at 94.1 MHz relative to external capillary of T.F.A.

Table 3.4

N-trifluoroacetyl meta fluoro phenyl alanine with pH

pH	CF_3 Shift	Aromatic Shift
6.0	-304.4 Hz	+ 3330.8 Hz
6.5	-304.4 Hz	+ 3330.9 Hz
7.0	-304.3 Hz	+ 3330.8 Hz
7.5	-304.5 Hz	+ 3330.7 Hz
8.0	-304.3 Hz	+ 3330.8 Hz

Chemical shifts are relative to the same external capillary using the same n.m.r. tube.

Aromatic Resonance was decoupled Ratio = 94.079403

The solution concentrations were all 1.7×10^{-2} M.

Table 3.5

Fluorine Shifts of phenyl alanine derivatives without enzyme

Compound	Aliphatic CF_3	Aromatic F
(DL) N-trifluoroacetyl *A phenyl alanine	-306.2 Hz	-
(DL) N-trifluoroacetyl *A ortho fluorophenyl alanine	-305.0 Hz	+3744.8 Hz
(DL) N-trifluoroacetyl *A meta fluorophenyl alanine	-304.9 Hz	+3330.0 Hz
(DL) N-trifluoroacetyl *A para fluorophenyl alanine	-304.9 Hz	+3591.1 Hz
(DL) N-trifluoroacetyl *B para nitrophenyl alanine	-309.6 Hz	-
(DL) N-trifluoroacetyl *B para hydroxyphenyl alanine	-308.7 Hz	-
(L) " " " "	-308.5 Hz	-
(L) N-trifluoroacetyl *A phenyl alanine	-306.2 Hz	-

*A = Phosphate Buffer *B = Tris HCl Buffer

Shifts are relative to an external capillary of Trifluoroacetic acid at 94.1 MHz.

Table 3.6

Experiments with N-Trifluoroacetyl-L-Phenyl Alanine at pH 7.5

(1) Dilution Study

Solution	Substrate Concentration	Shift
1	7.8×10^{-2} Ml^{-1}	-306.7 Hz
2	3.9×10^{-2} Ml^{-1}	-305.5 Hz
3	1.95×10^{-2} Ml^{-1}	-305.4 Hz
4	9.97×10^{-3} Ml^{-1}	-305.5 Hz
5	4.48×10^{-3} Ml^{-1}	-305.3 Hz

(2) + α chymotrypsin

Solution	Shift with Enzyme
1	-306.4 Hz
2	-306.2 Hz
3	-306.3 Hz
4	-306.3 Hz
5	-306.2 Hz

Enzyme concentration 7.3×10^{-3} M

Shifts are relative to same external capillary of T.F.A.

Table 3.7

Variation of binding of (D) N-trifluoroacetyl phenyl alanine to α-chymotrypsin with pH

pH	Temp.	K_s	Δ
6.0	31°C	4.3×10^{-2} M	-46.0 Hz
6.5	31°C	3.9×10^{-2} M	-39.0 Hz
7.0	31°C	not calculable	2.5 Hz [A] ± 0.4
7.5	31°C		
8.0	31°C		

[A] Vary randomly with concentration.

Chemical shifts were measured relative to an external capillary of trifluoroacetic acid but converted to a scale relative to L enantiomer. Δ is the chemical shift of the bound inhibitor relative to the chemical shift of the free inhibitor. A phosphate buffer system was used.

Table 3.8

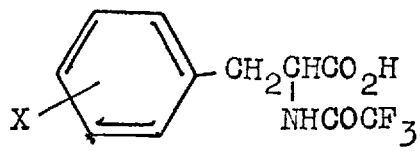
Effect of N-trans Cinnamoyl Imidazole on Enzyme Binding as shown by n.m.r.

Compound	con-centration	Shift	Enzyme con-centration	Shift with Enzyme D	Shift with Enzyme L	Shift with Enzyme + N-trans Cinnamoyl Imidazole	pH	Temp.
N-trifluoro-acetyl meta fluorophenyl alanine	3.7×10^{-2} M	-305.7 +3329.7	4.75×10^{-4} M	- 306.8 +3326.7	- 305.8 +3328.3	- 304.7 +3328.6	8.0 (phos-phate)	31°C
N-trifluoro-acetyl para nitrophenyl alanine	4×10^{-3} M	- 309.8	4.25×10^{-4} M	- 320.3	-309.6	-308.7	7.5 (Tris HCl)	31°C

Shifts are in Hz relative to external capillary of trifluoroacetic acid at 94.1 Hz

Table 3.9

Summary of K_s and Δ values for phenyl alanine derivatives binding to α-chymotrypsin



X	pH	Δ	K_s
p F	6.0	CF_3 = - 38.0 Hz Aromatic = -130.0 Hz	$1.6^{\pm 1.0} \times 10^{-3}$ M
	6.5	CF_3 = - 47.0 Hz Aromatic = -124.9 Hz	$2.2^{\pm 1.0} \times 10^{-3}$ M
	7.0	CF_3 = - 73.0 Hz Aromatic = -133.0 Hz	$3.9^{\pm 1.0} \times 10^{-3}$ M
	7.5	CF_3 = - 55.0 Hz Aromatic = -119.0 Hz	$7.2^{\pm 1.0} \times 10^{-3}$ M
	8.0	CF_3 = - 81.0 Hz Aromatic = -127.6 Hz	$9.3^{\pm 1.0} \times 10^{-3}$ M
m F	6.0	CF_3 = 33.4 Hz Aromatic = 50.0 Hz	8.8×10^{-4} M
	6.5	CF_3 = 40 Hz Aromatic = 100 Hz	2.5×10^{-3} M

/ contd. .....

Table 3.9 (contd.)

X	pH	Δ (Hz.)	K_s
m F	7.0	CF_3 obs = 1.1 ± .1*	
		Aromatic " = 1.7±0.1*	
	7.5	CF_3 " = 1.1±0.1*	
		Aromatic" = 1.9±0.1*	
	8.0	CF_3 " = 1.1±0.1*	
		Aromatic" = 1.7±0.1*	
o F	6.0 - 8.0	CF_3 = 1.0±0.2*	
		Aromatic = 1.9±0.3*	
H	6.0	-46.0	4.3×10^{-2} M
	6.5	-39.0	3.9×10^{-2} M
	7.0	obs	
	7.5	2.5 ± 0.4	
	8.0		
p No_2	7.5	490	1.6×10^{-2} M
p OH	7.5	no observable change	

* Shift in Hz concentration independent

Table 3.10

Dependence of N-trifluoroacetyl-D-phenyl alanine/α-chymotrypsin interaction on buffer system used

Buffer	pH	K_s	Δ
Phosphate	6.0	4.3×10^{-2} M	-46.0 Hz
Citrate	6.0	5.1×10^{-2} M	-38.0 Hz
Phosphate	7.5	-	2.5 Hz [A] $\pm$ 0.4 Hz
Tris HCl	7.5	-	2.7 Hz [A] $\pm$ 0.2 Hz

[A] vary randomly with concentration over stated range.

Chemical shifts were measured relative to an external capillary of Trifluoroacetic acid, but were converted to a scale based on the shift of the L enantiomer.

Table 3.11

Results calculated for (L) N-trifluoroacetyl para fluorophenyl alanine with α-chymotrypsin using the method of Sykes(9)

Solution	S_o/E_o	Observed Chemical Shift
1	50	-306.3 Hz
2	25	-305.2 Hz
3	12.5	-305.2 Hz
4	6.25	-305.2 Hz
5	3.125	-305.2 Hz

The measurements were made at pH 6.0 and 31°C.

Computer Calculated Results

$K_s = 2.5 \times 10^{-2}$ M

$\Delta = +55.5$ Hz

$\delta_{fit} = -306.8$ Hz

CHAPTER FOUR

Discussion

4.1 The α-chymotrypsin system as studied by Fluorine-19 resonance

There have been several nuclear magnetic resonance studies involving the interaction of α-chymotrypsin with fluorinated substrates, but unfortunately these experiments have tended to confuse rather than clarify the situation. The earliest work using a fluorinated substrate was described by Spotswood and his co-workers[8]. They studied the interaction of α-chymotrypsin with phenyl alanine derivations of the type shown in figure 4.1 (with X = H, meta F, para F, $R_1 = CH_3$).

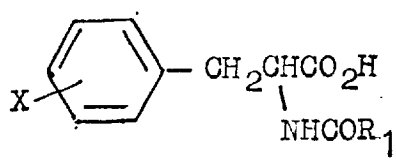


Figure 4.1 Generalised Phenyl Alanine Derivative

The fluorine resonance spectra of the meta and para fluorine derivatives appear as complex multiplets due to spin-spin interaction with the four non-equivalent

protons of the aromatic ring. In the presence of α-chymotrypsin the complex multiplet is doubled because the D and L enantiomers interact differently with the enzyme, it is very difficult to obtain quantitative data from these spectra because of their complexity. The experimental results suggest that for all the compounds studied the L-enantiomer does not bind to the enzyme, whereas the D-enantiomer does. They also show that in a chemically modified system (α-chymotrypsin in which Serine 195 is phosphorylated with diisopropyl fluorophosphate (figure 1.3, Compound XII) no spectral changes are observed.

These experiments of Spotswood used a deuterium oxide/citrate buffer solution (pD 6.0) with a solution temperature of 40°C; the use of deuterium oxide solutions makes comparison with other work difficult, since Bender[67] has shown a two-to three-fold decrease in the rate of α-chymotrypsin catalysed hydrolyses when deuterium oxide was used as solvent (it is not clear, however, whether this is due to an isotope dependence of the binding stage of the interaction, involving k_s, or the hydrolytic part of the reaction involving k_2 and k_3). Spotswood used both the aromatic fluorine and the acetyl hydrogens for observation.

The values of Δ for the D - enantiomer binding to the enzyme are - 85 Hz (at 56.4 MHz) for the aromatic fluorine and ~10 Hz (at 60 MHz) for the acetyl hydrogens. These differences between the value of Δ for proton and fluorine give an indication of the relative sensitivity of fluorine resonance chemical shifts compound with proton chemical shifts for similar small changes in the magnetic environment.

The next series of papers all involve similar derivatives of phenyl alanine with the site of fluorination changed (figure 4.1 X = H, $R_1 = CF_3$). Zeffern and Reavill[22] studied the interaction of N-trifluoroacetyl-DL-phenyl alanine with α-chymotrypsin using the fluorine-19 resonance of the trifluoroacetyl group. Their measurements were made at pH 6.0 using a citrate buffer system. The results obtained suggest that both the D and L enantiomers bind to the enzyme with similar values of K_s(4×10^{-2} M) but different values of Δ (-1.75 p.p.m. for the D enantiomer and -1.18 p.p.m. for the L enantiomer)*. The observed chemical shifts were measured relative to an external reference capillary of trifluoroacetic acid and were claimed to be accurate to "within 0.001 p.p.m.". In the course of

* The values of Δ are relative to the resonance position of the substrate without enzyme.

the present work a recalculation of these experimental results was made using the method of Groves, Huck and Homer[10] and suggests that the D-enantiomer binds to the enzyme with $K_s = 9.8 \times 10^{-2}$M, $\Delta = -1.98$ p.p.m. but the L-enantiomer does not bind. The conflict between these two interpretations of the same experimental results illustrates the difficulty of working with measurements which are comparable in magnitude to the experimental error, a point which is discussed later (Section 4.3). Zeffern and Reavill attempted to find a mixture of organic solvents for N-trifluoroacetyl-DL-phenyl alanine which gives the same observed chemical for the trifluoroacetyl group as that calculated for the trifluoroacetyl group in the enzyme-substrate complex, thereby gauging the "polarity" of the enzyme active centre. This approach overlooks the fact that many types of interaction other than those due to "polarity" have important consequences upon nuclear shielding[68]. They used solvents ranging from ethanol to benzene, with the extremes of chemical shift (relative to external T.F.A.) being -1.64 p.p.m. in ethanol and -2.25 p.p.m. in 50/50 benzene dioxane mixture. The bound chemical shifts for the D and L enantiomers, on the same scale, are -5.04 p.p.m. and -4.47 p.p.m. respectively. They were surprised to find that they were unable to

repeat these bound shifts with a mixture of organic solvents, but the best artificial systems for reproducing these shifts are solutions of 6.5 m sodium chloride in pH 6.0 citrate buffer for the L - enantiomer complex and 9.75 m sodium chloride in pH 6.0 citrate buffer for the D - enantiomer complex. It is rather difficult to assess the importance of these observations as a means of gaining information about the environment of the substrate molecule; the phenomenon is probably related to that of "salting out" of organic molecules from aqueous solutions[69].

The situation is made more complicated by Sykes[35] who has studied the interaction of N-trifluoroacetyl-D-phenyl alanine with α -chymotrypsin at pH 7.8 (using a tris HCl buffer); he obtained values of $K_s = 4.9 \times 10^{-2}$ M, Δ = + 0.43 p.p.m. which do not agree with any of the previous workers. These results were calculated using the method of Groves, Huck and Homer[10] cited earlier. The difference in Δ for the same compound obtained by Zeffern and Reavill at pH 6.0 (-1.75 p.p.m.) and Sykes at pH 7.8 (+0.43 p.p.m.) has been attributed to the "major conformational change of the enzyme which is known to occur at about pH 7.0". The results of Sykes are worth considering in detail, however, as this is the only study which has suggested an upfield shift for Δ and these results

were used as the basis of his transient method to obtain k_1 and k_{-1} for the equilibrium. The actual experimental values are shown in table 4.1. As the measurements are relative to an external capillary of trifluoroacetic acid, the accuracy is $\pm$ 0.1 Hz. A recalculation of these results taking into account error bars suggests that K_s is 4.2×10^{-2} M $\pm$ 2.4×10^{-2} M and Δ is in the range 30 - 56 Hz. This uncertainty in the value of K_s means that little reliance can be placed on the absolute values of k_1 and k_{-1} given in a later paper[9]. In this second paper, the binding of N-trifluoroacetyl-D-phenyl alanine to α-chymotrypsin modified with diisopropyl fluorophosphate at Serine 195 was also studied. The results for the binding of N-trifluoroacetyl-D-phenyl alanine to both α -chymotrypsin and DFP chymotrypsin are summarised in table 4.2.

These are a little surprising because they suggest that the differences in K_s for the two systems are mainly due to k_{-1} (the rate constant for the breakdown of the ES complex). In other words the rate of formation of the enzyme substrate complex is the same for the enzyme and the derivatised enzyme, indicating that there are similar forces affecting the binding in each case, but once the complex is formed there are repulsive forces in the

Table 4.1

Experimental results used for calculation of K_s and Δ by Sykes[9] for N-trifluoroacetyl-D-phenyl alanine at pH 7.8

So/Eo	$\frac{1}{\delta_{obs} - \delta_{I\ fit}}$	$\delta_{obs} - \delta_{I}^{fit}$
16	0.95	1.05
10	0.75	1.33
7	0.74	1.35
5	0.65	1.54
3	0.63	1.5
2	0.6	1.67

Shifts were measured from an external trifluoroacetic acid capillary.

Accuracy $\pm$ 0.1 Hz

Temperature: $33^{o} \pm 1$

Table 4.2

N-trifluoroacetyl-D-phenyl alanine binding to α-chymotrypsin and DFP chymotrypsin as studied by Sykes[9]

Enzyme	K_s M	Δ Hz.	k_1 M^{-1} sec^{-1}	k_{-1} M^{-1} sec^{-1}
chymotrypsin	4.9×10^{-2}	+24	1.0×10^{4}	4.9×10^{2}
DFP chymotrypsin	1.06×10^{-1}	+64	1.6×10^{4}	16.6×10^{2}

Shifts are relative to external T.F.A. at 56.4 MHz.

DFP chymotrypsin complex which cause the stability of the complex to be reduced. A recalculation of the N-trifluoroacetyl-DFP chymotrypsin results taking errors into account suggests that Δ may lie somewhere in the region 60 - 300 Hz, whereas K_s may be in the range 1.06×10^{-1} to infinity! The differences between K_s and Δ for the two systems may therefore be due to experimental error.

The literature results obtained by n.m.r. experiments for the phenyl alanine /α-chymotrypsin system are summarised in table 4.3. The wide variation in the values of Δ and K_s is not surprising when the different experimental conditions are considered. For example, buffer systems ranging from citrate to tris HCl have been used, with the pH ranging from 6.0 to 7.8 and in one case a D_2O/citrate system was used. The enzyme concentrations have been assessed in a variety of different ways, temperatures of experimental systems have varied and the methods of calculating K_s and Δ have varied. The use of an external reference capillary with observed shifts in the range 0 - 1.5 Hz leads to great inaccuracy (see section 4.3). One of the biggest criticisms of the published work is the lack of convincing evidence that a specific interaction between the enzyme and the substrate is responsible for the changes observed in the n.m.r. experiments described.

Table 4.3

Summary of Published Work on α -chymotrypsin - phenyl alanine systems

Substrate	pH	Concentration range	Concentration of enzyme	Method of calculating enzyme concentration	Method for obtaining K_s and	K_s	Δ
N-trifluoroacetyl[9] D-phenyl alanine	7.8	not stated	not stated	Erlanger and Edel[75]	Computer method	4.9×10^{-2} M	+ 41 Hz
N-Acetyl-D-para[8] fluorophenyl alanine	pD 6.0	$1 - 8 \times 10^{-2}$ M	3×10^{-3} M	not stated	Double Reciprocal plot	$(6 \pm 2) \times 10^{-3}$	- 83[a]
N-Acetyl-D-meta[8] fluorophenyl alanine						$(11 \pm 5) \times 10^{-3}$	- 85[a]
N-Acetyl-D-phenyl[8] alanine						10^{-3}	10[b]

a = aromatic fluorine

b = acetyl hydrogens

/contd. .....

Table 4.3 (contd.)

N-trifluoroacetyl-D-phenyl alanine[22]	6.0	3×10^{-3}M 8×10^{-2}M	2×10^{-3}M	Bender et al (63)	Double Reciprocal Plot	3.4×10^{-2}M	-1.75 ppm
N-trifluoroacetyl-L- phenyl alanine[22]						5.1×10^{-2}M	-1.18 ppm

4.2 Discussion

The work described in section 4.1 was the motivating force behind the present study, the first priority obviously being a systematic reinvestigation of the system studied by Sykes[35] and Zeffern and Reavill[22] under standardised experimental conditions. The study was extended to gain information about the aromatic binding pocket by considering substituted phenyl alanine derivatives of the type shown in figure 4.1, when X is F, OH or NO_2 and R_1 is CF_3. The use of N-trifluoroacetyl-fluoro phenyl alanine allows the aromatic binding and the amide group binding to be studied in the same molecule using ^{19}F resonance.

(i) Referencing and Enzyme Estimation

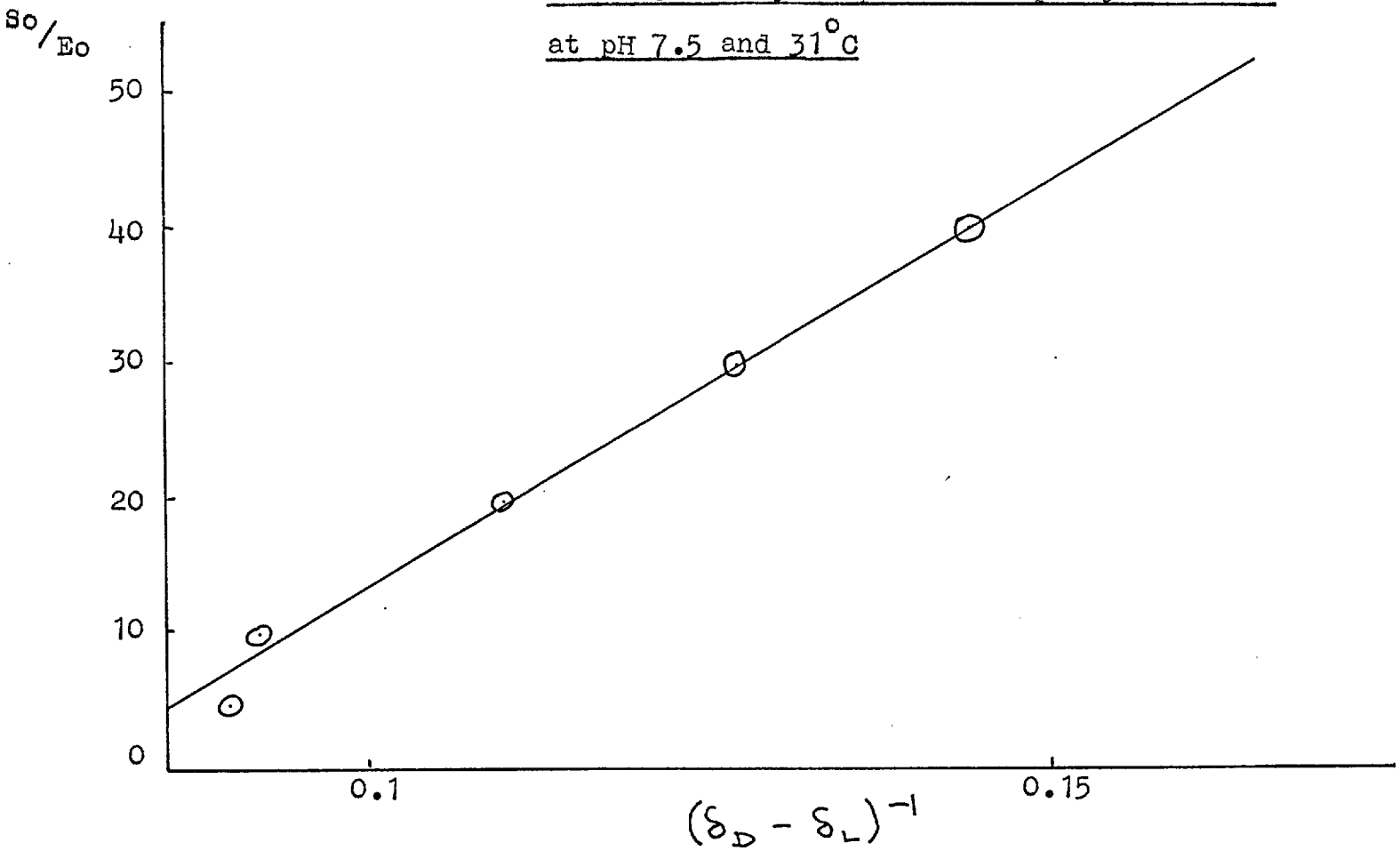
The chemical shifts of the fluorinated substrates described in the literature (section 4.1) were measured relative to an external reference capillary of trifluoroacetic acid. This is unsatisfactory when such small chemical shift changes are being observed for the small shifts (1 - 2 Hz) could merely be due to the change in the magnetic susceptibility of the solution on addition of enzyme. When using an external reference, changes in the bulk magnetic susceptibility of the solution will affect the substrate molecules which are dissolved in the solution, but will not affect the reference compound, which is physically separated from the experimental solution.

If an external reference is used, it is essential to keep the same n.m.r. tube and reference capillary for each measurement in a particular series. The effect of changing the n.m.r. tube with the same solution and reference capillary is shown in table 3.1. Our early attempts to find a suitable internal reference compound for fluorine-19 studies on α -chymotrypsin and substrates are summarised in table 3.2. The experimental results obtained with N-trifluoroacetyl-DL-phenyl alanine and α-chymotrypsin (discussed later on page 112) suggest

that the L- enantiomer does not interact with the enzyme (or the value of K_s is outside the range detectable by n.m.r.). It may be shown experimentally that the L -enantiomer does not bind to α -chymotrypsin and this is an ideal reference compound for n.m.r. experiments. This was demonstrated by adding pure L- enantiomer to the DL - mixture in the presence of α-chymotrypsin, and the experimental observations for one experiment are given in table 3.3. This table gives the results obtained when N-trifluoroacetyl-L-phenyl alanine is added to N-trifluoroacetyl-DL-phenyl alanine in the presence of α-chymotrypsin. The chemical shift of the D - enantiomer does not change when the L - enantiomer is added, which indicates that the L - enantiomer does not bind to α - chymotrypsin. The L - enantiomer was therefore used as an internal reference for the DL - mixture, while trifluoro-acetic acid capillary was used as a lock signal for the spectrometer.

The chemical shift difference between D and L enantiomers in the presence of α -chymotrypsin enabled a quick and convenient method of estimating enzyme concentration to be developed. Figure 4.1 shows a plot of the substrate/ enzyme ratio ($^{So}/_{Eo}$) against the reciprocal of the observed

Figure 4.1: Estimation of α-chymotrypsin using N-trifluoroacetyl-D-para-nitrophenyl alanine at pH 7.5 and 31°C



chemical shift difference between D and L enantiomers of N-trifluoroacetyl-DL-para nitro phenyl alanine. It is possible to measure the chemical difference for a standard N-trifluoroacetyl para nitro phenyl alanine solution in the presence of α -chymotrypsin of unknown concentration and thus obtain the enzyme concentration from this graph.

ii) pH dependence of the N-trifluoroacetyl-X-phenyl alanine/ α -chymotrypsin system

It is possible to obtain useful information from the pH dependence of enzyme binding[29], and an example of this was given in Chapter 1 for the lysozyme/N-acetyl glucosamine system[27]. Before considering the dependence of the enzyme/substrate interaction on pH, however, it is necessary to eliminate any pH dependence of the chemical shift of the substrate in the absence of enzyme. Experiments show that there is no pH dependence of the chemical shift of either the trifluoromethyl group resonance or the aromatic fluorine resonance in the N-trifluoroacetyl-X-phenyl alanines. This is shown in table 3.4. The pH profiles for the N-trifluoroacetyl-X-phenyl alanines with α -chymotrypsin are listed in table 3.5. All the compounds studied (with the exception of ortho fluoro and para hydroxy derivatives) show

a pH dependence of enzyme binding, although because of the inherent limitations of the technique N-trifluoroacetyl-D-para fluorophenyl alanine is the only compound which has measurable values of K_s and Δ over the full range of pH studied.

(a) Trifluoromethyl Resonance

The results for the binding of N-trifluoroacetyl-D-phenyl alanine to α -chymotrypsin over the pH range 6.0 - 8.0 are shown in table 3.5. When α -chymotrypsin is added to a buffered solution of N-trifluoroacetyl-DL-phenyl alanine at pH 6.0, two separate resonances are observed as shown in figure 4.2. One of these is attributable to the D enantiomer, the other to the L -enantiomer. Addition of the pure L - enantiomer shows that the high-field resonance corresponds to the L -enantiomer and the low-field resonance corresponds to the D -enantiomer. The chemical shift of the low-field resonance (D - enantiomer) varies with the enzyme/substrate ratio, whereas the chemical shift of the high-field resonance (L - enantiomer) does not do so. This suggests that the D -enantiomer binds to the enzyme whereas the L - enantiomer does not bind (or the value of K_s for the L - enantiomer is out of the range detectable by n.m.r.) Experiments with pure N-trifluoroacetyl-L-phenyl

α-chymotrypsin concentration 5×10^{-4} m

-trifluoroacetyl-DL-phenylalanine concentration 5.6×10^{-3} m

Shifts are relative to an external T.F.A. capillary.

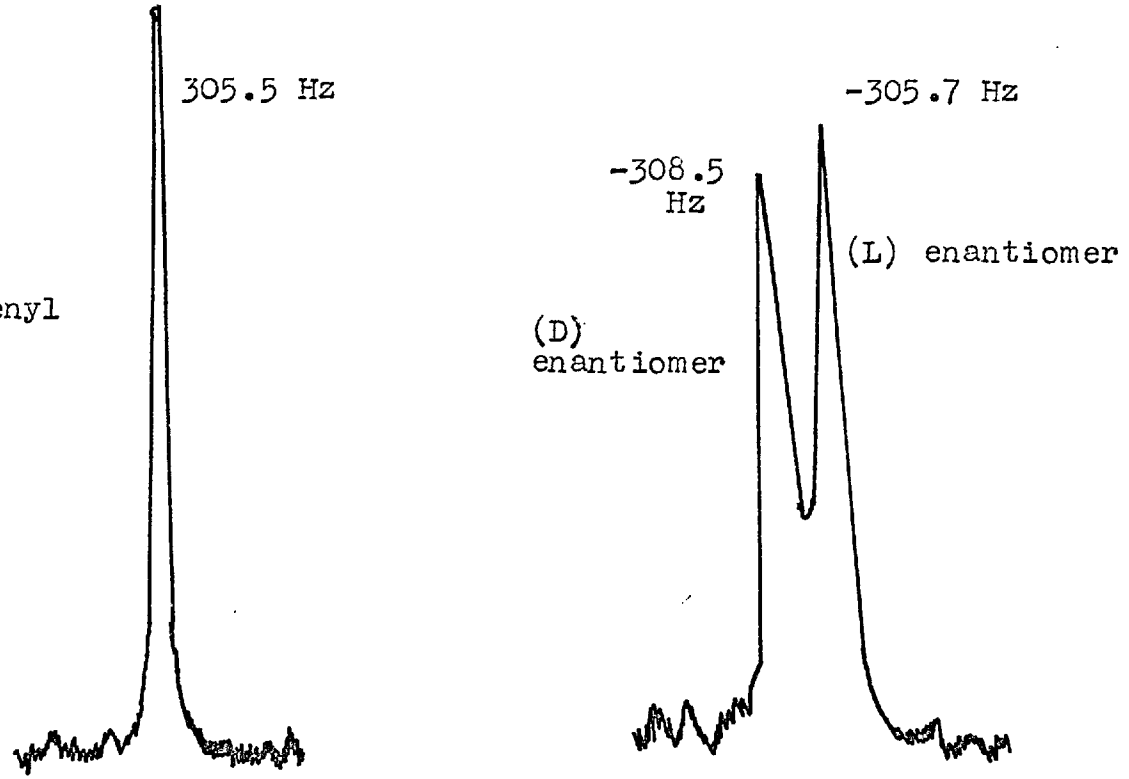


Figure 4.2: The effect on the trifluoromethyl resonance of addition of α-chymotrypsin to N-trifluoroacetyl-DL-phenyl alanine at pH 6.0 and 31°C.

alanine and α -chymotrypsin indicate that the chemical shift of the trifluoromethyl resonance is independent of the enzyme/substrate ratio over the pH range 6.0 - 8.0. The experimental results obtained at pH 7.5 are given in table 3.6.

The results observed with D and L - N-trifluoro-acetyl phenyl alanine binding to α -chymotrypsin are at variance with those of Sykes[9] and those of Zeffern and Reavill[22] for the same system. As the present study was made using a phosphate buffer system whereas Zeffern and Reavill used a citrate buffer system and Sykes used a tris/HCl buffer system, this may have caused the discrepancy. This is not the case, however, as shown in table 3.7, the citrate buffer at pH 6.0 and the tris/HCl buffer at pH 8.0 give the same values of K_s and Δ (within experimental error) as observed with the phosphate buffer at the same pH values. The failure to repeat the work of Zeffern and Reavill may be explicable in terms of the experimental errors involved, but the failure to repeat the work of Sykes is very difficult to explain. An alternative explanation is that changes in pH during the experiment cause the discrepancy between this work and that of Zeffern and Reavill, although this latter possibility again fails to explain the work of Sykes as the chemical shift of the

trifluoromethyl group is concentration-independent in the presence of α-chymotrypsin over the pH range 7.0 - 8.0 as shown in table 3.7. Hence small changes in pH would have no bearing upon the shift observed by Sykes.

The pH variation of the trifluoromethyl resonance in the N-trifluoroacetyl-D-para fluorophenyl alanine/ α-chymotrypsin complex is shown graphically in figure 4.3. Although there is an obvious upward trend in the value of Δ over the pH range 8.0 - 6.0 (i.e. Δ becomes less negative), the experimental errors (as shown) mean that it is not possible to interpret this curve as a specific type.

(b) Aromatic fluorine resonance

The chemical shift of the aromatic fluorine in the N-trifluoroacetyl-para fluorophenyl alanine/ α-chymotrypsin complex is constant (125 Hz $\pm$ 10 Hz at 94.1 MHz) over the pH range studied, which suggests that there is no major perturbation of the magnetic environment of this fluorine atom over this pH range.

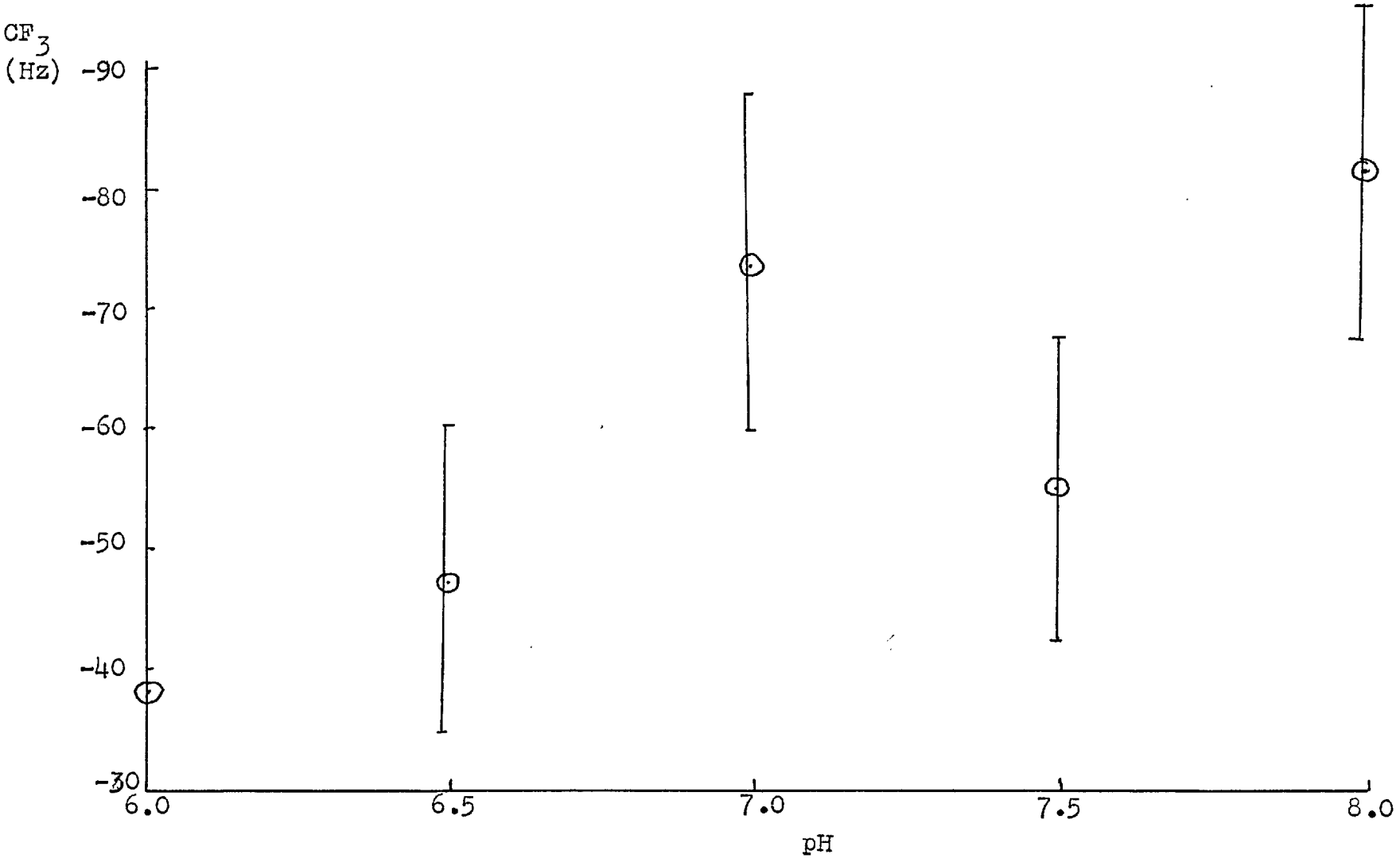


Figure 4.3: Variation of ΔCF_3 for N-trifluoroacetyl-D-para fluoro phenyl alanine with pH

(c) Variation of K_s

The pH variation of K_s is shown graphically in figure 4.4 for the same experimental system. Once again the errors involved in the measurements mean that it is not valid to interpret this as a curve of a specific type.

The order of K_s values for the substituted N-trifluoroacetyl-D-X-phenyl alanine derivatives binding to α-chymotrypsin at pH 7.5 is p NO_2 > pF > mF, oF, p OH, H. It has been suggested that the specificity of the binding of aromatic amino acids to α-chymotrypsin is due to the interaction of the aromatic portion of the substrate with an aromatic residue at the active centre of the enzyme[34]. This is termed an apolar or hydrophobic interaction (molecules which are 2 - 3.1 Å apart are considered to be involved in a polar interaction and molecules which are 3.1 - 4.1 Å apart are considered to be involved in an apolar or hydrophobic bond[70].

The sequence of K_s for the substituted N-trifluoroacetyl-X-phenyl alanine derivatives suggest that para substituents (especially nitro and fluoro) tend to enhance the stability of the enzyme - substrate complex. This may indicate that there is a residue suitably situated in the aromatic binding pocket of the enzyme which can interact with these para

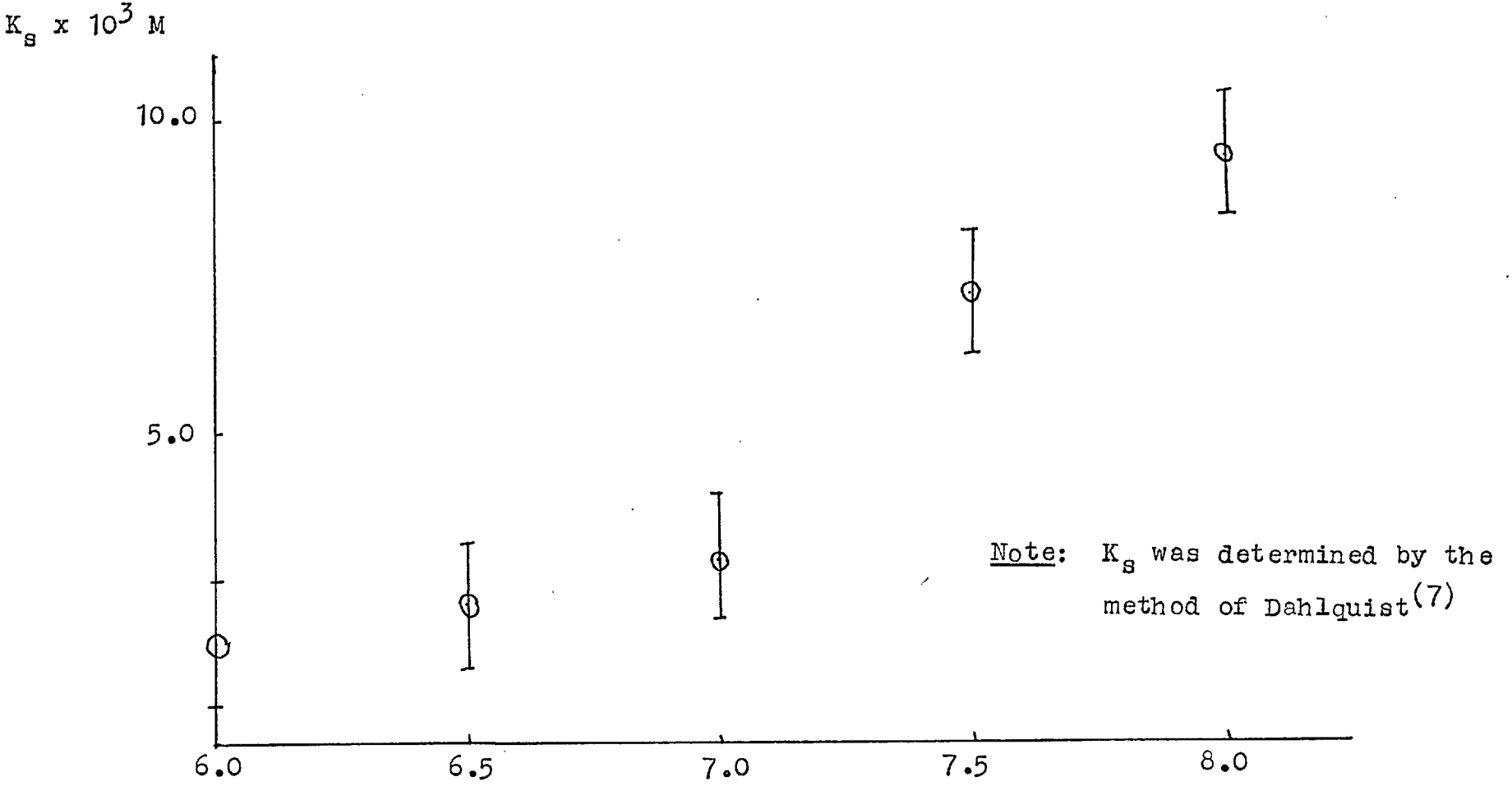
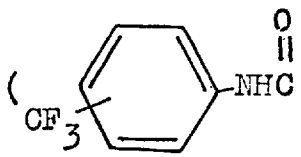


Figure 4.4: pH variation of K_s for N-trifluoroacetyl-D-parafluoro-phenyl alanine/ ∝ -chymotrypsin interaction.

substituents, which is in accord with the recent spin-echo experiments of Bittner et alia[71], who studied α - chymotrypsin which had been irreversibly inhibited with trifluoromethyl-bromo-acetanilide derivatives



(CF_3 -NHC - CH_2Br). They measured the T_2 values for the trifluoro methyl group in the irreversibly inhibited enzyme, giving results shown in table 4.4

Table 4.4

T_2 values for trifluoromethyl bromo acetanilide derivatives of α -chymotrypsin

	T_2		
	o	m	p
Derivatised -chymotrypsin	.04	0.016	0.006
Free Inhibitor	1.4	1.0	0.9

The correlation time τ_c for the para trifluoromethyl group in the derivatised enzyme is $\approx 3.2 \times 10^{-8}$ sec (which is very close to the calculated value for the

enzyme itself $\approx 4 \times 10^{-8}$ sec.); therefore in the complex the trifluoromethyl group appears to have stopped rotating about its local C_3 axis and the motion is due to the overall Brownian motion of the enzyme. The experiments of Bittner give an indication of the "strength" of this para interaction as it is sufficient to stop the rotation about the trifluoromethyl/aromatic ring carbon-carbon bond.

An alternative explanation may be that the aromatic binding mechanism involves interactions of the π charge transfer type (see, for example, Foster and Fyffe[72]). The dependence upon substituted orientation may then be explained in terms of their effects upon the stability of such complexes.

(iii) Evidence that n.m.r. changes are due to a specific interaction

Without corroborating evidence the observed chemical shifts of the trifluoro methyl group of N-trifluoroacetyl-X-phenyl alanines in the presence of α-chymotrypsin at any particular pH may equally well be explained as a generalised non-specific interaction between the enzyme and the substrate. Other workers have attempted to demonstrate that the observed changes are due to binding

to the active centre of the α-chymotrypsin by chemical modification of Serine-195 by phosphylation with di-isopropyl fluorophosphate which is an irreversible inhibitor of the enzyme. The biochemical evidence shows that D.F.P. stops the catalytic activity of α-chymotrypsin but this compound only blocks the hydrolytic part of the active site, and phenyl alanine derivatives of the type studied can still bind to D.F.P.-chymotrypsin as the part of the process under study is the binding interaction

$$E + S \rightleftharpoons ES$$

The chemical modification could well induce a change in the conformation of the enzyme, but it has been shown[63] that N-trans-cinnamoyl imidazole irreversibly inhibits α-chymotrypsin by forming the cinnamoyl derivative of Serine 195, thereby blocking the active centre of the enzyme. The carbon framework of cinnamic acid is sufficiently similar to phenyl alanine to infer that if the n.m.r. chemical shifts disappear when N-trans-cinnamoyl imidazole is added to the enzyme substrate mixture, the original effect was due to binding at the active centre of the enzyme. This test was used with all the substrates listed in table 3.5 in the presence of α-chymotrypsin. The results of two

such experiments are given in table 3.8. In all cases the n.m.r. spectrum reverted to the spectrum of the substrate without enzyme present, thus suggesting that for all cases studied, the observed n.m.r. changes are due to a specific interaction between the substrate and the active centre of the enzyme.

4.3 Limitations of the n.m.r. technique

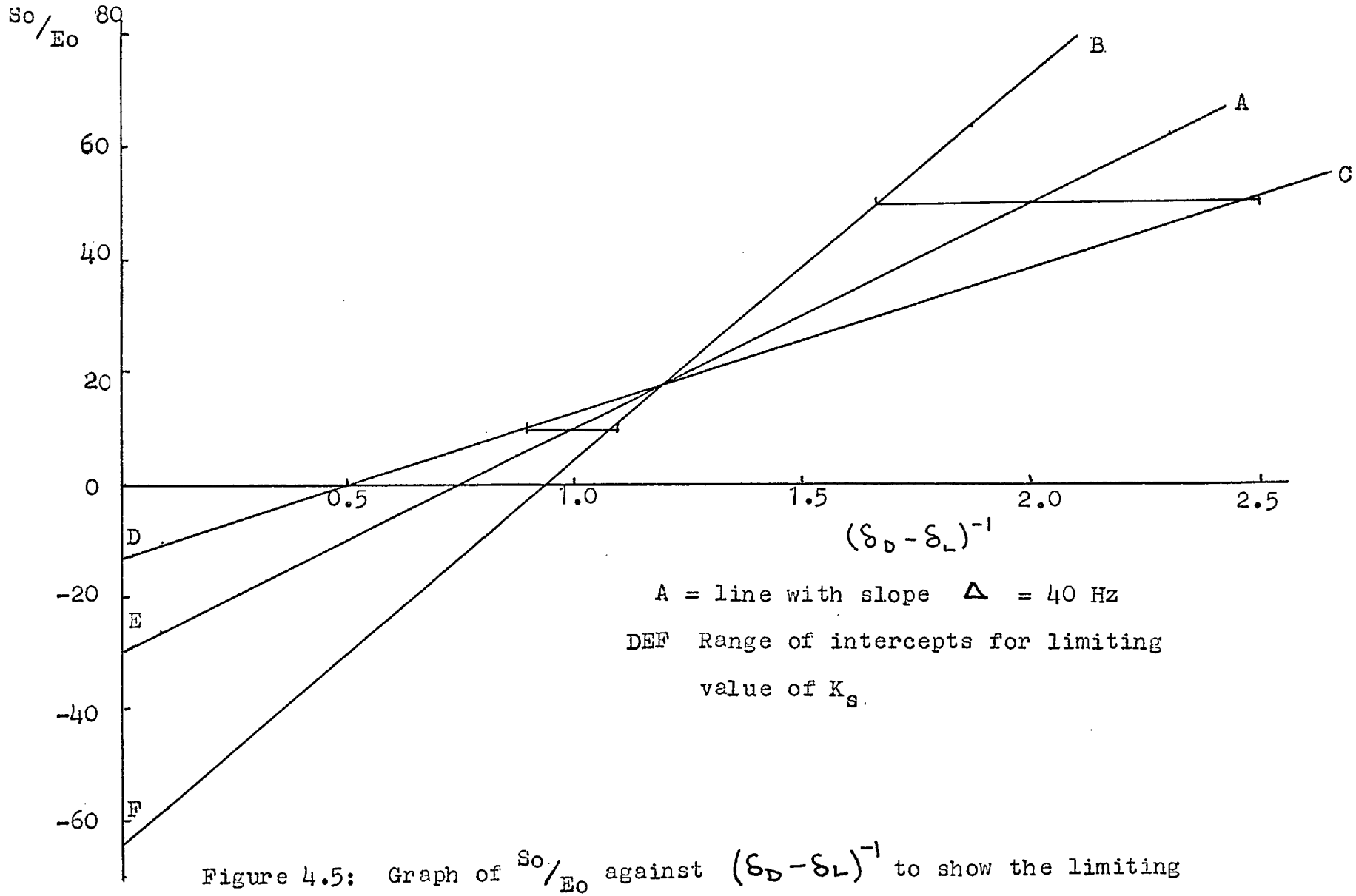
N.M.R. is a valuable tool for studying biological systems if its inherent limitations and inaccuracies are realised. Considering the simple Michaelis-Menten scheme for enzymic reactions (which was outlined in Chapter 1), it is obvious that a big disadvantage of the method is the small part of the overall enzymic process which is amenable to study by n.m.r. It is necessary to assume that the simple pseudo-substrate used for the n.m.r. experiment binds to the enzyme in an analagous manner to the true substrate, and furthermore that the complex formed between the enzyme and pseudo-substrate in the binding stage is similar to the "active complex" formed between the enzyme and true substrate during the enzymic reaction. Another general disadvantage of the technique is its lack of sensitivity even using techniques of signal enhancement, which means that high concentrations of enzyme and substrate (compared with the concentration found in nature) are required in order to observe effects. This introduces the possibility of other equilibria, for example dimerisation of the enzyme

$$E + E \rightleftharpoons E_2$$

or more than one substrate molecule binding to the enzyme

$ES + S \rightleftharpoons ES_2$. Results must therefore be treated with caution as they cannot necessarily be extrapolated to more dilute solutions where there is less chance of the other equilibria. When high concentrations of impure enzymes are used (for example, Kato used Horse Serum Cholinesterase[23] at concentrations of 150 mg/ml) trace impurities could easily cause spurious effects.

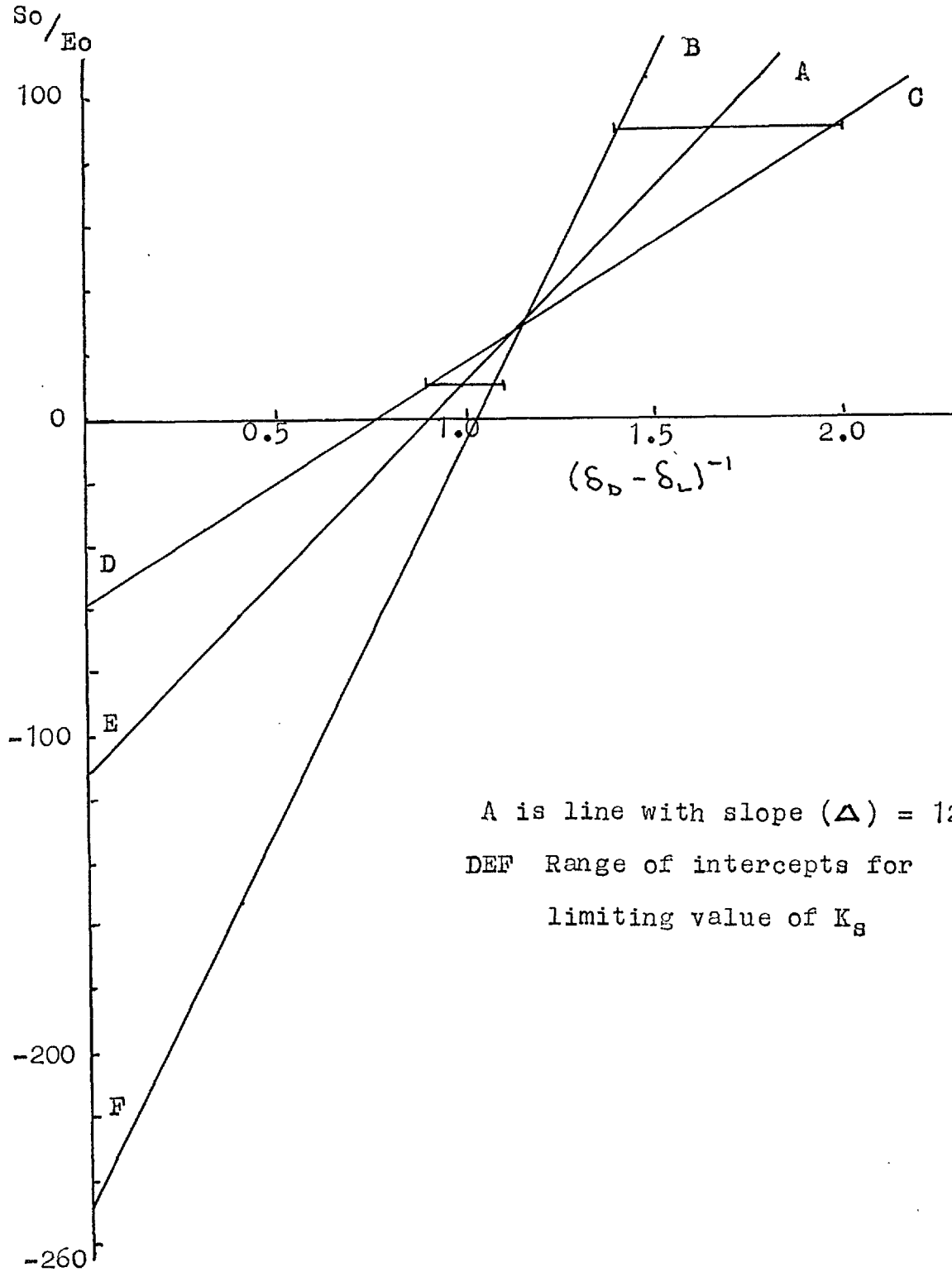
The useful range of K_s detectable by n.m.r. depends mainly on the value of the bound shift Δ. The experiments normally vary the substrate/enzyme ratio over the range 10 - 100 with the lower limit of substrate concentration attainable depending upon the absolute sensitivity of the spectrometer. Figures 4.5 and 4.6 show the range of K_s measurable when Δ = 40 Hz and 120 Hz respectively. These graphs were computed by assuming the minimum useful range of chemical shifts corresponding to the $^{So}/_{Eo}$ ratio change of 10 - 100 is 0 - 1.0 Hz. The error bars for these extreme cases are shown, assuming an accuracy of $\pm$ 0.1 Hz on the chemical shift measurement. These calculations are summarised in table 4.5.



A = line with slope Δ = 40 Hz

DEF Range of intercepts for limiting value of K_s.

Figure 4.5: Graph of $^{So}/_{Eo}$ against $(\delta_D - \delta_L)^{-1}$ to show the limiting value of K_s when Δ = 40 Hz.



A is line with slope (Δ) = 120 Hz

DEF Range of intercepts for limiting value of K_s

Figure 4.6: Graph of $^{So}/_{Eo}$ vs $(\delta_D - \delta_L)^{-1}$ to show limiting value of K_s when Δ = 120 Hz

Table 4.5

Dependence of K_s on Δ

Δ	Range of K_s measurable	Error on limiting measurements
40 Hz	Eo → 30 Eo	10 Eo — 60 Eo
120 Hz	Eo → 120 Eo	50 Eo — 200 Eo

The errors are rapidly reduced as the range of the observed chemical shift increases above 1.0 Hz; this is evident from table 4.6 which shows the error in the reciprocal of various (hypothetical) observed chemical shift.

Table 4.6

Error range for various observed shifts

δ Observed Shift (Hz)	$\frac{1}{\delta}$	Range of $1/\delta$ allowing for possible $\pm$ 0.1 Hz
0.2	5.0	3.33 → 10
0.5	2.0	1.67 → 2.5
1.0	1.0	0.91 → 1.1
4.0	0.25	0.24 → 0.26

Every effort must be made to find a suitable internal reference for the system under study. When this is not possible certain precautions must be taken, for example, the enzyme solution concentration should be kept constant to minimise solution bulk magnetic susceptibility changes, and the same n.m.r. tube and external reference capillary should be used for all measurements in a particular series. The method of Sykes[9] mentioned earlier which eliminates the necessity for an experimental blank solution must be used with great caution. Table 3.9 shows the values of K_s and Δ for the N-trifluoroacetyl-L-para fluoro phenyl alanine interacting with α-chymotrypsin at pH 6.0 calculated by the computer method of Sykes, together with the experimental results used for the calculation. The calculated values of K_s and Δ seem reasonable for the system under study (if it is reasonable for Δ to have a positive value) but nevertheless the results obviously indicate that the substrate does not bind to the enzyme.

NOTE

Since the completion of this work, a paper has appeared by Ashton and Co-workers[74] involving ^{19}F studies and α-chymotrypsin. Although Ashton was mainly

studying the N-trifluoroacetyl tryptophan/ α -chymotrypsin complex, he reported some values of K_s and Δ for the trifluoroacetyl-D-phenyl alanine/ α -chymotrypsin system which agree with those obtained in this study and disagree with those obtained by Sykes[9] and Zeffern and Reavill[22].

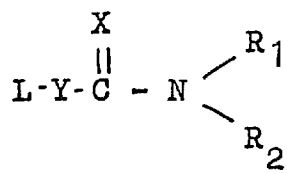
PART II

CONFORMATIONAL STUDIES OF SOME ENZYME INHIBITORS

CHAPTER ONE

Introduction

Carbamic acid derivatives of the type shown in figure 1.1 have been shown to be potent inhibitors of the enzyme acetyl cholinesterase[1].



X = O, S

Y = O, S

Figure 1.1: A generalised carbamic acid derivative

For a binding interaction to occur between an enzyme and a substrate (or inhibitor) both molecules must adopt a suitable conformation and nuclear magnetic resonance is a useful technique for probing subtle conformational changes of organic molecules in solution. It was decided to examine the possibility that the energy changes required to induce conformational changes in various substrates in free solution would correlate with the binding coefficient of the same substrate to the enzyme, and hence with its inhibitive properties. In principle, it should be

possible to study the conformational changes occurring in both the substrate (or inhibitor) and the enzyme, but the long correlation time of the enzyme molecule makes it rather difficult to study, and work has been restricted to the smaller molecule.

Nuclear magnetic resonance can be used to elucidate the conformation of a rigid system, e.g. an alkaloid[2] or it can equally well be applied to a molecule interconverting between different conformations; in the latter case it is possible to calculate the activation parameters for the interconversion process.

N.M.R. has been successfully applied to conformational studies of many different systems, e.g. substituted ethanes[3]; ring interconversions of cyclic compounds such as cyclohexane derivatives[4]; carbohydrates[5]; dithianes[6]; hindered rotation about carbon-nitrogen bonds in amides[7]; anilides[8]; urethanes[9], and ureas[10]. In the present work, the study will be restricted to the problem of rotation about a C - N bond.

1.1 General Theory

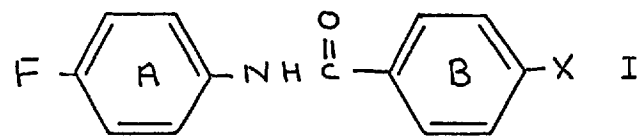
Theory suggests that there is a significant amount of double bond character in the carbon-nitrogen bond of amides

I II

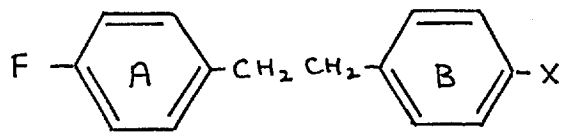
Figure 1.2: Double bond character of the amide linkage

Considered from a valence bond point of view, canonical forms such as II (figure 1.2) contribute significantly to the resonance hybrid. Pauling[11] has calculated that canonical form II contributes about 40% to the total structure. The double bond character of the amide linkage has been invoked to suggest explanations for several different n.m.r. observations; Haake and co-workers[12], for example, have used Carbon-13-hydrogen coupling constants to suggest the same proportions of I and II as calculated by Pauling. Pews[13] has recently demonstrated the same effect with a series of experiments involving fluorine-19 resonance. He studied the transmission of electronic

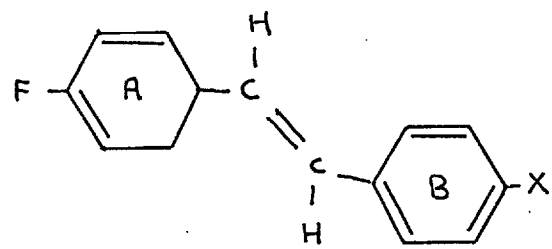
effects between the two aryl rings of stilbene, dibenzyl and benzanilide derivatives of the type shown in figure 1.3. The effect of the substituent X in ring B on the chemical shift of the fluorine atom in ring A was studied.



I Substituted Benzanilide



II Substituted Dibenzyl



III Substituted Stilbene

Figure 1.3: Compounds used to show double bond character of amide linkage[13]

If these assumptions are made, it transpires that there is 40% transmission in the case of the amide derivative (I). This is interpreted as being due to the 40% "double bond" character of the amide linkage. This particular series requires a rather deeper study as recent work suggests that the σ framework is important for the transference of electronic effects[15].

The partial double bond character of the carbon-nitrogen bond of amides manifests itself in the nuclear magnetic resonance spectra of most molecules of this type by the magnetic non-equivalence of groups R_1 and R_2. As in the case of enzyme equilibria cited earlier, the exact effect on the n.m.r. spectrum will depend on the difference in chemical shift of the magnetic group in each environment, and the exchange rate for the interconversion. These limits are shown in table 1.1.

Table 1.1

Dependence of Spectral Appearance on lifetime (τ) and chemical shift difference ($\delta\nu_{AB}$) for the system A $\rightleftharpoons$ B [14]

	Condition	Spectral Appearance
(a)	$\tau \gg (\delta\nu_{AB} 2\pi)^{-1}$	two separate resonance lines situated at ν_A and ν_B respectively.
(b)	$\tau \ll (\delta\nu_{AB} 2\pi)^{-1}$	one sharp line situated at $\frac{\nu_A - \nu_B}{2}$
(c)	$\tau \approx (\delta\nu_{AB} 2\pi)^{-1}$	two overlapping lines at a position intermediate between ν_A and ν_B

A series of experimental spectra showing these limits is shown in figure 1.4.

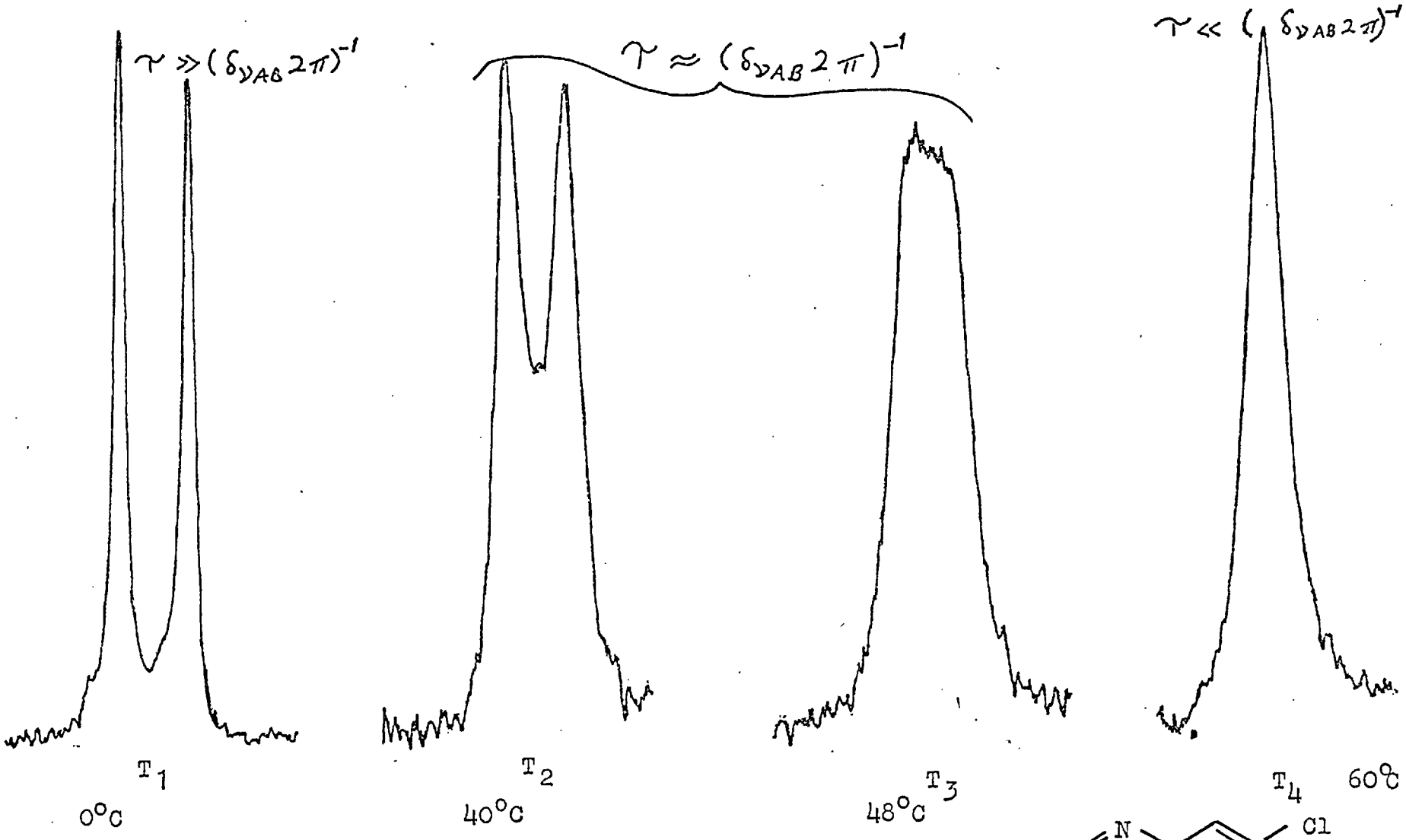
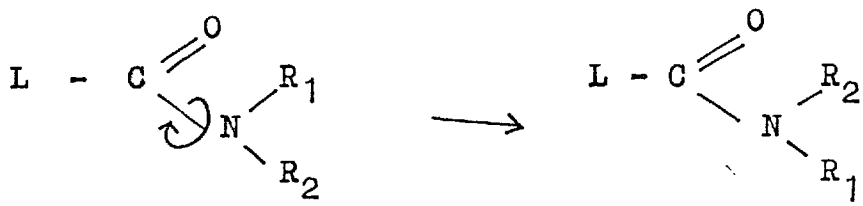


Figure 1.4: Variable temperature n.m.r. spectrum of methyl region of

The simplest possible case to consider theoretically is the situation in which groups R_1 and R_2 show no mutual spin-spin coupling, and there are equal proportions of I and II in the equilibrium mixture (the so-called AB uncoupled case). The two magnetic groups R_1 and R_2 exchange identity as shown in figure 1.5.



I II

Figure 1.5: Possible Conformations of the Amide Group

In conformation I, the group R_1 is *cis* to the carbonyl oxygen (in the shielding region) and the group R_2 is *trans*; in conformation II these positions are reversed. The simplext examples of this type of system are when $R_1 = R_2$ = Me or H.

One theory for the influence of the exchange process on the shape of the nuclear magnetic resonance signals obtained from two exchanging nuclei has been derived by

Gutowsky et al[16], a theory which was later simplified by McConnell[17]. According to this, the line-shape of the signals from protons exchanging between two sites A and B is determined by the parameters P_A, P_B, T_{2A}, T_{2B}, $\delta\nu$, τ_A and τ_B, where

P_A = population of state A
P_B = population of state B
} fractions i.e. $P_A + P_B = 1$

T_{2A}, T_{2B} = spin-spin relaxation times for A and B
for convenience, $T_{2A} = T_{2B} = T_2$ ..... (1)

τ_A and τ_B = mean life times of protons in sites A and B.

The exchange process interchanges the protons between sites A and B, so if the number of protons N_A and N_B at each site are labelled N_A^* and N_B^* at some instant

$$\frac{dN_A^*}{dt} = -k_A N_A^* \quad \text{and} \quad \frac{dN_B^*}{dt} = -k_B N_B^* \qquad \ldots\ldots\ldots (2)$$

$$k_A P_A = k_B P_B \qquad \ldots\ldots\ldots (3)$$

The average lifetime of the proton at each site is therefore

$$\tau_A = \frac{1}{k_A} = \frac{\tau}{P_B} \quad \text{and} \quad \tau_B = \frac{1}{k_B} = \frac{\tau}{P_A} \qquad \ldots\ldots (4)$$

$$\tau = \frac{\tau_A \tau_B}{\tau_A + \tau_B} \qquad \ldots\ldots\ldots (5)$$

This leads to an equation which describes the line-shape

$$\nu = \frac{\omega_1 Mo\left[(1 + \tau/T_2)P + QR \right]}{P^2 + R^2} \qquad \ldots\ldots (6)$$

where

$$P = \tau\left[(1/T_2^{\,2}) - (\Delta\omega)^2 + (\delta\omega/2)^2 \right] + \frac{1}{T_2} \qquad \ldots\ldots (7)$$

$$Q = \tau\left[\Delta\omega - (\delta\omega/2)(P_A - P_B) \right]$$

$$R = \Delta\omega\left[1 + (2\tau/T_2) \right] + (\delta\omega/2)(P_A - P_B) \qquad \ldots\ldots (8)$$

Another approach uses the density matrix methods of Binsch[18] and Forsen[19]. These usually require computer programs to calculate the spectral line-shape, e.g. the program Densmat written by Forsen et al[19] will calculate the spectral line-shape for a system of up to six spins undergoing exchange.

There have been several approximate equations developed for the AB uncoupled exchange; these equations relate a characteristic feature of the exchange broadened spectrum to the exchange rate τ. The commonest are

Peak Separation[20]

$$\tau = \frac{\sqrt{2}}{\pi \sqrt{(\delta\nu)^2 - (\delta\nu_e)^2}} \quad \ldots\ldots (9)$$

where τ = mean lifetime for exchanging protons in each site

$\delta\nu$ = the peak separation in absence of exchange

$\delta\nu_e$ = the peak separation at temperature under consideration.

Intensity Ratio[21]

$$\tau = \frac{\sqrt{2}\sqrt{r + \sqrt{r^2 - r}}}{\pi\,\delta\nu} \quad \ldots\ldots (10)$$

where r is the ratio of the peak maximum to the central minimum.

These two equations can be used in the region between the slow exchange limit and the coalescence temperature. Other equations which may be used at the

coalescence temperature and above are those of

Piette and Anderson[22]

$$\tau = \frac{1}{\pi(\delta\nu_{\frac{1}{2}} - \delta\nu^{o})} \qquad \ldots\ldots (11)$$

$\frac{1}{2}$ = line width at half peak height.

$$\tau = \frac{2(\delta\nu_{\frac{1}{2}} - \delta\nu^{o})}{\pi\,(\delta\nu)^{2}} \qquad \ldots\ldots (12)$$

$$\tau = \frac{2\,(\delta\nu_{\frac{1}{2}})}{\pi\left[(\delta\nu)^{4} + 2(\delta\nu\,\delta\nu_{\frac{1}{2}})^{2} - (\delta\nu_{\frac{1}{2}})^{4}\right]^{\frac{1}{2}}} \qquad \ldots\ldots (13)$$

The exchange rate for the interconversion is

$$k = \frac{\tau_A\,\tau_B}{\tau_A + \tau_B} \qquad \ldots\ldots (14)$$

where τ_A , τ_B are lifetimes in state A and state B respectively.

For AB exchange $\tau_A = \tau_B = \tau$

i.e. exchange rate $$k = \frac{1}{2\tau} \qquad \ldots\ldots (15)$$

The thermodynamic activation parameters for the exchange process may be calculated using the absolute rate theory developed by Eyring[24]

$$\ln k = \frac{-\Delta H^{\ddagger}}{RT} + \frac{\Delta S^{\ddagger}}{R} + \ln x \frac{kT}{h} \qquad \ldots\ldots (16)$$

where $\Delta H^{\ddagger}$ enthalpy of activation

$\Delta S^{\ddagger}$ entropy of activation

E_A energy of activation

$\Delta G^{\ddagger}$ free energy of activation

This assumes that the exchange approximates to a first order rate process. The other activation parameters may be calculated from standard equations

$$\Delta G^{\ddagger} = -RT \ln k \qquad \ldots\ldots (17)$$

$$\Delta H^{\ddagger} = E_A - RT \qquad \ldots\ldots (18)$$

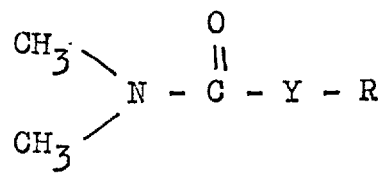
Marquardt[25] has developed an equation for the determination of the free energy of activation ($\Delta G^{\ddagger}$) from coalescence data

$$\Delta G^{\ddagger} = R \times T_c \times 2.303\left(\log \frac{R}{N \times h} + \log \frac{T_c}{k_c}\right) \quad \ldots\ldots (19)$$

where $$k_c = \frac{\pi \times \delta\nu}{\sqrt{2}} \quad \ldots\ldots (20)$$

1.2 Review of Literature

Most previous studies have considered rotation about the C - N bond in amides, but there has been little published work on carbamic acid derivatives; however, Valega[26] has discussed the room temperature proton magnetic resonance spectra of 26 different N,N dimethyl carbamates of the type shown in figure 1.5.



Y = O or S

R = propyl, butyl, substituted phenyl (substituents of a wide range of polarity were used)

Figure 1.5: Carbamic Acid Derivatives studied by Valega[26]

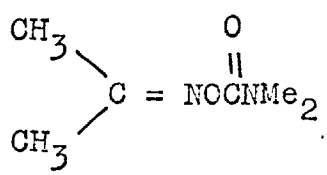
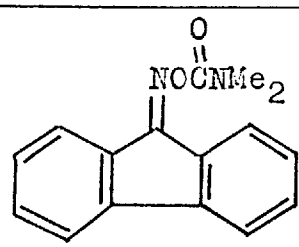
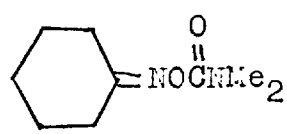
He found that the N,N dimethyl resonance only showed non-equivalence ("splitting") at room temperature when there was a strongly electron withdrawing group in the aromatic ring (e.g. nitro, bromo, chloro present at ortho, meta or para positions all showed this non-equivalence at room temperature). These results were explained by the lowering

of energy of canonical forms in which the heteroatom Y is conjugated with the aromatic ring, a situation which would be enhanced by an electron withdrawing substituent. The results were also rationalised using Hammett σ values.

Bushweller[27] has reported the free energy of activation ($\Delta G^{\neq}$) for the compounds shown in table 1.2.

Table 1.2

Carbamic acid derivatives studied by Bushweller[27]

Compound	Coalescence Temperature T_c (°K)	$\Delta G^{\neq}$ kcal/mole	Solvent
	264	14.4	CS_2
	262	14.3	CS_2
	270	14.2	CS_2

The results were merely reported without any constructive comment by the author.

It is worth considering some of the relevant lessons which are evident from the plethora of work which has appeared on the amide group itself. This has been one of the most confused areas studied by nuclear magnetic resonance, a fact which is obvious when it is realised that there are calculated values ranging from E_A = 7.0 kcal/mole[28] to E_A = 22.0 kcal/mole[28] for the barrier to rotation for dimethyl formamide. Several reviews cover the early work in the field (up to 1968), e.g. those of Binsch[29], Siddall and Stewart[30], Stewart and Siddall[28].

A comprehensive analysis of the factors causing these wide variations in the reported data for the same compound has been made by Gutowsky[31]. He examined the inherent errors in the experimental technique (rate of sample spinning and sample depth are just two factors which produce inaccuracies in temperature measurement) and he examined the general applicability (or otherwise!) of the approximate equations (9 - 14) together with the limitations on their use. Drakenberg et alia[32] have calculated the activation parameters for N,N dimethyl trichloroacetamide using each of the approximate equations in turn and the results were compared with the values obtained from full

line-shape analysis. Their results are summarised in table 1.3.

Table 1.3

Calculation of Activation Parameters for N,N dimethyl trichloroacetamide by different methods

Eqn. No.	Page	log A	E_A kcal/ mole	$\Delta H^{\ddagger}$ kcal/ mole	$\Delta G^{\ddagger}$ kcal/ mole	$\Delta S^{\ddagger}$ e.u.
Full line shape	141	13.3	15.7 ±.6	15.1 ±.6	15.0 ±0.1	0.3
9	142	12.0	13.9	13.3	15.0	-5.7
10	142	11.2	12.8	12.2	15.0	-9.2
11	143	16.1	19.2	18.6	14.7	+13.3
12	143	14.6	17.6	17.0	15.7	+6.4
13	143	19.9	15.1	14.5	15.0	-1.5

Table 1.3 illustrates the futility of attempting to compare experimental results with published results calculated by a different method. The only parameter which is insensitive to these systematic errors is the

free energy of activation ($\Delta G^{\ddagger}$). The parameter most sensitive to these errors is the one which is potentially the most useful, namely the entropy of activation ($\Delta S^{\ddagger}$). Even the use of the full line-shape analysis is not without certain inherent difficulties, however; for example the spectral line-shape is insensitive to the exchange lifetime τ at both very slow and very fast rates of exchange, and the line shape changes rapidly over a very short temperature range in the intermediate region of exchange. This tends to give an Arrhenius plot of dubious significance.

Another problem with the full line shape equation arises from the computer programs used for the calculation. Typically the experimental spectrum is input as a series of manually digitised points at fixed frequency increments; the program then obtains a least squares fit of the experimental and theoretical points and outputs the "best fit" value of the exchange rate ($\frac{1}{2\tau}$). The limit of accuracy for this procedure is the number of manually digitised points input, the practical limit for a typical spectrum being about 50. Ideally for greater accuracy many more points are required. This procedure is obviously more accurate than using the approximate equations, however, in spite of the limitations.

Riddell and Williams[33] have overcome the problem outlined in the previous paragraph by a rather ingenious method. The experimental spectrum is accumulated in the computer of average transients (CAT) attached to the spectrometer. The information in the memory of the CAT is then transferred to a paper tape which is used as an input to their full line shape program Chem 21. This gives 1024 digitised points for each spectrum.

Despite the limitations of the n.m.r. method for studying conformational changes it is a very useful technique providing that emphasis is placed on the observed trends for a particular series rather than the absolute values obtained. The experimental limitations can be overcome to some extent by using (as far as possible) identical conditions for each member of a particular series.

CHAPTER TWO

Experimental

2.1 Nuclear Magnetic Resonance Measurements

The spectra were recorded on a Varian Associates Model A60 spectrometer operating at 60 MHz. The spectrometer was equipped with a V6040 variable temperature system.

The samples were dissolved in deuterochloroform (except where indicated) at a concentration of 10 - 12% (w/v).

The spectrometer variable temperature system was calibrated before and after each experimental sample. The range -100°C to +40°C was calibrated using the standard methanol sample[34], and the range +40°C to +200°C was calibrated with the standard ethylene glycol sample[35]. After each temperature adjustment, the sample was left in the probe for at least ten minutes to attain equilibrium at that temperature before the spectrum was recorded. Temperature readings are accurate to ± 1°C. The spectrum of each sample was recorded ten times at each temperature, five times in a downfield direction, five times in an upfield direction. Chemical shifts were measured directly from the pre-calibrated charts.

2.2 Preparation of Compounds

The compounds prepared are listed in table 2.1. All compounds which are described in the results section which do not appear in this table were kindly supplied by Shell Research Ltd. The methods for purification and identification of compounds were described in Part I, Section 2, page 49.

Table 2.1

Compounds Prepared for Variable Temperature Study

I	2-nitrophenyl-N,N dimethyl carbamate
II	3-nitrophenyl-N,N dimethyl carbamate
III	4-nitrophenyl-N,N dimethyl carbamate
IV	Phenyl - N,N dimethyl carbamate
V	2-fluorophenyl N,N dimethyl carbamate
VI	3-fluorophenyl N,N dimethyl carbamate
VII	4-fluorophenyl N,N dimethyl carbamate
VIII	2-t . butyl phenyl N,N dimethyl carbamate
IX	3-t . butyl phenyl N,N dimethyl carbamate
X	4-t . butyl phenyl N,N dimethyl carbamate
XI	2-chlorophenyl N,N dimethyl carbamate

/contd. .....

XII	3-chlorophenyl N,N dimethyl carbamate
XIII	4-chlorophenyl N,N dimethyl carbamate
XIV	2-fluorophenacetyl chloride
XV	N-methyl-2-fluorophenyl acetamide
XVI	N,N dimethyl-2-fluorophenyl acetamide
XVII	3-fluorophenacetyl chloride
XVIII	N-methyl-3-fluorophenyl acetamide
XIX	4-fluorophenacetyl chloride
XX	N-methyl-4-fluorophenyl acetamide
XXI	3-trifluoromethyl phenacetyl chloride
XXII	N-methyl-3-trifluoromethyl phenyl acetamide
XXIII	4-trifluoromethyl phenacetyl chloride
XXIV	N,N dimethyl-4-trifluoromethyl phenyl acetamide
XXV	N-methyl-4-trifluoromethyl phenyl acetamide

I Preparation of 2-nitrophenyl N,N dimethyl carbamate

2-nitrophenol (1.39 g; .01 m) was dissolved in dry redistilled benzene (10 ml)/dry pyridine (5 ml); N,N dimethyl carbamoyl chloride (1.07 g; .01 m) was added dropwise with stirring. The solution was stirred at room temperature (two hours). Distilled water (10 ml) was added, and the layers were separated. The organic layer was washed with dilute sodium hydroxide solution (5 ml of 0.1 m solution) twice to remove excess phenol, then it was washed twice with dilute hydrochloric acid (5 ml of 0.1 m) to remove excess pyridine. The layers were separated. The organic layer was dried over magnesium sulphate. The drying agent was filtered off, the solvent was evaporated under vacuum to yield 1.30 g of pale yellow oil. This oil crystallised on trituration with 40 - 60 petrol. The product was recrystallised from benzene/hexane to yield pale yellow crystals, m.p. 55°C (Lit. value 57°C[36]), yield = 46% of theoretical.

II Preparation of 3-nitrophenyl N,N dimethyl carbamate

3-nitrophenol (6.95 g; .05 m) was dissolved in dry redistilled dimethyl formamide (20 ml). Sodium hydride (2.5 g of 50% dispersion in oil) was added carefully with

cooling and shaking; a blood red colour developed in the solution. N,N dimethyl carbamoyl chloride (6.0 g; .05 m + excess) was added dropwise with stirring. The solution was heated on an oil bath at 80°C for one hour. The hot solution was poured on to ice (100 g). The crude solid product was filtered off at the pump. It was washed with distilled water (5 x 50 ml), and dried under vacuum. Yield = 8.7 g (83% of theoretical). It was recrystallised from benzene/hexane to yield pale yellow crystals, m.p. 60 - 61°C (lit. value[36] 63°C).

Analysis

	C	H	N
Anticipated	51.5%	4.7%	13.3%
Found	51.2%	4.7%	13.3%

III Preparation of 4-nitrophenyl N,N dimethyl carbamate

4-nitrophenol (1.39 g; .01 m) was dissolved in dry benzene (10 ml)/dry pyridine (5 ml). N,N dimethyl carbamoyl chloride (1.07 g; .01 m) was added dropwise with stirring. The solution was stirred at room temperature (two hours). Distilled water (10 ml) was added and the layers were separated. The organic layer was washed with 0.1 m sodium hydroxide solution (2 x 5 ml portions) to remove the

excess phenol. It was then washed with 0.1 m hydrochloric acid (2 x 5 ml portions) to remove excess pyridine. The layers were separated. The organic layer was dried over magnesium sulphate, filtered, evaporated under vacuum to yield 0.96 g of crystalline product (44% of theoretical). The product was recrystallised from benzene/hexane to yield pale yellow crystals, m.p. = 106°C (Lit. 109°C[36]).

IV Preparation of Phenyl N,N dimethyl carbamate

Phenol (4.7 g, .05 m) was dissolved in dry redistilled formamide (20 ml). Sodium hydride (2.5 g, 50% dispersion in oil) was added portionwise with stirring and cooling. N,N dimethyl carbamoyl chloride (6.0 g) was added dropwise with stirring. The mixture was heated (80°C for one hour) on an oil bath. It was then poured on to ice (100 g). The crude solid product was filtered off, it was washed at the pump with cold distilled water (5 x 5 ml). The solid product was dried under vacuo. Yield = 7.0 g (84.8% of theoretical). The crystalline product was recrystallised from benzene/hexane, m.p. = 44 - 45°C.

Analysis for $C_9H_{11}NO_2$

	C	H	N
Anticipated	65.4	6.7	8.5
Experimental	65.3	6.8	8.4

V Preparation of 2-fluorophenyl N,N dimethyl carbamate

2-fluorophenol (3.36 g; 0.03 m) was dissolved in dry benzene (30 ml) and dry pyridine (15 ml); the solution was cooled. N,N dimethyl carbamoyl chloride (3.21 g; 0.03 m) was added dropwise with cooling and stirring. The solution was stirred at room temperature overnight. Distilled water (30 ml) was added and the layers were separated. The organic layer was washed with distilled water (3 x 20 ml), hydrochloric acid (3 x 10 ml of 0.05 m solution), sodium hydroxide (3 x 10 ml of 0.05 m solution). The organic layer was dried over magnesium sulphate, filtered and the solvent was evaporated off under reduced pressure. Yield of crude product 3.2 g. The product was distilled, b.p. 60 - 62°C @ 0.05 mm.

VI Preparation of 3-fluorophenyl N,N dimethyl carbamate

3-fluorophenol (1.12 g; 0.01 m) was dissolved in dry benzene (10 ml) and dry pyridine (15 ml); the solution was cooled. N,N dimethyl carbamoyl chloride (1.07 g; 0.01 m) was added dropwise with cooling and stirring. The solution was stirred at room temperature (two hours). Distilled water (10 ml) was added, and the layers were separated. The organic layer was washed with distilled water (3 x 5 ml),

sodium hydroxide solution (2 x 5 ml, 0.1 m solution) and hydrochloric acid (2 x 5 ml, 0.1 m solution). The layers were separated, and the organic layer was dried over magnesium sulphate. The solution was filtered. The solvent was removed under water-pump vacuum to yield crude product (1.0 g) (55%). This was distilled, b.p. = 66°C @ 0.05 mm.

VII Preparation of 4-fluorophenyl N,N-dimethyl carbamate

4-fluorophenol (5.6 g; 0.05 m) was dissolved in dry, redistilled dimethyl formamide (20 ml). Sodium hydride suspension (2.5 g of 50% dispersion in oil ~ 0.05 m) was added portionwise with stirring and cooling. N,N dimethyl carbamoyl chloride (5.0 ml, 0.05 m + 10% excess) was added dropwise with cooling and shaking. The resulting mixture was heated on an oil bath (80°C, one hour). It was poured on to ice (100g). The aqueous layer was extracted with chloroform (4 x 50 ml portions). The layers were separated. The chloroform layer was dried over magnesium sulphate and filtered. The chloroform and excess dimethyl formamide were distilled off under water-pump vacuum. The crude product (7.5g, 83.5%) was distilled, b.p. 80°C @ 0.1 mm.

Microanalysis

Calculated for $C_9H_{10}NO_2F$ C 59%, H 5.4%, N 7.6%

Found C 58.6%, H 5.4%, N 7.5%

VIII Preparation of 2-t-butylphenyl N,N dimethyl carbamate

2-t-butylphenol (7.2 g, 0.05 m) was dissolved in dry, redistilled dimethyl formamide (20 ml). Sodium hydride (2.5 g of 50% dispersion in oil) was added portionwise with stirring and cooling. N,N dimethyl carbamoyl chloride (5.0 ml, 0.05 m + 10% excess) was added dropwise. The resulting mixture was heated on an oil-bath (80°C, one hour). It was poured on to ice. The crude crystalline product was filtered off and dried in a vacuum dessiccator. (Yield = 11.5 g, 100% of theoretical). The product was recrystallised from hexane to yield salmon pink crystals, m.p. 105 - 7°C.

Analysis for $C_{13}H_{19}NO_2$

Calculated C 70.5%, H 8.6%, N 6.3%

Found C 70.4%, H 8.6%, N 6.3%

IX Preparation of 3-t-butylphenyl N,N dimethyl carbamate

3-t-butylphenol (7.2 g, 0.05 m) was dissolved in dry, redistilled dimethyl formamide (20 ml). Sodium hydride

(2.5 g of 50% dispersion in oil) was added portionwise with stirring and cooling. N,N dimethyl carbamoyl chloride (5.0 ml, 0.05 m + 10% excess) was added dropwise. The resulting mixture was heated on an oil bath (80°C, one hour). It was poured on to ice. The crude crystalline product was filtered off and dried in a vacuum dessiccator. (Yield = 11.2 g, 97.4% of theoretical). The product was decolourised with charcoal and recrystallised from hexane, m.p. 55 - 56°C.

Analysis for $C_{13}H_{19}NO_2$

Calculated C 70.5%, H 8.6%, N 6.3%

Found C 70.5%, H 8.7%, N 6.3%

X Preparation of 4-t-butylphenyl N,N dimethyl carbamate

4-t-butylphenol (7.2 g, 0.05 m) was dissolved in dry, redistilled dimethyl formamide (20 ml). Sodium hydride (2.5 g of 50% dispersion in oil) was added portionwise with stirring and cooling. N,N dimethyl carbamoyl chloride (5.0 ml, 0.05 m + 10% excess) was added dropwise. The resulting mixture was heated on an oil bath (80°C, one hour) It was poured on to ice. The crude crystalline product was filtered off and dried in a vacuum dessiccator. (Yield = 8.7 g, 76% of theoretical). The crude product was

recrystallised from hexane after decolourisation with charcoal, m.p. 91 - 91°C.

Analysis for $C_{13}H_{19}NO_2$

Calculated C 70.5%, H 8.6%, N 6.3%

Found C 70.5%, H 8.7%, N 6.3%

XI Preparation of 2-chlorophenyl N,N dimethyl carbamate

2-chlorophenol (850 mgs, .005 m) was dissolved in dry, redistilled dimethyl formamide (2.0 ml). Sodium hydride suspension (0.25 g, 50% dispersion in oil ~ 0.005 m) was added with cooling. N,N dimethyl carbamoyl chloride (0.5 ml, 0.005 + 10% excess) was added with shaking and cooling. The resulting mixture was heated on an oil bath (80°C, one hour). It was poured on to ice (10 g). The aqueous layer was extracted with chloroform (4 x 10 ml). The layers were separated. The chloroform layer was dried over magnesium sulphate, filtered, and evaporated to yield 910 mgs (65 % of theoretical) of crude product. 100 mgs of this were purified by T.L.C. (attempts at distillation resulted in decomposition of product). The chromatographic system used was Silica GF 254 preparative plate eluted twice with chloroform. The pure 2-chlorophenyl N,N dimethyl carbamate was isolated as

the band with RF 0.25. The product was characterised by n.m.r.

XII Preparation of 3-chlorophenyl N,N dimethyl carbamate

3-chlorophenol (850 mgs, 0.005 m) was dissolved in dry, redistilled dimethyl formamide (2.0 ml). Sodium hydride suspension (0.25 g, 50% dispersion in oil $\sim$ 0.005 m) was added with cooling. N,N dimethyl carbamoyl chloride (0.5 ml, 0.005 + 10% excess) was added with shaking and cooling. The resulting mixture was heated on an oil bath (80°C, one hour). It was poured on to ice (10 g). The aqueous layer was extracted with chloroform (4 x 10 ml). The layers were separated. The chloroform layer was dried over magnesium sulphate, filtered, and evaporated to yield 890 mgs (63.5% of theoretical) of crude product. 100 mgs of this were purified by T.L.C. (attempts at distillation resulted in decomposition of product). The chromatographic system used was Silica GF 254 preparative plate eluted twice with chloroform. The product was characterised by n.m.r.

XIII Preparation of 4-chlorophenyl N,N dimethyl carbamate

4-chlorophenol (850 mgs, 0.005 m) was dissolved in dry, redistilled dimethyl formamide (2.0 ml). Sodium hydride suspension (0.25 g, 50% dispersion in oil $\sim$ 0.005 m) was added with cooling. N,N dimethyl carbamoyl chloride (0.5 ml, 0.005 + 10% excess) was added with shaking and cooling. The resulting mixture was heated on an oil bath (80°C, one hour). It was poured on to ice (10 g). The aqueous layer was extracted with chloroform (4 x 10 ml). The layers were separated. The chloroform layer was dried over magnesium sulphate, filtered, and evaporated to yield 900 mgs (64 % of theoretical) of crude product. 100 mgs of this were purified by T.L.C. (attempts at distillation resulted in decomposition of product). The chromatographic system used was Silica GF 254 preparative plate eluted twice with chloroform. The product was characterised by n.m.r.

XIV Preparation of 2-fluorophenacetyl chloride

2-fluorophenyl acetic acid (5.1 g, .033 m) was refluxed with redistilled thionyl chloride (15 ml, one hour). The excess thionyl chloride was distilled off. Dry benzene (20 ml) was added to azeotrope off the remains of the

thionyl chloride. This crude acid chloride was then diluted to 20 ml with dry benzene. Aliquots were then used for experiments.

XV Preparation of N-methyl-2-fluorophenyl-acetamide

Crude 2-fluorophenacetyl chloride (5 ml of solution prepared in XIV) was cooled in an ice bath. A saturated aqueous solution of methylamine was added dropwise until the violent exothermic reaction ceased. The layers were separated. The benzene layer was washed with distilled water (2 x 5 ml portions). The layers were separated. The benzene layer was dried over magnesium sulphate, filtered, and evaporated to yield 1.30 g crude product (100% of theoretical). The product was recrystallised from ether to give 1.15 g pure product, m.p. 99°C.

Microanalysis for $C_9H_{10}NOF$	C	H	N	F
Calculated	64.67	5.98	8.38	11.37
Experimental	64.87	6.24	8.70	11.40

XVI Preparation of N,N dimethyl-2-fluorophenyl-acetamide

Crude 2-fluorophenacetyl chloride (5 ml of solution prepared in XIV $\equiv$ 1.25 g acid) was cooled in an ice bath. Aqueous dimethylamine solution was added dropwise with

shaking until no further reaction was evident. The layers were separated. The benzene layer was washed with distilled water (2 x 5 ml portions). The layers were separated. The benzene layer was dried over magnesium sulphate, filtered, and evaporated under water pump vacuum. The crude product was triturated with petroleum ether (boiling range 60 - 80°C). This was filtered to yield 1.5 g product (100% of theoretical) which was recrystallised from ether, m.p. = 59°C.

Microanalysis for $C_{10}H_{12}NOF$

	C	H	N	F
Calculated	66.29	6.62	7.73	10.49
Experimental	66.18	6.68	7.80	10.26

XVII Preparation of 3-fluorophenacetyl chloride

3-fluorophenyl acetic acid (5.1 g, .033 m) was refluxed with redistilled thionyl chloride (15 ml, one hour). The excess thionyl chloride was removed by distillation. Dry benzene (20 ml) was added and distilled off (to azeotrope traces of thionyl chloride).

The crude acid chloride solution was diluted to 20 ml with dry benzene and used for subsequent experiments.

XVIII Preparation of N-methyl-3-fluorophenyl-acetamide

3-fluorophenacetyl chloride solution (5 ml of solution prepared in XVII) was cooled in an ice bath. A saturated aqueous solution of methylamine was added dropwise until the violent reaction subsided. The layers were separated. The benzene layer was washed with distilled water (2 x 10 ml). The layers were separated. The organic layer was dried over magnesium sulphate, filtered, and evaporated under water pump vacuum to yield 1.2 g of crude product. This was re-crystallised from ether to yield 1.0 g (77% of theoretical), m.p. = 82°C.

XIX Preparation of 4-fluorophenacetyl chloride

4-fluorophenyl acetic acid (5.1 g, .033 m) was refluxed with redistilled thionyl chloride (15 ml, one hour). The excess thionyl chloride was removed by distillation. Dry benzene (20 ml) was added and distilled off (to azeotrope traces of thionyl chloride).

The crude acid chloride solution was diluted to 20 ml with dry benzene and used for subsequent experiments.

XX Preparation of N-methyl-4-fluorophenyl-acetamide

4-fluorophenacetyl chloride (5 ml of solution prepared in XIX) was cooled in an ice bath. A saturated aqueous solution of methylamine was added dropwise with shaking until the violent reaction subsided. The layers were separated. The benzene layer was washed with distilled water (2 x 10 ml). The layers were separated. The organic layer was dried over magnesium sulphate, filtered, and evaporated under water pump vacuum to yield 1.2 g of crude product. This was recrystallised from ether to yield 0.9 g (72% of theoretical), m.p. = 101°C.

Microanalysis for $C_9H_{10}NOF$

	C	H	N	F
Calculated	64.67	5.98	8.38	11.37
Experimental	63.93	6.21	8.22	11.28

XXI Preparation of 3-trifluoromethyl-phenacetyl chloride

3-trifluoromethyl phenyl acetic acid (2.04 g, .01 m) was refluxed with thionyl chloride (10 ml, one hour). The excess thionyl chloride was removed by distillation. Dry benzene (5 ml) was added and distilled off (to azeotrope off excess thionyl chloride). The solution was diluted to 20 ml with dry benzene. Aliquots were used for subsequent experiments.

XXII Preparation of N-methyl 3-trifluoromethyl phenyl acetamide

3-trifluoromethyl phenacetyl chloride (5 ml of solution prepared in XXI) was cooled in an ice bath. A saturated aqueous solution of methylamine was added dropwise with stirring until the exothermic reaction subsided. The layers were separated. The organic layer was washed with distilled water (2 x 5 ml), the layers were separated and the organic layer dried over magnesium sulphate, filtered, and evaporated under water pump vacuum to yield 0.7 g crude product which crystallised on trituration with hexane. The product was recrystallised from benzene/hexane to yield 0.4 g (75% of theoretical), m.p. = 65 - 66°C.

Microanalysis for $C_{10}H_{10}F_3ON$

	C	H	N	F
Calculated	55.29	4.6	6.4	26.27
Experimental	54.59	4.52	6.20	25.86

XXIII Preparation of 4-trifluoromethyl phenacetyl chloride

4-trifluoromethyl phenyl acetic acid (2.04 g, .01 m) was refluxed with thionyl chloride (10 ml, one hour). The excess thionyl chloride was removed by distillation. Dry benzene (5 ml) was added and distilled (to remove excess

thionyl chloride). The solution was diluted to 20 ml with dry benzene. Aliquots were used for subsequent experiments.

XXIV Preparation of N,N dimethyl-4-trifluoromethyl phenyl acetamide

4-trifluoromethyl phenacetyl chloride (5 ml of the solution prepared in XXIII) was cooled in an ice bath. An aqueous solution of dimethylamine was added dropwise with stirring until the vigorous effervescence ceased. The layers were separated. The benzene layer was washed with distilled water (2 x 5 ml), the layers were separated. The organic layer was dried over magnesium sulphate, filtered, and evaporated under water pump vacuum to yield 0.6 g crude product. This was recrystallised from ether to give 0.45 g (78% of theoretical), m.p. = 97°C.

Microanalysis for $C_{11}H_{12}F_3ON$

	C	H	N	F
Calculated	57.14	5.19	6.06	24.67
Experimental	56.21	5.00	6.10	24.00

XXV Preparation of N-methyl-4-trifluoromethyl phenyl acetamide

4-trifluoromethyl phenacetyl chloride (5 ml of the solution prepared in XXIV) was cooled in an ice bath. A saturated aqueous solution of methylamine was added dropwise

with shaking until the vigorous exothermic reaction subsided. The layers were separated. The benzene layer was washed with distilled water (2 x 5 ml), the layers separated, and the organic layer dried over magnesium sulphate, filtered, and evaporated under water pump vacuum to yield 0.51 g crude product which crystallised on trituration with hexane. The crude product was recrystallised from benzene/hexane to yield 0.35 g (62% of theoretical), m.p. = 107°C.

Microanalysis for $C_{10}H_{10}NOF_3$

	C	H	N	F
Calculated	55.29	4.6	7.37	26.27
Experimental	54.89	4.67	7.26	25 68

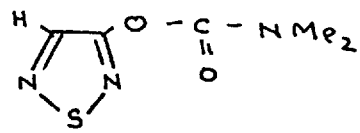
CHAPTER THREE

Results

3.1 Calculation of Activation Parameters

The free energy of activation ($\Delta G^{\ddagger}$) for each compound was calculated at its coalescence temperature using equation

The other thermodynamic activation parameters (E_A, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$) posed rather more of a problem. Initially the results were calculated using the computer program ABEXCH (listed in the Appendix) which utilises the approximate intensity ratio[21] and peak separation[20] equations. The parameters calculated using this program are listed in tables 3.4 and 3.5. Intuitively, the low values for the energy and enthalpy of activation together with the large negative values for the entropies of activation seem unreasonable. As the complete line-shape equation overcomes the inherent mathematical inadequacies of the approximate equations[31] program CHEM23 was developed. (This is also listed with full details in the Appendix.) The parameters obtained from ABEXCH are compared with the values obtained from CHEM23 for various compounds and the results are summarised in table 3.7. In order to determine the activation parameters, an Arrhenius plot is made. When the graph of logk against $^1/_T$ is drawn for each compound,

however, it is apparent that for these systems the full line shape equation offers little advantage over the approximate equations. Figure 3.1 shows this plot for N,N dimethyl carbamic acid - 1,2,5 thiadazol -3-yl ester () using the values of log k calculated from ABEXCH (symbolised ⊙) and CHEM23 (symbolised X). The line-shape only changes markedly with the exchange rate over the relatively narrow temperature range of 20°C. Small errors in the measurement of the sample temperature and in the calculation of log k can cause appreciable errors in both the slope (which gives E_A and $\Delta H^{\ddagger}$) and the intercept (which yields log A, hence $\Delta S^{\ddagger}$).

There is increasing evidence from combined equilibration, spin echo and line-shape measurements on a single compound[37,38] that the entropy of activation for these systems is very close to zero. The combination of the three techniques has the considerable advantage that a much larger range of k and temperature is accessible than with any of the techniques used individually. The small entropy of activation can to some extent be predicted from the absolute rate theory[24]. When an amide molecule goes into its activated state for rotation, the 40% contribution

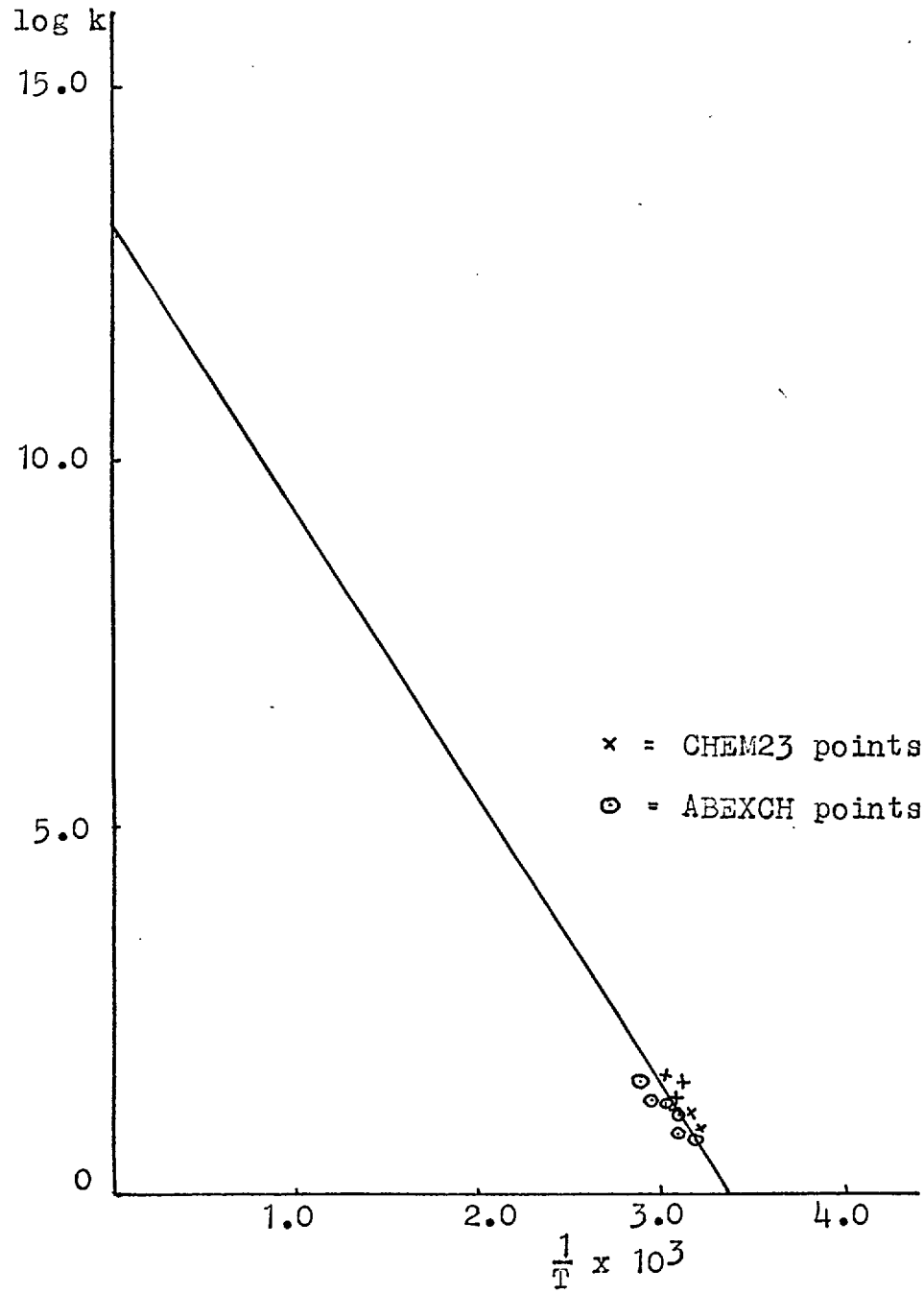


Figure 3.1: Comparison of Arrhenius plots from CHEM23 and ABEXCH

from the dipolar form ($-\overset{O^{\ominus}}{\underset{|}{C}}=\overset{\oplus}{N}$) must tend to zero, because in the activated complex the nitrogen atom and its substituents are in a plane perpendicular to the plane of the carbonyl group (the delocalisation of the lone pair of electrons of the nitrogen atom will depend on $\cos^2 \phi$ (39), where ϕ is the angle between planes). The decreasing contribution of the dipolar form to the activated complex means that the complex is less polar than the ground state molecule (hence less susceptible to association with other amide or solvent molecules), resulting in a gain in entropy. This gain, however, will be very slight because even in the activated complex the amide carbonyl group must have a similar polarity to a ketone carbonyl group (hence the polar interactions will not be entirely absent). A set of corrected values for the energy and enthalpy of activation was calculated by assuming a constant frequency factor of $\sim 10^{13}$ and using the Arrhenius equation

$$\log k = \log A - \frac{E_A}{2.303RT}$$

i.e. $E_A = (\log k - 13.0) \times 2.303 \times RT$

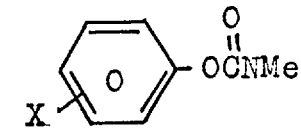
It is reasonable to assume that the entropy of activation will not vary very much from member to member of a particular series, e.g. $R - O - \overset{O}{\overset{\|}{C}} - N\begin{matrix}CH_3\\CH_3\end{matrix}$

carbamates

so even if the value of log A used above is incorrect it will only lead to a constant error in the values of E_A and $\Delta H^{\ddagger}$ calculated by this method. It is obvious, however, that the values of E_A and $\Delta H^{\ddagger}$ calculated by this method for a given series cannot be compared directly with the values calculated by the same method for a different series. Whilst it is clearly reasonable to assume that the members of the N,N dimethyl carbamate series shown above have similar entropies of activation, it is <u>not</u> reasonable to assume that amides, thioamides, carbamates and thiocarbamates all have similar entropies of activation.

3.2 Experimental Results.

Table 3.1

Free Energy of Activation ($\Delta G^{\ddagger}$) for the rotation about the carbon-nitrogen bond of carbamic acid derivatives of the type 

X	Coalescence Temperature T_c (^{o}K)	Maximum Separation ν (Hz)	$\Delta G^{\ddagger}$ (k.cal/mole)
H	302	5.4	14.8 (2)
2 NO_2	319	6.9	15.5 (4)
3 NO_2	316	5.5	15.5 (3)
4 NO_2	317	5.5	15.5 (8)
2,4 di NO_2	322	7.2	15.6 (6)
2F	312	6.0	15.2 (7)
3F	309	5.1	15.2 (2)
4F	302	5.0	14.8 (7)
2Cl	314	7.0	15.2 (8)
3Cl	307	4.6	15.1 (8)
2 t.butyl	307	6.8	14.7 (0)
3 t.butyl	300	5.1	14.7 (6)
4 t.butyl	301	5.1	14.8 (1)

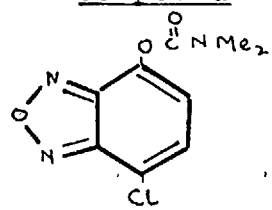
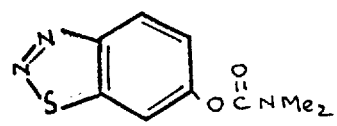
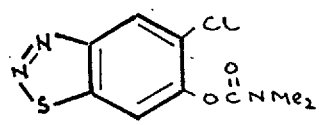
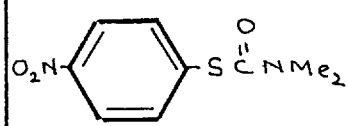
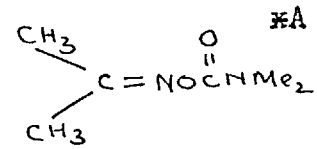
/contd. .....

Table 3.1 (contd.)

4 CN	312	5.2	15.3 (6)
3 Me 4 Cl	305	4.9	15.0 (4)
3 Me 4 NMe_2	298	4.6	14.7 (2)
3 iso propyl	293	5.2	14.3 (9)

Table 3.2

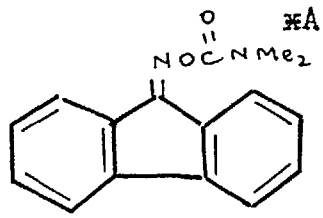
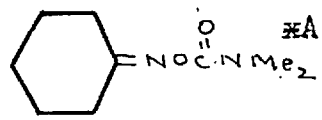
Free Energy of Activation ($\Delta G^{\ddagger}$) for the rotation about the carbon-nitrogen bond of various carbamic acid derivatives

Compound	Coalescence Temperature T_c (°K)	Maximum Separation ν (Hz)	$\Delta G^{\ddagger}$ (k.cal/mole)
	322	7.8	15.6 (1)
	314	6.2	15.2 (7)
	317	9.0	15.3 (5)
	276	4.2	13.6 (4)
 ⁂A	264	3.2	14.4

⁂A This data was taken from literature reference (27); the solvent used was carbon disulphide.

/contd. .....

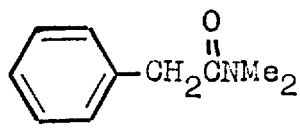
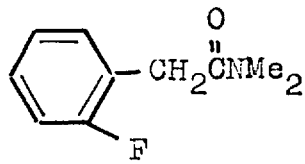
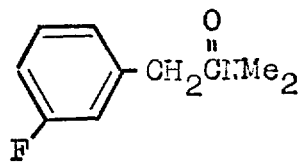
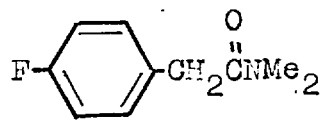
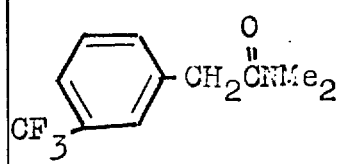
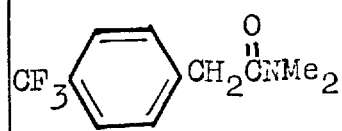
Table 3.2 (contd.)

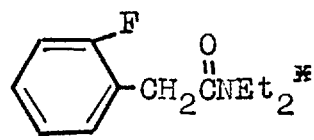
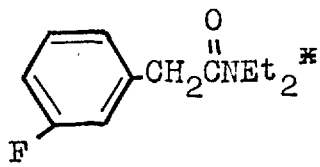
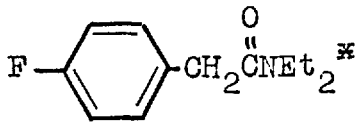
 ⁂A	262	2.7	14.3
 ⁂A	270	8.9	14.2

⁂A This data was taken from literature reference (27); the solvent used was carbon disulphide.

Table 3.3

Free Energies of Activation ($\Delta G^{\ddagger}$) for various amides

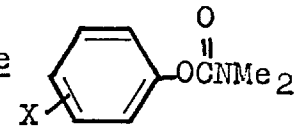
Derivative	Coalescence Temperature (^{o}K)	Maximum Separation (Hz)	$\Delta G^{\ddagger}$ (k.cal/mole)
	309	1.8	17.2
	323	2.4	17.6
	314	2.0	17.6
	311	1.8	17.3
	319	2.2	18.0
	325	1.5	17.9

	308	3.0	17.4
	312	3.0	17.6
	309	3.0	17.5

* The exchange rates for the diethyl compounds were obtained at the coalescence temperatures using the equation

$$k_c = \frac{\pi \left[|\nu_A - \nu_B|^2 + 6J_{AB}^2 \right]^{\frac{1}{2}}}{\sqrt{2}} \quad (52)$$

Table 3.4

Activation parameters for N,N dimethyl carbamic acid derivatives of the type  using the Intensity Ratio Method

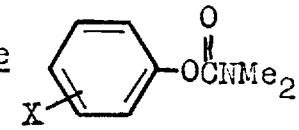
X	E_A kcal/mole	$\Delta H^{\ddagger}$ kcal/mole	$\Delta S^{\ddagger}$ eu	log A
H	6.7	6.1	-32.4	6.15
2 Cl				
3 Cl	8.3	7.7	-27.7	7.18
4 Cl	9.3	8.7	-24.2	7.92
2 NO_2	7.9	7.3	-29.4	6.79
3 NO_2	8.9	8.3	-26.4	7.45
4 NO_2	6.9(8)	6.31	-32.9	6.04
2,4 di NO_2	12.9	12.3	-13.7	10.22
2 t.butyl	9.0	8.4	-24.9	7.8
3 t.butyl	9.61	9.01	-22.6	8.28
4 t.butyl	7.31	6.72	-30.3	6.59
2F				
3F	8.14	7.5	-28.23	7.05
4F	8.5	7.9	-26.7	7.39

/contd. .....

Table 3.4 (contd.)

4 CN	5.7	5.11	-36.9	5.16
3 Me 4 NMe_2	7.1	6.5	-30.9	6.47
3 Me 4 Cl	8.1	7.5	-27.9	7.07
3 i propyl	9.3	8.7	-22.9	8.22
S-4-NO_2	6.1	5.5	-32.96	6.02

Table 3.5

Activation Parameters for N,N dimethyl carbamic acid derivatives of the type  using the Peak Separation Method

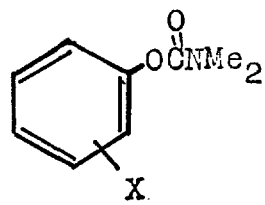
X	E_A kcal/mole	$\Delta H^{\neq}$ kcal/mole	$\Delta S^{\neq}$ eu	log A
H	7.8	7.2	-30.0	6.65
2 Cl				
3 Cl	9.9	9.3	-23.9	7.99
4 Cl	9.6	9.0	-29.4	6.8
2 NO_2	13.1	12.5	-13.96	10.17
3 NO_2	9.4	8.8	-26.0	7.5
4 NO_2	6.2	5.6	-36.3	5.29
2,4 di NO_2	11.7	11.1	-18.8	9.12
2 t.butyl	11.3	10.7	-18.8	9.12
3 t.butyl	13.1	12.5	-12.3	10.5
4 t.butyl	6.3	5.7	-34.9	5.59

/contd. .....

Table 3.5 (contd.)

2F				
3F	7.3	6.7	-32.1	6.2
4F	10.9	10.4	-19.8	8.9
4 CN	5.9	5.3	-37.4	5.04
3 Me 4 NMe_2	6.3	5.6	-35.2	5.5
3 Me 4 Cl	8.8	8.2	-27.1	7.3
3 i propyl	7.7	7.1	-29.0	6.77

Table 3.6

Activation parameters for N,N dimethyl carbamates of the type  calculated assuming a constant frequency factor (log A = 13.0)

X	E_A kcal.mole^{-1}	$\Delta H^{\neq}$ kcal.mole^{-1}	$\log_{10} k$ (25°C)
H	17.0	16.4	0.959
2Cl	16.8	16.2	1.09
3Cl	17.4	16.8	0.74
4Cl	17.0	16.4	0.96
$2NO_2$	17.5	16.9	0.5639
$3NO_2$	17.2	16.6	0.9120
$4NO_2$	17.2	16.6	0.9198
2,4 di NO_2	17.7	17.1	0.5
2 t.butyl	16.8	16.2	1.15
3 t.butyl	16.7	16.1	1.23
4 t.butyl	16.8	16.2	1.19

/contd. .....

Table 3.6 (contd.)

2-F	16.7	16.1	1.19
3-F	16.9	16.3	1.08
4-F	16.8	16.2	1.15
4CN	17.4	16.8	0.7
3 Me 4 NMe_2	16.6	16.0	1.3
3 Me 4 Cl	17.1	16.5	0.93
3 i Propyl	16.7	16.1	1.2

Table 3.7

Comparison of Activation Parameters obtained from CHEM23 (line shape) and ABEXCH (Approximate Equations)

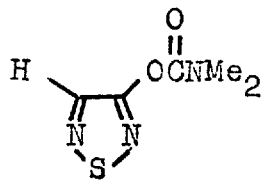
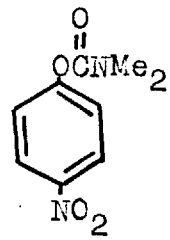
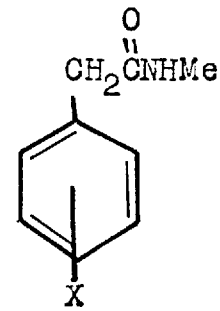
Compound	Program	E_A kcal mole^{-1}	$\Delta H^{\ddagger}$ kcal mole^{-1}	$\Delta S^{\ddagger}$ eu	log A
	ABEXCH	13.7	13.1	-12.8	10.44
	CHEM23	13.9	13.3	-13.7	10.23
	ABEXCH	6.2	5.6	-36.3	5.29
	CHEM23	9.6	9.0	-22.3	8.25

Table 3.8

I.R. Spectral Data for  derivatives

X	NH Vibration	Amide I	Amide II
2-F	3200 cm^{-1}	1620 cm^{-1}	1540 cm^{-1}
3-F	3300 cm^{-1}	1620 cm^{-1}	1560 cm^{-1}
4-F	3250 cm^{-1}	1630 cm^{-1}	1550 cm^{-1}
3-CF_3	3300 cm^{-1}	1620 cm^{-1}	1550 cm^{-1}
4-CF_3	3300 cm^{-1}	1620 cm^{-1}	1560 cm^{-1}

CHAPTER FOUR

Discussion

Introduction

The discussion is restricted to a consideration of $\Delta G^{\ddagger}$ as this is the parameter which is least susceptible to systematic errors, and when the entropy of activation is assumed to be constant $\Delta G^{\ddagger}$ is proportional to $\Delta H^{\ddagger}$ and E_A. Any discussion of the effects on $\Delta G^{\ddagger}$ are therefore equally applicable to $\Delta H^{\ddagger}$ and E_A.

4.1 The effect of the aromatic substitution pattern on the carbon-nitrogen bond rotation of N,N dimethyl phenyl carbamates

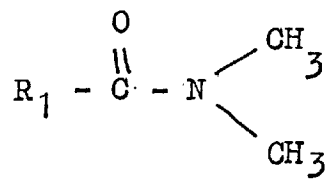


Figure 4.1: Generalised N,N dimethyl amide

The effect of varying the group R_1 (figure 4.1) upon the barrier to rotation of the carbon-nitrogen bond is readily seen from the published data shown in table 4.1.

Table 4.1

Barriers to Rotation for the N,N dimethyl amides, published by Neumann and Jonas[40]

R_1	$\Delta G^{\neq}$ (kcal.mole^{-1})	Solvent
H	21.7	None
CH_3	17.5	"
CF_3	18.6	"
CCl_3	14.8	"
$CH_2 = CH$	16.1	"
C_6H_5	15.7	chloroform

Neumann and Jonas[40] correlated the rotational barrier of the six N,N dimethyl amides shown in this table with Tart's inductive parameters, σ^* and E_S[41]. Although the correlation with Taft's parameters is not good, it gives an indication that both steric and polar effects are important for the rotation process. It is very difficult, however, to estimate the relative importance of the steric and polar effects from

this approach (the Taft equation is $\log {}^{k_1}/_{k_0} = \rho^*\sigma^* + sE_s$, having two "adjustable" constants ρ^* and s).

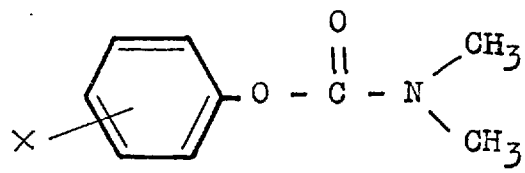


Figure 4.2: Phenyl Carbamic Acid Derivative

The phenyl carbamic acid derivatives (figure 4.2) are potentially useful for studying the electronic effects alone, since it is quite reasonable to assume that for the meta and para substituted molecules the main steric effect will be due to the oxygen atom and the phenyl ring.

Ager and Phillips[42] have recently demonstrated that the Hammett reactivity parameter, σ para, effectively measures the total interaction of the substituent with the aromatic pi-system and it does not encompass any sigma bonded interaction. The free energies of activation for the rotation process in the substituted phenyl carbamates were plotted against the substituent constants suggested by Jaffe in his extensive review[43] and the graph is shown in figure 4.3. There is an excellent correlation with a value of $\rho = +1.12$, indicating that the rotational barrier is

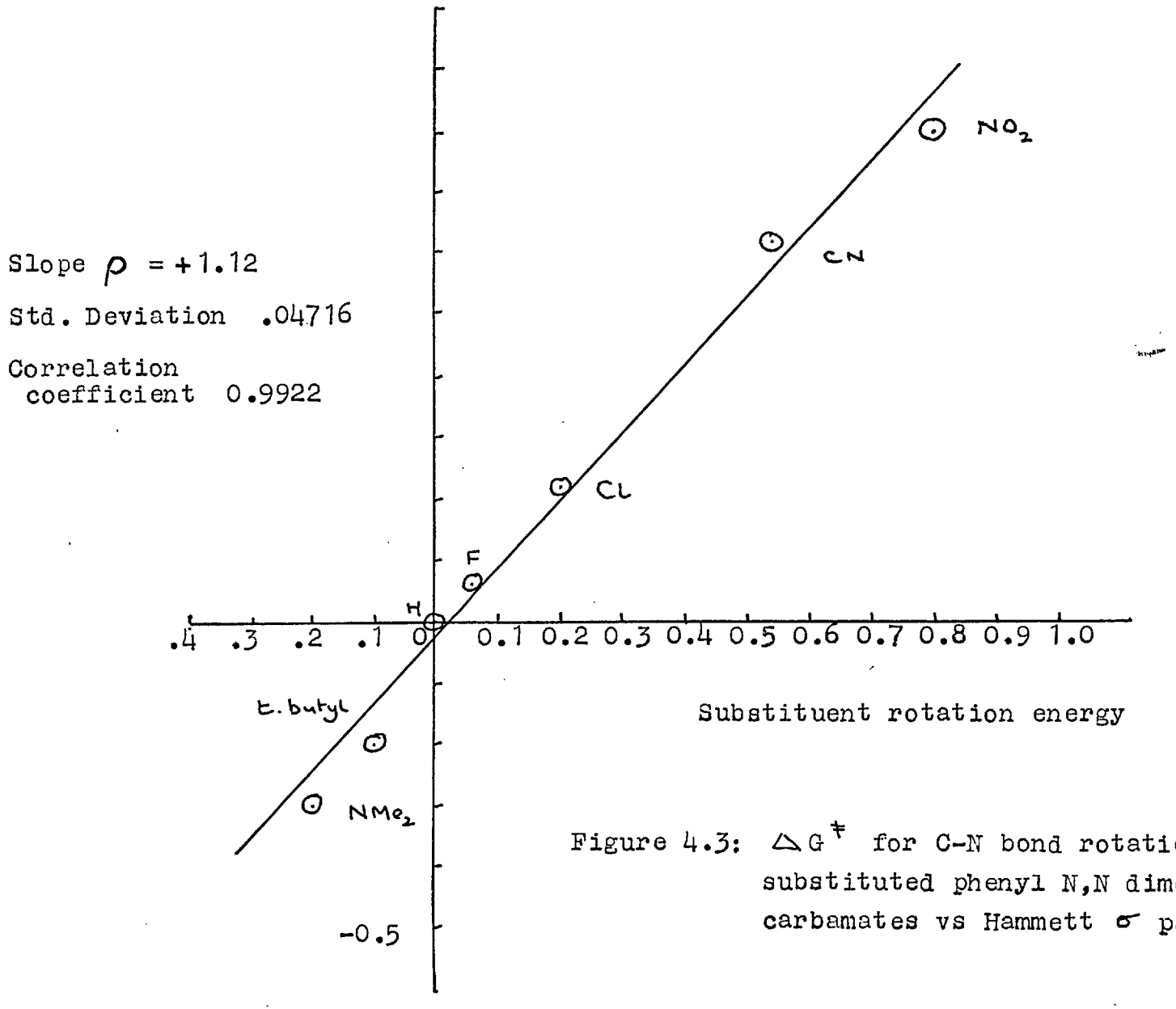
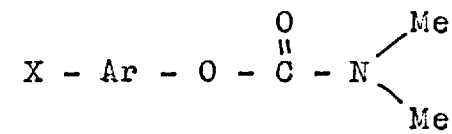


Figure 4.3: $\Delta G^{\neq}$ for C-N bond rotation in 4-substituted phenyl N,N dimethyl carbamates vs Hammett σ para.

increased by electron-withdrawing substituents and furthermore that the effects are transmitted through the aromatic pi-system.

Carbamic acid esters are formed by the elimination of water between carbamic acid and a hydroxy compound. Those considered so far are phenyl esters, and there exists a satisfactory correlation between the pK_a of the phenol from which they are derived and the Hammett σ values (figure 4.4) of the substituent in the phenol. Thus, a correlation between the phenol pK_a and the values of $\triangle G^{\ddagger}$ for rotation about the C - N bond in the compounds



is realised (figure 4.5). A method is therefore suggested for extending the analysis to esters other than the phenyl type by means of a general correlation between $\triangle G^{\ddagger}$ and the pK_a of the "hydroxy component" of the ester.

Figure 4.6 shows the plot of pK_a against $\triangle G^{\ddagger}$ for a wide variety of such hydroxy compounds and the result is linear. This observation supports the thesis that the barrier to rotation changes as a result of the electron withdrawing ability of the group bonded to oxygen, since the effect of

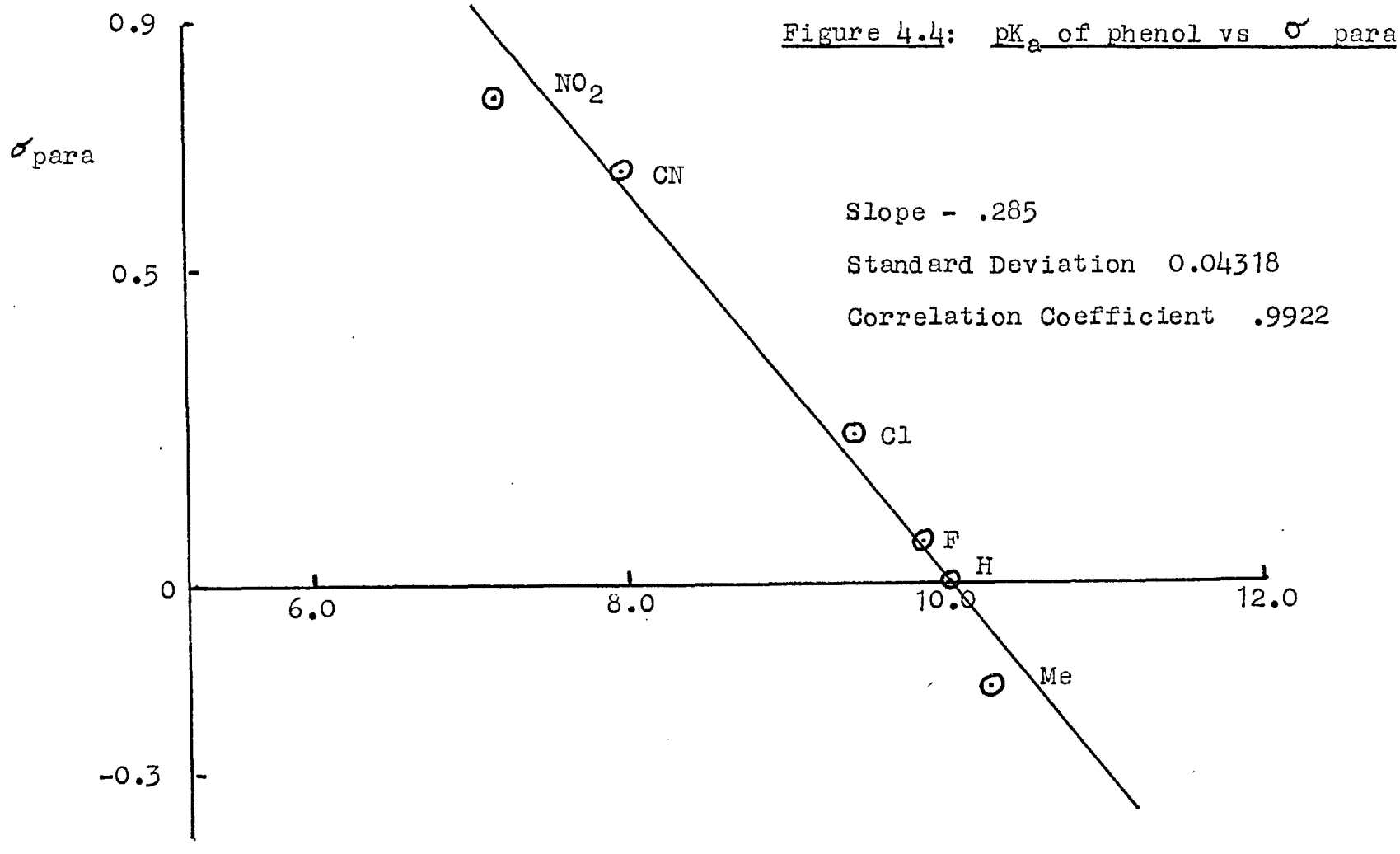


Figure 4.4: pK_a of phenol vs σ para

Slope - .285

Standard Deviation 0.04318

Correlation Coefficient .9922

Figure 4.5: $\Delta G^{\ddagger}$ for C-N bond rotation in N,N dimethyl phenyl carbamates vs pK_a of phenol

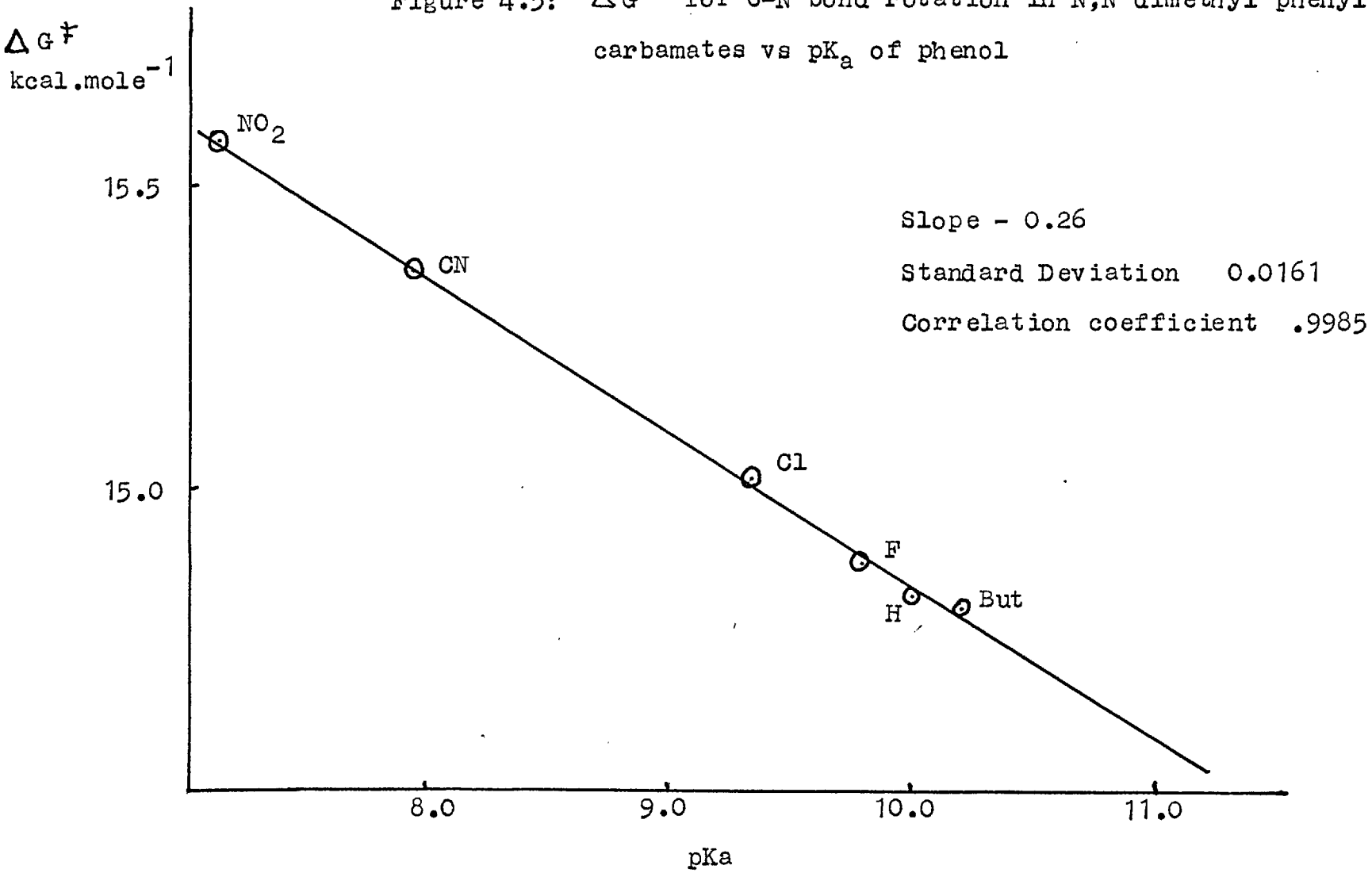
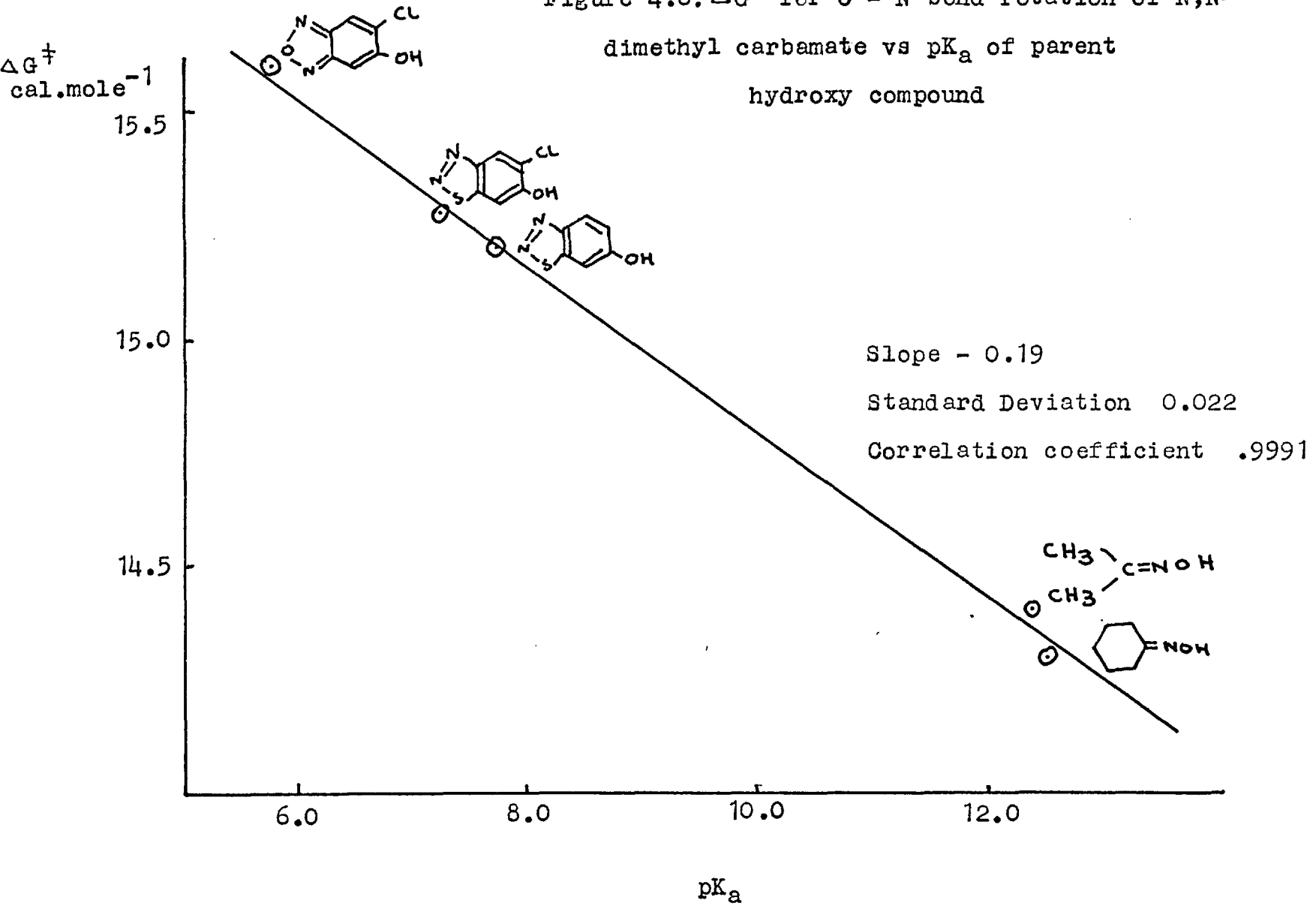


Figure 4.6: $\Delta G^{\ddagger}$ for C - N bond rotation of N,N-dimethyl carbamate vs pK_a of parent hydroxy compound



substitution upon the pK_a of ROH is presumably related to the ability of the group R to stabilise the negative charge upon the oxyacid anion. It further demonstrates the absence of significant steric effects in this series.

4.2 The Variation of the Carbonyl Substituent

In the preceding section, it was shown that substituent effects are transmitted by the aromatic pi-system and intermediate oxygen atom to the amide carbon-nitrogen bond in the substituted N,N dimethyl phenyl carbamates. The results obtained can now be used as a basis for the examination of the effect of substituents on the barrier to rotation of the amide linkage in other aromatic systems in which the phenyl ring is separated from the carbonyl group with other atoms, viz: benzamides, phenyl acetamides, thiocarbamates.

The free energies of activation ($\Delta G^{\ddagger}$) for the carbon nitrogen bond rotation process are given in table 4.2 for a series of unsubstituted aromatic amide derivatives of the type shown in figure 4.7.

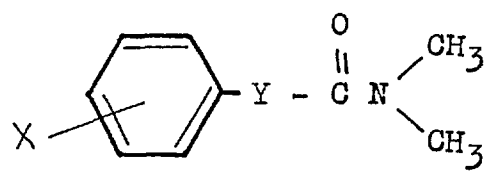
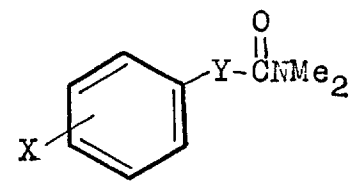


Figure 4.7: Generalised aromatic N,N dimethyl amide

Table 4.2

$\Delta G^{\ddagger}$ for C-N bond rotation in aromatic amides of the type



Series	X	Y	$\Delta G^{\ddagger}$ kcal.mole^{-1}	Ref.
benzamide	H	-	15.5 ± 0.1	44
phenyl acetamide	H	CH_2	17.2 ± 0.1	this study
thiocarbamate	H	S	12.8 ± 0.1	" "
carbamate	H	O	14.8 ± 0.1	" "

If it is assumed that there is 100% transmission of pi-electronic effects in meta and para substituted benzamide derivatives in which the amide carbonyl group is directly conjugated to the aromatic pi-system, it is possible to compare the barriers to rotation for members of the series under study with the corresponding benzamide derivative.

The results can then be fitted to the general equation

$$\Delta G^{\ddagger}_{\text{benzamide}} = \chi \, \Delta G^{\ddagger}_{\text{derivative}} + c$$

where χ^{-1} is a transmission coefficient, $\Delta G^{\ddagger}_{\text{benzamide}}$ is the free energy of activation for a particular substituted benzamide and $\Delta G^{\ddagger}_{\text{derivative}}$ is the free energy of activation for the same substituent in the series under study.

χ is obtained as the slope from a graph of $\Delta G^{\ddagger}_{\text{benzamide}}$ vs $\Delta G^{\ddagger}_{\text{derivative}}$.

A graph of this type is illustrated in figure 4.8 for the free energy of activation of the benzamides plotted against the free energy of activation for the carbamates. The transmission coefficients for the different series are summarised in table 4.3.

It seems surprising at first sight that the order for the transmission of pi-electronic effects is

$$S > O > \text{direct bond} > CH_2$$

and that there is such a large transmission of these effects through a nominally sp^3 hybridised methylene group.

Figure 4.8: $\Delta G^{\ddagger}$ for substituted carbamates vs $\Delta G^{\ddagger}$ for substituted amides.

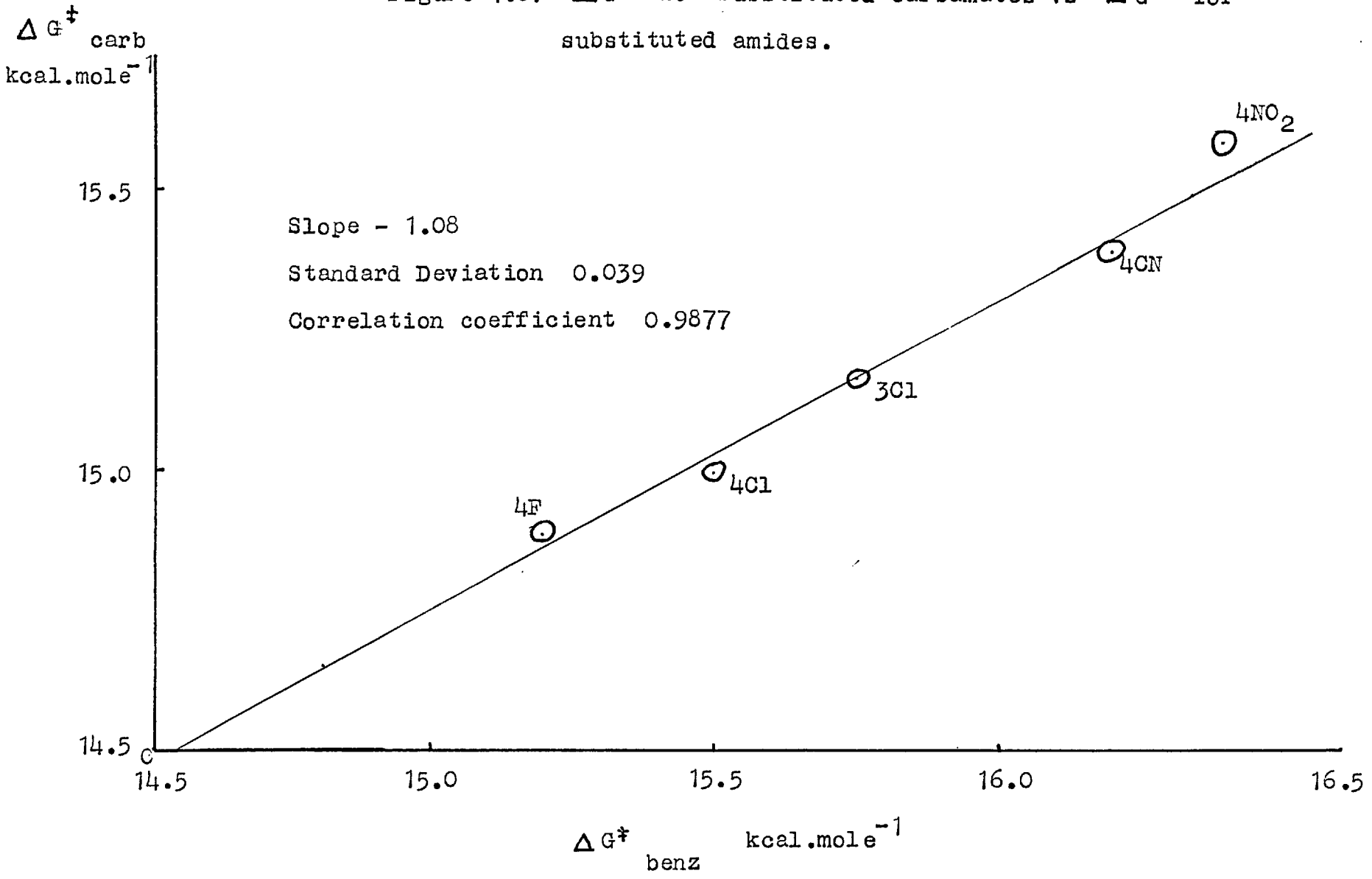
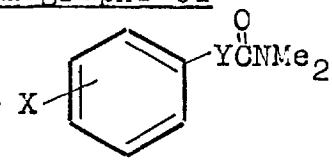


Table 4.3

Transmission coefficients obtained from graphs of $\Delta G^{\neq}_{\text{benzamide}}$ against $\Delta G^{\neq}_{\text{derivative}}$ for 

Series	Y	Transmission Coefficient
carbamates	O	1.05
phenyl acetamides	CH_2	0.8
benzamides	-	1.0
thiocarbamates	S	1.21

The order is in accord, however, with the recent results obtained by Ager and Phillips[42] for bicyclic aromatic systems of the type shown in figure 4.9.

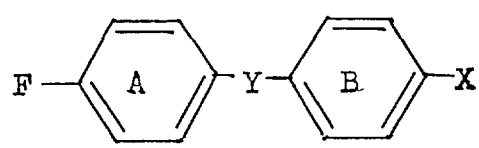


Figure 4.9: Bicyclic Aromatic Systems of the type studied by Ager and Phillips[42]

They studied the effect on the ^{19}F chemical shift of the fluorine atom in ring A of varying substituents X in ring B and concluded that the effects are largely transmitted by the pi-system. Consequently, their results can be interpreted in a similar way to the amide results above. The transmission coefficients calculated from their work are summarised in table 4.4.

Table 4.4

Transmission of pi-electronic effects calculated from ^{19}F measurements in bicyclic aromatic systems(42)

Series	Y	Slope A	Slope B
Biphenyls	-	1.36	1.0
Diphenyl ethers	O	1.506	1.11
Diphenyl thioethers	S	2.07	1.52
Diphenyl methanes	CH_2	0.645	0.475

Slope A is relative to Stilbene = 1.0

Slope B is relative to biphenyl = 1.0

It can be seen that the order of transmission coefficients is the same for the bicyclic aromatic compounds and the aromatic amides, although the absolute values of the coefficients are different.

Carbamates

The effect of a substituent on the barrier to rotation of the amide linkage in these molecules is slightly larger than in the corresponding benzamide ($\chi^{-1} = 1.05$). This is a little surprising as the method of transmission is not so obvious since the two groups are not directly conjugated and the molecular geometry is not well defined. The obvious explanation is that the oxygen atom is sp^2 hybridised and the pi-system of the aromatic ring and the carbonyl group overlap with the P_z orbital, which is not involved in hybridisation.

Thiocarbamates

The magnitude of the transmission coefficient ($\chi^{-1} = 1.21$) is significantly larger than for the benzamides and carbamates and it is tempting to conclude that the increase in conjugation is due to sulphur 'd' orbital participation.

Although this suggestion is reasonable, the conjugation could be explained in a manner similar to than invoked for oxygen, the differences being caused by the longer sulphur carbon bond length ($\sim$ 0.4 Å) which allows the system to obtain a more "planar" conformation.

Phenyl Acetamides

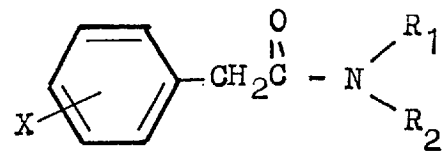
This system is analogous to the thiocarbamates, the hybridisation of the methylene carbon atom can be assumed to be sp^3 with a bond angle close to 108^o. The overlap in this case, however, must occur between the carbonyl and aromatic pi-systems and a carbon hydrogen sigma bond rather than a 'lone pair' "hyperconjugation". The small transmission coefficient (0.8) could either be due to the shorter carbon-carbon bond leading to increased steric repulsion or by a smaller overlap between the aromatic and carbonyl pi-systems and the carbon-hydrogen sigma bond than with the oxygen and sulphur lone pairs. The latter explanation seems most plausible as an increase in steric repulsion would lead to a general decrease in the barrier to rotation[40] and this is not observed.

4.3 Variation of Nitrogen Substituent

At the present time computer programs are only available for six-spin systems undergoing exchange between two magnetically non-equivalent environments[45]. Consequently, this study was confined to monomethylamino, dimethylamino and diethylamino derivatives. The barriers to rotation for the fluorophenyl acetamide derivatives are summarised in table 4.5.

Table 4.5

Free energy of activation for derivatives of the type



X	R_1	R_2	$\Delta G^{\ddagger}$ (kcal.mole^{-1})
ortho F	Me	H	-
	Me	Me	17.6
	Et	Et	17.4

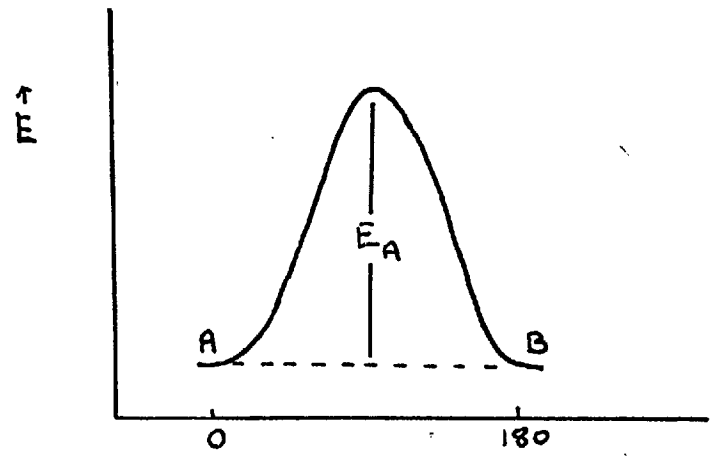
/contd. .....

Table 4.5 (contd.)

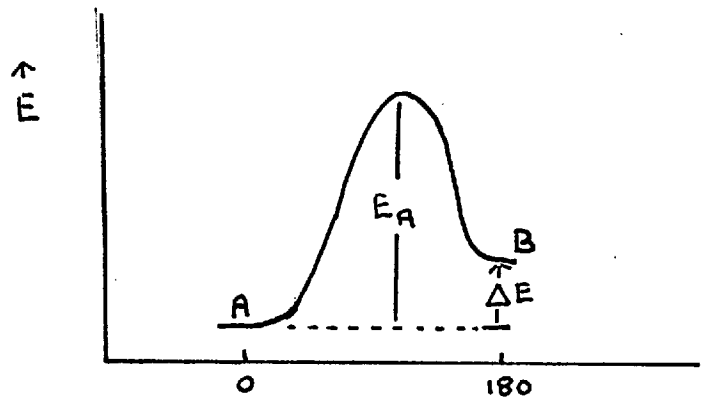
meta F	Me	H	-
	Me	Me	17.6
	Et	Et	17.6
para F	Me	H	-
	Me	Me	17.3
	Et	Et	17.5

The diethyl substituents are not large enough to cause any steric hindrance to the rotation process, consequently the barriers to rotation for the dimethyl and diethyl amino compounds are the same with experimental error. It is not possible to calculate the free energies of activation for the monomethyl derivatives as their n.m.r. spectra are invariant over the temperature range -60°C to +60°C. They are apparently stabilised in one conformation with no evidence for the existence of the other isomer. One possible interpretation of this phenomenon is that for some reason there is an exceptionally high barrier to rotation in these compounds. The other, more plausible,

interpretation is that the inherent energy difference between the cis and trans conformers is greater than 3 kcal/mole and the higher energy state is not populated to any great extent, with a similar barrier to rotation to that observed for the dimethyl amide. These possibilities are shown diagrammatically in figure 4.10.



(a) Large barrier to rotation with conformations A and B of similar energy

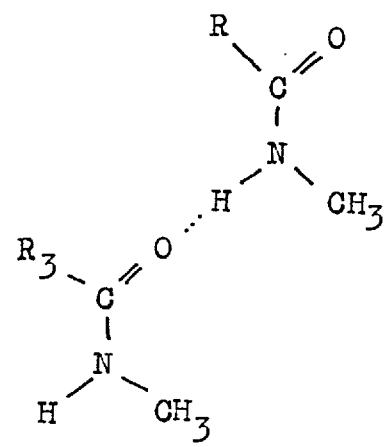


(b) Difference in inherent energy of conformations A and B.

Figure 4.10: Possible reasons for non-observation of barrier to rotation in monomethyl amides

Evidence for the latter explanation comes from I.R. studies which show that the barrier to rotation for N-methyl acetamide is 14 ± 2 kcal.mole^{-1} with an inherent difference ($\triangle$ E) between the two conformations of 7±2 kcal.mole^{-1} (46).

It is possible to assign conformations A and B to either cis or trans isomers by considering their infra-red spectra; Mizushima[47] has assigned certain I.R. bonds to either cis or trans isomers by combining dipole moment and I.R. measurements. The I.R. spectral data for the mono-methyl compounds in this study is given in table 3.8. The existence of the amide II band at ~ 1550 cm^{-1} is strong evidence for a <u>trans</u> CONH group[47]. The (NH) vibration frequencies suggest that these molecules are associated in solution. Linear oligomers analagous to the type suggested by Mizushima for N-methyl acetamides are an obvious possibility and these are shown diagrammatically in figure 4.11.



<u>Figure 4.11:</u> <u>Possible representation of oligomer formation with the N-methyl phenyl acetamide derivatives</u>

This energy difference between conformers does not exist when the nitrogen atom is symmetrically substituted (for example by methyl groups) and the possible conformations are therefore equally populated.

4.4 Empirical Correlation of Activation Parameters for C-N bond rotation in carbamic acid derivatives with Enzymic Activity

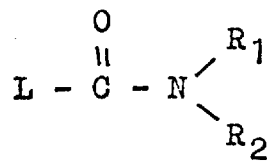


Figure 4.12: Generalised amide

Several workers have attempted to correlate some measurable or calculable property of an organic molecule with its biological activity. For example, McFarlane and co-workers[48] have studied the interaction of carbamic acid and derivatives (figure 4.12, L = -OR, -SR) with acetyl-cholinesterase. They have found that the 'in vitro' enzymic activity depends on the nature of the leaving group L and on the nature of the head substituents R_1 and R_2. There is an indication that the acidity of the leaving group (pK_a) governs the rate of enzymic hydrolysis.

Hansch and co-workers have even attempted to put their observations on a quantitative basis for phenyl substituted carbamates interacting with acetyl cholinesterase[49]

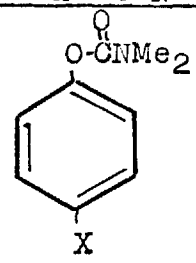
They developed multi-parameter equations but these have little practical use because they are so specific for a particular series of compounds. They do give some idea, however, of the complexity of the enzyme binding process. The electronic nature of the substrate and its molecular size and geometry are obviously important for the binding process.

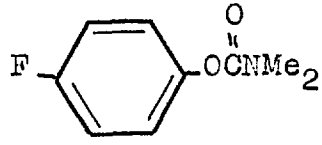
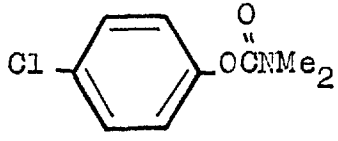
One big difficulty with studies of the type described above is the sensitivity of the A.Ch.E. system to slight changes in the experimental procedure. The enzyme activity depends on the temperature and pH of the measurements as well as the actual source of the enzyme - a molecule will show different activity with A.Ch.E. extracted from Bovine Erythrocytes and flyheads for example.

Table 4.6 lists the enzymic activity of some para-X-phenyl N,N dimethyl carbamic acid derivatives and also the free energy of activation ($\Delta G^{\ddagger}$) for the rotation about the C-N bond as determined by n.m.r. These are illustrated graphically in figure 4.13. As the currently accepted mechanism for A.Ch.E.[50] action postulates the presence of the anionic site on the enzyme where the substrate molecule is anchored, it is tempting to suggest that both the enzymic activity and the carbon-nitrogen bond rotation

Table 4.6

Comparison of Acetyl Cholinesterase Inhibition with free energy of Activation ($\Delta G^{\ddagger}$) for rotation about the C-N bonds of N,N dimethyl carbamates of the type(31)



Substrate	Enzyme	pH	Temp.	k_2/K_I $lmole^{-1}min^{-1}$	$\Delta G^{\ddagger}$ (kcal/mole)
	Bovine Erythrocyte A.Ch.E.	7.0	38°C	3.0×10^1	14.8 (7)
	"	7.0	38°C	3.2×10^2	15.0 (2)

/contd. .....

Table 4.6 (contd.)

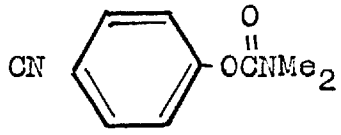
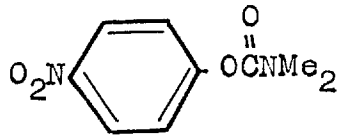
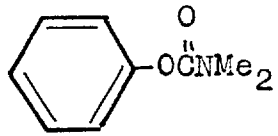
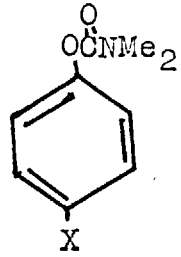
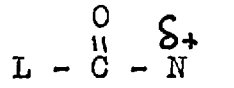
	Bovine Erythrocyte A.Ch.E.	7.0	38°C	4.8×10^2	15.3 (6)
	"	7.0	38°C	7.5×10^2	15.5 (8)
	Fly Head	7.5	35°C	1.5×10^5	14.8

Figure 4.13: Correlation of Acetyl Cholinesterase Inhibition with free energy of activation ($\Delta G^{\ddagger}$) for rotation about the carbon-nitrogen bond of N,N dimethyl carbamates of the type



must depend on a common factor, namely



the positive charge induced on the nitrogen atom by the substituent L. Although this explanation is plausible, data for many more compounds is needed to show that a genuine correlation exists.

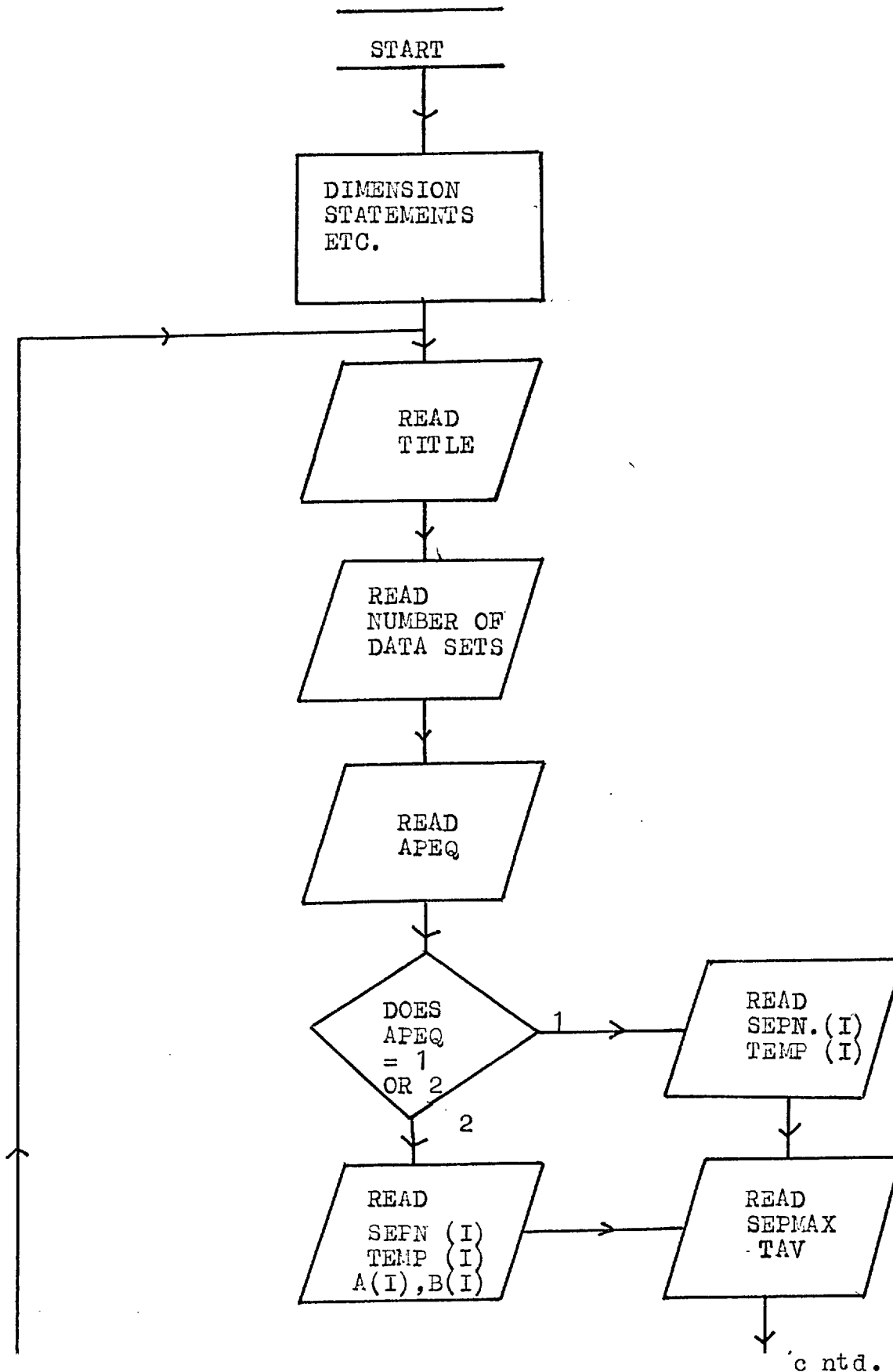
APPENDIX

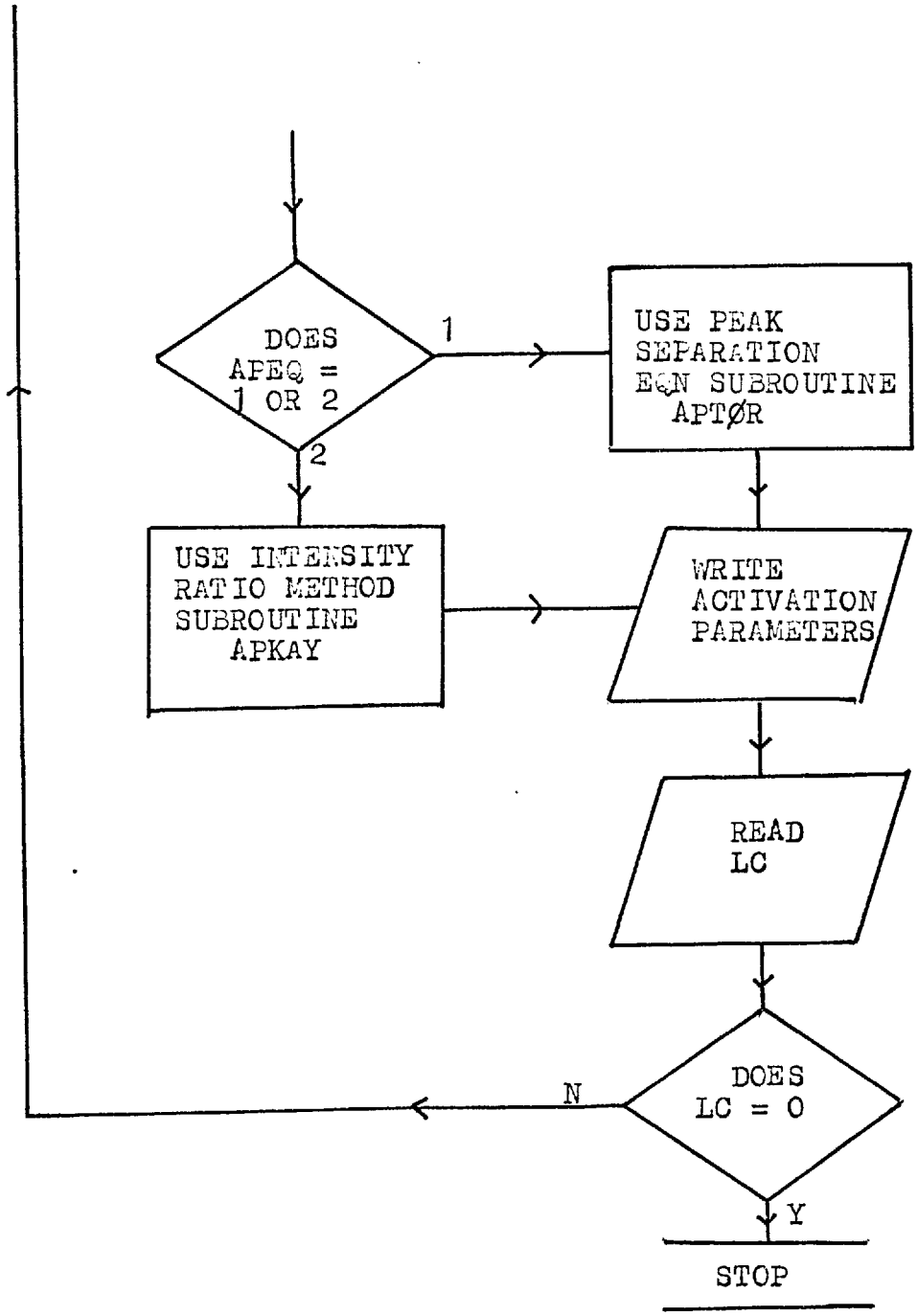
Computer Programs developed for Part II

(i) ABEXCH

(ii) CHEM23

PROGRAM ABEXCH




DOES APEQ = 1 OR 2
1
2
USE PEAK SEPARATION EQN SUBROUTINE APTØR
USE INTENSITY RATIO METHOD SUBROUTINE APKAY
WRITE ACTIVATION PARAMETERS
READ LC
DOES LC = 0
N
Y
STOP

ABEXCH

This is a program written in FORTRAN IV by D.P. Leworthy which calculates the exchange rate ($^1/_{2\tau}$) for a magnetic group undergoing exchange between two non-equivalent uncoupled magnetic sites (AB exchange). It uses the approximate intensity ratio[21] and peak separation[20] equations incorporating the limit of use of these equations suggested by Gutowsky ($^1/_{2\tau} \times \delta\nu \leqslant 5.0$). If this condition is not satisfied an error message is produced. The exchange rate at each temperature is automatically passed to a Least Squares Arrhenius Plot program which gives E_A, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ with their standard deviations.

Ordering of Data Deck

FIRST CARD TITLE (80 alpha numeric characters/card). Up to 11 cards may be used for the title. A blank card must be placed after the last title card.

NEXT CARD N - the number of sets of data to be input and used for activation parameter calculation. FORMAT I5.

NEXT CARD APEQ - this flag decides which equation will be used to calculate the exchange rate

APEQ = 1 Peak Separation Equation

APEQ = 2 Intensity Ratio Equation

FORMAT I1

IF APEQ = 1

NEXT CARD SEPN(I), TEMP(I). SEPN(I) is the separation (Hz) between the coalescing signals at temperature (^{o}C) TEMP(I)

FORMAT = 4(F10.0, F10.0)

IF APEQ = 2

NEXT CARD TEMP(I), A(I), B(I). A(I) is the average height of the coalescing peaks (cms). B(I) is the height of the valley between coalescing peaks (cms) at temperature (^{o}C) TEMP(I).

FORMAT = 2(10X, 3F10.0)

NEXT CARD (Regardless of value of APEQ)

SEPMAX, TAV. SEPMAX is the maximum separation between the two peaks in the absence of exchange. TAV is the temperature at which the activation parameters are to be calculated.

FORMAT F10.0, 10X, F10.0.

NEXT CARD - LC - if further sets of data are to be input L = 1 (column 1) followed by data deck ordering as above. After the last set of data place a blank card.

```
      PROGRAM  ABEYCH     OPL/1971


C PROGRAM TO CALCULATE ACTIVATION PARAMETERS FOR SIMPLE TWO SITE EXCHANGE
C NMR DATA
C PROGRAMMED BY DR LEWORTHY ON 19/2/71
      DIMENSION TEMP(100),SEPN(100),A(100),B(100),EXKAY(100),TITLE(14)
      COMMON TEMP,SEPN,A,B,EXKAY,TITLE,TAV,N,SEPNAV,APEQ
       INTEGER APEQ
    1 CALL INPUT
       GO TO (2,3),APEQ
    2 CALL APTOR
       GO TO 4
    3 CALL APKAY
    4 READ(5,1000)LC
 1000 FORMAT(I5)
      IF(LC.NE.0) GO TO 1
      STOP
      END
```

```
      SUBROUTINE INPUT
      INTEGER TITLE
      DATA IBLNK/6H      /
      INTEGER AREA
      DIMENSION TEMP(100),SERN(100),A(100),B(100),EXRAY(100),TITLE(14)
      COMMON TEMP,SERN,A,B,EXRAY,TITLE,TAV,N,SERMAX,AREA
C READ AND PRINT TITLE
   20 WRITE(6,200)
  200 FORMAT(1H1)
      DO 27 I=1,11
      READ(5,10)TITLE
   10 FORMAT(20A4)
      IF (TITLE.EQ.IBLNK) GO TO 12
      WRITE(6,201)TITLE
  201 FORMAT(1X,20A4)
   27 CONTINUE
C READ NUMBER OF DATA SETS
   12 READ(5,111)N
  111 FORMAT(I5)
      READ(5,1001)AREA
 1001 FORMAT(I1)
      GO TO (13,14),AREA
   13 READ(5,112)((SERN(I),TEMP(I)),I=1,N)
  112 FORMAT(8(F10.4,F10.2))
      GO TO 15
   14 READ(5,113)((SERN(I),TEMP(I),A(I),B(I)),I=1,N)
  113 FORMAT(6(4F10.4))
      GO TO 15
```

```
      PROGRAM ABEXCH  SUBROUTINE INPUT

      IS RETURN
         END
```

```
C PROGRAM AREXCH  SUBROUTINE LSARP

C SUBROUTINE LSARP IS USED AS THE LAST ROUTINE OF CHEM 23

      SUBROUTINE ERROR
      WRITE(6,10)
   10 FORMAT(36HTHIS APPROXIMATE EQUATION IS NOT VALID )
      RETURN
      END
```

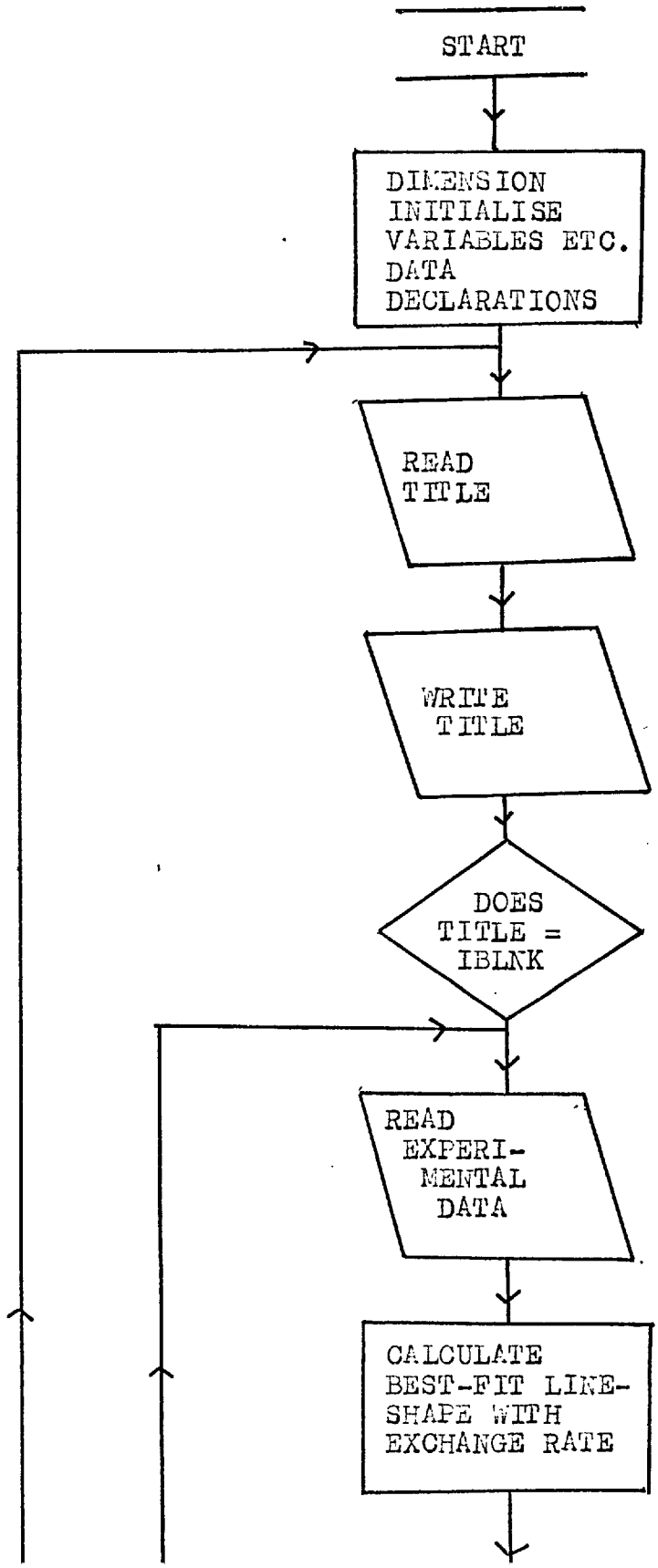
PROGRAM ABEXCH SUBROUTINE &PTOP

```
      SUBROUTINE &PTOP
      DIMENSION NRUN(20)
      DIMENSION TEMP(100),SEPN(100),A(100),B(100),EXKAY(100),TITLE(14)
      COMMON TEMP,SEPN,A,B,EXKAY,TITLE,TW,N,SEPMAX,&PEO
      DO 29 I=1,N
      D=(SEPMAX**2.-SEPN(I)**2.)
      NRUN(I)=1
      D=SQRT(D)
      PI=3.14159
      EXKAY(I)=(1.0/SQRT(2.0))*D*PI
      TOT=(1.0/EXKAY(I))*SEPN(I)
      IF(TOT.GE.5.0) CALL ERROR
   29 CONTINUE
      WRITE(6,233)
  233 FORMAT(////1HY,7HRESULTS)
      WRITE(6,234)
  234 FORMAT(////A2H   SAMPLE      TEMPERATURE(*C)      SEPARATION(HZ)
     1K(SEC-1)    )
      WRITE(6,235)(NRUN(I),TEMP(I),SEPN(I),EXKAY(I)),I=1,N)
  235 FORMAT(//1X,I5,8X,F10.2,8X,F10.2,8X,F10.2)
      WRITE(6,236)SEPMAX
  236 FORMAT(1HY,21HMAXIMUM SEPARATION = ,F10.2,3H47.)
      CALL ISSRP
      RETURN
      END
```

PROGRAM ABEXCH SUBROUTINE ARRAY

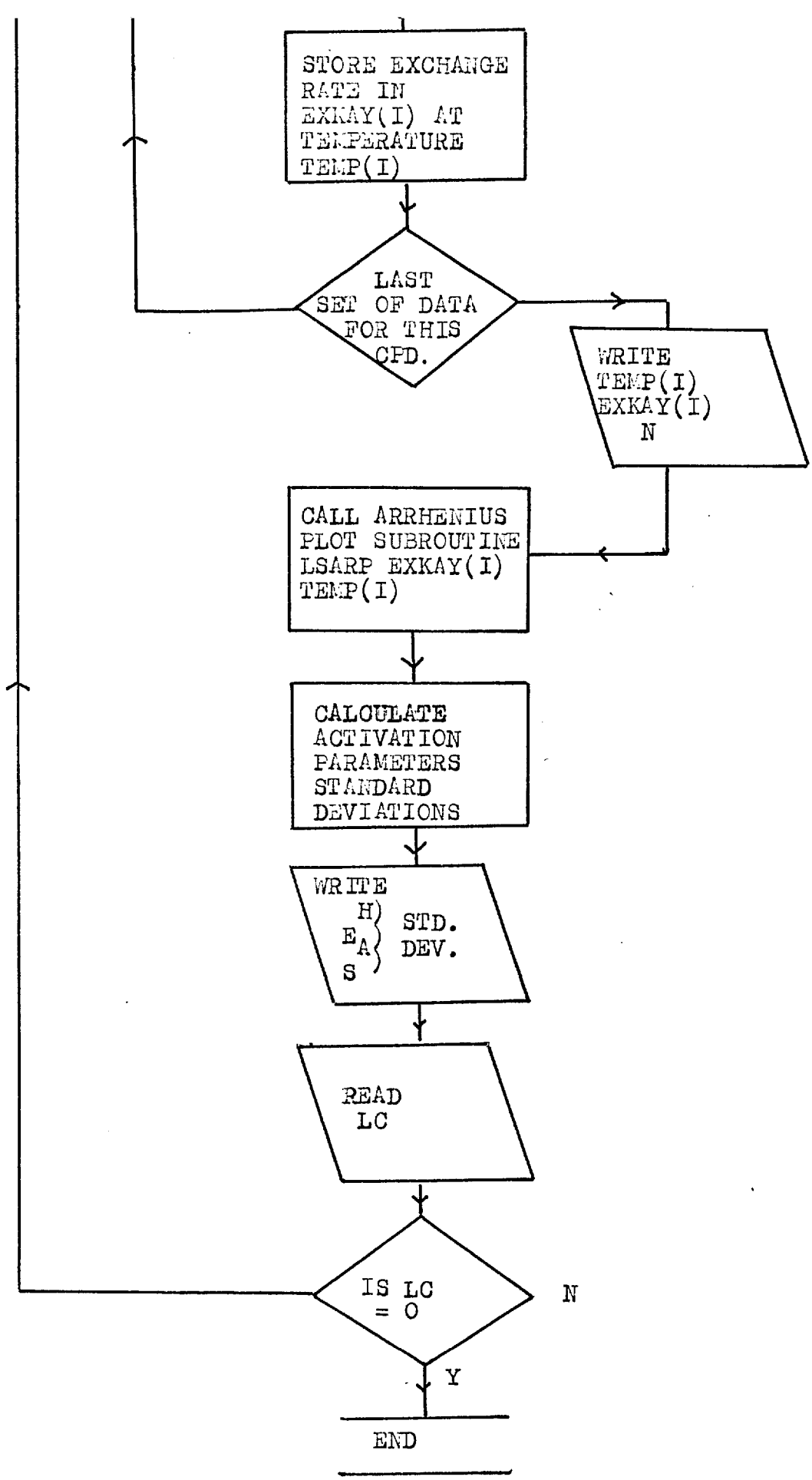
```
      SUBROUTINE ARRAY
      DIMENSION TEMP(100),SEPN(100),A(100),B(100),EXRAY(100),TITLE(14)
      COMMON TEMP,SEPN,A,B,EXRAY,TITLE,TAV,N,SEPMAX,IPEO
      DIMENSION RATIO(100)
      DIMENSION NRUN(50)
      PI=3.14159265359
      DO 29 I=1,N
      RATIO(I)=A(I)/B(I)
      NRUN(I)=I
      C=PI*SEPMAX*(SQRT(2.0))
      D=.5*SQRT(RATIO(I)+SQRT(RATIO(I)**2.0-RATIO(I)))
      EXRAY(I)=C*(1.0/D)
      TOT=(1.0/EXRAY(I))*SEPN(I)
      IF(TOT.GE.5.0)CALL ERROR
   29 CONTINUE
      WRITE(6,213)
  213 FORMAT(////10X,7HRESULTS)
      WRITE(6,214)
  214 FORMAT(////79H   SAMPLE    TEMPERATURE(*C)     SEPARATION(HZ)      A
     1      B    RATIO     K(SEC-1)    )
      WRITE(6,215)(NRUN(I),TEMP(I),SEPN(I),A(I),B(I),RATIO(I),EXRAY(I)
     1,I=1,N)
  215 FORMAT(//1X,I5,8X,F10.2,8X,F10.2,6X,F5.2,1X,F5.2,1X,F5.2,3X,F10.2)
      CALL ISAGP
      RETURN
      END
```

CHEM23



CHEM 21

/contd. .....


STORE EXCHANGE RATE IN EXKAY(I) AT TEMPERATURE TEMP(I)
LAST SET OF DATA FOR THIS CPD.
WRITE TEMP(I) EXKAY(I) N
CALL ARRHENIUS PLOT SUBROUTINE LSARP EXKAY(I) TEMP(I)
CALCULATE ACTIVATION PARAMETERS STANDARD DEVIATIONS
WRITE H E A S STD. DEV.
READ LC
IS LC = 0
N
Y
END

CHEM23

This program is a modified version of Chem21. Chem21 was written in Algol by Drs. Riddell and Williams of the University of Stirling. It was converted to Fortran by Mr. R.J. Smart. This program was then modified by D.P. Leworthy and renamed Chem23. Chem23 calculates the best fit theoretical n.m.r. spectrum for a magnetic group undergoing exchange between two non-equivalent sites in the absence of spin-spin interactions (AB exchange). It takes digitised spectra (either manually digitised or as the paper tape output of a computer of average transients) at a series of different temperatures and calculates the exchange rate ($^1/_{2\tau}$) at each temperature. These exchange rates are passed automatically to least squares Arrhenius Plot subroutine (LSARP) to yield the thermodynamic activation parameters with their standard deviations.

ORDERING OF INPUT DATA

Card 1 TITLE (80 alphanumeric characters). Up to 11 cards may be used for the title. A blank card must be placed after the last title card.

Next Card TAV - the temperature (^{o}C) at which activation parameters are to be calculated. Format F10.0.

Next Card NDAT - the number of sets of data to be input and used for Arrhenius plot calculation. Format I10

Next Card TEMP(L) - temperature at which particular set of measurements was made (^{o}C). Format F10.0

Next Card N- number of digitised points used for spectrum. Format I10

Next Card XSTART, INCX - XSTART is the starting value of X in Hz (note that the origin is taken as the centre of the spectrum to be input, XSTART is a negative number representing the lowfield extreme of measurement). INCX is the increment in X. Format 2F10.5, i.e. XSTART cols. 1 - 10 and INCX cols. 10 - 20.

Next Card NUM 1, NUM 2 - number of points at beginning and end of spectrum respectively, required for base-line calculation. Format 2I10.

Next Card J, B, BA - J is the separation of the peaks (Hz). B and BA are the line-widths at half peak height in the absence of exchange (Hz). Format 3F10.5

Next Card Y(I) - the intensities of the experimental spectrum, commencing at XSTART at intervals of INCX. Format 4 (F5.0, 1x)

If further sets of data are to be input put a card with 1 in col. 1 after last data card and repeat. Otherwise put a blank card after the last data card. This will stop the program.

```
      PROGRAM CHEM 23      03/1971


      DIMENSION TAU(21),SQUSUM(21)
      DIMENSION X(2100),YOBS(2,50),YTWO(2500),BX(100),BY(100)
      DIMENSION TITLE(18)
      DIMENSION TEMP(101),AK(100)
      COMMON SUBT,TEMP,AK,TAU
      DATA IBLNK/6H      /
      REAL MINIM
      REAL TMPX
      REAL J,K
      INTEGER SUM
      INTEGER N
       INTEGER TITLE
C
    1 FORMAT(I10)
    2 FORMAT (2F10.5)
    5 FORMAT(5(F5.2,1X) )
    6 FORMAT(2I10)
   11 FORMAT(3F10.5)
   17 FORMAT(/'TAU =',G11.4,6X,'SUM OF SQUARES =',G15.8,6X,'K =',G15.8)
   19 FORMAT(8F10.5)
   23 FORMAT( /  'NEXT STARTING VALUE OF TAU = ',G15.5)
   21 FORMAT(// ' VALUE OF TAU USED = 'G11.4 )
   25 FORMAT( /,' FINAL K = ' G15.8)
   11 FORMAT(1316,A2)
  101 FORMAT (F10.0)
  102 FORMAT(F10.0)
  201 FORMAT(10X,1316)
  221 FORMAT(I5)
C
```

```
      PROGRAM CHEM 23      OPL/1971


C
C  READ AND PRINT TITLE
   76 DO 38 I=1,11
      READ(5,100)TITLE
      WRITE(6,200)TITLE
      IF(TITLE(I).EQ.IBLNK) GO TO 40
   38 CONTINUE
   40 READ(5,102)TAV
      READ(5,1)NDAT
      DO 36 I=1,NDAT
      READ(5,102) TEMP(I)
      READ(5,1)N
      WRITE(6,204)N
  204 FORMAT(52HNUMBER OF DIGITISED POINTS USED FOR CALCULATION  =  ,I10
     ")
      NPTAPE = 5
      NMONE = N-1
      READ(5,2) XSTART,INCX
      X(1) = XSTART
      DO 3 I=2,N
      X(I) = X(I-1) + INCX
    3 CONTINUE
      READ(5,6) NUM1,NUM2
      READ(5,1)) J,B,BA
      READ(NPTAPE,5) (YONE(I),I=1,N)
C
C
      SUM = NUM1 + NUM2
      NMSUM = N-SUM
C
```

```
      DO 7 I=1,NUM1
      BX(I) = X(I)
      BY(I) = YONE(I)
    7 CONTINUE
      NUM1X = NUM1+1
C* ORDER OF INPUT
      DO 8 I= NUM1X,SUM
      BX(I) = X(NMSUM+I)
      BY(I) = YONE(NMSUM+I)
    8 CONTINUE
      CALL LSFIT(BX,BY,SUM,C,XM)
      DO 9 I=1,N
      YONE(I) = YONE(I) - YS(XM,X(I),C)
    9 CONTINUE
C
C
C
C
      EXAREA = AREA(YONE,N,IMCX)
C
      PI    = 3.14159265359
      B=B*PI
      BA = BA * PI
      PISQ = PI*PI
      CA = (B+BA) /2.0
      CB = B*BA + PISQ*.1*.1
      CC = 4.0 * PISQ
      CD = D/2.0
      CE = BA-B
      GS = 0.0
```

```
      GI = 1.0
      GF = 10.1
      P = 0
   22 M = 0
      H = 0
C
C ALGOL REAL DO LOOP
C
      G=GS
   11 CONTINUE
      M=M+1
      K=1
      TAU(M) = G
      IF(.NOT.TAU(M)._T.10.0E-09) GO TO 12
      DO 13 I=1,N
      YTWO(I) = 0.0
   13 CONTINUE
      GO TO 14
C
   12 CALL YMAIN(K,CA,CB,CC,CD,CE,X,YTWO,TAU(M),N)
      SL = (YTWO(N)-YTWO(1)) /(X(N)-X(1))
      CEPT = YTWO(1) - SL*X(1)
      DO 15 I=1,N
      YTWO(I) = YTWO(I) -YS(SL,X(I),CEPT)
   15 CONTINUE
      CAAREA = AREA(YTWO,N,INCX)
      K = EXAREA / CAAREA
      DO 16 I=1,N
      YTWO(I) = YTWO(I)*K
   16 CONTINUE
```

```
   14 SQUSUM(M) = SUMSQ (YONE,YTWO,N)
C END OF LOOP
C
      G = G+GI
      IF(G.LT.GF) GO TO 11
C
C
C
      GS = MINIM(TAU,SQUSUM,M)
      TMIN = GS+GI
      WRITE(6,20)GS
      IF(P.GE.6) WRITE(6,21) TMIN
      IF(P.GE.6) GO TO 50
       GO TO 51
   50 AK(L)= 1.0/(2.0*TMIN)
   51 GI=GI/10.0
      GF = 21.1 * GI + GS
      P=P+1
      IF(P.LT.7) GO TO 22
      K=1
      CALL YMAIN(K,C4,CB,CC,CD,CE,X,YTWO,TMIN,N)
      SL = (YTWO(N) - YTWO(1))/(X(N)-X(1) )
      CEPT = YTWO(1) -SL * X(1)
      DO 23 I=1,N
      YTWO(I) = YTWO(I) - YS(SL,X(I),CEPT)
   23 CONTINUE
      CAREA = AREA(YTWO,N,INCX)
      K = EXAREA / CAREA
      DO 24 I=1,N
      YTWO(I) = YTWO(I)*K
```

```
 24 CONTINUE
    WRITE(6,25) K
 36 CONTINUE
    WRITE(6,202)
202 FORMAT(////20X,16HTEMPERATURE(*C)  ,10X,8HK(SEC-1)  )
    WRITE(6,203)(TEMP(L),AK(L),L=1,NDAT)
203 FORMAT(16X,F10.5,13X,F10.5)
    CALL ISARP
 26 READ(5,211)IC
    IF(IC.NE.0)GO TO 35
    STOP
    END
```

```
      FUNCTION   EXMIN(A,N)
      DIMENSION A(N)
      REAL MIN
      DO 1  I=1,N
      IF(A(I).LT.MIN) MIN=A(I)
    1 CONTINUE
      EXMIN = MIN
      RETURN
       END


      FUNCTION WYMAX(A,N)
      DIMENSION A(N)
      REAL MAX
      MAX = 0.
      DO 1 I=1,N
      IF(A(I).GT.MAX) MAX=A(I)
    1 CONTINUE
      WYMAX = MAX
      RETURN
       END


      FUNCTION SUMS2(A,B,N)
```

```
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      DIMENSION A(N),B(N)
      AMS = 0.0
      DO 1 I=1,N
      AMS = AMS + (A(I)-B(I))*(A(I)-B(I))
    1 CONTINUE
      SUMSQ = AMS
      RETURN
       END



      FUNCTION AREA(A,N,STEP)
      DIMENSION A(N)
      SUM = 0.0
      DO 1 I=1,N
      SUM = SUM + A(I) + A(I)
    1 CONTINUE
      SUM = SUM - A(1) - A(N)
      AREA = SUM * STEP * 0.5
      RETURN
       END



      FUNCTION YS(M,X,C)
      REAL M
```

```
      YS = M*X + C
      RETURN
       END



      REAL FUNCTION MINIM(A,B,N)
      DIMENSION A(N),B(N)
      TINY  = 10.**30
      DO 1 I=1,N
      IF(B(I).GE.TINY) GO TO 1
      TINY = B(I)
      IF(I.EQ.1) CORRT = A(I)
      IF(I.NE.1) CORRT = A(I-1)
    1 CONTINUE
      MINIM = CORRT
      RETURN
      END
```

PROGRAM CHEM 23 SUBROUTINE LSLFIT

```
      SUBROUTINE LSLFIT(A,B,N,C,GRAD)
      DIMENSION A(N),B(N)
      TOT = 0.0
      SUM = 0.0
      DO 1 I=1,N
      SUM = SUM + A(I)
      TOT = TOT + B(I)
    1 CONTINUE
      XBAR = SUM/N
      YBAR = TOT/N
      SUM = 0.0
      TOT = 0.0
      DO 2 I=1,N
      SUM = SUM + (A(I)-XBAR) * (B(I)-YBAR)
      TOT = TOT + (A(I)-XBAR) *(A(I)-XBAR)
    2 CONTINUE
      GRAD = SUM/TOT
      C = YBAR - GRAD*XBAR
      RETURN
      END
```

PROGRAM CHEM 23 SUBROUTINE YMAIN

```
      SUBROUTINE YMAIN(K,CA,CB,CC,CD,CE,X,Y,T,N)
      REAL K
      DIMENSION X(N),Y(N)
      FA = 1.0 + T*CA
      FB = T*CB + CA
      FC = T*CC
      FD = 1.0 + 2.0*T*CA
      FE = T*CB*CE
      VA = FA*FB
      VB = FC*FE
      VC = FC*FD - FA*FC
      VD = FB*FB + CC*FE*FE
      VE = 2.0 * CC * FD * FE
      VF = CC *FD *FD - 2.0 *FB*FC
      VG = FC *FC
      DO 1 I=1,N
      XX1 = VA+VB*X(I) + VC*X(I)**2
      XX2 = VD+VE*X(I) + VF*X(I)**2 + VG*X(I)**4
      Y(I) = K*(XX1 / XX2)
    1 CONTINUE
      RETURN
      END
```

```
   PROGRAM CHEM 23   SUBROUTINE  LSARP



   SUBROUTINE LSARP
   DIMENSION T(100),AK(100),TR(20),GK(20),ALK(20)
   COMMON N,T,AK,TAV
   WRITE(6,8)TAV
 8 FORMAT(1HO,14HTEMPERATURE = ,F7.3,20H  DEGREES CENTIGRADE )
 3 ST = 0.0
   STS=0.
   SK=0.
   SKS=0.
   STK=0.
   DO 40 I=1,N
   TR(I)=1./(T(I)+273.16)
   GK(I) = ALOG(AK(I))
   ALK(I) = ALOG10(AK(I))
   ST=ST+TR(I)
   STS=STS+TR(I)*TR(I)
   SK=SK+GK(I)
   SKS=SKS+GK(I)*GK(I)
40 STK=STK+TR(I)*GK(I)
   BN=N
   DSTK=BN*STK-ST*SK
   DSTS=BN*STS-ST*ST
   B=DSTK/DSTS
   BT=B/2.303
   EA= -1.9865*B/1000.
   A=(SK-B*ST)/BN
   GA=A/2.303
48 VAR=(SKS-SK*SK/BN-B*DSTK/BN)/(BN-2.)
   IF(VAR)55,55,50
```

```
50 S=SQRT(VAR)/2.303
   SB=SQRT(VAR*BN/DSTS)/2.303
   SA=SQRT(VAR*STS/DSTS)/2.303
   SEA=(4.5749*SB)/1000.
   SDS=4.5749*SA
55 TAVA=TAV + 273.16
   DH = EA - 1.9865*TAVA/1000.
   DS = 1.9865*(1-ALOG(TAVA)) - 49.203
   WRITE(6,9)
 9 FORMAT(1H0,4H   T,11X,1HK,12X,3H1/T,9X,5HLOG K)
   WRITE(6,59)(T(I),AK(I),TR(I),ALK(I),I=1,N)
59 FORMAT(1H ,0PF7.2,5X,1PE9.3,5X,1PE9.3,5X,0PF7.4)
   WRITE(6,60)
60 FORMAT(1H0,52HACTIVATION PARAMETERS FROM LEAST SQUARES CALCULATION
  1)
   WRITE(6,62) EA, DH, DS
62 FORMAT(5H EA =,F8.4,10H DELTA H =,F8.4,10H DELTA S =,F8.4/)
   WRITE(6,64)
64 FORMAT (25H CONSTANTS OF LOG A + B/T)
   WRITE(6,66) GA, BT
66 FORMAT(8H LOG A =,F10.5,4H B =,F10.3/)
   IF(VAR)68,68,72
68 WRITE(6,69)
69 FORMAT(32H STD DEVS TOO SMALL TO CALCULATE//)
   GO TO 78
72 WRITE(6,73) SEA,SDS
73 FORMAT(18H STD DEV DELTA H =,F8.4,18H STD DEV DELTA S =,F8.4)
   WRITE(6,75) SA, SB
75 FORMAT(16H STD DEV LOG A =,F10.5,12H STD DEV B =,F10.3)
   WRITE(6,77) S
```

```
      PROGRAM CHEM 23   SUBROUTINE  LSAPP


77 FORMAT(16H STD DEV LOG K =,F10.5///)
78 RETURN
   END
```

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