^{19}F NMR Studies of the Interaction of α-Chymotrypsin with N-Trifluoroacetyl Amino Acids.

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Summary.

Several aromatic α-amino acids and their N-trifluoroacetates, as well as cinnamic acids and 3-carboxy-7-fluorodihydroisocarbostyril were synthesised. The interactions of these compounds as inhibitors with α-chymotrypsin were studied by ^{19}F NMR spectroscopy at 56.4 MHz. These studies allowed the dissociation constant of the enzyme-inhibitor complex, K_D, and the chemical shifts of the fluorine nuclei in the enzyme-inhibitor complex, Δ, to be calculated. These results were interpreted in terms of the orientation of the inhibitor in the active site of the enzyme, the model of the active site used being that determined previously by X-ray diffraction studies of the crystal enzyme. A halogen atom on the phenyl ring of N-trifluoroacetylphenylalanine greatly increased the binding affinity, the degree depending on its nature and position. The position of the halogen atom surprisingly influenced the position of the N-trifluoroacetyl group in the active site as evidenced by different values of the chemical shift of these ^{19}F nuclei for different enzyme-inhibitor complexes. The D and L isomers of a particular inhibitor were also shown to bind in different ways with respect to the N-trifluoroacetyl group.

K_D and Δ were also calculated with the inclusion of an inactive enzyme dimer model in these systems, the enzyme dimer dissociation constant being taken from the literature. Such an inclusion had the effect of greatly decreasing K_D but hardly affecting Δ.

Statement.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, no material previously published or written by another person, except where due reference is made in the text of the thesis.

B.C. NICHOLSON.

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INTRODUCTION

1. Applications of NMR Spectroscopy in the Study of Biological Problems.

(a) Historical and general.

Although the first reports of nuclear magnetic resonance (NMR) of hydrogen nuclei in solids were made in 1946,[1,2] it is only in the last few years that NMR spectroscopy has really developed into a powerful tool in the elucidation of problems of biological interest. However NMR spectroscopy was applied to biological systems as early as 1950 by Shaw and coworkers.[3,4,5] Materials such as proteins, carbohydrates, and vegetable tissues, were investigated, but all that was recorded was a fairly sharp water resonance superimposed on a broad envelope due to the rest of the protons. This allowed little more than the water content to be estimated. Odeblad et al.[6,7] observed the same phenomena in their studies of tissues[6] and human cervical mucus.[7] More recently it has been shown in studies of tissues that the relaxation rates of water protons in normal and malignant tumours are different, and may serve as a diagnostic tool.[8]

Wertz and Jardetzky[9-12] recorded the ^{23}Na NMR spectra of sodium ions in solution and drew conclusions regarding the extent of their binding with metabolites, and state of ionisation; ^{23}Na NMR has similarly been used recently to investigate sodium ion transport in tissues.[13,14,15]

Although few meaningful results had been obtained, interest in this new technique was such, that, by the end of 1957, two reviews on

the subject had appeared.[16,17] The reasons for this early lack of success with this technique, when applied to protons, are at once apparent. The extremely complex materials, studied in the solid state in some cases, and at low frequency would be expected to give broad resonances with a large water resonance perhaps predominating. Had fine structure been observed, the assignment of resonances of particular nuclei could not have been made because the basic principles had not been established. This necessitated the study of simple molecules, the building blocks of the complex biological molecules, and the elaboration of such concepts as chemical shifts and coupling constants. It can be said that, as these basic principles became apparent, then the study of biological molecules started to yield meaningful results. Progress in this field has now begun to parallel the development of commercial spectrometers of higher frequency which are capable of better resolution and greater sensitivity. Now that 100 MHz machines are readily available, the number of papers in the literature employing this technique in biological research is enormous; Jardetzky[18] estimates over 800 in his review of 1971. With the ready availability of 220 and 300 MHz instruments, the rapid rise of pulsed Fourier transform (PFT) spectroscopy,[19,20,21] especially ^{13}C PFT spectroscopy,[22] the number should increase even more rapidly.

The theory of NMR spectroscopy and its application to organic chemistry is well documented[23-27] and theory will not be discussed except where necessary. The basic theory is relevant to all facets of

chemistry, but the theory, pertinent to biological studies, has been discussed separately.[28-34]

(b) The spectra of amino acids, proteins, and nucleic acids.

The information necessary for the interpretation of the NMR spectra of proteins is, in part, embodied in the spectra of their building blocks, the amino acids. The first report of the spectra of amino acids appeared in 1957,[35] followed closely by the more detailed studies of the Jardetzkys[36] and Bovey and Tiers.[37] The following years saw many more papers appear on amino acids; these have been extensively reviewed by Roberts and Jardetzky[38] and Rowe et al.[39] Recently the 220 MHz spectra of the amino acids have been reported,[40] as well as their ^{13}C spectra.[41] Even with the spectra of amino acids known, the interpretation of protein spectra is still complex. Because of its three-dimensional structure, the protons of seemingly equivalent residues are usually not in magnetically equivalent environments and hence their resonances are not coincidental. The large number of overlapping resonances thus gives the spectrum of a protein the appearance of a broad envelope.

The first reported spectrum of a protein, the enzyme ribonuclease, appeared in 1957.[42] Very few assignments could be made and subsequent work by Kowalsky,[43] Mandel,[44] and others on proteins did not clarify the situation. The later work of McDonald and Phillips[45] at 220 MHz was more informative, but the amount of information available from protein spectra was much less than that desired. In some cases there are a small number of resonances to high or low field of the main resonance envelope which

may be very informative. Thus the study of the histidine residues of ribonuclease,[46,47] of which the resonances of some protons occur to lowfield of the main resonance envelope, has resulted in their assignment[46] and the proposal of a ribonuclease mechanism.[48] Cytochrome c has resonances to high and low field of the main resonance envelope[45,49] as do other heme proteins.[50-53] These can be monitored in conformational and other studies.

In some proteins, hydrogen bonding shifts the resonances of the protons involved to low field of the main resonance envelope.[54,55] The low field resonance in δ-chymotrypsin has been attributed to the hydrogen bonded proton between histidine-57 and aspartic acid-102 inferred from the crystallographic data.[55]

With nucleic acids, the complex structures once again lead to broad uninformative spectra.[56,57,58] In order to interpret these complex spectra, most work has been directed towards their nitrogenous bases, with evidence being found for base stacking and hydrogen bonding.[59-64] This work has been reviewed.[38,39,65]

It seems that should instruments operating at frequencies higher than 300 MHz become available, the direct observation of a protein spectrum may not be much more informative. ^{13}C NMR spectroscopy has been applied recently to ribonuclease[66] and lysozyme;[67] the preliminary results indicate that this technique may yield more information. The applications of NMR spectroscopy in the study of proteins has been extensively reviewed.[18,28-34,38,39,68,69,70]

In order to overcome the difficulties inherent in interpreting NMR spectra of proteins, several methods have been devised. Difference spectroscopy has yielded more information on ribonuclease,[71] but perhaps the best method is the study of selectively deuterated proteins. Deuterated proteins have been isolated from algae grown in D_2O; the residues containing hydrogen atoms can be regulated by the addition of ^1H amino acids to the culture medium. In this way phycocyanin[72] and staphylococcal nuclease[73,74] have been studied.

(c) Small molecule-macromolecule interactions.

(i) Spectral changes induced by small molecules.

In some cases the spectral changes in the macromolecule, induced by a small molecule (reporter molecule) exchanging between free solution and a macromolecule-bound state, can be informative. In this way the enzymes lysozyme, ribonuclease, carbonic anhydrase, and staphylococcal nuclease have been studied by the spectral changes induced by inhibitors. In lysozyme the interactions of an inhibitor were investigated by monitoring the change in the histidine[75] and aromatic[76,77] resonances. Similarly with ribonuclease the histidine resonances were studied in detail,[78,79,80] assigned to specific residues[46] and a mechanism for the action of the enzyme proposed.[48] With staphylococcal nuclease, the binding of inhibitors was studied by employing the selectively deuterated enzyme.[81] The techniques employed with carbonic anhydrase[82,83] were similar to those used with ribonuclease. Once again observation of the

macromolecule spectrum was feasible only when there were resonances to low field of the main resonance envelope or selectively deuterated proteins could be employed.

(ii) Spectral changes induced by ion probes and the use of ions as reporter ions.

As mentioned earlier, ^{23}Na NMR spectroscopy has been used to study sodium ion transport in biological systems.[13,14,15] ^{35}Cl NMR spectroscopy has also been used to determine the environment of chloride ions in several biological systems.[84-87] Those enzymes which contain a paramagnetic ion have been extensively studied by observing the influence of the ion on the relaxation rates of protons in the enzyme or substrate. This work has been recently reviewed by Mildvan and Cohn.[88] Kayne and Reuben[89] have used thallium-205 as a probe in the study of the pyruvate kinase reaction by monitoring the thallium-205 resonance.

(iii) Spectral changes of reporter molecules due to their association with macromolecules.

(α) Relaxation rate and line width studies.

The most fruitful application of NMR spectroscopy in the elucidation of biological problems has been the study of the reporter molecule exchanging between free solution and a macromolecule-bound state. Such applications have several advantages. The reporter molecule usually has a small number of resonances which can be uniquely assigned. In the presence of a macromolecule they will be discernible above the macromolecule

signal envelope if the exchange is rapid and the concentration of the reporter molecule is sufficiently high. Such criteria have usually been met in the systems so far studied. Rosenberg et al.[90] overcame the problem of protein resonances masking the reporter molecule resonances by employing a fully deuterated protein. In this way it was possible to study the interaction of the surfactant, sodium dodecyl sulphate, with deuterated phycocyanin.

Jardetzky and coworkers[91-94] studied antibiotic-protein interactions by monitoring changes in the line widths of the antibiotic protons on binding with proteins. In this way penicillin-serum albumin interactions[91] and others were shown to involve the aromatic ring of the antibiotic in hydrophobic binding. The nature of catecholamine-nucleotide complexes was also examined by NMR spectroscopy.[95] In these cases, the interactions would produce different relaxation rates in different parts of the molecules, depending on which parts were involved in the interaction. The increased relaxation rate of protons in the phenyl ring was evidenced by the selective broadening of their resonances. The effect of molecular interactions on relaxation rates and hence line widths and the applications to pharmacology have been reviewed by Jardetzky.[96,97]

Also studied by relaxation rate processes, and to a small extent chemical shift differences have been hapten-antibody interactions,[98,99] enzyme-coenzyme interactions,[100,101] binding of sulphoacetamides and sulphonamides to carbonic anhydrase,[102,103] binding of ribonuclease S

to ribonuclease S peptide,[104] binding of succinate to aspartate transcarbamylase,[105] the interactions of reporter molecules with nucleic acid helices,[106] and substrate binding to α-chymotrypsin,[107-111] carboxypeptidase A,[112] and alcohol dehydrogenases.[113,114] The interaction of nucleotides with ribonuclease has been studied using proton[115] and ^{31}P NMR spectroscopy.[116] Sykes and coworkers[117-122] have employed relaxation rate studies along with chemical shift differences to determine exchange rates of inhibitors with lysozyme,[117,118,119] aspartate transcarbamylase,[120] α-chymotrypsin,[121] and serum albumin.[122]

(β) Chemical shift differences.

In 1966, Thomas[123] reported that the chemical shift of the acetamido resonance of N-acetyl-D-glucosamine was a function of concentration in the presence of lysozyme, and that this was due to binding between the enzyme and sugar. Subsequent work investigated the binding process in detail using mainly chemical shift differences, but some relaxation effects.[124-130] Apart from this, and the ^{19}F studies of lysozyme and other enzymes to be discussed later, there appears to be little work to date in the biological field which is based primarily on chemical shift differences due to association. Perhaps the only other quantitative study has been the investigation of the binding of serotonin and tryptamine to nucleic acids.[131] Of course chemical shift differences have been used extensively in solute-solvent studies to determine binding constants,[132-137] and the treatment of data is essentially the same.

(γ) The information obtainable from line width, relaxation rate, and chemical shift data.

From the preceding two sections it is clear that line width and chemical shift studies have been carried out on a large number of systems. The parameters attainable are binding constants, chemical shifts of particular nuclei in the reporter molecule-macromolecule bound state and, in some cases, exchange rates. The binding constant and shift on binding for a particular system are obtained in the following way:

For the equilibrium

$$a + b \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} c \qquad K$$

where a is the reporter molecule, b the macromolecule, and c the complex, then the dissociation constant for the complex

$$K_D = 1/K = \frac{a.b}{c}$$

$$K_D = \frac{(a^o-c)(b^o-c)}{c} \qquad (1)$$

where a^o = initial concentration of a

b^o = initial concentration of b

If the exchange is rapid, then the observed chemical shift, δ, will be weighted average of that in free solution, δ_o, and that in the complex, Δ. Thus

$$\delta = P\Delta + \delta_o$$

where P is the mole fraction bound and Δ is relative to δ_o as 0. If δ

is also measured relative to $\delta_o = 0$, then

$$\delta = \frac{c}{a^o} \Delta$$

$$c = \frac{\delta}{\Delta} a^o$$

If this is substituted in (1) with the assumption that the term in c^2 is much less than the other terms and can be ignored, then

$$a^o = \frac{1}{\delta} (b^o \Delta) - (K_D + b^o) \qquad (2)$$

Thus Δ, the shift on binding and K_D, the dissociation constant of the complex, can be found from a knowledge of a^o, b^o, and δ. As Deranleau[138] points out, the accuracy of this method depends on which form of the expression relating a^o, b^o, δ, Δ, and K_D [of which (2) is one form] is treated graphically. Sykes[121] has overcome this by using a computer method, involving no approximations and based on the method of Groves et al.[139] for the treatment of data obtained in solvent-solute interactions studied by NMR spectroscopy. This will be discussed in more detail later.

The use of line width and relaxation rate data to determine parameters for the system is more complex since it is often difficult to distinguish between exchange broadening and that due to molecular association. Jardetzky[96] has discussed in detail the use of line width and relaxation rate data to determine association parameters. His assumption that the spin-lattice relaxation time, T_1, and the spin-spin relaxation time, T_2, are equal leads to the simple relationship,

$\Delta\nu_{1/2} = 1/\pi T_2$ where $\Delta\nu_{1/2}$ is the peak width at half height. The method of Sykes for the determination of the exchange rates is based on spin-lattice relaxation times in the rotating frame,[140,141] coupled with chemical shift differences.

(d) ^{19}F NMR Studies of Biological Systems.

In 1967, Spotswood et al.[142] first reported the use of ^{19}F NMR spectroscopy in the biological field. The interaction of fluorophenylalanines with α-chymotrypsin was studied and the binding constants and shifts on binding of the fluorine nuclei in N-acetyl-m- and p-fluorophenylalanine reported. In the above case, the preferential binding of the D isomer to the enzyme resulted in the fluorine resonance separating into two resonances, corresponding to the D and L enantiomers of the DL inhibitor used. A similar result was obtained by Zeffren and Reavill[143,144] with N-trifluoroacetylphenylalanine in the presence of α-chymotrypsin. Sykes[121,145] investigated the system in more detail and reported exchange rates. Trifluoroacetylated inhibitors and their ^{19}F chemical shifts have recently been used in studies of lysozyme[146,147,148] and further studies of α-chymotrypsin.[149-153] ^{19}F NMR spectroscopy has also been used in the study of the interaction of fluoride ions with the active site of carboxypeptidase A,[154] the interaction of 8,8,8-trifluorooctylbenzene-p-sulphonate ions with serum albumin,[155] the fluorokinase reaction,[156] micelle structure,[157] and the binding of trifluoroacetate ions to aspartate transaminase.[158]

Raftery and coworkers[148,159] have studied conformational changes in proteins using ^{19}F NMR spectroscopy. Selected residues were modified to include fluorine atoms and the spectral changes of these nuclei as a function of pH, and in the presence of inhibitors, measured. These were then interpreted in terms of conformational changes within the protein. In a similar manner, Bittner and Gerig[160] have modified α-chymotrypsin by reaction with the three trifluoromethyl-substituted α-bromoacetanilides (reaction presumably occurs at methionine-192). The resulting proteins were inactive with the introduced groups most likely near, or in, the active site. Relaxation rate measurements then gave the order of restricted molecular rotation of the trifluoromethyl groups in the three proteins.

Hunkapiller and Richards[161] have followed the reaction between pepsin and N-trifluoroacetyl amino acids and determined rate constants using ^{19}F NMR specroscopy.

The use of ^{19}F NMR spectroscopy has many advantages. There are no proton resonances of the macromolecules to interfere with the resonances under investigation. Thus the problem of the broad macromolecule spectrum envelope masking reporter molecule resonances is removed. The small size of the fluorine atom makes its incorporation into a protein ideal as such incorporations should not, then, drastically affect the molecular structure. This appears to be the case, as modified ribonuclease S was shown to be fully active,[148,159] and modified hemoglobin A had essentially the same properties as the unmodified protein.[148] It

is unfortunate that there are no naturally occurring fluorine containing macromolecules. Introduction of fluorine then produces a system which only approximates a natural system. However, the results so far, indicate that this is an excellent approximation. ^{19}F Chemical shifts are also spread over a much wider range[162,163,164] (150 p.p.m. as against 15 p.p.m. for protons) and this reduces the problem of assignment due to overlapping resonances. However the most important advantage is the sensitivity of ^{19}F chemical shifts to environment.[25] Small changes in the environment of a fluorine nucleus can have a significant effect on its chemical shift. Thus ^{19}F chemical shifts serve as a sensitive probe in the orientation of an inhibitor in the active site of an enzyme.

2. The Chemistry of α-Chymotrypsin.

(a) General.

The enzyme chymotrypsin A_α occurs in mammalian pancreatic juice as its inactive precursor (zymogen), chymotrypsinogen A. The zymogen is enzymatically activated in the duodenum by trypsin to give the various forms of chymotrypsin A (α, β, γ, δ, and π forms).[165,166,167] For simplicity the α, β, γ, δ, and π forms of chymotrypsin A will be referred to as α-chymotrypsin, β-chymotrypsin, and so on instead of chymotrypsin A_α chymotrypsin A_β. Also occurring in the pancreas are precursors of a number of other enzymes; trypsinogen, chymotrypsinogen B, and proelastase, which are also activated in the duodenum. Those enzymes which have been found to contain a reactive serine residue, important in the catalytic process have been called the serine proteinases,[168] this group includes the chymotrypsins, elastase, and thrombin. Other serine proteinases are found in other organisms and plants, and some, such as subtilisin BPN[1], are found in microorganisms.

In the main these are digestive enzymes; they hydrolyse peptide bonds in proteins with remarkable activity and specificity. α-Chymotrypsin hydrolyses the peptide bonds on the carboxyl side of residues with aromatic side chains, i.e. the carboxyl peptide bonds of phenylalanine, tyrosine, and tryptophan residues. Trypsin is specific for the carboxyl peptide bonds of basic amino acid residues, i.e., lysine and arginine residues, while elastase is specific for small, uncharged amino acid residues. Thus these three enzymes are complementary in

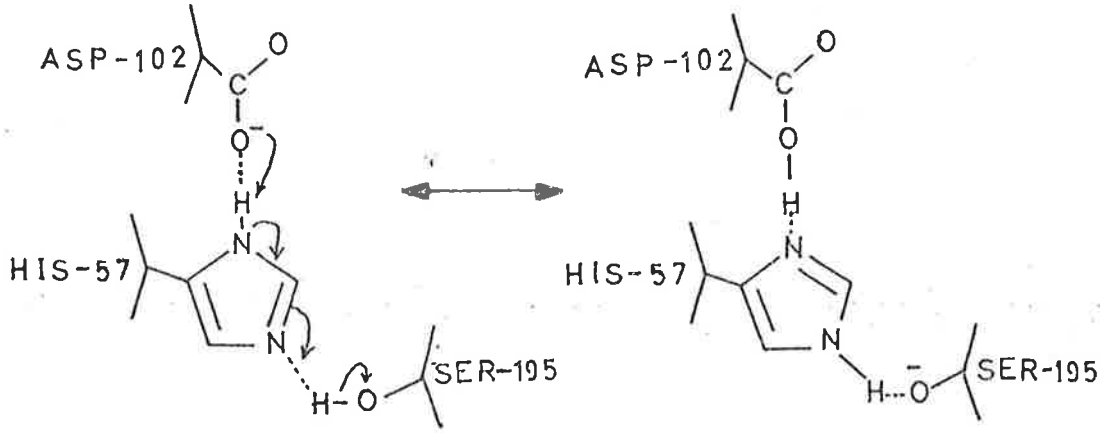
their specificities. Some serine proteinases have similar specificities, for example subtilisin BPN[1] and α-chymotrypsin. Some have similar amino acid sequences[169,170] and similar three-dimensional structures, especially in the region of the active site.[171,172,173] It would seem then, that the difference in specificities must be inherent in subtle differences in the residues around the active site. Such similarities have also invoked the idea of enzyme evolution.[174,175] The homologies of amino acid sequence and three-dimensional structure, as well as the subtle structural differences between the zymogen and free enzyme,[176] should be of great importance in the elucidation of the catalytic processes. In fact, a consideration of these processes is best carried out in terms of these homologies.

(b) Amino acid sequence and three-dimensional structure of α-chymotrypsin.

α-Chymotrypsin is a large molecule; it has 241 amino acid residues and a molecular weight of 23,000.[177] The primary structure, the amino acid sequence, was determined by Hartley[178,179] from studies of chymotrypsinogen, and apparently confirmed by Meloun et al.[180] However a correction has been made in which residue 102, originally designated asparagine, was found to be aspartic acid.[181] This was of great importance as the catalytic activity of the enzyme could then be explained as due, in part, to the increased nucleophilicity of serine-195 brought about by the charge relay system (Fig. 1) which was invoked; the residues involved being in bonding distance (from the X-ray diffraction data).

Fig. 1.

Charge relay system, pH8.[181]



canonical forms

α-Chymotrypsin is one of a number of enzymes of which the three-dimensional structure has been determined by X-ray diffraction;[182-185] these include elastase,[171,186,187] trypsin,[188] two subtilisins,[172,173,189-192] and γ-chymotrypsin.[193,194] The structure of chymotrypsinogen has also been determined.[176] The three-dimensional structure of α-chymotrypsin was determined as its tosyl derivative by Blow and co-workers[195-199] using X-ray diffraction techniques. In this derivative the catalytically important residue, serine-195, had been tosylated. The benzene ring of the tosyl group was found to occupy a cleft in the molecule which has been called the "tosyl hole". The "tosyl hole" has also been shown to be the site of the indole ring of N-formyl-L-tryptophan in the X-ray diffraction studies of the N-formyl-L-tryptophan:

α-chymotrypsin complex.[200] These studies also showed the probable existence of a hydrogen bond between the amido hydrogen of N-formyl-L-tryptophan and the carbonyl oxygen of serine-214 in the enzyme.[199,200] The "tosyl hole" was also occupied by the indole ring of indoleacryloyl-α-chymotrypsin[201] and the aromatic rings of other inhibitors in enzyme-inhibitor complexes.[200] N-Formyl-L-tryptophan can be considered a virtual substrate by analogy with N-acetyl-L-tryptophan which forms an acyl-enzyme as evidenced by ^{18}O exchange between the carboxyl group and the enzyme.[202,203] From the X-ray diffraction data it is difficult to say whether the N-formyl-L-tryptophan:α-chymotrypsin complex is an acyl-enzyme; the coordinates of the carboxyl group of the virtual substrate are not sufficiently well defined to determine whether a bond exists between it and serine-195. Indoleacryloyl-α-chymotrypsin is, however, a stable acyl-enzyme which has been characterised.[204,205] The crystalline acyl-enzyme, when dissolved, was shown to be the same as the acyl-enzyme which can be prepared in solution. Also determined have been the structures of α-chymotrypsin in which histidine-57 had been methylated[206] and carbamyl-α-chymotrypsin.[207] Quite recently, Birktoft and Blow,[208] by refinement of the X-ray diffraction data, have determined the structure of α-chymotrypsin in much more detail.

The homologies of amino acid sequence and crystal structure of α-chymotrypsin, trypsin, and elastase make it possible to explain their different specificities, by reference to the nature of the binding pocket in each.[174,188] The hydrophobic binding pocket ("tosyl hole") of α-

chymotrypsin is large enough to accommodate an aromatic ring. In trypsin, the carboxyl side chain of aspartic acid-189 is at the bottom of the cleft. Under the conditions where trypsin is active, this will be negatively charged and hence capable of bonding the ammonium ion of the substrate side chain. In elastase, the residue at the mouth of the pocket is valine-216. The steric size of its side chain will exclude all but the smallest side chain. This is represented in Fig. 2 (after Shotton[174]).

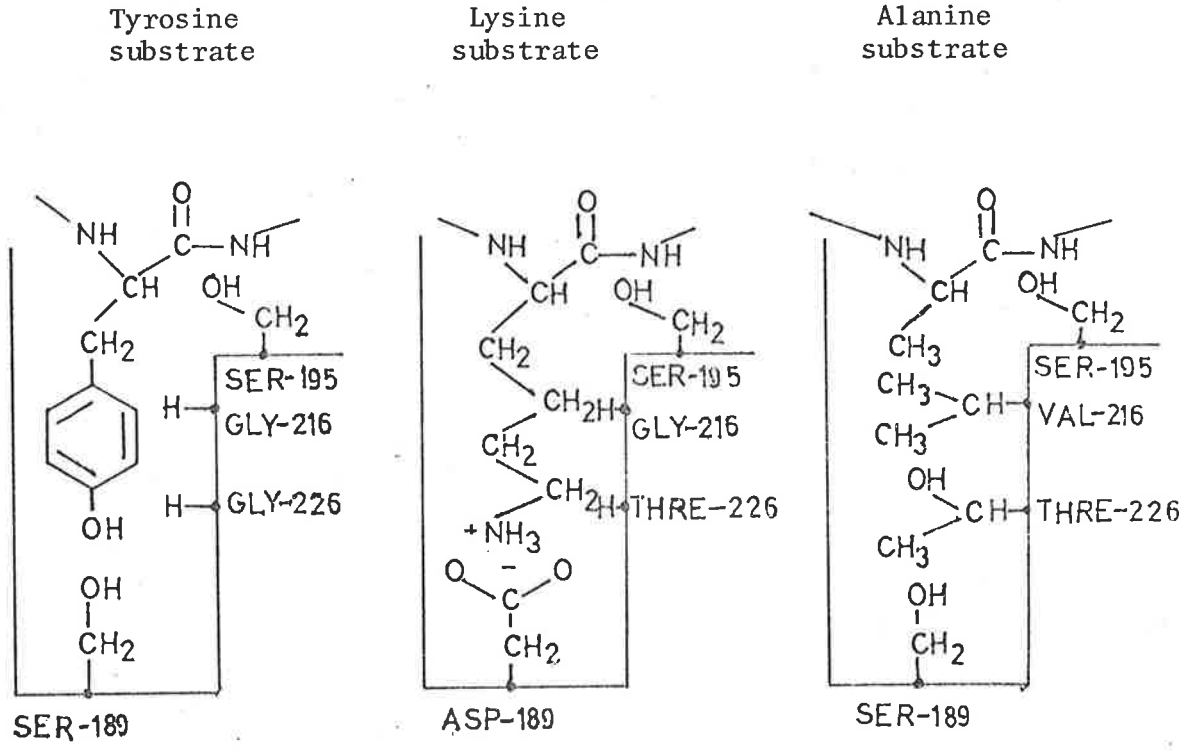


Fig. 2.

The preceding structures involving α-chymotrypsin were all determined at pH~4. Vandlen and Tulinsky[209] have reported X-ray diffraction studies of α-chymotrypsin at pH 3.9 and pH 6.7.

The enzyme-small molecule complexes only approximate the complexes which must occur in the hydrolysis of large peptide substrates *in vivo*. In these cases secondary binding sites probably become important.[210] To this end, Huber et al.[211,212] have determined the structure of a naturally occurring peptide, bovine pancreatic trypsin inhibitor, which inhibits both trypsin and α-chymotrypsin. From a knowledge of this structure and that of α-chymotrypsin, a model for the association between the inhibitor and enzyme has been proposed.[213]

(c) The relevance of crystallographic data to the mechanism of α-chymotrypsin catalysed reactions in solution.

The large amount of information on the primary, secondary, and tertiary structure of α-chymotrypsin is of a comparatively recent nature. Workers have been trying for over thirty years to elucidate the mechanism of α-chymotrypsin catalysed reactions, and determine the amino acid residues important in the catalytic process using physico-chemical means. The information gained from crystallographic studies, especially that from the enzyme-virtual substrate, enzyme-inhibitor, and acyl-enzyme complexes, is complementary to the physico-chemical results if the enzyme has the same structure in solution during reaction as it has in the solid state. Kallos[214] reported low activity for reactions catalysed by solid α-chymotrypsin, but more recently Sluytermann and de Graaf,[215] and Rossi

and Bernhard[216] have concluded that the solid enzyme has comparable activity to that in solution. Comparable activity would most certainly suggest comparable geometry of the active site. The X-ray diffraction studies also indicated only small differences in the position of residues around the active site between the native and inhibited enzymes.[200,201] There seems little doubt then, that the structures in the solid state and in solution are similar, and that the "tosyl hole" is the aromatic binding site.

However as mentioned earlier, these results apply to the enzyme at pH∿4 whereas the catalytic activity is greatest around pH 7.5.[217] Vandlen and Tulinsky[209] have reported changes in crystal structure between pH 3.9 and pH 6.7 comparable with the changes which occur on binding, but neither details nor the magnitude of these changes were given. Shiao and Sturtevant[218,219] have concluded from their calorimetric studies that binding of an inhibitor induces conformational changes in the enzyme. This conclusion has been reached by other workers employing different techniques.[220-223] Since binding of inhibitors most certainly occurs at the active site, these conformational changes must involve the active site. This, however, is difficult to reconcile with the small changes which occur on binding, as evidenced from the crystallographic data.

Evidence also exists for the occurrence of two conformations of α-chymotrypsin in solution with the inactive form predominating at high pH.[222,224-228] The X-ray diffraction results of Vandlen and Tulinsky[209] support this change of conformation with pH.

A problem which is not solved by X-ray diffraction is that of association of the enzyme in solution. There is much evidence for the existence of dimers and higher oligomers in solution.[218,229-235] Different workers have reached different conclusions regarding the properties of these oligomers. Faller and LaFond[233,234] concluded that only the monomer was active and could bind an inhibitor. Martin and Niemann[236] concluded that the dimer was capable of binding substrate, but such complexes could not lead to product. The conclusion of Inagami and Sturtevant[237] was that the oligomers were partly active while Sarfare et al.[238] concluded that oligomerisation did not affect access to the active site, i.e., the dimer contains two active sites of normal activity, the trimer three active sites, and so on. Kézdy and Bender[235] concluded that the dimer was inactive but readily activated in the presence of substrate. These points will be further mentioned in the Discussion.

(d) The activity and specificity of α-chymotrypsin towards organic molecules.

α-Chymotrypsin in solution hydrolyses, with marked activity and specificity, the esters and amides of the acyl derivatives of the naturally occurring aromatic amino acids. These can be considered chemical substrates as distinct from the more complex natural or physiological substrates, the proteins. Attempts to elucidate the mechanism of α-chymotrypsin controlled catalysis have, for the most part, involved the study of these chemical substrates. As such, any proposed mechanism

really only applies to chemical substrates. Being smaller and simpler molecules than the natural substrates, they provide information only about the catalytic site. With the more complex peptide substrates, secondary binding sites (subsites) probably become important in binding the peptide to the enzyme surface, with the bond to be cleaved at the active site.[210,239,240] Morihara et al.[241,242] have shown the existence of subsites in α-chymotrypsin and subtilisin BPN[1]. Subsites have also been shown to be important in the cleavage of peptides by papain,[243] pepsin,[210,244] carboxypeptidase A,[245] and elastase.[246,247] Nevertheless the seemingly ideal situation of using simple chemical substrates, instead of the larger peptides or proteins, still leads to complex results.

The reactivity and specificity of chemical substrates towards α-chymotrypsin has been extensively reviewed,[165,168,248-255] but the salient features will be discussed. As mentioned earlier, the enzyme hydrolyses the esters and amides of the acyl derivatives of the naturally occurring amino acids with high activity. The L isomers are rapidly hydrolysed, while the D antipodes are virtually unreactive. The specificity is relative, not absolute; the D antipodes are hydrolysed at rates of the order of 10^4 slower than their L counterparts. Also the esters are hydrolysed more rapidly than the amides. The aromatic ring and the amido hydrogen are necessary for this high reactivity and specificity. Replacement of either of these groups leads to greatly reduced reactivity with rather unpredictable specificity. This has been summarised in Tables I and II (due to Cohen[248]).

TABLE I.

Hydrolysis of methyl β-substituted α-acetamidopropionates R $CH_2CHCO_2CH_3$ (NHCOCH$_3$) by α-chymotrypsin.

β-substituent, R	k_{cat}/K_M ($M^{-1}sec^{-1}$)	Reference
CH_3-, L	∿ 2	256
C_6H_5-, L	6.2×10^4	257

TABLE II.

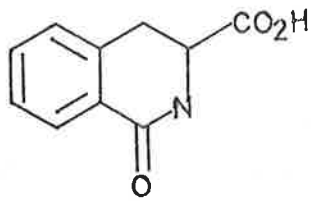
Hydrolysis of methyl α-substituted β-phenylpropionates $C_6H_5CH_2CHCO_2CH_3$ (R) by α-chymotrypsin

α-substituent, R	k_{cat}/K_M ($M^{-1}sec^{-1}$)	Reference
-$NHCOCH_3$, L	6.2×10^4	257
-H -	15[a]	258
-$OCOCH_3$, L	26[b]	259
-CH_2CO_2R, L	8[a,c]	258
-CH_3, L	3[a,c]	258
-OH, L	∿ 1x 10^3 [b]	259
-OH, D	11[b]	259
-Cl, D,L	35[d]	260

a 10% ethanol.
b 20% ethanol.
c hydrolysis of L enantiomer of DL mixture.
d 20% methanol.

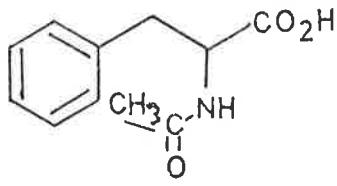
A similar table describing the effect of the β-substituent on the rate of reaction has been compiled by Knowles.[255] The constants k_{cat} and K_M are defined and their meaning discussed in section II (f).

Of particular interest are the esters of 3-carboxydihydroisocarbostyril (3-carboxy-1-keto-1,2,3,4-tetrahydroisoquinoline) (I) of



(I)

which the D antipodes are hydrolysed at a rate comparable with the esters of N-acetyl-L-phenylalanine.[261,262] This surprising reversal of specificity has led to much work being done with this and similar compounds which can be designated substrates of restricted conformational freedom. A comparison of the structure of (I) with that of N-acetylphenylalanine (II) shows the similarity of structure, with structure (I) having much less conformational freedom and hence a restricted reactive conformation. The similar reactivity, but different specificity, may then enable a study of the behaviour of (I) to assist in the elucidation of the reactive



(II)

conformation of (II). In order to define the conformation of (I) more precisely, Abdullaev et al.[263] have measured the isoquinoline ring coupling constants in order to determine the dihedral angles, and hence conformation, in non-polar solvents (analogous to an hydrophobic active site). Cohen et al.[248,258] and Silver et al.[264] have examined the hydrolyses of other cyclic substrates in the presence of α-chymotrypsin in order to elucidate the catalytic mechanism. Cohen and coworkers[248,258] have interpreted these results in terms of an active site model with five loci. Niemann and coworkers[252,262,265] have explained the specificity of α-chymotrypsin in terms of microscopic binding sites. These loci and microscopic binding sites have yet to be identified with the functional groups and residues important in the catalytic as determined by other means.

The activity of α-chymotrypsin towards substrates is inhibited

by a large number of compounds. A number of these react with the enzyme in a 1:1 ratio to give a stable, inactive protein,[266,267,268] for example the dialkyl fluorophosphates,[269] sulphonyl halides,[270] sulphonic acid sulphones,[271] α-bromoacetanilides,[272,273] chloromethyl ketones,[274] and carbamyl chlorides.[275] These are irreversible inhibitors. Apart from these, the remainder of inhibitors fall into the category of competitive, non-competitive, or uncompetitive;[276] by far the largest number falling into the category of competitive inhibitor. These bind at the active site in competition with the substrate, i.e., they compete effectively with the substrate for the active site. Since the enzyme-inhibitor complex is non-productive; it does not lead to products, the reaction between enzyme and substrate is slowed. The most important are derivatives of the N-acyl aromatic amino acids[277-280] and a large number of aromatic compounds.[281-284]

(e) The nature of the functional groups and residues important in the catalytic process.

Evidence so far has shown the functional groups of five residues to be important in the catalytic process;[254,285] this evidence also correlates well with the stereochemistry and nature of residues around the "tosyl hole" as determined by X-ray diffraction.

α-Chymotrypsin reacts with dialkyl fluorophosphates in a 1:1 ratio. The product of the reaction, the DFP-chymotrypsin, is an enzymatically inactive protein.[266-269] Degradation of this protein yields a phosphorylated serine peptide.[286] Comparison of the sequence of this

peptide with the known sequence of the enzyme leads to the result that this is serine-195. Kinetic experiments implied the existence of a catalytically important residue of which the side chain had a pKa ∿ 7. This was thought to be the imidazole ring of an histidine residue. Reaction of the enzyme with the chloro- and bromomethylketones derived from N-tosyl-L-phenylalanine produced an inactive protein. Degradation yielded a peptide in which one histidine had reacted with the ketones.[274,286] This was found to be histidine-57. The fact that acetylation of the free amino group, formed in the activation step, results in the destruction of enzymatic activity makes this residue important in the catalytic process. This had also been inferred from kinetic studies - a group with pKa ∿ 9 was found to be important and could only be a free amino group.[287,288] This is isoleucine-16. X-ray diffraction studies indicate that the α-ammonium ion of the residue forms a salt bridge with the carboxylate ion of aspartic acid-194, thus forming an hydrophobic binding pocket for aromatic side chains.[289] Deprotonation of this amino group above pH 9 would thus lead to the destruction of the salt bridge and hence the hydrophobic binding site. Thus the high pH, inactive form[224] of the enzyme could be produced.[225] Modification of a methionine residue (shown to be methionine-192) was found to affect the enzyme-substrate affinity but the enzyme was still appreciably active.[285] It appears, then, that this residue may not be critically important. The crystal structure of the enzyme indicates that modification of this residue may hinder access to the active site, as it is at the entrance. The importance

of a proton donor in α-chymotrypsin catalysis has been proposed.[292,293] The recent amendment to the amino acid sequence and a consideration of the three-dimensional structure of the enzyme makes this proton donor most likely aspartic acid-102. The X-ray diffraction results show that aspartic acid-102 is in the correct orientation to aid acylation of serine-195 by increasing its nucleophilicity (the charge relay system[181] Fig. 1).

Weiner and Koshland[294] have tested the hypothesis that, in the chemical modification of the serine residue, loss of activity may be the result of the bulkier modified group preventing access to the active site (as is most certainly the case in the modification of methionine-192). To this end, they have removed the elements of water from the serine residue and found the resulting enzyme to have appreciable binding activity. This result has recently been reported by other workers.[295] As access could not have been restricted relative to the native enzyme, the hydroxymethyl group cannot be critically important in binding of inhibitors.

From these results, it is clear that the enzymatic activity results from folding of the three protein chains which brings the catalytically important residues, serine-195, histidine-57, and aspartic acid-102 into the correct orientation. Serine-195 is an important catalytic residue, but may not be critical for binding of inhibitors. The conformation of the aromatic binding site and hence the active site, is controlled by the isoleucine-16-aspartic acid-194 salt bridge. Methionine-192 is at

the entrance to the active site.

(f) A description of α-chymotrypsin catalysed reactions and proposed mechanisms.

A good representation of the reaction of substrates with α-chymotrypsin is the double displacement reaction,[223,296,297,298] (Scheme I) where E represents enzyme, S the substrate, ES' the enzyme-substrate

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES' \qquad K_S$$

$$ES' \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} ES'' + P'$$

$$ES'' \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} E + P''$$

Scheme I.

complex, ES'' the acyl-enzyme intermediate, P' the leaving group (an alcohol or amine depending on whether the substrate was an ester or amide), and P'' the carboxylic acid. Such a description is, however, not a mechanism. A mechanism, by definition, applies on an atomic level and is a description of all atomic parameters over the whole course of the reaction. This description is important, however, as thorough kinetic evidence serves as the basis for reaction mechanisms. In this case, which is rare in mechanistic studies, the physico-chemical evidence can

be directly compared with information on one of the reactants (the enzyme) gained by X-ray diffraction studies.

In the case of competitive inhibition by N-acyl amino acids, the enzyme-inhibitor complex probably approximates an enzyme-substrate complex or, in some cases an acyl-enzyme intermediate, for example, inhibition by virtual substrates.[202,203] The major binding interactions involve the aromatic ring and the amido group, and it seems logical that D and L isomers of a particular inhibitor bind in a similar way at the active site with regard to these substituents.

In α-chymotrypsin controlled catalysis of esters, deacylation of the acyl-enzyme intermediate is the rate-determining step.[299,300,301] This may not be true for natural substrates; in fact in the hydrolysis of amides, acylation of the enzyme is the rate-determining step.[302,305] With esters, then, the acyl-enzyme is a very important intermediate which is reasonably stable - in some cases it can be isolated. Thus enzyme-inhibitor studies, even those involving inhibitors of the unnatural (D) configuration, should be of great benefit in elucidating the first step of the mechanism.

The double displacement reaction can be considered in a simpler form (Scheme II).

$$E + S \underset{}{\overset{K_M}{\rightleftarrows}} ES \xrightarrow{k_{cat}} E + P$$

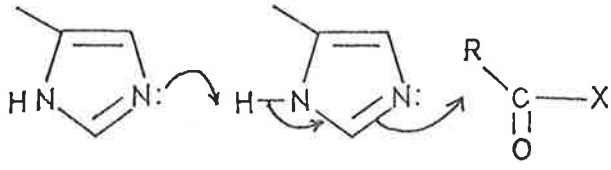
Scheme II.

In this case, the measured Michaelis-Menten constant is an apparent

constant so that K_M (app) = K_M ($\frac{k_3}{k_2 + k_3}$) and $k_{cat} = \frac{k_2}{(1 + k_2/k_3)}$, [297] where k_{cat} is the overall rate constant. This is the definition of the parameters used in section II(d) to show the relationship between structure and reactivity of substrates. k_{cat}/K_M is measure of the efficiency of the hydrolysis.

Recent work has shown that, in the hydrolysis of amides, there exist other eintermediates before the acyl-enzyme intermediate.[222,301, 306-309] A similar situation has been shown in the hydrolysis of N-acetyl-N-methyltyrosine methyl ester.[310]

From the wealth of data available there seems little doubt that the catalytic reaction involves acylation of serine-195. Early evidence which purported to show acylation of imidazole[311,312] can be repudiated. The involvement of the imidazole ring of histidine-57 has been postulated to occur in several ways. In conjunction with the imidazole ring of the neighbouring histidine-56, a concerted acylation of histidine-57 was proposed[312] (Scheme III).



Scheme III.

Bender and Kézdy[313] proposed the involvement of the two histidine residue imidazole rings as charge transfer complexes. These two postulates can be ruled out however. It has also been suggested that activation of serine-195 involves Δ^2-oxazoline formation.[314] This, and other mechanisms involving heterocyclic active sites,[315] can also be dismissed. The occurrence of oxazolinone intermediates has, however, been postulated.[316]

As mentioned earlier, the X-ray diffraction studies explain the high nucleophilicity of serine-195 (the charge relay system). The use of this charge relay system then overcomes a weakness of the pre-transition state protonation theory in the mechanism of Wang and Parker.[317,318,319] Nevertheless, the mechanistic proposals are still fairly complex, especially in the case of amides, and reference is made to the individual papers.[302,306,307,313,316,320]

RESULTS AND DISCUSSION.

Objectives.

From the preceding introduction it can be seen that, although a vast amount of work has been carried out in attempting to elucidate the mechanism of α-chymotrypsin catalysis and much has been achieved, there are still many questions to be answered. NMR spectroscopy has been shown to be a powerful tool in the elucidation of problems of biological interest; especially in regard to small molecule-macromolecule interactions of which enzyme-inhibitor interactions are one example. Quantitation of chemical shifts and line widths of inhibitors binding to enzymes, then, can potentially give the environment of the nuclei under investigation and hence the orientation of the inhibitor in the active site. Thus enzyme inhibition can be studied by NMR spectroscopy and, if the inhibition can be shown to resemble the first stage in the catalytic mechanism of the hydrolysis of substrates, then information on this mechanism can be gathered.

^{19}F NMR spectroscopy has also been shown to be of use in the elucidation of these problems if fluorine atoms can be introduced as probes without serious disruptions. Moreover the use of ^{19}F NMR spectroscopy removes the nuisance of the background "envelope" of the protein resonances and, in some cases, the solvent resonances.

The aim, then, was to synthesise various fluorinated inhibitors of α-chymotrypsin, for example N-trifluoroacetylated phenylalanine, tryptophan and their derivatives as well as several cinnamic acids, and to study their interaction with the enzyme by monitoring the ^{19}F reso-

nances. From these studies it was hoped to be able to formulate a model for the enzyme-inhibitor complex formation.

1. Synthetic Work.

(a) Preparation of inhibitors - acetates and trifluoroacetates.

Phenylalanine was most conveniently acetylated with acetic anhydride in dilute acetic acid according to the method of Town.[322] The reaction carried out over 30 minutes at 40°, gave comparable yields of products to those reported in the acetylation reactions under basic conditions at 0°.[323] However with tryptophan, this reaction was unsatisfactory as the product was often coloured and of a low purity. Acetylation of tryptophan under weakly alkaline conditions at 0° according to du Vigneaud and Sealock[324] gave excellent yields of the N-acetyltryptophans. The results are summarised in Table (III).

TABLE III.

Compound	Method of Preparation	Yield (%)
N-acetylphenylalanine	Ref. 322	74
N-acetyl-D- "	Ref. 322	72
N-acetyl-L- "	Ref. 322	68
N-acetyltryptophan	Ref. 322	81
N-acetyl-D- "	Ref. 324	70
N-acetyl-L- "	Ref. 324	73

Trifluoroacetylation of phenylalanine and its derivatives was best achieved using trifluoroacetic anhydride in dry benzene.[325,326,327] The reactions were rapid; the yields in most cases were 70-80% and

purity was excellent as evidenced by the fact that further recrystallisation did not raise the melting points more than two degress, if at all. With tryptophan, the acid instability of the indole ring was again encountered and this method was unsatisfactory. The method of Weygand and Geiger[328] employing trifluoroacetic anhydride in trifluoroacetic acid/ether, at 0^{o}, was also unsatisfactory. However the method of Schallenberg and Calvin,[329] employing ethyl thioltrifluoroacetate as the acylating agent gave excellent yields of the desired compounds when the reaction time was lengthened from 24 to 72 hours. The results are summarised in Table IV. With tyrosine, it was found that a twofold excess of trifluoroacetic anhydride was necessary to obtain good yields when the reaction was carried out in benzene. Using one equivalent of trifluoroacetic anhydride, or trifluoroacetic anhydride in trifluoroacetic acid/ether according to the literature,[328] the yields were low. With the two equivalents of acylating agent, no evidence was found for O-acylation. Recently Weyand and Rüpsch[330,331] have reported the conversion of most amino acids to their N-trifluoroacetates in good yields using phenyl trifluoroacetate.

An attempt was made to prepare N-trifluoroacetyl-D-p-methoxyphenylalanine by the silver oxide catalysed methylation of N-trifluoroacetyl-D-tyrosine with methyl iodide in acetone. Under the reaction conditions, the trifluoroacetate moiety was retained, but the product was the methyl ester. This is not surprising when compared to the work of Mehta,[332] who has employed methyl iodide in the preparation of methyl esters of a number of carboxylic acids.

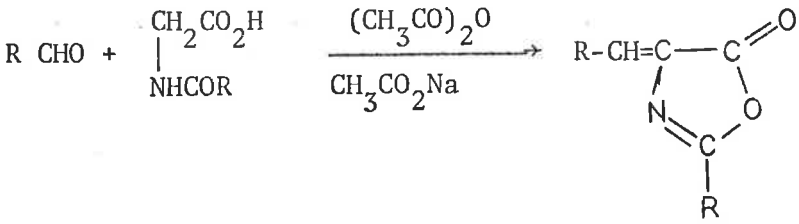
TABLE IV.

N-trifluoroacetate of	Method of preparation	Yield (%)	m.p.
phenylalanine	Ref. 327	74	123-125°
phenylalanine	Ref. 329	76	126-128°
D-phenylalanine	Ref. 327	68	120-121°
L-phenylalanine	Ref. 327	72	119-120°
m-methylphenylalanine	Ref. 327	71	116-117°
p-methylphenylalanine	Ref. 327	76	134.5-136°
o-fluorophenylalanine	Ref. 327	68	132-133°
m-fluorophenylalanine	Ref. 327	73	119-120°
p-fluorophenylalanine	Ref. 327	73	144.5-145.5°
m-bromophenylalanine	Ref. 327	88	135-137°
p-bromophenylalanine	Ref. 327	85	160-162°
2,4-difluorophenylalanine	Ref. 327	67	131-133°
m-trifluoromethylphenylalanine	Ref. 327	82	143-145°
p-trifluoromethylphenylalanine	Ref. 327	76	142-144°
phenylglycine	Ref. 327	67	159-160°
tryptophan	Ref. 329	75	155-157°
D-tryptophan	Ref. 329	73	163-164°
L-trptophan	Ref. 329	66	163-164°

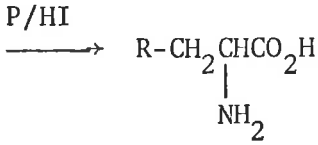
(b) Preparation of substituted phenylalanines.

(i) The azlactone method.

The fact that benzaldehyde condenses with hippuric acid in the presence of sodium acetate and acetic anhydride to give 4-benzylidene-2-phenyl-5-oxazolone (I) was reported by Plöchl[333] in 1883. Erlenmeyer[334] subsequently discovered that the oxazolone (azlactone) could be reduced to give phenylalanine (Scheme I).

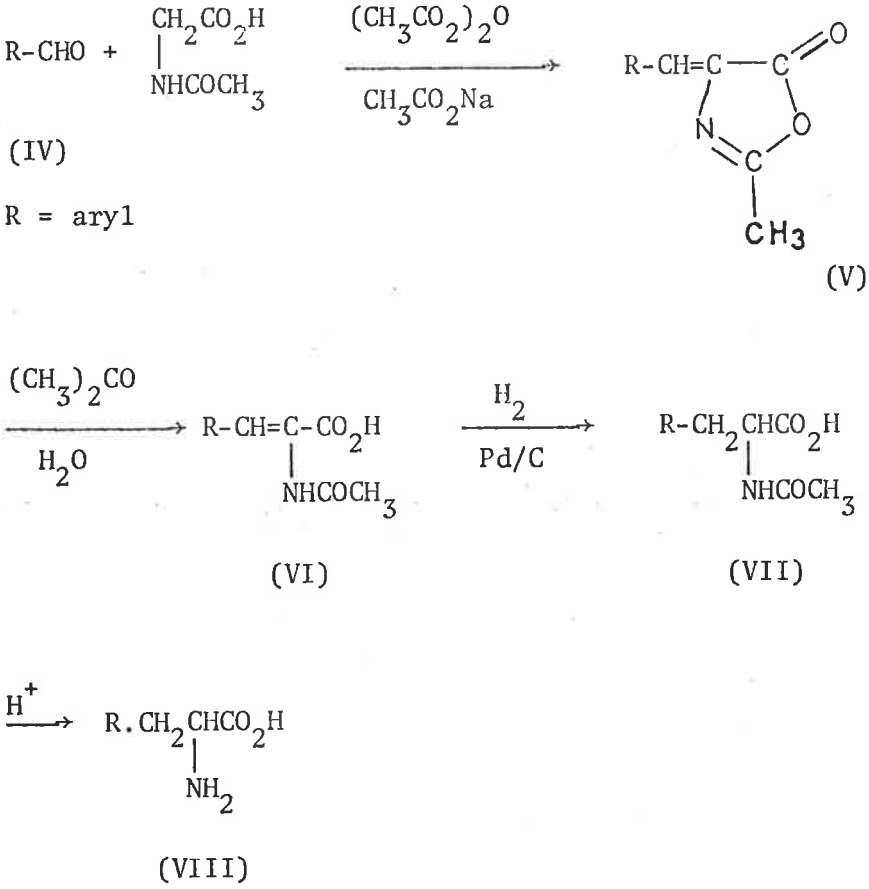


R = C_6H_5- (III)



Scheme I.

The reaction also occurs between benzaldehyde, aceturic acid (acetylglycine), sodium acetate, and acetic anhydride to give 4-benzylidene-2-methyl-5-oxazolone,[335,336] which is best converted to the amino acid in 3 steps; namely hydrolysis to the α-acetamidocinnamic acid with aqueous acetone, hydrogenation to give the N-acetyl amino acid, and finally acid hydrolysis (Scheme II).



Scheme II.

This procedure was adopted initially as the aldehydes were available, or easily prepared, and some α-acetamidocinnamic acids and acetyl amino acids were required. As the stability of the 2-methyl-5-oxazolones is considered less than that of the 2-phenyl analogues,[337,338] this probably accounts for the lower yields obtained in the first step of the reaction (of the order of 50%). Attempts to improve the yields of azlactones were unsuccessful and purification was difficult as they had to be freed of water and unreacted aldehyde. The IR spectra also indicated that hydrolysis to the α-acetamidocinnamic acid had occurred to some extent.

As this was formed in the next step of the reaction sequence, its removal was not necessary. The next step proceeded readily with the crude azlactone and the high melting point of the product formed made purification by recrystallisation simple. This, and subsequent steps, proceeded in excellent yields. The methylphenyl and m-trifluoromethylphenylalanines, as well as α-acetamidocinnamic and α-acetamido-β-(p-fluorophenyl)-acrylic acid were prepared by this method. The results are summarised in Table V.

TABLE V.

Yields (%) of compounds formed in azlactone synthesis (Scheme II)

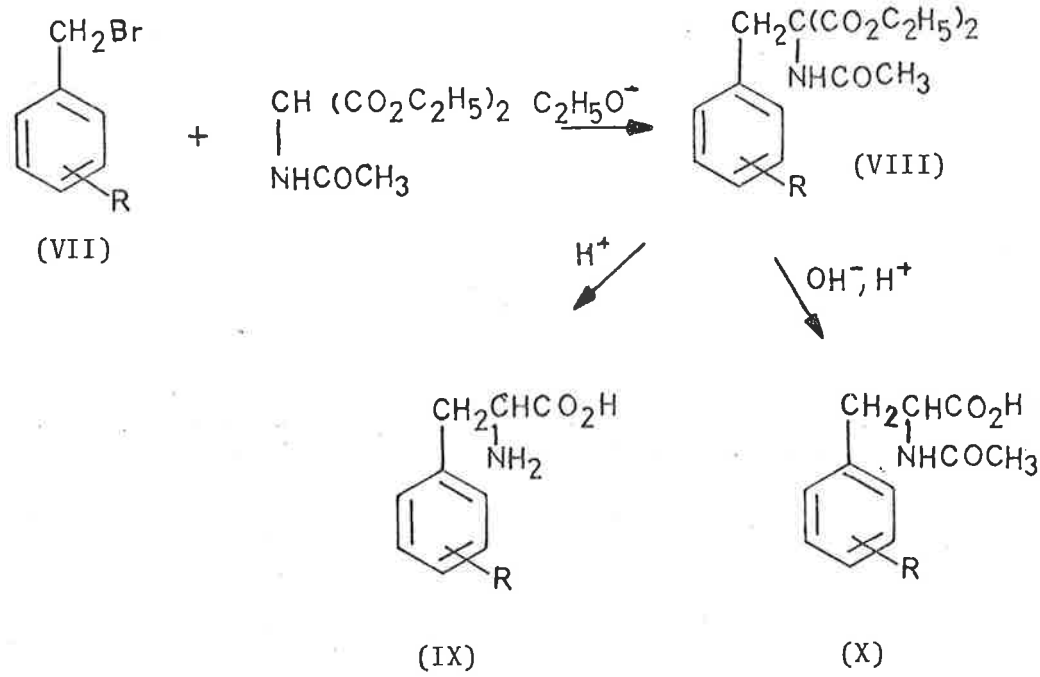
Aldehyde II	Azlactone III	Acetamido-cinnamic acid IV	Acetyl-phenylalanine V	Phenyl-alanine VI
Benzaldehyde	52	82	-	-
m-tolualdehyde	50	85	77	91
p-tolualdehyde	54	77	81	98
m-trifluoromethyl-benzaldehyde	67	76	86	53
p-fluorobenzal-dehyde	56	81	-	-

(ii) The malonic ester method.

In 1945 it was simultaneously reported by Albertson and Archer,[339] and Snyder et al.[340] that benzyl chloride reacted with diethyl acetamido-

malonate in the presence of sodium ethoxide and that the product of the reaction underwent acid hydrolysis to give phenylalanine. As both starting materials were readily available and the yield in each step was excellent, this constituted probably the simplest and best method for preparing phenylalanine. It has been shown by Bennett and Niemann[341] that this method is superior to the azlactone method for the synthesis of fluorophenylalanines. These workers have also shown that in the preparation of substituted phenylalanines by the azlactone method, transacylation occurs in the step leading to the oxazolone.[342] This lowers the yield of the desired oxazolone still further and makes the malonic ester synthesis more attractive. The majority of amino acids used in this work were prepared by this method which is outlined in Scheme III.

Of these, only p-bromophenylalanine had been prepared previously by this method but no details were given.[343] p-Trifluoromethylphenylalanine had previously been prepared by the azlactone method,[344] but this present method produced better yields in a smaller number of steps. The synthesis of p-trifluoromethylphenylalanine is represented in Scheme IV. Conversion of (XI) to (XII) and (XIII) to (XIV) both involve strongly acidic conditions. Under these conditions, no hydrolysis of the trifluoromethyl group to a carboxyl group was detected. This agrees with the results of Filler and Novar[344] who prepared m-trifluoromethylbenzyl bromide from m-trifluorobenzyl alcohol under acidic conditions. However several workers have reported hydrolysis of this group with

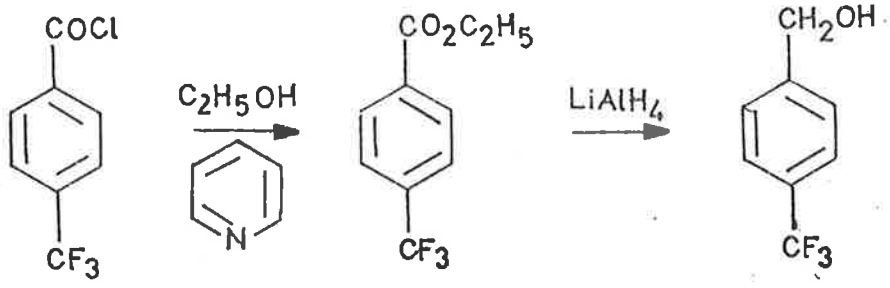


Scheme III.

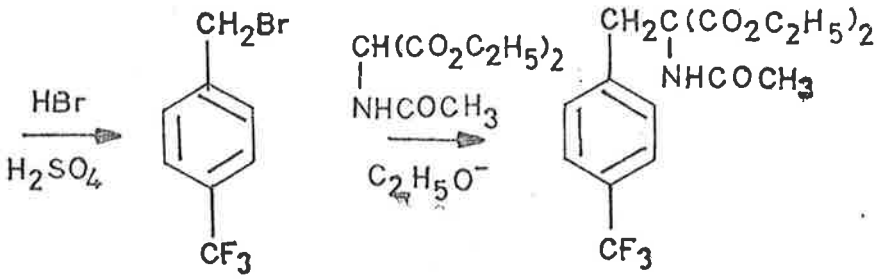
concentrated sulphuric acid[345,346,347] and concentrated hydrobromic acid,[348,349] but it seems more forcing conditions are required than those used here.

The results of Scheme III are summarised in Table VI.

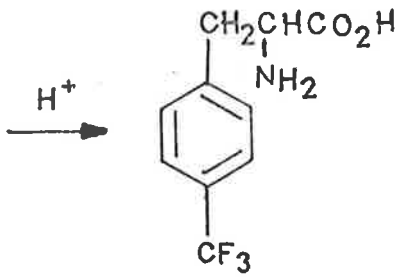
Phenylalanines have also been widely synthesised using cyano-acetic ester instead of diethyl acetamidomalonate[350] and other less general methods which have been recorded by Greenstein and Winitz.[351] More recently Kidwai and Devasia[352] have reported the synthesis of phenylalanine using 2,4-disubstituted-2-imidazolin-5-ones, the nitrogen equivalent of the oxazolone, but it is doubtful whether there are any



(XI)



(XII) (XIII)



(XIV)

Scheme IV.

TABLE VI.

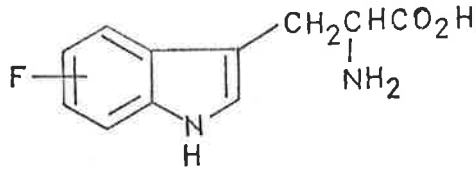
R (VII)	Yield (%) of the diethyl α-acetamido-α-benzylmalonate (VIII)	Yield (%) of the N-acetylphenylalanine (X)	Yield (%) of the phenylalanine (IX)
o-fluoro-	74	-	58
2,4-difluoro-	74	65	76
m-bromo-	79	-	64
p-bromo-	75	-	100
p-trifluoromethyl-	64	-	60

advantages over other syntheses. Yamada et al.[353] have reported α-amination of carboxylic acids via the α-lithio derivative but this, too, does not seem to have any advantages in the synthesis of phenylalanines.

(c) The preparation of fluorotryptophans.

(i) General and methods of preparation of tryptophan.

The synthesis of tryptophan derivatives, in which a fluorine atom was substituted in the aromatic ring (XV) presented an interesting challenge. The approaches involve introduction of fluorine into tryptophan itself or a precursor which can be readily converted into a fluorotryptophan. Tryptophan can be synthesised from indole-3-aldehyde by an azlactone method[354,355] (similar to Scheme I for the preparation of phenylalanines). Diethyl acetamidomalonate reacts with gramine

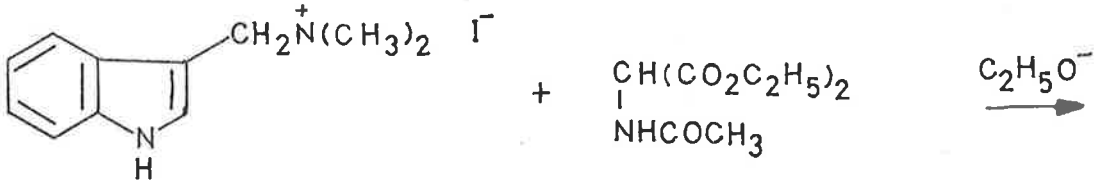


(XV)

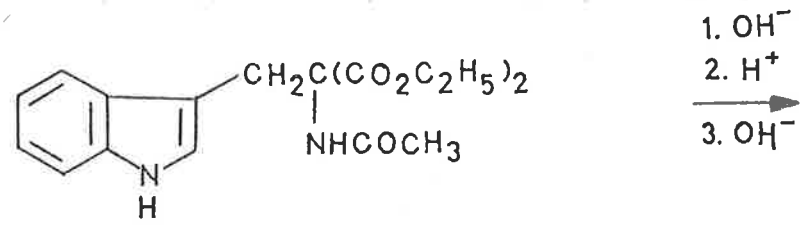
methiodide (XVI)[356] (or ethiodide[357]) in the presence of sodium ethoxide to give a product (XVII) which can be readily converted to tryptophan (XVIII) (Scheme V). Gramine methiodide is readily prepared from gramine, and both indole-3-aldehyde and gramine are readily prepared from indole. Thus the synthesis of fluorotryptophans could also involve introduction of fluorine into gramine, indole-3-aldehyde, or indole on synthesis using a fluoroindole.

A method for the preparation of tryptophan, which does not require the preformed indole ring, is that reported by Moe and Warner.[358,359] The phenylhydrazone of the condensation product between acrolein and diethyl acetamidomalonate also gives (XVII) (Scheme VI).

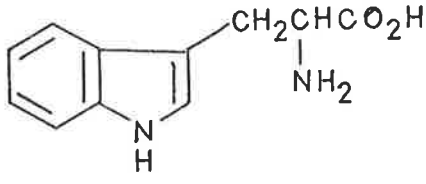
Thus the p-fluorophenylhydrazone of (XIX) should give 5-fluorotryptophan, the m-fluorophenylhydrazone should give a mixture of the 4- and 6-isomers, while the o-fluorophenylhydrazone should give 7-fluorotryptophan. Such an approach had been reported by Rinderknecht and



(XV)

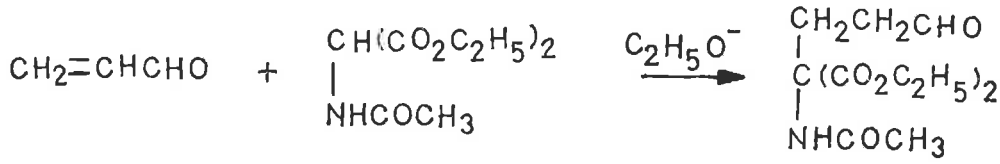


(XVII)

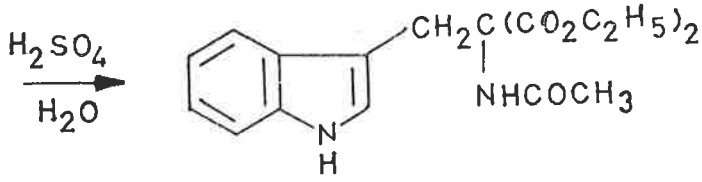


(XVII)

Scheme V.



(XIX)



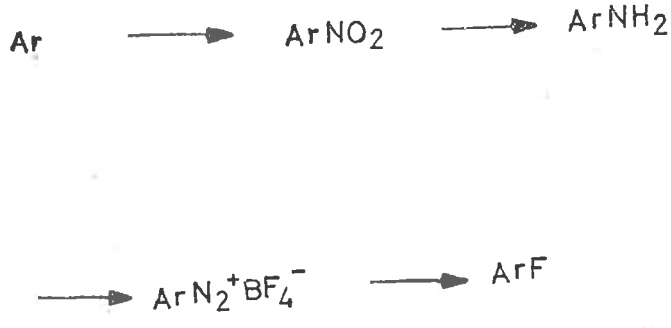
(XVII)

Scheme VI.

Niemann[360] for the synthesis of 5-fluorotryptophan and was utilised in this work. As a mixture of isomers most probably would be formed in the cyclisation of the m-phenylhydrazone of (XIX), this method was deemed unsatisfactory for the preparation of 4- and 6-fluorotryptophan, and was not investigated as a method for the preparation of these compounds. The approaches which were investigated were those involving introduction of fluorine into tryptophan, indole-3-aldehyde, gramine or indole, and the synthesis of fluoroindoles.

(ii) Approaches involving introduction of fluorine into tryptophan, indole-3-aldehyde, or gramine.

The usual method for synthesis of an aromatic fluoro compound is nitration, reduction of the nitro group to an amino group, conversion of this group to a diazonium fluoborate, followed by thermal decomposition (Scheme VII).



Scheme VII.

Conversion of (XXIII) to (XXIV) is the Schiemann reaction.[361,362,363] This, and other methods for the introduction of fluorine into organic compounds, have been reviewed.[364,365] Methods other than the Schiemann reaction are not feasible in any of the approaches towards the synthesis of fluorotryptophans.

The nitration of tryptophan has been reported, the nitro group entering the 6-position.[366] The reported yield was, however, low. Reduction of this nitro group to an amino group should proceed in good yield; reduction of the 2-3 bond of the indole ring would probably not be a competing reaction as the reduction of this bond in indole itself requires fairly forcing conditions.[367] However conversion of the amino group to the diazonium fluoborate would necessitate protection of the α-amino group. It has also been shown that diazonium fluoborates containing carboxyl groups are formed in low yield and their subsequent decompositions proceed in poor yield.[362] The yield of both steps could possibly be increased by esterification of the carboxyl group. Overall, the predicted low yields of some steps if they occurred at all, coupled with the protection and deprotection reactions, made this approach unattractive for the synthesis of 6-fluorotryptophan and it was not carried out experimentally.

Nitration of indoles, substituted in the 2- and 3-positions proceeds readily in good yield,[368,369,370] while substitution in the 3-position only leads to a mixture of isomers,[366,369-373] the yield of each depending on the nitrating agent. Indole-3-aldehyde can be

nitrated to give the 5- or 6-isomer, depending on reaction conditions.[372,373] 5-Nitroindole-3-aldehyde would lead to 5-fluorotryptophan which can be more conveniently synthesised by a different route. 6-Nitroindole-3-aldehyde (which would lead to 6-fluorotryptophan) is unfortunately formed in low yield.[373] Similar results are obtained for gramine. Thus syntheses of fluorotryptophans involving gramine or indole-3-aldehyde as starting materials were also unattractive and not carried out experimentally.

(iii) Approaches involving cyclisation of phenylhydrazones - the synthesis of 5-fluorotryptophan.

The synthesis of 5-fluorotryptophan according to Scheme VI was the method employed in this work. p-Fluorophenylhydrazine was readily prepared in high yield from p-fluoroaniline by sodium sulphite reduction of the diazonium salt.[374] The use of stannous chloride as reported by Suschitzky[375] was cumbersome. No product was obtained, however, when an attempt was made to reduce the diazonium salt of o-fluoroaniline (a logical precursor of 7-fluorotryptophan) with sodium sulphite. This agrees with the result of Suschitzky[375] who found sodium bisulphite ineffective, and Bullock and Hand,[376] who found that different reducing agents had different reactivities. Suverov et al.[377] have reported improved yields in the cyclisation of phenylhydrazones of the type (XXII) by employing sulphosalicylic acid instead of dilute sulphuric acid but this modification was not attempted.

Cyclisation of phenylhydrazones is a method of great versatility

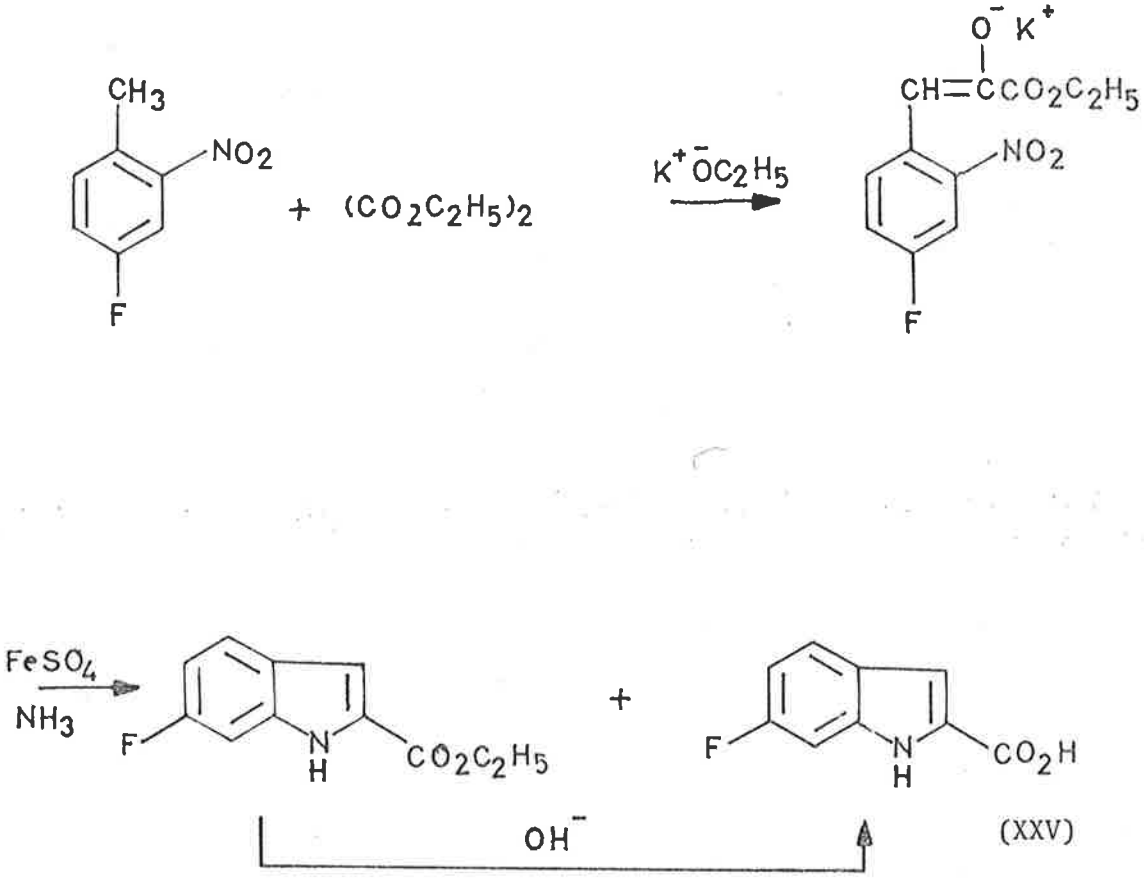
in the synthesis of indoles.[378,379,380] These methods require the phenylhydrazone to have a structure such that an indole, substituted in the 2- or 3-position, is formed. Without this requirement, the reaction fails. Ethyl pyruvate phenylhydrazones can undergo this Fischer indole-type cyclisation to give ethyl indole-2-carboxylate, which can be saponified and decarboxylated to give indole. Indole is readily converted to gramine methiodide from which tryptophan is best synthesised (Scheme V). Once again, the p-substituted phenylhydrazone would lead to a 5-substituted indole, an o-substituted phenylhydrazone would lead to an indole substituted in the 7-position while a mixture of the 4- and 6-isomers would be formed from the meta-substituted phenylhydrazone. This method would thus be satisfactory for the synthesis of 5- and 7-fluorotryptophan, but these two compounds would most certainly be more conveniently prepared as already discussed (Scheme VI).

(iv) Other syntheses of fluoroindoles.

As mentioned earlier, indole is readily converted to tryptophan via gramine. One method of synthesis of indole, the cyclisation of ethyl pyruvate phenylhydrazone and subsequent reactions, has also been discussed. Although this is not the method of choice in the synthesis of 5- and 7-fluorotryptophan, it has been used for the synthesis of 5- and 7-fluoroindole.[381] 6-Fluoroindole and 6-fluorotryptophan had been prepared by a reaction sequence involving oxidation of 4-fluoro-2-nitrotoluene to 4-fluoro-2-nitrobenzaldehyde.[382,383] The yield of this step was reported to be low by either of the methods employed for the oxidation.

6-Fluoroindole had also been prepared from indoline.[384] Nitration of indoline gave the 6-nitro derivative[385] which could be smoothly converted to 6-fluoroindoline essentially as described in Scheme VII. Dehydrogenation was reported to give 6-fluoroindole in good yield.[385] Indole itself could not be used, as the products of nitration were reported to be tars and polymeric materials when the reaction was carried out under acidic conditions.[367] Nitration using benzoyl nitrate was reported to give 3-nitroindole[386] and not nitration in the benzene ring. Reaction of potassamide with halogenoindoles was reported to give the amino indoles in good yields,[387] a mixture of isomers indicated an aryne-type mechanism. It would be interesting to see whether these amino indoles would undergo the Schiemann reaction. An attempt was made to synthesise 6-fluoroindole from indoline but the near explosive decomposition of the diazonium fluoborate led to its abandonment.

Attention was then directed towards the Reissert synthesis,[388] an approach which had been used previously for the synthesis of 6-fluoroindole[381] (Scheme VIII). Conversion of (XXV) to (XXVI) by thermal decarboxylation proceeded in a yield of 52%. A later method employing copper chromite in quinoline[389] as the decarboxylating agent failed in this case. The failure of this reaction has, however, been reported to be due to impurities in the system.[390] Nevertheless, the yield of indole from the thermal decarboxylation was sufficiently high to make this procedure worthwhile. Others steps proceeded smoothly. Another procedure reported for the preparation of 6-fluoroindole involved 1,4-

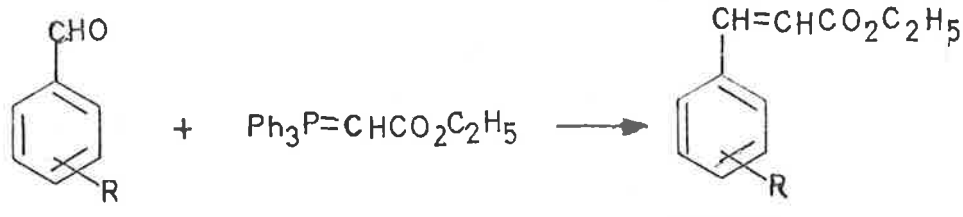

CH3
NO2
F
+ (CO2C2H5)2
K+ ⁻OC2H5
O⁻ K+
CH=CCO2C2H5
NO2
F
FeSO4
NH3
F
N
H
CO2C2H5
+
F
N
H
CO2H
OH⁻
(XXV)
Δ
F
N
H
(XXVI)

Scheme VIII.

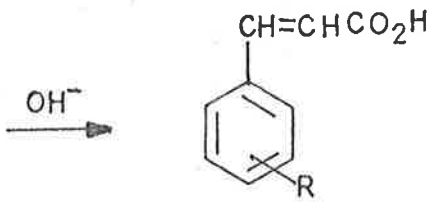
difluoro-2-nitrobenzene as starting material, but as the reported yields were very low,[391] it was not investigated. The preparation of 4-fluoro-indole from 6-fluoro-2-nitrobenzaldehyde had been reported,[392] but was not investigated.

(d) Preparation of cinnamic acids.

For the purpose of this work, it was desirable to synthesise fluorinated cinnamic acids. o-Fluorocinnamic acid had been prepared as early as 1885 by the reaction of diazotised o-aminocinnamic acid with hydrofluoric acid.[398] This reaction was utilised by Kindler[394,395] for the preparation of m- and p-fluorocinnamic acids but no yields were reported. p-Fluorocinnamic acid had also been prepared by the Perkin reaction[396] and from a Schiemann reaction of ethyl p-aminocinnamate.[397] o-Fluorocinnamic acid had been prepared by the Knoevenagel reaction[398] while the method employed for m-trifluoromethylcinnamic acid had been the Doebner modification of the Knoevenagel reaction.[399] The method employed in the literature for the preparation of 2,4-difluorocinnamic acid was the Perkin reaction.[400] The long reaction times and low yields in some cases made these approaches unsuitable for the synthesis of a series of cinnamic acids. It was reported that the Wittig reaction of benzaldehyde with carbomethoxymethylidenetriphenylphosphorane gave a quantitative yield of ethyl cinnamate.[401] As saponification of the ester should give the corresponding acid in good yield, and each step is easily carried out, this method was chosen (Scheme IX).



(XXVII)



(XXVIII)

Scheme IX.

The results are summarised in Table V.

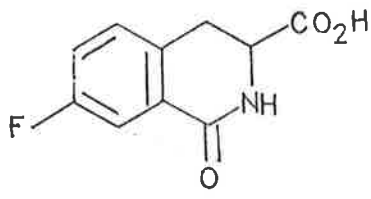
TABLE V.

R	Yield (%) of (XXVII)	Yield (%) of (XXVIII).
p-methyl	87	73
o-fluoro	86	83
m-fluoro	84	80
p-fluoro	83	82
2,4-difluoro	82	81
m-trifluoromethyl	86	74

The preparation of α-fluorocinnamic acid was also investigated. This acid had been prepared in 2% yield by the Perkin reaction[402] and in fair yield by the reaction of ethyl fluoroacetate and benzaldehyde in the presence of sodium hydride.[403] The ethyl ester of this acid had also been prepared in poor yield by the Reformatsky reaction of benzaldehyde and ethyl bromofluoroacetate,[404] and in good yield by the Claisen reaction of benzaldehyde, ethyl oxalate, and ethyl fluoroacetate.[405] This latter method was chosen. The ester was prepared in 65% yield and the acid in 75% yield.

p-Fluorophenylpropionic acid was prepared from p-fluorocinnamic acid by catalytic hydrogenation. This appears to be superior to the reported method[406] involving a variation of the Willergodt reaction.

(e) The synthesis of 3-carboxy-7-fluorodihydroisocarbostyril.

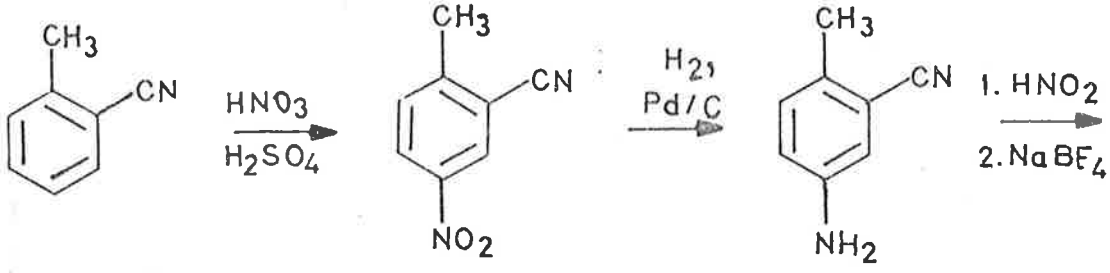


(XXIX)

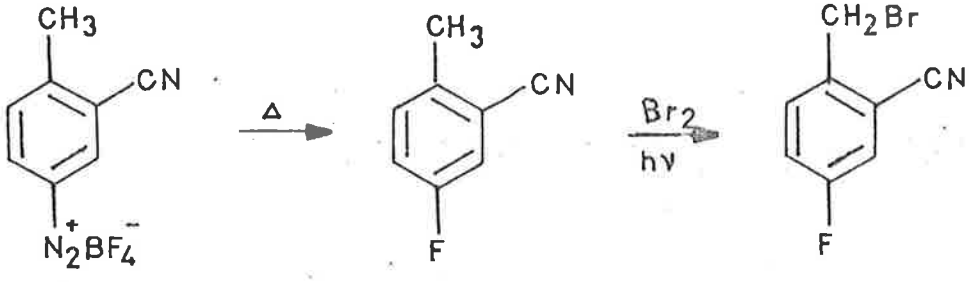
As an example of a fluorine labelled substrate of restricted conformational geometry,[261,262,264] 3-carboxy-7-fluorodihydroisocarbostyril (XIX)

(3-carboxy-7-fluoro-1-keto-1,2,3,4-tetrahydroisoquinoline) was synthesised. The conditions followed those for the parent compound[262] very closely. The synthesis can be represented by Scheme X.

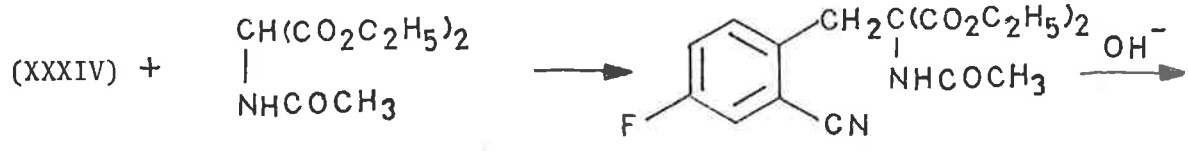
Nitration of o-tolunitrile with nitric acid/sulphuric acid[407] gave 2-cyano-4-nitrotoluene (XXX) in 77% yield. This was catalytically reduced using palladium on charcoal as catalyst to give 4-amino-2-cyano-toluene (XXXI) in 84% yield. Conversion of this proceeded smoothly to give the diazonium fluoborate (XXXII), thermal decomposition of which gave 2-cyano-4-fluorotoluene (XXXIII) in 52% yield. This is quite a good yield for fluoro compounds prepared by the Schiemann reaction. Also there appear to be no reports in the literature of the Schiemann reaction being carried out with a compound containing a cyano group. Free-radical bromination of 2-cyano-4-fluorotoluene gave 2-cyano-4-fluoro-benzyl bromide (XXXIV) in 65% yield. This compound was found to be a powerful lachrymator and skin irritant. Condensation of this, with diethyl acetamidomalonate, produced diethyl α-acetamido-α-(2-cyano-4-fluorobenzyl)-malonate (XXXV) in 76% yield. The next step of the sequence, saponification and cyclisation of (XXXV) to give (XXXVI) proceeded with a better yield (45%) than that reported in the literature for the parent compound (35%). 3-Carboxy-7-fluorodihydroisocarbostyril was readily esterified, using thionyl chloride in methanol (92% yield).



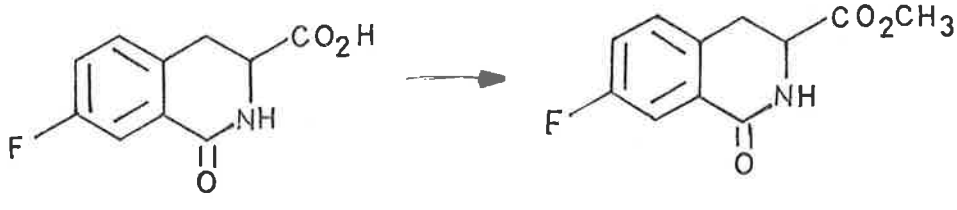
(XXX) (XXXI)



(XXXII) (XXXIII) (XXXIV)



(XXXV)



(XXXVI)

Scheme X.

2. Quantitation of Chemical Shifts.

For an inhibitor, I, exchanging between solution and the active site of an enzyme, E,

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES$$

if a 1:1 complex is formed, and the dissociation constant for the enzyme-inhibitor complex is related to the enzyme and inhibitor concentrations by the expression

$$K_D = \frac{E.I.}{EI}$$

If the exchange is rapid, then the observed chemical shift, δ, will be the weighted average of the shift in the complexed form, Δ', and that in free solution, δ_{free}

$$\text{Thus,} \quad \delta = \frac{EI}{I_o} \Delta' + \frac{I_o - EI}{I_o} \delta_{free} \qquad (1)$$

If the chemical shift of the complexed form is to be determined relative to $\delta_{free} = 0$, then $\Delta = \Delta' - \delta_{free}$ and (1) simplifies to

$$\delta = \frac{EI}{I_o} \Delta + \delta_{free} \qquad (2)$$

If δ is also measured relative to $\delta_{free} = 0$ then

$$\delta = \frac{EI}{I_o} \Delta$$

$$K_D = \frac{E.I}{EI} \qquad (3)$$

$$= \frac{(E_o - EI)\ (I_o - EI)}{EI} \qquad (4)$$

From (2) $$EI = \frac{\delta}{\Delta} I_o$$

$$\therefore \quad K_D = \frac{[E_o - (\delta/\Delta)I_o]\ [I_o - (\delta/\Delta)I_o]}{(\delta/\Delta)I_o}$$

$$= \frac{[E_o - (\delta/\Delta)I_o]\ [1-\delta/\Delta]}{\delta/\Delta}$$

$$= \frac{\Delta}{\delta} E_o - E_o - I_o + I_o \frac{\delta}{\Delta}$$

If $\delta << \Delta$, then $\frac{\delta}{\Delta} I_o$ << other terms

Hence $$K_D = \frac{\Delta}{\delta} E_o - (E_o + I_o)$$

$$I_o = \frac{1}{\delta} (\Delta E_o) - (E_o + K_D) \qquad (5)$$

Thus a plot of I_o vs $\frac{1}{\delta}$ will be a straight line of slope ΔE^o and intercept $-(K_D + E_o)$. Hence Δ and K_D can be found by graphical methods. The approximation by Spotswood et al.,[142] that, in the equilibrium expression, $EI << I_o$ and can be ignored leads to the expression

$$I^o = \frac{1}{\delta} (\Delta E^o) - K_D$$

However this approximation is not particularly valid as it was found that $K_D \sim E_o$. Hence using this approximation can lead to large errors in K_D.

Equation (5) can also be derived from (4) and (3) by making the

assumption that in (4), terms in EI^2 are sufficiently small that they may be ignored.

The calculation of K_D and Δ requires a knowledge of δ_{free}, the chemical shift of the resonance in free solution. As this is often difficult to determine, use was made of the method of Sykes[117,121] for the calculation of K_D and Δ. This method has the advantage that no approximations are involved. The method is as follows:

(4) can be expanded to give

$$K_D = \frac{E_o I_o - E_o EI - I_o EI + EI^2}{EI} \qquad (6)$$

Solving for EI in (6) gives

$$EI = \frac{(E_o + I_o + K_D \pm \sqrt{(E_o + I_o + K_D)^2 - 4E_o I_o}}{2} \qquad (7)$$

K_D is assumed and EI calculated according to (7) with the condition $o \leq \frac{EI}{E_o} << 1$ for each value of I_o. The value of EI is used to calculate the least squares error in (2) for the experimental values of δ and I_o. The value of K_D then, which best fits the values of δ and EI/I_o to a straight line in (2) is chosen as the true value. This also allows Δ and δ_{free} to be calculated.

This was most conveniently carried out by computer. K_D was assumed and EI calculated. The RMS error corresponding to this value of K_D and hence EI for the data points was calculated according to (2). K_D was then incremented (or decremented) until the RMS error reached a

minimum. This corresponds to the best value of K_D and hence Δ. K_D and Δ were also calculated assuming inactive enzyme dimer formation was also taking place. The calculation was essentially the same, except that the added restriction of the enzyme dimer equilibrium had to be maintained. The value of the enzyme dimer dissociation constant was taken from the literature. In this system, the added equation is

$$E + E \rightleftarrows EE \qquad K_{DIM} \tag{8}$$

$$K_{DIM} = \frac{E.E}{EE}$$

where EE is enzyme dimer

$$E_o = E + EI + 2.EE$$

$$K_{DIM} = \frac{(E_o - EI - 2EE)^2}{EE} \tag{9}$$

Solving for EE in (9) gives

$$EE = \frac{(E_o - EI + \frac{K_{DIM}}{4}) \pm \sqrt{(E_o - EI + \frac{K_{DIM}}{4})^2 - (E_o - EI)^2}}{2} \tag{10}$$

Similarly (3) with $E_o = E + EI + 2EE$ becomes

$$K_D = \frac{(E_o - EI - 2EE)\ (I_o - EI - 2EE)}{EI}$$

Solving for EI in (10) gives

$$EI = \frac{(E_o - 2EE + I_o + K_D) \pm \sqrt{(E_o - 2EE + I_o + K_D)^2 - 4I_o(E_o - 2EE)}}{2} \qquad (11)$$

EI and EE are calculated by the following set of operations. K_D is set.

1. Calculate EI from (11) with EE = 0
2. Calculate EE from (10) with EI value from step 1.
3. Calculate EI using previous calculated EE value.
4. Continue until there is a convergence to 0.0001 in EI.

K_D is iterated and Δ calculated from (2) as described earlier. The value of K_D for which there is the minimum least squares deviation in (2) is the true value.

3. The Interaction of Inhibitors with α-Chymotrypsin - NMR Studies.

(a) Bulk susceptibility effects.

It has been shown in the previous section that a computer treatment of the data requires only an accurate determination of the chemical shift relative to a fixed reference. For a graphical treatment of data it is necessary to know δ_{free}. Most of the inhibitors studied here were used as DL racemates. In most cases, in the presence of α-chymotrypsin, the ^{19}F NMR spectrum consisted of two resonances. Whereas the position of the resonance to low field was a function of inhibitor concentration, it was found that the position of the resonance to high field was virtually unmoved by changing inhibitor concentration. This phenomenon was also reported by Zeffren and Reavill[143] and by Spotswood et al.[142] As the chemical shift of this high field resonance was considerably different between solutions with and without enzyme, and subsequent shifts with changing inhibitor concentrations were very small, it was thought that this initial large shift on the addition of enzyme to an inhibitor solution may have been a bulk susceptibility effect. The chemical shift of the ^{19}F resonance of the inhibitor N-trifluoroacetyl-p-bromophenylalanine (XXXVII) was monitored in the presence of various enzymes (Table VI). In the presence of α-chymotrypsin there were two resonances. By analogy with the results obtained with N-trifluoroacetylphenylalanine to be discussed later, this was due to preferential binding of the D enantiomer of the racemate used.

TABLE VI.

Chemical shifts of the ^{19}F resonances of N-trifluoroacetyl-p-bromophenylalanine (XXXVII) in the presence of enzyme (0.1M citrate buffer, pH 5.3) at 56.4 MHz.

Enzyme	Concentration (mM)	Conc. of (XXXVII) (mM)	Chemical shift of ^{19}F resonance (Hz)[a]	Change in shift (Hz)
-	-	30	186.3	-
pepsin	2.3	30	188.6	2.2
		9	188.5	2.3
lysozyme	2.4	30	188.0	1.5
		9	187.8	1.7
trypsin	2.5	30	189.6	3.8
		9	190.1	3.3
DFP-α-chymotrypsin	2.6	30	188.3	1.9
		9	188.2	2.0
α-chymotrypsin	2.6	30	188.4, 193.7	2.1, 14.1
		9	188.7, 203.4	2.4, 7.4

a Relative to trifluoroacetic acid lock.

It can be seen that addition of an enzyme to an inhibitor solution causes a shift to low field of the ^{19}F resonance of the inhibitor. For a particular enzyme concentration, this shift is virtually independent of the inhibitor concentration, but depends on the nature of the enzyme.

If the enzyme is α-chymotrypsin, this result applies only to the high field resonance, the low field resonance position depends markedly on inhibitor concentration. Thus it appears that the L antipode of the racemic inhibitor is only weakly binding to α-chymotrypsin, or that the chemical shift of the enzyme-inhibitor complex is not significantly different from that of the inhibitor in free solution. In this case it appears valid to use the chemical shift of the L enantiomer as δ_{free}, the initial shift of 2.1 Hz being a bulk susceptibility effect. This is amplified by the results obtained with the other enzymes.

With the other enzymes, it is unlikely that the observed shift could be due to N-trifluoroacetyl-p-bromophenylalanine functioning as an inhibitor. Lysozyme is an enzyme which hydrolyses molecules containing sugar moieties.[130] It also binds sugars; in fact these lysozyme-sugar complexes have been extensively studied by NMR spectroscopy.[123-130, 146, 147,148] The likelihood of its binding an acyl amino acid is extremely remote. The change in shift measured, then, can only be explained in terms of a bulk susceptibility change. The fact that the change is not concentration dependent supports this conclusion; the formation of an enzyme-small molecule complex would be evidenced by a concentration dependent shift.

In the case of pepsin and trypsin, the conclusions are not as clear cut. Pepsin is a peptidase which hydrolyses peptide bonds on the amino side of residues with aromatic side chains,[210] c.f., α-chymotrypsin. The fact that the initial observed shift is not then concentration depen-

dent makes it most likely due to the nature of the enzyme's specificity, but apparently is not occurring to an extent whereby it can be detected by NMR techniques. With trypsin binding is again a possibility. X-ray diffraction results show the binding site to be somewhat larger than that of α-chymotrypsin.[188] Naturally occurring bovine pancreatic trypsin inhibitor also inhibits α-chymotrypsin;[213] as such it seems possible that inhibitors of α-chymotrypsin may inhibit trypsin. Also trypsin is inhibited by aromatic compounds.[408] These compounds are amines, however, which fulfil the requirement of the basic side chain necessary for binding of substrates. In this case there is probably hydrogen bonding between the ammonium ion group of the inhibitor, and the carboxylate anion of aspartic acid-189 at the bottom of the binding pocket. The concentration independent shift once again suggests only a bulk susceptibility effect.

Thus any attempt to measure a chemical shift of an inhibitor resonance in the presence of an enzyme must make allowances for a chemical shift due to solution properties different for solutions with, and without, enzyme.

(b) The interaction of inhibitors with DFP-α-chymotrypsin.

The results obtained with DFP-α-chymotrypsin, in which the catalytically important residue of α-chymotrypsin, serine-195, has been esterified with diisopropyl fluorophosphate, were similar to those obtained with the enzymes pepsin, lysozyme, and trypsin. That is, there was a significant change in the chemical shift of the ^{19}F resonance on

addition of the enzyme to the inhibitor solution. Subsequent changes in inhibitor concentration, with the same concentration of DFP-α-chymotrypsin, failed to affect the chemical shift further. Once again, only a bulk susceptibility effect was operating; there was no evidence of binding.

The interaction between an inhibitor and DFP-α-chymotrypsin was investigated at pH 7.7 in 0.1M Tris buffer, using N-trifluoroacetyl-p-fluorophenylalanine as the inhibitor. With this inhibitor, under the same conditions but with α-chymotrypsin, the NMR spectrum consisted of two resonances. For a 20mM solution of the inhibitor, the peak separation was 5.9 Hz, due to the preferential binding of the D enantiomer. In the presence of DFP-α-chymotrypsin there was no separation, indicating no binding or, more correctly, no preferential binding of either isomer. As it was possible that both enantiomers could bind equally well to DFP-α-chymotrypsin, and this binding could be evidenced by almost identical shifts on binding, the interaction of N-trifluoroacetyl-D-tryptophan was studied.

Results with this inhibitor and α-chymotrypsin at pH 7.6 showed a substantial concentration dependent chemical shift (∿ 5Hz over the concentration range studied). However in the presence of DFP-α-chymotrypsin, the chemical shift was concentration independent. There was, however, a bulk susceptibility shift of 2.1 Hz on addition of the enzyme to a 50mM inhibitor solution; decreasing this concentration to 10mM effected only a further 0.1 Hz change. Sykes[121] has reported ^{19}F

NMR studies of the binding of N-trifluoroacetyl-D-phenylalanine to DFP-α-chymotrypsin at pH 7.8. The results reported depended on the measurement of very small chemical shift changes (< 0.5 Hz). With the large errors thus involved, the report appears dubious; such small changes in chemical shift could easily arise from a small concentration effect due to the inhibitor slightly affecting bulk susceptibility.

The wealth of information on DFP-α-chymotrypsin leaves no doubt that this enzyme is totally inactive.[269] Whether or not it is capable of binding an inhibitor or substrate is another question. Modification of serine-195 would preclude its acylation by a substrate (or inhibitor, if the inhibitor is a virtual substrate). However the aromatic binding site, the "tosyl hole", is still intact in tosyl-α-chymotrypsin[195-199] and most probably would be so in other serine-195 modified enzymes. Binding could be precluded by the diisopropyl phosphorylo group either being in, or preventing access to the aromatic binding site. A consideration of the three-dimensional structure of N-formyl-L-tryptophan:α-chymotrypsin shows the aromatic ring of the virtual substrate to occupy the "tosyl hole". The amido hydrogen forms a hydrogen bond with the carbonyl oxygen of serine-214 and the carboxyl group is near serine-195.[200] Steric interactions between the carboxyl and diisopropyl phosphorylo groups are then inevitable. Thus binding at the active site becomes unlikely. Conformational changes of a degree necessary to relieve these interactions would most certainly disrupt the aromatic binding site. On these bases, binding to DFP-α-chymotrypsin by N-acyl aromatic amino acid inhibitors is unlikely.

Proflavine has been shown to bind weakly to DFP-α-chymotrypsin and chymotrypsinogen by equilibrium dialysis studies.[294] However the studies using visible absorption spectroscopy demonstrated no binding.[409] With the dye thionine, an inhibitor, binding both to α-chymotrypsin and chymotrypsinogen was reported, and postulated to occur at sites other than the catalytic site.[410] The catalytic site was shown to be the only site of binding for the dye, Biebrich scarlet[411] however. Thus it seems there are other binding sites in α-chymotrypsin which may or may not be used if the catalytic site is unavailable, for example in chymotrypsinogen. However, as our ^{19}F NMR studies failed to demonstrate any binding at all to DFP-α-chymotrypsin, these secondary sites could not be further elucidated.

Anhydro α-chymotrypsin, in which the elements of water had been removed from serine-195, was found to be capable of binding inhibitors.[294,295] It is clear, then, that phosphorylation of serine-195 has the effect of preventing access to the aromatic binding site. Serine-195 cannot be important in binding by forming an acyl-enzyme as many strong inhibitors such as indole and proflavine lack a carboxyl group. Thus binding to the aromatic binding site must be of major importance.

To test this further, the binding of N-trifluoroacetyl-p-bromophenylalanine, a strongly bound inhibitor, to DFP-α-chymotrypsin was studied at pH 5.3 where the binding of both inhibitors[279,412,413] and substrates[217,414] is known to be stronger than at alkaline pH's. No separation of the ^{19}F signal into two resonances due to preferential binding of one isomer could be detected. Apart from a small initial

bulk susceptibility change, there was no concentration dependent shift which indicates no detectable binding by this method.

(c) Proton magnetic resonance studies.

The interaction of several inhibitors with α-chymotrypsin was studied by proton magnetic resonance spectroscopy. The resonances of the protons in the acetyl groups of N-acetyl-D,L and DL-phenylalanine, N-acetyl-D,L and DL-tryptophan, and α-acetamidocinnamic acid, and the acetyl and methyl groups of α-acetamido-β-(m-methylphenyl)-acrylic acid were observed. Addition of α-chymotrypsin to 50mM solutions of these inhibitors in 0.1M citrate buffer in D_2O, pD 5.4, such that the concentration of the enzyme was 2.6mM, caused shifts of 0.5-1.0 Hz in these resonances. The changes in chemical shifts on decreasing the inhibitor concentration to 10mM, in the presence of the same concentration of enzyme, varied. Over this range, the acetyl proton resonances of N-acetyl-D and L-phenylalanine, and α-acetamidocinnamic acid moved to low field. However this shift was $\leq$ 0.5Hz, which was within the limits of error of there being no change in shift. The same result was obtained with the acetyl and methyl proton resonances of α-acetamido-β-(m-methyl-phenyl)-acrylic acid. With N-acetyl-DL-phenylalanine and N—acetyl-DL-tryptophan, no separation of the acetyl proton resonances due to preferential binding of one isomer was observed. The initial low field shift of the resonances on the addition of enzyme is, once again, most probably due to bulk susceptibility changes. Such shifts were also observed in

the ^{19}F studies and are described in section 3a.

A chemical shift change of a particular resonance in the presence of enzyme (after bulk susceptibility effects have been allowed for) is a sufficient, but not a necessary criterion for binding. N-acetyl-D-phenylalanine has been shown to ben an inhibitor of α-chymotrypsin by these techniques.[142] The low value of Δ obtained implies that very small chemical shift changes must have been measured, and hence must be subject to large errors due to the possibility of small concentration dependent shifts arising from bulk susceptibility changes. As a change in the observed chemical shift of a nucleus will occur only if the binding is strong and there is a reasonable different between the free solution and enzyme bound shifts, these criteria must be satisfied if accurate results are to be obtained.

N-Acetyl-D and L-tryptophan have been shown to be inhibitors of α-chymotrypsin,[277,278,271,412,416] although N-acetyl-L-tryptophan has been termed a virtual substrate by virtue of its ^{18}O exchange properties which have been interpreted in terms of acyl-enzyme formation.[203] The changes in chemical shifts of the acetyl proton resonances were small over the concentration range studied (10-50mM). These shifts were too small (1.0 Hz to low field and 0.7 Hz to high field for the D and L isomers respectively) to allow a quantitative determination of K_D and Δ. Recently, Gerig and Rimerman[111] have reported studies of this system at 100 MHz and pD 6.6. The reported values of Δ were -0.13 and 0.17 ppm for the D and L enantiomers. This corresponds to shifts on binding of

7.8 Hz to low field and 10.2 Hz to high field at 60 MHz. Once again the measured changes of chemical shift must have been small. These workers have also investigated the binding of N-acetyl-D and L-tryptophan to tosyl-α-chymotrypsin. As discussed in section 3b, any binding which presumably occurs with modified enzymes, does so at sites other than the active site when access to the aromatic binding pocket is precluded. In tosyl-α-chymotrypsin, this pocket is undoubtedly occupied by the phenyl ring of the tosyl group. Their reported values of Δ are so small that any measured changes in chemical shift must have been so small that they could easily be due to the bulk susceptibility changes which occur on varying the inhibitor concentration. The reported values for the tosyl-α-chymotrypsin system must, then, be accepted with reservation.

Overall, the rather small changes on binding of the proton resonances which could be readily observed with these inhibitors ($\leq$ 1 Hz) made it impossible to study quantitatively the binding process at 60 MHz. Also any data reported to have been obtained by these techniques must be treated cautiously.

(d) The interaction of N-trifluoroacetylphenylalanine with α-chymotrypsin.

The interaction of N-trifluoroacetylphenylalanine with α-chymotrypsin had been studied by Sykes[121,145] and Zeffren and Reavill.[143,144] The 94.1 MHz studies of Zeffren and Reavill[143] were carried out at pH 6 with the racemic inhibitor. They reported that the ^{19}F NMR spectrum of

N-trifluoroacetylphenylalanine, in the presence of α-chymotrypsin, consisted of two resonances, the positions of which were concentration dependent. Their interpretation of the data neglected bulk susceptibility effects; the shifts were measured relative to the chemical shift in solution in the absence of enzyme. In this system, the change in chemical shift due to bulk susceptibility changes is a major contribution to the observed shift. Hence the quantitative interpretations are incorrect.

The work of Sykes[121,145] at 94.1 MHz and pH 7.7 involved very small chemical shift differences; this was carried out using the D enantiomer. An attempt was made to repeat this work but failed; within the limits of error (± 0.2 Hz), there was no trend of the ^{19}F resonance to move to high or low field as a function of inhibitor concentration, except for the usual bulk susceptibility phenomenon.

This system was then investigated at pH 5.3 in 0.1M citrate buffer. The changes in chemical shift of the N-trifluoroacetyl ^{19}F resonance were small, but could be determined more precisely by averaging a number of results. The results obtained are shown in Table VII. From these data, K_D and Δ were calculated according to the method described in section 2. The values were 8 ± 1 mM and -9 ± 1 Hz* respectively. The shift on binding, Δ, was to low field which is in the opposite direction to that found by Sykes[121,145] at pH 7.8. However, the value reported is considerably different in his two papers purporting to describe the same system under the same conditions (41 vs 24 Hz).

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* Negative values indicate shifts on binding to low field.

TABLE VII.

Chemical shift[a] of ^{19}F resonance of N-trifluoroacetyl-D-phenylalanine in the presence of α-chymotrypsin, E_o = 2.6 mM; pH 5.3 0.1M citrate, at 56.4 MHz.

Concentration of N-trifluoroacetyl-D-phenylalanine, I_o (mM)	Chemical shift of ^{19}F trifluoroacetyl resonance (Hz)[a]
25	188.1
20	188.3
15	188.5
10	188.7
5	189.2

a relative to trifluoroacetic acid lock

The activity of α-chymotrypsin has been shown to be a maximum at pH 7.5-8.0.[217,413,414,415] As this activity is probably associated with the conformational changes (and also with the state of ionisation of histidine-57) it is possible that the trifluoroacetyl group has a different environment between pH 5.3 and pH 7.8. As the shifts measured by Sykes[121,145] were very small and his work could not be repeated, coupled with the fact that the shifts measured at pH 5.3 were also small, detailed interpretation of these results can only be made with reservation.

Ashton and Capon[149] have reported studies of this system also.

They found a concentration dependent shift at pH 6.34 but no evidence of binding at pH 7.96. Their values of K_D (43 mM from the NMR studies and 30 mM from kinetic studies) are some fivefold larger than those determined here. It is difficult to know whether bulk susceptibility effects were included in the consideration of NMR data by these workers.

The interaction of N-trifluoroacetyl-DL-phenylalanine with α-chymotrypsin was also studied at pH 5.3. In the presence of enzyme, two separate resonances were observed. The chemical shift of the resonance to high field was independent of the inhibitor concentration, while the separation of the resonances, and the chemical shift of the low field resonance were concentration dependent. Addition of N-trifluoroacetyl-D or L-phenylalanine to a solution of this inhibitor in the presence of the enzyme showed that the resonance at lower field was due to the D enantiomer while that at higher field was due to the L isomer. As the position of the resonance of the L isomer was invariant, its position was a convenient reference and the position of the D isomer resonance was measured relative to it, i.e., the separation was measured. The position of the L isomer resonance was thus assumed to be equivalent to δ_{free} for the D antipode for graphical treatment of the data. It was also assumed that the L isomer was binding much more weakly and its effect could be ignored. Clearly this assumption is only a first approximation, but it is strongly supported by the fact that the separation of the D and L isomer resonances at constant D isomer and enzyme concentration was independent of the concentration of L isomer, within experimental error.

The results obtained are shown in Table VIII.

TABLE VIII.

Interaction of N-trifluoroacetyl-DL-phenylalanine with α-chymotrypsin, E_o = 2.6 mM; pH 5.3 in 0.1M citrate buffer at 56.4 MHz.

Concentration of D isomer of N-trifluoroacetyl-DL-phenyl-alanine, I_o (mM)	Separation of resonances (Hz)
10	1.25 ± 0.1
7.5	1.55
6.25	1.65
5.0	1.75
4.0	2.00
2.5	2.25

The graph of this data (I_o vs $1/\delta$) is shown in the appendix (Fig. 1). From this graph K_D = 5.4 mM, Δ = -9.0 Hz.

K_D and Δ were also calculated using the method utilised for the D isomer alone. If the L isomer were binding weakly, a change in shift may not be apparent but its measured shift may still be slightly different from the free solution shift. The position of the L enantiomer resonance was thus taken as a convenient reference, and K_D, Δ, and δ_{free} were calculated by the iterative procedure described in section 2. δ_{Free}

was found to be within ± 0.5 Hz of the L isomer resonance position in this, and all the other systems involving racemic derivatives of phenylalanine. The values of K_D and Δ obtained were 8.5 ± 0.3 mM and -11 ± 2 Hz respectively. The good agreement of these results, with those obtained by graphical methods and the iterative computer procedure, on the D isomer alone confirm that the assumptions are valid and the approximations excellent.

It is difficult to interpret these results in absolute terms; the effect of the environment on ^{19}F chemical shifts is not sufficiently well documented to allow these results to interpreted in terms of specific interactions. These results are best discussed in terms of relative effects by comparison with results obtained with other inhibitors.

(e) The interaction of N-trifluoroacetyltryptophan with α-chymotrypsin.

The interaction of N-trifluoroacetyl-D-tryptophan with α-chymotrypsin gave rise to phenomena similar to those discussed earlier for N-trifluoroacetyl-D-phenylalanine. In this case, at pH 5.3, the observed shift to low field of the ^{19}F resonance over the concentration range studied was much larger (14.3 Hz *vs* 1.1 Hz for N-trifluoroacetyl-D-phenylalanine) which allowed the system to be studied in greater detail. The results are shown in Table IX. Treatment of these data according to the method described earlier gave $K_D < 0.1$ mM and $\Delta = -30.4 \pm 0.2$ Hz. Under essentially the same conditions, Ashton and Capon,[149] have subsequently reported $K_D = 6.5$ mM and $\Delta = -112$ Hz (at 94.1 MHz) respectively. This corresponds to a shift of 67 Hz to low field at 56.4 MHz.

TABLE IX.

Interaction of N-trifluoroacetyl-D-tryptophan with α-chymotrypsin, E_o = 2.6 mM; pH 5.3 in 0.1M citrate buffer at 56.4 Hz.

Concentration of N-trifluoroacetyl-D-tryptophan, I_o (mM)	Chemical shift[a] of ^{19}F trifluoroacetyl resonance (Hz).
50.0	184.0 ± 0.2
40.0	184.7
30.0	185.0
25.0	185.4
20.0	186.4
15.0	187.5
10.0	190.2
5.0	198.3

a relative to trifluoroacetic acid lock.

Under the same conditions, the change in chemical shift of the ^{19}F resonance of the L isomer (1.7 Hz) was much smaller over the concentration range studied. The results are shown in Table X. The calculated values of K_D and Δ by the iterative procedure are 5.6 ± 1.6 mM and -7.8 ± 0.6 Hz respectively. As the measured shifts were small, the calculated constants are subject to the same uncertainties as those encountered with N-trifluoroacetyl-D-phenylalanine.

TABLE X.

Interaction of N-trifluoroacetyl-L-tryptophan with α-chymotrypsin, E_o = 2.6 mM; pH 5.3 in 0.1M citrate buffer at 56.4 MHz.

Concentration of N-trifluoroacetyl-L-tryptophan, I_o (mM)	Chemical shift of N-trifluoroacetyl resonance (Hz)[a]
50	182.9 ± 0.2
40	183.0
30	183.2
25	183.3
20	183.4
10	183.7
5	184.3

a relative to trifluoroacetic acid lock

This system was also investigated at pH 7.6. At this pH, no binding of the L isomer was detected; i.e., the chemical shift was concentration independent. For the D isomer, the concentration dependent change in chemical shift was smaller than that observed at pH 5.3 (6.9 vs. 14.3 Hz to low field). This is still sufficiently large for reliable and accurate data for the system to be calculated. The results are shown in Table XII. K_D and Δ were calculated from these data to be 4.3 ± 1.6 mM and -34 ± 1 Hz respectively. Ashton and Capon[149] report values of 28.6 mM and 148 Hz respectively (at 94.1 MHz and pH 7.72).

the shift on binding with pH, will be discussed in a subsequent section.

The interaction of N-trifluoroacetyl-DL-tryptophan with α-chymotrypsin was studied at pH 5.3. The separation of the ^{19}F resonance into two, in the presence of enzyme, showed the preferential binding of one isomer or the possible differentiation of the environment of the fluorine nuclei between the enzyme-D-inhibitor and enzyme-L-inhibitor complexes. The separation, over the concentration range 5-50 mM of the racemic inhibitor, varied between 1.8 and 19.0 Hz. The resonance to lower field was shown to be that of the D enantiomer by the fact that its intensity was increased by the addition of this enantiomer. The chemical shift of the L enantiomer resonance also moved to low field on decreasing the inhibitor concentration. This shift was small (1.1 Hz over the concentration range studied). The concentration dependent chemical shift, although small, indicated that the L isomer was binding to some extent; this was also proved by the fact that peak separation was also dependent on the concentration of the L enantiomer present at constant D isomer and enzyme concentration. Thus, in this case, the L enantiomer must be competing effectively with the D enantiomer for the enzyme, although there is only a small shift on binding. A graph of the separation of resonances as a function of the concentration of the D enantiomer was made according to equation (5) (Appendix, Fig. 2) although the assumption that the L isomer is not binding is not good. Δ Was found to be -22 Hz for the D isomer by this method, while the dissociation constant calculated was less than zero. As this is impossible,

it also indicates that the assumption is invalid and/or the approximation of ignoring terms in EI^2 is poor. As the work showed with the D enantiomer alone, K_D to be very small, the approximation involving the removal of the term in EI^2 will be poor at low concentrations of I_o as $EI^2 \sim E_oEI$.

Treatment of the data, according to the method described for N-trifluoroacetyl-DL-phenylalanine, involves the assumption of the L isomer not binding, but no mathematical approximations. K_D and Δ were found to be < 0.1 mM and -22 ± 1 Hz respectively. The agreement between these results, and those obtained for the D enantiomer alone, is still not good, and shows that the assumption of the L isomer not binding is not valid.

(f) The interaction of N—trifluoroacetyl substituted phenylalanines with α-chymotrypsin.

The interactions of a number of N-trifluoroacetyl substituted phenylalanines with α-chymotrypsin were studied. These inhibitors were used as their racemic mixtures. The ^{19}F resonances separated into two resonances in the presence of α-chymptrypsin. The low field resonance, the position of which was concentration dependent, was assumed to be that of the D enantiomer by virtue of its preferred binding. There is little doubt of this being so; it has been reported previously in observations of the ring fluorine nuclei on binding[142] and has been shown to be the case in this work with the N-trifluoroacetylphenylalanine and N-trifluoroacetyltryptophan. The L enantiomers of these substituted phenylalanines

were assumed not to bind. Their resonance positions (the high field resonance) did not change with changing inhibitor concentration and it may reasonably be assumed that the L isomer is ineffective as a competitive inhibitor.

The N-trifluoroacetyl fluorinated phenylalanines were mainly studied, as these inhibitors had at least two fluorine labels. The ^{19}F resonances of the ring fluorine nuclei were the expected multiplets. In the presence of enzyme, extremely complex spectra were produced due to the overlapping multiplets of the D and L isomer resonances. The complex spectra were an indication that there was preferential binding of the D isomer. However, the complexity of the spectra did not allow the separation of the resonances to be measured with any certainty. With the aid of an heteronuclear spin decoupler, operating at the ^{1}H frequency of 60 MHz, the multiplets were collapsed into two singlets, the separations of which were measured as a function of inhibitor concentration for the para isomer. Instability in the heteronuclear spin decoupler, as well as the time needed to accumulate sufficient spectra in the CAT, made this a very tedious and frustrating procedure which, because of this, was not applied to other systems. The separation of the ring fluorine resonances of N-trifluoroacetyl-DL-p-fluorophenylalanine varied from 4.8 - 10.4 Hz over the concentration range 20-50 mM for the racemic mixture at pH 5.3. From these data, K_D and Δ were calculated to be 1.5 ± 1 mM and -55 ± 2 Hz respectively. K_D and Δ for the corresponding N-acetyl compound were reported as 6 ± 2 mM and -83 ± 5 Hz respectively

at pD 6.[142] The limited data available from kinetic studies indicate enzyme-inhibitor dissociation constants for acetates and trifluoroacetates of a particular inhibitor to be similar,[278,279,425,420] which a comparison of these results also shows.

The trifluoroacetyl ^{19}F NMR spectrum of the above compound was also monitored in the presence of α-chymotrypsin at pH 5.3. The separation of the resonances varied from 3.1 to 13.6 Hz over the concentration range 5-50 mM of the racemic inhibitor. K_D and Δ were calculated to be 1.3 ± 0.2 mM and -26 ± 1 Hz respectively. The values of the dissociation constant calculated from the two kinds of fluorine nuclei are in good agreement, and of an order expected, while the ring fluorine undergoes a substantially greater shift on binding to the enzyme (Table XIV). Gammon et al.[150] report values of 2.19 mM, -65 Hz, and -115 ± 15 Hz for K_D, Δ_{TFA}^{*}, and Δ_{RING} respectively for this system at pH 5.5 and 94.1 MHz. These values were obtained from calculations which included a model for dimerisation. Such an inclusion in a calculation of the binding parameters has the effect of markedly altering K_D but hardly altering Δ. This will be further discussion in section (h). This corresponds to shifts on binding of -39 and -69 Hz at 56.4 MHz. When this system was studied in D_2O instead of water, values of 0.7 ± 1.5 mM and -26 ± 4 Hz were found for K_D and Δ respectively at pD 5.4. As these parameters are the same, within experimental error, for the two systems, there does

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* Δ_{TFA} and Δ_{RING} refer to the shifts on binding for the trifluoroacetyl and ring fluorine nuclei respectively.

not appear to be any change between the state of ionisation of any residue important in binding, or any conformational differences of the enzyme in the two systems. A similar conclusion was reached by Bender et al.[217] from kinetic studies.

At pH 7.7, the separation of the ^{19}F trifluoroacetyl resonances was much less (3.5 - 9.4 Hz) over the concentration range studied (10-30 mM for the racemic inhibitor). The calculated values were 2.5 ± 0.5 mM and -34 ± 3 Hz for K_D and Δ respectively. Once again the weaker binding at alkaline pH was observed.

With N-trifluoroacetyl-m-fluorophenylalanine in the presence of α-chymotrypsin, the complex spectra of the overlapping multiplets of the ring fluorine nuclei could not be satisfactorily resolved into those of the D and L isomers. At pH 5.3 the separation of the N-trifluoroacetyl ^{19}F resonances varied from 1.7 - 4.3 Hz over the concentration range 5-30 mM of the racemic inhibitor in the presence of 2.6 mM α-chymotrypsin. K_D and Δ were calculated to be 2.7 ± 0.2 mM and -11 ± 0.4 Hz respectively. The values from the graph (Appendix, Fig. 4) were found to be 3.4 mM and -14 Hz respectively indicating the usefulness of the graphical method when K_D is not too small.

From these results it can be seen that the ring fluorine atom influences both the enzyme-inhibitor complex dissociation constant (K_D) and the chemical shift of N- trifluoroacetyl fluorine nuclei in the enzyme-inhibitor complex (Δ). The decreased values of K_D relative to that found with N-trifluoroacetylphenylalanine show that the presence of the ring

fluorine atom aids binding to the enzyme. Presumably formation of the enzyme-inhibitor complex is favoured by added interaction of the ring fluorine atom with the hydroxyl hydrogen of serine-189 which the crystallographic data shows to be at the bottom of the aromatic binding pocket, the "tosyl hole". This hydrogen bonding could also orient the inhibitor in the active site. Hydrogen bonding of the meta and para ring fluorine atoms would, by necessity, place the trifluoromethyl group of the respective inhibitors in different environments which the different values of Δ show. Such a conclusion would also explain the stronger binding of N-acetyl-p-aminophenylalanine methyl ester relative to N-acetylphenylalanine methyl ester at pH 8.[421] At this pH, the free amino group with a $pK_b \sim 4.6$[422] would most certainly be unprotonated.

With N-trifluoroacetyl-o-fluorophenylalanine, no separation of the ^{19}F trifluoroacetyl resonances could be detected by NMR methods. The ^{19}F trifluoroacetyl resonance remained a singlet over the concentration range 5-50 mM of the racemic inhibitor. It has been reported that the ring fluorine resonances of N-acetyl-o-fluorophenylalanine separate into those due to the D and L isomers in the presence of α-chymotrypsin, but quantitative results could not be obtained.[423] Thus one isomer is preferentially binding to the enzyme. It appears then, that binding is occurring, but that the environment of the trifluoromethyl group in the enzyme-inhibitor complexes must be very close to its free solution environments for both enantiomers.

In order to overcome the problem of overlapping multiplets in

the spectra of the ring fluorine nuclei of inhibitors in the presence of α-chymotrypsin, the interactions of N-trifluoroacetyl-m- and p-trifluoromethylphenylalanine with this enzyme were investigated. With these probable inhibitors, the aromatic ring is now labelled with fluorine nuclei of which the ^{19}F NMR resonances are singlets. Thus in the present of α-chymotrypsin, preferential binding of one enantiomer should result in ^{19}F NMR spectra consisting of two resonances. However, over the concentration range studied (5-20 mM of racemic inhibitor at pH 5.3, enzyme concentration = 2.6 mM) the ^{19}F resonance remained a single peak both for the aromatic ring and trifluoroacetyl trifluoromethyl groups of both compounds. Once again, either weak binding or binding of each enantiomer equally well such that the environment of the nuclei in the enzyme-inhibitor complex approximates that in free solution is possible. However the aromatic ring, which is of major importance in binding, must occupy the "tosyl hole". By analogy with the results obtained with N-trifluoroacetyl-p-fluorophenylalanine, the shift in the enzyme-inhibitor complex could not be similar to that in free solution. Thus the only conclusion that can be reached is that these two compounds do not function as inhibitors. This conclusion is strengthened by recent work on the α-chymotrypsin catalysed hydrolysis of tyrosine derivatives. It was found that replacement of the phenolic hydrogen atom by a bulky alkyl or alkyloxy group led to the abolition of reactivity.[424] Hence steric requirements of a functional group on the aromatic ring of a substrate severely influence its binding ability.

In the case of substitution by an alkyl group, however, these results are in contradiction to those of Peterson et al.[425] who reported high reactivity.

However the trifluoromethyl group is not much larger than a methyl group or bromine atom, the incorporation of either of which onto the aromatic ring of N-trifluoroacetyl phenylalanine actually increases the binding affinity. It seems unlikely that the slightly larger size of the trifluoromethyl group relative to the methyl group would preclude its binding altogether. The trifluoromethyl group has more hydrophobic character than the methyl group or bromine atom.[426] Its insertion into the hydrophobic binding pocket should be favoured. The possibility of both isomers preferentially binding at a site other than the active site such that the environment is essentially the same as that in aqueous solution seems fairly remote. Thus the lack of binding can only be attributed to the unfavourable steric size of the aromatic ring with a trifluoromethyl group.

N-Trifluoroacetyl-m- and p-methylphenylalanine were found to bind strongly to α-chymotrypsin. The para isomer showed a separation of 1.8 - 5.9 Hz of the ^{19}F trifluoroacetyl resonances in the concentration range 10-50 mM of racemic inhibitor. K_D and Δ were calculated to be 1.5 ± 0.6 mM and -16 ± 1.5 Hz respectively. The values from the graph (Appendix, Fig. 5) were 1.0 mM and -19 Hz respectively. With the meta compound the separation varied from 1.8 - 4.5 Hz over the same concentration range. K_D and Δ were calculated as 4.5 ± 0.2 mM and -20.1 ± 0.2 Hz respectively.

The values from the graph (Appendix, Fig. 6) were 2.4 mM and -18 Hz respectively. Once again meta substitution leads to weaker binding. In this case, however, the substituent does not orientate the inhibitor in the active site; the shifts on binding are virtually the same for both inhibitors. The presence of the methyl group does strengthen the binding relative to the unsubstituted inhibitor (Table XIV). Presumably interaction between the residues constituting the "tosyl hole" and the methylphenyl group of the inhibitor, are enhanced relative to the unsubstituted inhibitor, by virtue of the former's increased hydrophobicity. The hydrophobicity of inhibitor side chain with an attached substituent decreases in the order CF_3 > Br > Cl > CH_3 > diF > F.[426] This is not quite the order found for binding constants indicating the hydrophobic interactions are not the only factors influencing binding. No indication of the trifluoromethyl substituted inhibitors binding to the enzyme could be demonstrated. The binding of fluoro substituted inhibitors was stronger than expected, with the ring fluorine atom also orienting the trifluoroacetyl group indicating interaction of the ring fluorine with some residue of the enzyme. Thus the binding of inhibitors with essentially non-polar substituents paralleled their hydrophobic character indicating the importance of this interaction.

N-Trifluoroacetyl-2,4-difluorophenylalanine was shown to be an inhibitor of α-chymotrypsin, using this NMR technique. The separation of the ^{19}F trifluoroacetyl resonances varied from 1.9 - 4.5 Hz over the concentration range 10-40 mM of racemic inhibitor. K_D and Δ were

calculated as 4.5 ± 1.5 mM and - 20 ± 1 Hz respectively. The graphed values (Appendix, Fig. 9) were 3.9 mM and -21 Hz respectively. These results show that, with N-trifluoroacetyl-o-fluorophenylalanine, a failure to bind cannot be due to the steric interference of the ortho fluorine atom with a residue side chain of α-chymotrypsin. In fact N-trifluoroacetyl-2,4-difluorophenylalanine has a very similar shift or binding to that of N-trifluoroacetyl-p-fluorophenylalanine, and the binding is stronger than that of N-trifluoroacetylphenylalanine itself (Table XIV). It appears, then, that in N-trifluoroacetyl-o-fluorophenylalanine, the ortho ring fluorine has an unfavourable interaction with the enzyme which results in very weak binding. A further fluorine in the para position compensates this effect, by interacting strongly with, perhaps, the hydroxyl hydrogen of serine-189. The resulting complex is orientated by this para ring fluorine-serine-189 hydrogen bond. Thus the enzyme-inhibitor complexes involving the N-trifluoroacetates of p- and 2,4-difluorophenylalanine would have similar geometry. This is evidenced by the similar shifts on binding, Δ (Table XIV).

Both N-trifluoroacetyl-m- and p-bromophenylalanine bound strongly to α-chymotrypsin. At pH 5.3, over the concentration range 9-30 mM of racemic inhibitor, the separation of the ^{19}F resonances of the meta isomer varied between 4.4 and 9.5 Hz. K_D and Δ were calculated as 1.8 ± 0.6 mM and -26 ± 3 Hz respectively. The graphed values (Appendix, Fig. 8) were 2.2 mM and -32 Hz respectively. For the para compound at this pH, the measured separation was 3.8 - 14.2 Hz over the concentration

range 5-40 mM of the racemic inhibitor. K_D and Δ were calculated as < 1 mM and -26 ± 1 Hz respectively. The graphed value (Appendix, Fig. 7) of Δ was -33 Hz. K_D was determined as < 0 from the graph, indicating the approximation involved to be only fair. Relative to N-trifluoroacetyl-phenylalanine, the addition of a bromine atom to the aromatic ring greatly enhances the binding of the inhibitor. The para compound binds much more strongly than the meta isomer; in fact the para compound probably binds as strongly as tryptophan. With these bromophenylalanines, hydrogen bonding to the bromine atom cannot be of major importance in binding as the trifluoromethyl group of each inhibitor occupies a very similar environment, c.f., binding of N-trifluoroacetyl-m- and p-fluoro-phenylalanine where the environment of the trifluoromethyl groups was quite different.

At pH 7.6 the binding of both N-trifluoroacetyl-m- and p-bromo-phenylalanine was much weaker. The resonance separations varied from 4.1 - 8.1 Hz and 5.1 - 10.7 Hz for the meta and para isomers respectively over the concentration range 9-30 mM of racemic inhibitors. K_D and Δ were calculated as 4.6 ± 0.5 mM and -33 ± 1.5 Hz, and 3.2 ± 0.4 mM and -37 ± 2 Hz for the meta and para isomers respectively. At pH 7.6, then, the environments of the trifluoromethyl groups in the two complexes are still very similar. There are slight increases in the shifts on binding for both compounds, indicating perhaps that the trifluoromethyl groups are more buried at the higher pH.

The interaction of N-trifluoroacetyl-p-bromophenylalanine with α-chymotrypsin was also studied at a number of other pH's; in all cases

the concentration of α-chymotrypsin was 2.6 mM. The results are shown in Table XIII.

TABLE XIII.

Binding parameters of N-trifluoroacetyl-D-p-bromophenylalanine as a function of pH

pH	K_D (mM)	Δ (Hz at 56.4 MHz)
5.0	< 1	-30 ± 2
5.3	< 1	-26 ± 1
6.0	< 1	-31 ± 2
6.6	< 1	-28 ± 3
7.6	3.2 ± 0.4	-37 ± 2
8.2	9.0 ± 1.5	-25 ± 1.5

At all pH's < 7, the enzyme-inhibitor dissociation constant was small (< 1 mM). At pH 7.6 and 8.2, K_D was quite large, indicating that the binding was much weaker at these alkaline pH's. At pH 8.2, the shift on binding, Δ, was substantially smaller than at the other pH's. As has been mentioned, considerable amounts of the inactive enzyme are believed to exist at high pH.[222,224-228] The different value of Δ at pH 8.2 may be an artifact of the ignorance of this phenomenon in a consideration of the data obtained.

The values of K_D as a function of pH are plotted in Fig. 3. The graph of K_D vs pH for N-trifluoroacetyl-D-p-bromophenylalanine shows

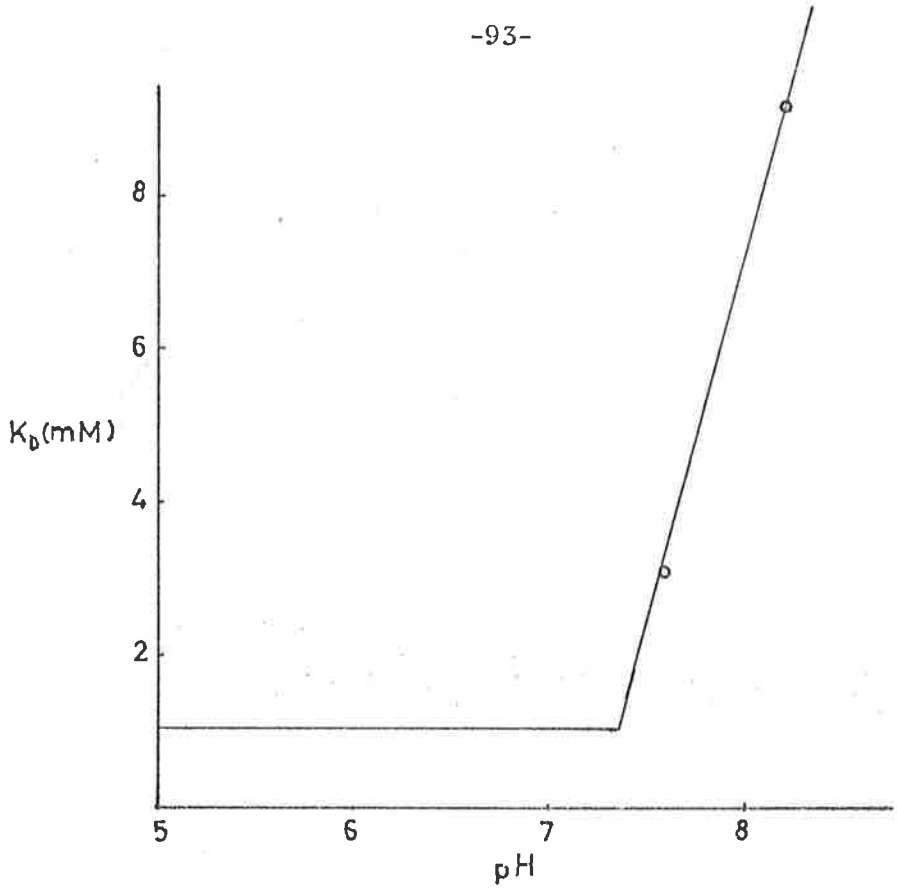


Fig. 3. Variation of K_D with pH for N-trifluoroacetyl-D-p-bromophenylalanine.

a break point at pH ∿ 7.5 consistent with the ionisation of a group in the enzyme with a pKa of this value. This is presumably the imidazole ring of histidine-57.

The interaction of N-trifluoroacetyl-D-tyrosine with α-chymotrypsin was studied at pH 5.3. Except for the initial shift due to bulk susceptibility effects, the chemical shift of the ^{19}F trifluoroacetyl resonance was concentration independent indicating very weak binding, or the shift of the fluorine nuclei in the enzyme-inhibitor complex to be similar to the free solution shift. As mentioned earlier, the acetate and trifluoroacetate of a particular amino acid have similar enzyme-

inhibitor disscciation constants.[278,279,420,427] On this basis, and with a consideration of the trends in K_D for amino acids and their amides,[278,279,280] K_D for N-trifluoroacetyl-D-tyrosine would be expected to be ∿ 50 mM at pH 7.9. At pH 5.3 this could be expected to be ∿ 10 mM. If the shift on binding were also small, then there would be no apparent change in shift. The pKa of the phenolic hydroxyl would ∿10.[428] Thus it is the phenolic, and not the anionic phenoxide form which is binding at pH's < 8. The weaker interaction of this inhibitor with α-chymotrypsin must be due to unfavourable interactions of the hydroxyl group with the aromatic binding site.

Another compound with which no interaction with α-chymotrypsin could be detected, was N-trifluoroacetylphenylglycine. There was neither change in the position of the resonance over the concentration range studied, nor did the resonance separate into two. The β-methylene group in the derivatives of phenylalanines is therefore important in binding. Without this β-methylene group, binding of the aromatic ring, if it occurred, would place the amido hydrogen too far from serine-214 for hydrogen bonding to further stabilise the complex. The size, rather than the nature of the β-methylene group has been shown to be important by the fact that its replacement by sulphur or an amino group does not decrease a substrate's binding ability; in fact it is slightly enhanced.[429] However the rate of hydrolysis of the esters of the L enantiomers is greatly decreased.[429] A similar lack of reactivity when the β-hydrogens are replaced by methyl groups has been observed.[430]

The values of K_D and Δ obtained for the series of compounds studied are summarised in Table XIV.

TABLE XIV.

Binding parameters of substituted N-trifluoroacetyl-D-phenylalanines at pH 5.3 and 56.4 MHz, 0.1M in citrate buffer (determined from DL racemates)

Substituent	K_D (mM)	Δ (Hz) of N-trifluoroacetyl ^{19}F nuclei.
H	8 ± 1	-9 ± 1
p-F	1.3 ± 0.2	-26 ± 1
m-F	2.7 ± 0.2	-11 ± 0.4
2,4-diF	4.5 ± 1.5	-20 ± 1
p-Br	< 1	-26 ± 1
m-Br	1.8 ± 0.6	-26 ± 3
p-CH_3	1.5 ± 0.5	-16 ± 1.5
m-CH_3	4.5 ± 0.2	-20 ± 0.2
p-CF_3	no binding detected	
m-CF_3	no binding detected	
o-F	no binding detected	

(g) Interaction of the cinnamic acids with α-chymotrypsin.

The previous reports of the inhibition of α-chymotrypsin by cinnamic acid[108,282] and dihydrocinnamic acid,[219,431,432] suggested that interaction of their fluoro derivatives with α-chymotrypsin could be

studied using ^{19}F NMR spectroscopy. These inhibitors were not sufficiently soluble in a buffer of pH 5.3. Since binding should be stronger at this pH, the solubility in a 30% methanol citrate buffer of pH 5.3 was investigated, as α-chymotrypsin was known to be fully active in this solvent system.[433] A sufficiently high concentration (50 mM) of the inhibitor could be obtained, but addition of α-chymotrypsin to this solution such that the concentration of enzyme was 2.6 mM, gave a flocculent solution, unsuitable for quantitative studies. No further investigations were carried out.

(h) The effect of enzyme dimerisation and activity on the binding parameters.

As mentioned in the introduction, the properties of the dimers and oligomers of α-chymotrypsin is still an open question. Evidence has been presented in favour of inactive oligomers,[233,234] partially active oligomers,[237,434] oligomers with the number of binding sites equal to the number of associated monomers,[238] dimers binding substrate but unable to decompose to give products,[236] and oligomers dissociating in the presence of substrate.[235] The methods used for determining the activity, or concentration of active sites, of the enzyme, depend on the measurement of the concentration of a leaving group formed in the initial burst of the catalysed hydrolysis of a substrate.[435,436,437] Under the conditions, this may be considered as irreversible formation of an acyl-enzyme. If conversion of oligomers to monomers is reasonably rapid,

then all the potentially available enzyme will be present as the acyl-enzyme. Rao and Kegeles[231] have concluded from their studies of oligomerisation of α-chymotrypsin that the equilibrium is truly reversible. Thus the methods reported for the measurement of activity actually determine the concentration of potential monomer, i.e., the amount of molecules present as monomer, dimer, and trimer. Irreversibly formed oligomers are, of course, implicit in the determined activity.

A consideration of reversible enzyme inhibition is thus complex, as either of the models for oligomerisation can be chosen with equal justification. By far the simplest is that pertaining to inactive oligomers. The data obtained were treated by a computer method, the details of which are described in section 2. The treatment of the data using other models is much more complex and not worthwhile until the real model is known. The values of the dimer dissociation constant used were based on effective "dimerisation" constants reported by Gammon et al.[150] i.e., the constant is weighted average of all oligomer dissociation constants. They were 0.05, 0.1, and 0.4 mM at pH's 5.0 - 5.3, 6.0 - 6.6, and 7.6 - 8.2 respectively.

A comparison of the results obtained, with and without the inactive dimer model at pH 5.3, are shown in Table XV. It can be seen that inclusion of such a model in the calculations reduces K_D by about a factor of ten but has little effect on Δ. A similar result is obtained with the parameters of N-trifluoroacetyl-D-p-bromophenylalanine at various pH's (Table XVI) and N-trifluoroacetyl-D-tryptophan (Table XVII).

TABLE XV.

A comparison of the binding parameters of N-trifluoroacetyl substituted D-phenylalanines with and without an inactive enzyme dimer model; at pH 5.3 and 56.4 MHz, trifluoroacetyl ^{19}F nuclei, dimer dissociation constant = 0.05 mM.

Substituent	Dimer not included		Dimer included	
	Δ (Hz)	K_D (mM)	Δ (Hz)	K_D (mM)
H	-9 ± 1	8 + 1	-5.9 ± 0.2	0.32 ± 0.03
	-10.6 ± 1.4[a]	8.0 ± 0.9[a]	-6.1 ± 0.5[a]	0.45 ± 0.05[a]
m-F	-11 ± 0.4	2.7 ± 0.2	-9.7 ± 0.4	0.25[c]
p-F	-26 ± 1	1.3 ± 0.2	-24 ± 1.2	0.15[c]
		1.5 ± 2[b]		0.4[b,c]
2,4-diF	-20 ± 1	4.5 ± 1.5	-16 ± 0.5	0.3 ± 0.03
m-CH_3	-20 ± 0.2	4.5 ± 0.2	-13.6 ± 0.1	0.3[c]
p-CH_3	-16 ± 1.5	1.5 ± 0.5	-15 ± 0.8	0.2[c]
m-Br	-26 ± 3	1.8 ± 0.6	-11 ± 2	0.18 ± 0.05
p-Br	-26 ± 1	< 1	-27 ± 1.7	< 0.1

(a) From pure D isomer.

(b) From ring fluorine studies.

(c) Error < step range (0.1 mM)

TABLE XVI.

Binding parameters of N-trifluoroacetyl-D-p-bromophenylalanine as a function of pH with and without an inactive enzyme dimer model; at 56.4 mHz, trifluoroacetyl ^{19}F nuclei, dimer dissociation constants of 0.05 mM (pH 5.0 and 5.3), 0.1 mM (pH 6.0 and 6.6), and 0.4 mM (pH 7.6 and 8.2).

pH	Dimer not included		Dimer included	
	Δ (Hz)	K_D (mM)	Δ (Hz)	K_D (mM)
5.0	-30 ± 2	< 1	-28 ± 1.4	< 0.1
5.3	-26 ± 1	< 1	-27 ± 1.7	< 0.1
6.0	-31 ± 2	< 1	-29 ± 1.5	0.1 ± 0.02
6.6	-28 ± 3	< 1	-27 ± 2.2	< 0.1
7.6	-37 ± 2	3.2 ± 0.4	-32 ± 1.5	0.8 ± 0.1
8.2	-25 ± 1.5	9.0 ± 1.5	-19 ± 1.1	1.7 ± 0.1

TABLE XVII.

Binding parameters of N-trifluoroacetyl-D-tryptophan as a function of pH with and without an inactive enzyme dimer model; at 56.4 MHz trifluoroacetyl ^{19}F nuclei, dimer dissociation constants of 0.05 mM (pH 5.3) and 0.4 mM (pH 7.6).

pH	Dimer not included		Dimer included	
	Δ (Hz)	K_D (mM)	Δ (Hz)	K_D (mM)
5.3	-30.4 ± 0.2	< 0.1	-30 ± 0.8	< 0.1
7.6	-34 ± 1	4.3 ± 1.6	-28 ± 1	0.92 ± 0.06

K_D and Δ for N-trifluoroacetyl-D-m-bromophenylalanine at pH 7.6 were 1.02 ± 0.10 mM and -27 ± 1.3 Hz respectively. For N-trifluoroacetyl-L-tryptophan at pH 5.3 the values were 0.4 ± 0.1 mM and -5.5 ± 0.7 Hz respectively. The inclusion of the enzyme-dimer model in the calculations does not, for the most part, affect the relative values of K_D and Δ. As such, conclusions reached on binding modes and affinities from results calculated in the presence, or absence, of the dimer model are the same.

(i) A model for α-chymotrypsin inhibition.

By far the greatest factor influencing binding of inhibitors (and substrates) to α-chymotrypsin is the hydrophobic interaction of the inhibitor or substrate side chain with the enzyme.[248,255,438-443] The energy changes on binding have been found to be similar to those which occur on transferring the substrate from water to certain organic solvents, e.g. n-octanol.[440,444,445,446] This, and other partition models[447-450] have prompted suggestions that the active site polarity is comparable to that of these organic solvents. The hydrophobic interactions of the inhibitor (or substrate) enzyme result from the more favourable interaction of the inhibitor (or substrate) side chain with a non-polar environment rather than with the polar water molecules of the solvent.[451,452]

The amido group is probably only important in orienting the labile group of the substrate in the correct position for hydrolysis to occur. The catalytically important residues are serine-195 and

histidine-57. Modification of either residue side chain may or may not affect binding of inhibitors and substrates. Greatly decreased binding appears to be due to the modification of the residue side chain preventing access to the hydrophobic binding site. Of course the binding site for the hydrophobic side chain will, itself be hydrophobic. Its hydrophobicity will be evidenced by lack of reactive polar functional groups. As such, it is virtually impossible to identify residues in the hydrophobic pocket by chemical means. Fortunately X-ray diffraction data have identified the hydrophobic pocket as the "tosyl hole" and allowed the residues comprising it to be determined. Yet the down field shift of the ring fluorine nuclei on binding, suggests a more polar environment. This, and other spectrophotometric evidence purporting to be the result of a polar,[300,453] or non-polar active site,[220,223,409,454,455,456] really only applies to that part of the site which is being investigated. It seems logical that, as the stereospecificity of α-chymotrypsin demands a three point interaction of the substrate with the enzyme, these three points could be associated with loci of greatly different nature. The negligible difference in Δ of the inhibitors studied between pH 5.0 and 7.6 infers that there is negligible change in the environment over this pH range. No change in environment suggests possibly no change in conformation of the active site. It seems that electrostatic interactions play a major part in the binding of inhibitors to α-chymotrypsin. Whereas the binding of neutral inhibitors (amides) is virtually pH independent, that of charged inhibitors acyl amino acids at pH's above

5 (where the anion will be the predominant form[428]) is markedly so. As the binding decreases markedly above pH 7, this has been interpreted as due to a change of ionisation of a residue side chain of the enzyme with this pKa. Presumably this is deprotonation of the imidazole ring of histidine-57. As this phenomenon also occurs with neutral substrates [217,414,457,458,459] and inhibitors,[419] but to a lesser extent, the decreased binding of charged inhibitors at alakline pH's cannot be due to purely electrostatic interactions.

Changes in conformation of the active site with pH[220,224,222, 291,414] or on binding of inhibitors[218,219] have been the interpretations of many workers from results obtained by other methods. The similar Δ values at these two pH's, then, must be due to the trifluoromethyl group of the inhibitor occupying a similar environment, but the remaining part of the molecule could be experiencing a different environment. The work of Gammon et al.[150] shows that the ring fluorine nuclei of N-trifluoroacetyl-D-p-fluorophenylalanine have a similar environment over the pH range 5.0 - 8.0. Also, the possibility exists that the conformational changes of the active site do not involve the residues important in binding an inhibitor or substrate, but do involve the orientation of the groups important for the catalytic activity.

(j) The origin and cause of the down field shift on binding of the ^{19}F nuclei and the nature of the aromatic and acylamino binding sites.

Whereas factors influencing proton chemical shifts are well

documented, such is not the case with fluorine chemical shifts.[162,163,164] The large amount of information which has been gathered in studies of proton NMR spectroscopy has allowed fairly precise explanations of factors influencing chemical shifts in terms of susceptibilities, hydrogen bonding, ring currents, and so forth. However, the amount of information available on ^{19}F chemical shifts is much less, and the influence of external factors such as those cited above has not been elaborated. Also a simple relationship between chemical shift and structure cannot be established by references to the trends in proton chemical shifts as a function of structure. ^{19}F Chemical shifts as a function of structure bear no relationship to those of protons in a comparison of what type of nuclei resonate at high or low field. Whereas protons on aromatic rings resonate at low fields relative to those in methyl groups, the reverse is the case for fluorine nuclei in similar structures.

The down field shift on binding of the ring fluorine nuclei of the inhibitors has tentatively been attributed to hydrogen bonding of the fluorine atom to the hydroxyl hydrogen of serine-189. This was discussed in section (e). However, hydrogen bonding effects have been the explanation of shifts both to low and high field in ^{19}F NMR studies of fluorine containing ions.[460,461] It is also impossible to decide what influence the hydrophobic interaction of the aromatic ring of the inhibitor with the aromatic binding site would have on the ring fluorine chemical shift. Presumably this interaction would not influence the chemical shift of the trifluoroacetyl fluorine nuclei as they are removed

from the aromatic binding site. However the fact that both kinds of fluorine nuclei move to low field on binding suggests that the same factors are operating on these nuclei, but to a different extent.

Thus it is impossible from this, and previous work, to decide the origin of ^{19}F chemical shifts induced by binding. The magnitudes of these shifts can be used to discuss relative effects, i.e. the greater the induced shift, the more removed from the aqueous environment are the nuclei in question.

Zeffren[144] found that the trifluoroacetyl ^{19}F resonance of N-trifluoroacetylphenylalanine is upfield in an hydrophobic solvent, relative to its position in aqueous buffer. However in the presence of an electrolyte, the shift was to low field. His interpretation of this phenomenon was that the binding site was hydrophobic but a high charge density was conferred on it. However, as these shifts were measured relative to an external reference with no susceptibility corrections, the measurements are suspect and cannot be used as evidence. The work of Arrington et al.[462] shows that, for the ring fluorine of fluorobenzene, the chemical shift is upfield in aqueous solvents relative to that in non-polar solvents. Similar phenomena have been found in studies of fluoroform,[463,464] and other fluoro compounds.[465] This suggests that the factors causing the low field shift of the ring fluorine nuclei can be attributed to non-polar (hydrophobic) interactions. Such explanations however, must be accepted with reservation.

As mentioned earlier, evidence has been presented both for a

polar and non-polar active site. It seems logical though, that in the binding of substrates or inhibitors, the aromatic ring is bound in an hydrophobic pocket such that the carboxyl (or carboalkoxy) and amido groups are in the correct orientation at, or near the surface of the enzyme, and in the correct orientation to undergo hydrogen bonding with particular polar residue side chains in the enzyme. Martinek et al.[432, 444] have concluded that the polar moieties of the substrate do not interact with hydrophobic regions of the enzyme. These workers have shown that, if the polar group is on a hydrocarbon chain, however, it will readily enter the hydrophobic binding site.[466] This conclusion is well supported by the crystal structure of the N-formyl-L-tryptophan : α-chymotrypsin complex in which the amido hydrogen of the inhibitor and the carbonyl oxygen of serine-214 in the enzyme are in hydrogen bonding distance near the surface, and the aromatic ring of the inhibitor occupies a narrow cleft which extends towards the centre of the enzyme molecule.[199,200,201] Previous work which purported to show the active site to be polar or non-polar really applies only to that part of the site which is interacting with the inhibitor, or other molecule of which the change in properties due to the interaction is being monitored. Similar disadvantages apply to the work of Martinek, Berezin, and coworkers which demonstrates that the active site is as polar as n-octanol. Such measurements really give only the average of the interactions.

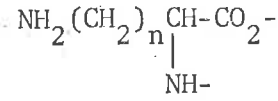
The specificity of α-chymotrypsin for binding substrates is due, in part, to hydrophobic as well as a charge-transfer interaction of the

phenyl ring of the aromatic side chain of the substrate with the enzyme. Substrates with alkyl side chains of the form $CH_3(CH_2)_n\underset{\underset{NHCOR}{|}}{CH}CO_2CH_3$ bind with the enzyme, especially when n = 4,[467] but not as tightly as the aromatic substrates. Derivatives of tryptophan and polycyclic hydrocarbons are very strongly bound suggesting that charge-transfer interactions may also be of importance. This shows that the alkyl group must be sufficiently long enough for binding to be at a maximum, but that an optimum length is required for the amido and carboethoxy group to hydrogen bond to the enzyme. That is when $n < 4$, the amido and carboxyl groups may bond to the enzyme, but the hydrophobic binding will be weak. If $n > 4$, the complex is destabilised because the hydrophobic pocket is limited in size, can accommodate only a side chain the size of the $CH_3(CH_2)_4$- unit, and no favourable interactions of the amido and carboxyl groups can occur.

Also the strength of binding of aliphatic alcohols to α-chymotrypsin is proportional to their chain length, i.e., their hydrophobicity.[446,468] If the acyl group is large in comparison with the side chain (i.e., more hydrophobic), kinetic data suggests that the acyl group may bind at the aromatic binding site.[469] Such a mode of binding has been called "wrong way binding" as such a complex is in the wrong orientation for hydrolysis to occur. This probably only occurs with aryl derivatives of aliphatic amino acids, e.g., hydrolysis of N-picolinylalanine methyl ester where some inversion of specificity occurs and is certainly negligible in the interaction of acyl derivatives of

aromatic amino acids. As such it can be neglected in these studies.

It also seems feasible that the walls of the binding pocket are hydrophobic, while the bottom is hydrophilic. This would explain the preferentially binding of the N-trifluoroacetylfluorophenylalanines (relative to N-trifluoroacetylphenylalanine). Such an explanation has precedent in the specificity of trypsin. Since the binding depends on a basic substrate side chain of a particular length, e.g.



where n = 4 for optimum binding (the substrate becomes a derivative of the naturally occurring basic amino acid, lysine, there appears to be hydrophobic interactions between the hydrocarbon part of the side chain and the enzyme, and also hydrogen bonding between the ammonium ion of the substrate side chain (at the optimum pH) and the enzyme. In fact the binding pockets of trypsin and α-chymotrypsin are of similar size and comprised of similar residues except at the bottom where the aspartic acid-189 of trypsin becomes the serine-189 of α-chymotrypsin. In the presence of ammonium ions, trypsin behaves remarkably like α-chymotrypsin.[470] Presumably aspartic acid-189 at the bottom of the aromatic binding pocket is neutralised, thus leaving the remaining part of the site like that of α-chymotrypsin. Trypsin also binds aromatic amines particularly well, again showing the homologies of the side chain binding pocket as regards size and length [e.g. Introduction 2(a)].

If, in the interaction of acyl amino acid substrates with α-

chymotrypsin, it is postulated that hydrophobic binding is the primary binding force, followed by hydrogen bonding of the amido hydrogen to the carbonyl oxygen of serine-214, the activity would arise from the ester or amide bond being in the correct orientation for catalytic scission of the labile bond to occur with the aid of the charge relay system. With the D antipode of a substrate, the hydrophobic and amido bonds would lead to the labile bond being in the wrong orientation, i.e., away from, instead of towards, the nucleophilic serine-195 of the charge relay system, for hydrolysis to occur. With acyl amino acids, an acyl-enzyme could thus form with the L, but not the D antipode.

However, the NMR results of this research show that this cannot be strictly correct. The different values of Δ of the ^{19}F N-trifluoroacetyl fluorine nuclei with different inhibitors show that the aryl side chain orients the trifluoroacetyl group. The vastly different values for the D and L enantiomers show that the environments of the trifluoroacetyl group is vastly different between these forms. For the L isomer, the small value for the shift on binding indicates an environment little different from that in free solution. However the trifluoroacetyl group of the D isomer is possible in a substantially more polar but at the same time hydrophobic environment.

(k) A model for enzyme-inhibitor complexes.

The nature of the residues forming the active site has been determined with some certainty for the crystalline enzyme. This appears to be a good model for the system in solution. The approximate size and

shape of the aromatic binding site had also been determined previously from the optimum, and limiting dimensions of molecules which can act as substrates,[471] or inhibitors.[283,472] Histidine-57, like serine-195, has been shown to be important in catalysis, but its modification does not affect binding of substrates and inhibitors.[473,474] Hence evidence gathered by chemical means has not demonstrated the importance of any polar residue in the enzyme critically important in binding of inhibitors or substrates, the main interaction is that of the hydrophobic substrate or inhibitor side chain with the hydrophobic pocket of the enzyme. Part of the inhibitor which is polar (the acyl or carboxyl groups) may interact near serine-195 as bulky modification of this residue prevents binding.

The X-ray diffraction results show that the amido hydrogen of the inhibitor probably forms a hydrogen bond with the carbonyl oxygen of serine-214. The carboxyl group of the inhibitor is then near histidine-57 and serine-195. Such an arrangement fulfils the requirement of stereospecificity and places the labile group of an ester in the correct situation for hydrolysis. For a D inhibitor, the hydrophobic interactions must stay the same; there is only one hydrophobic cleft. The interactions of the amido and carboxyl groups cannot be reversed, i.e., the amido group cannot interact with serine-195 and the carboxyl group with serine-214, as the polarities are reversed. At all the pH's studied, the inhibitor will be present as the carboxylate anion. This anion probably interacts with a neutral active site below pH 7 and a

negative active site above pH 7 when histidine-57 is deprotonated. The carboxyl oxygen of the L inhibitor is probably hydrogen bonded to the amido hydrogens of glycine-193 and serine-195. Such a model places the N-trifluoroacetyl group of an L amino acid in essentially an aqueous environment, i.e., it protrudes into the aqueous solution from the active site. Theoretical calculations have also shown this model to be most stable.[475,476] This is consistent with these NMR results which show the environment of the ^{19}F trifluoroacetyl nuclei to be in an environment very similar to a free solution environment.

With an inhibitor of the D configuration it is unlikely that the carboxyl group still interacts with serine-195. If its orientation were the same as that for the L enantiomer, then the ester of D antipodes should be appreciably active. Presumably the position of the carboxyl group is nearer to the imidazole ring of histidine-57. This then puts the acyl group near to glycine-193. If a hydrogen bond between the amido hydrogen of the inhibitor and the backbone carbonyl of glycine-193 were formed, the trifluoroacetyl group would be flanked on one side by part of the enzyme molecule. The N-trifluoroacetyl ^{19}F nuclei would thus be partly protected from the solvent, and such interactions could explain the down field shifts on binding of the D isomers.

EXPERIMENTAL.

(a) General.

^{19}F NMR spectra were recorded on a Varian DA-60 (I.L.) N.M.R. spectrometer operating at 56.4 MHz in the frequency sweep mode. Probe temperature was 29°. Spectra at low concentrations of inhibitor were obtained with the aid of a Varian C-1024 computer of average transients (CAT) which was used to drive a Hewlett-Packard 3310-A low frequency function generator. The spectra were obtained by sweeping a frequency range of 20-50 Hz at a rate of one scan per 25 seconds. Peak positions and peak separations were determined by electronic counting with a RACAL SA35 universal counter-timer and are accurate to ± 0.2 Hz in most cases.

The heteronuclear spin decoupler was a unit constructed by Mr. R.L. Paltridge of this department.

In order to observe the trifluoroacetyl ^{19}F signals of the inhibitors, a capillary of trifluoroacetic acid was used as the reference lock signal. A capillary of benzotrifluoride was used as the reference lock signal in the recording of the aromatic trifluoromethyl resonances. In order to observe the aromatic ^{19}F resonances, a capillary of 1,1,2,2-tetrachlorotetrafluorocyclobutane in carbon tetrachloride was used. The same capillary was used for each determination in a particular experiment; peak positions and peak separations were comparable only when the same capillary was used.

With some meta-fluoro compounds, the ^{19}F resonances were coincidental with the lock. In these cases, a capillary of trifluoroacetic

acid was used as the lock and the manual oscillator frequency was supplied by a Muirhead D-890A decade oscillator.

Solutions were prepared by dissolving a weighed amount of the enzyme in a known volume of a mixture of the inhibitor in buffer solution, and a solution of the buffer such that the total volume of the solution was 0.5 ml.

α-Chymotrypsin (three times crystallised), DFP-chymotrypsin and the other enzymes used were purchased from Worthington Biochemical Corporation. The activity of α-chymotrypsin was assumed to be 100%; no correction was made for inactive impurities. Although the activity is ∿80%, the values of K_D and Δ determined are important in relative, rather than absolute terms so are unaffected by such an assumption. It was also assumed that the activity remained constant throughout different batches.

m-Fluorophenylalanine and p-fluorophenylalanine were purchased from Sigma Chemical Co., Missouri.

The buffers used were prepared according to Wolff and Vandereau[477] or "Biochemists' Handbook"[478] and the pH checked with a Pye universal pH meter. For solutions prepared in D_2O, pD = meter reading +0.4 according to Glascoe and Long.[479]

Optical rotations were determined on a Hilger M412 polarimeter.

Petroleum ether fractions, b.p. 40-60° and 60-80° are X-4 and X-60 respectively. All melting points were determined on a Kofler hot stage melting point apparatus and are uncorrected.

Analyses were performed by the Australian Microanalytical Service, Melbourne.

In the naming of racemic inhibitors, the prefix DL was not always used.

(b) ACETYLATION OF THE AMINO ACIDS.

N-Acetylphenylalanine.

Phenylalanine was acetylated according to Town[322] to give N-acetylphenylalanine, 74%, m.p. 145-147° (lit.[322,480] 146°, 150-151°).

N-Acetyl-D-phenylalanine.

D-Phenylalanine gave, by the above method, N-acetyl-D-phenylalanine, 72%, m.p. 170-171°, $[\alpha]_D^{25}$ = -48.5°, c = 2% in absolute ethanol (lit.[323] 172°, $[\alpha]_D^{26}$ = -51° in absolute ethanol).

N-Acetyl-L-phenylalanine.

L-Phenylalanine gave, by the above method, N-acetyl-L-phenylalanine, 68%, m.p. 171-172°, $[\alpha]_D^{25}$ = 47.1°, c = 1.93% in absolute ethanol (lit.[323] 172°, $[\alpha]_D^{26}$ = -51° in absolute ethanol for the D isomer).

N-Acetyltryptophan.

Tryptophan was acetylated by the method of Town[322] to give N-acetyltryptophan, 81%, m.p. 204-206° (lit.[324] 205-206°).

N-Acetyl-D-tryptophan.

D-Tryptophan was acetylated according to du Vigneaud and Sealock[324] to give N-acetyl-D-tryptophan, 70%, m.p. 179-182°, $[\alpha]_D^{25}$ = -25°, c = 1% in water with 1 equivalent of sodium hydroxide (lit.[324,277] 189-190°; $[\alpha]_D^{31}$ = 29°, c = 1% in water with 1 equivalent of sodium hydroxide for the L isomer : 180-181°; $[\alpha]_D^{25}$ = 30 ± 1°, c = 1.23% in water with 1 equivalent of sodium hydroxide for the L isomer).

N-Acetyl-L-tryptophan.

L-Tryptophan similarly gave N-acetyl-L-tryptophan, 73%, m.p. 178-181°, $[\alpha]_D^{25}$ = 21° c = 1% in water with 1 equivalent of sodium hydroxide (lit.[324,277] as above).

(c)
PREPARATION OF THE N-TRIFLUOROACETATES.

N-Trifluoroacetylphenylalanine.

The method employed was essentially that of Weygand and Leising.[327] Phenylalanine (0.825 g, 0.005 mole) was suspended in dry benzene (20 ml) and, with vigorous stirring, trifluoroacetic anhydride (0.8 ml, 0.005 mole) added. The solution was warmed to 70°, the solvent, trifluoroacetic acid, and unreacted trifluoroacetic anhydride removed under reduced pressure and the residue crystallised from benzene. N-Trifluoroacetylphenylalanine, 0.96 g (74%) was obtained; m.p. 123-125° (lit.[481,329] 121-123°, 125.6-126.8°). This compound was also prepared by the method of Schallenberg and Calvin.[329] Phenylalanine (0.825 g, 0.005 mole) was dissolved in 1N sodium hydroxide (5 ml, 0.005 mole) water (20 ml), and ethyl thioltrifluoroacetate (1.0 ml, 0.008 mole) added. The mixture was shaken for 72 hours, made acid with concentrated hydrochloric acid, cooled in ice, and the solid filtered off. Recrystallisation from benzene gave N-trifluoroacetylphenylalanine, 0.98 g (76%) m.p. 126-128° (lit.[481,329] 121-123°, 125.6-126.8°).

Also prepared by the former method were:-

N-Trifluoroacetyl-m-methylphenylalanine, 71%, m.p. 116-117°. Calc. for $C_{12}H_{12}NO_3F_3$: C, 52.37; H, 4.36; N, 5.09; F, 20.7. Found: C, 52.33; H, 4.21; N, 5.20; F, 20.8%.

N-Trifluoroacetyl-p-methylphenylalanine, 76%, m.p. 134.5-136°. Calc. for $C_{12}H_{12}NO_3F_3$: C 52.37; H, 4.36; N, 5.09; F, 20.7. Found: C, 52.65; H, 4.39; N, 5.20; F, 20.4%.

N-Trifluoroacetyl-o-fluorophenylalanine, 68%, m.p. 132-133°. Calc. for $C_{11}H_9NO_3F_4$: C, 47.31; H, 3.23; N, 5.02; F, 27.2. Found: C, 47.48; H, 3.16; N, 4.78; F, 26.9%.

N-Trifluoroacetyl-m-fluorophenylalanine, 73%, m.p. 112-113°, followed by solidification and finally melting at 119-120°. Calc. for $C_{11}H_9NO_3F_4$: C, 47.31; H, 3.23; N, 5.02; F, 27.2. Found: C, 47.52; H, 3.27; N, 4.87; F, 27.2%.

N-Trifluoroacetyl-p-fluorophenylalanine, 73%, m.p. 144.5-145.5°. Calc. for $C_{11}H_9NO_3F_4$: C, 47.31; H, 3.23; N, 5.02; F, 27.2. Found : C, 47.51; H, 3.38; N, 4.96; F, 27.1%.

N-Trifluoroacetyl-m-bromophenylalanine, 88%, m.p. 135-137°. Calc. for $C_{11}H_9NO_3F_3Br$: C, 38.83; H, 2.65; N, 4.12; F, 16.7; Br, 23.5. Found: C, 38.84; H, 2.70; N, 4.23; F, 16.4; Br, 23.7%.

N-Trifluoroacetyl-p-bromophenylalanine, 85%, m.p. 147-148° and then resolidifying, finally melting at 160-162°. Calc. for $C_{11}H_9NO_3F_3Br$: C, 38.83; H, 2.65; N, 4.12; F, 16.7; Br, 23.5. Found: C, 38.98; H, 2.47; N, 4.25; F, 16.3; Br, 23.2%.

N-Trifluoroacetyl-2,4-difluorophenylalanine, 67%, m.p. 131-133°. Calc. for $C_{11}H_8NO_3F_5$: C, 44.44; H, 2.69; N, 4.71; F, 32.5. Found: C, 44.35; H, 2.70; N, 4.73; F, 32.5%.

N-Trifluoroacetyl-m-trifluoromethylphenylalanine, 82%, m.p. 143-145°. Calc. for $C_{12}H_9NO_3F_6$: C, 43.77; H, 2.74; N, 4.26; F, 34.7. Found: C, 44.00; H, 2.83; N, 4.27; F, 34.8%.

N-Trifluoroacetyl-p-trifluoromethylphenylalanine, 76%, m.p. 142-144°. Calc. for $C_{12}H_9NO_3F_6$: C, 43.77; H, 2.74; N, 4.26; F, 34.7. Found: C, 43.84; H, 2.63; N, 4.15; F, 34.9%.

N-Trifluoroacetylphenylglycine, 57%, m.p. 159-160°. Calc. for $C_{10}H_8NO_3F_3$: C, 48.59; H, 3.24; N, 5.67; F, 23.1. Found: C, 48.70; H, 3.18; N, 5.87; F, 22.9%.

N-Trifluoroacetyl-D-phenylalanine, 68%, m.p. 120-121°, $[\alpha]_D^{25} = -29°$, c = 1% in water with 1 equivalent of sodium hydroxide (lit.[329] 119.4-120.6° $[\alpha]_D^{25} = 36.4°$, c = 0.0187 g in 5.0 ml of glacial acetic acid for the L isomer).

N-Trifluoroacetyl-L-phenylalanine, 72%, m.p. 119-120°, $[\alpha]_D^{25}$ = 32°, c = 1% in water with 1 equivalent of sodium hydroxide (lit.[329] as above).

N-Trifluoroacetyl-D-tryptophan.

D-Tryptophan was reacted with ethyl thioltrifluoroacetate according to Schallenberg and Calvin,[329] but with a reaction time of 72 hours. N-Trifluoroacetyl-D-tryptophan was obtained in a yield of 73%, m.p. 163-164°, $[\alpha]_D^{25}$ = -31°, c = 1% in water with 1 equivalent of sodium hydroxide (lit.[329] 162-163° for the L isomer).

N-Trifluoroacetyl-L-tryptophan.

In the same manner, N-trifluoroacetyl-L-tryptophan was obtained in 66% yield, m.p. 163-164°, $[\alpha]_D^{25}$ =26°, c = 1% in water with 1 equivalent of sodium hydroxide (lit.[329] 162-163°).

N-Trifluoroacetyltryptophan.

This compound was likewise prepared in 75% yield, m.p. 155-157° (lit.[482] 152-154°).

N-Trifluoroacetyl-L-tyrosine.

L-Tyrosine (1.81 g, 0.01 mole) was suspended in dry benzene (20 ml) and trifluoroacetic anhydride (3.2 ml, 0.02 mole) added with stirring. The solution was warmed to 70°, the solvent, excess anhydride, and trifluoroacetic acid removed in vacuo, and the residue crystallised from benzene to give N-trifluoroacetyl-L-tyrosine, 79%, m.p. 195-196°, $[\alpha]_D^{25}$ =

43^{o}, c = 1% in water with 1 equivalent of sodium hydroxide (lit.[329] 192-193^{o}, $[\alpha]_D^{20} = 44.8^{o}$, c = 0.4% in water with 1 equivalent of sodium hydroxide.

N-Trifluoroacetyl-D-tyrosine.

D-Tyrosine gave in an analogous manner, N-trifluoroacetyl-D-tyrosine, 78%, m.p. 193-195^{o}, $[\alpha]_D^{25} = -52^{o}$, c = 1% in water with 1 equivalent of sodium hydroxide (lit.[329] 192-193^{o}, $[\alpha]_D^{20} = 44.8^{o}$, c = 0.47% in water with 1 equivalent of sodium hydroxide for the L isomer).

N-Trifluoroacetyl-p-methoxyphenylalanine methyl ester.

N-Trifluoroacetyl-D-tyrosine (0.227 g, 0.001 mole), methyl iodide (2.5 ml), and silver oxide (1 g) in dry acetone (10 ml) were stirred overnight. The silver oxide was removed by filtration and the residue, after removal of the solvent, was sublimed at 140^{o}/15 mm. N-Trifluoroacetyl-p-methoxyphenylalanine methyl ester, 0.210 g (69%), m.p. 76-78^{o} after a further sublimation was obtained; mass spectrum, M^{+}, m/e = 305 (m.wt. = 305); Calc. for $C_{13}H_{14}NO_4F_3$: C, 51.15; H, 4.59; N, 4.59; F, 18.7. Found: C, 51.05; H, 4.64; N, 4.37; F, 19.3%.

(d) PREPARATION OF THE AMINO ACIDS AND α-ACETAMIDOCINNAMIC ACIDS VIA THE AZLACTONE METHOD.

α-ACETAMIDOCINNAMIC ACID.

The method employed was that of Herbst and Shemin.[336] Benzal-

dehyde (21.2 g, 0.2 mole), aceturic acid (15.6 g, 0.134 mole), sodium acetate (8.2 g, 0.1 mole), and acetic anhydride (36 g, 0.34 mole) were heated on the water-bath until solution became complete (20-30 minutes). The resulting solution was heated under reflux for 1 hour and then cooled in the refrigerator overnight. Water (40 ml) was added to the solid mass which was broken up and filtered off. Recrystallisation from ethyl acetate/X-60 gave 4-benzylidene-2-methyl-5-oxazolone, 13.0 g (52%), m.p. 149-152°. The azlactone (10 g) was heated under reflux for 4 hours with acetone (35 ml) and water (90 ml), acetone added until solution was complete, and the solution boiled for a further few minutes in the presence of charcoal. The solution was filtered and α-acetamido-cinnamic crystallised on cooling. The yield was 9.5 g (86%) m.p. 196° (lit.[336] 191-192°).

α-ACETAMIDO-β-(p-FLUOROPHENYL)-ACRYLIC ACID.

2-Methyl-4-p-Fluorobenzylidene-5-oxazolone was similarly prepared from p-fluorobenzaldehyde. The yield was 56%, m.p. 152-153°. Calc. for $C_{11}H_8NO_2F$: C, 64.39; H, 3.90; N, 6.83; F, 9.3. Found: C, 64.49; H, 3.95; N, 6.91; F, 9.0%. Hydrolysis gave α-acetamido-β-(p-fluoro-phenyl)-acrylic acid, 81%, m.p. 216-220°. Calc. for $C_{11}H_{10}NO_3F$: C, 59.20; H, 4.48; N, 6.28; F, 8.5. Found: C, 59.07; H, 4.73; N, 5.99; F, 8.1%.

m-METHYLPHENYLALANINE.

m-Tolualdehyde.

This aldehyde, b.p. 87-89°/17 mm (lit.[483] 93-94°/17 mm) was

prepared in 42% yield from m-bromotoluene by the method of Smith and Bayliss.[484]

2-Methyl-4-m-methylbenzylidene-5-oxazolone was prepared in 50% yield from m-tolualdehyde according to Herbst and Shemin.[336] The m.p. was 105-108°. Further purification was difficult and was not persevered with.

α-Acetamido-β-(m-methylphenyl)-acrylic acid.

The crude azlactone (10 g) in water (35 ml) and acetone (100 ml) was heated under reflux for 4 hours. The solvent was removed in vacuo, and the residue crystallised from acetone/water to give the acrylic acid, 85%, m.p. 186-187° as yellow plates. Recrystallisation from chloroform/acetone or water gave white needles, m.p. 186-187°. Calc. for $C_{12}H_{13}NO_3$: C, 65.76; H, 5.98; N, 6.39. Found: C, 65.82; H, 5.90; N, 6.27%.

N-Acetyl-m-methylphenylalanine.

The acrylic acid (6.8 g) was suspended in 95% ethanol (150 ml) and hydrogenated in the presence of 5% palladium on carbon (1 g) at 4 atmospheres for 4 hours. The catalyst was removed by filtration through Celite and, after removal of the solvent in vacuo, the residue was crystallised from water to give N-acetyl-m-methylphenylalanine, 5.3 g (77%), m.p. 149-151° (lit.[485] 148-149°).

m-Methylphenylalanine.

The acetylated amino acid (5 g) was heated under reflux for 18 hours with 1N hydrochloric acid (100 ml). The acid and water were removed in vacuo, water (50 ml) added, and that removed in vacuo. The residue was dissolved in a little hot water, the pH brought to 6 with conc. ammonia, and the amino acid filtered off after cooling. The yield was 91%, m.p. 210-213° d (lit.[486] 242-246° d).

p-METHYLPHENYLALANINE.

2-Methyl-4-p-methylbenzylidene-5-oxazalone, 54%, m.p. 130-135° was similarly prepared. Further recrystallisation raised the melting point to 136-137°. Calc. for $C_{12}H_{11}NO_2$: C, 71.65; H, 5.47; N, 6.98. Found: C, 71.95; H, 5.47; N, 7.05%.

α-Acetamido-β-(p-methylphenyl)-acrylic acid was obtained in a yield of 77%, m.p. 225-230°. Recrystallisation from chloroform/acetone raised the melting point to 234-235°. Calc. for $C_{12}H_{13}NO_3$: C, 65.76; H, 5.98; N, 6.39. Found: C, 66.04; H, 5.83; N, 6.26%.

N-Acetyl-p-methylphenylalanine, m.p. 166.5-168° (lit.[485] 163.5-164.5°) was obtained in a yield of 81%.

p-Methylphenylalanine, m.p. 214-218° d (lit.[486] 276-279° d) was obtained in 98% yield.

m-TRIFLUOROMETHYLPHENYLALANINE.

m-Bromobenzotrifluoride.

Benzotrifluoride was brominated[487] to give the above compound in 55% yield, b.p. 154-157° (lit.[487] 52%, b.p. 151-152°).

m-Trifluoromethylbenzaldehyde.

m-Bromobenzotrifluoride was converted to its Grignard reagent by the method of Smith and Bayliss.[484] The Grignard reagent was reacted with N-methylformanilide according to Gilman et al.[488] to give m-trifluoromethylbenzaldehyde, 50%, b.p. 72-73°/17 mm (lit.[488] 51-59%, b.p. 64-66°/10 mm).

2-Methyl-4-m-trifluoromethylbenzylidene-5-oxazalone.

m-Trifluoromethylbenzaldehyde, aceturic acid, acetic anhydride, and sodium acetate were reacted according to Filler and Novar[344] to give the azlactone, 67%, m.p. 123-125° (lit.[344] 84%, m.p. 130-131°).

α-Acetamido-β-(m-trifluoromethylphenyl)-acrylic acid.

Hydrolysis of the azlactone according to Filler and Novar[344] gave the acrylic acid, 76%, m.p. 219-220° (lit.[344] 81%, m.p. 225-226°).

N-Acetyl-m-trifluoromethylphenylalanine.

α-Acetamido-β-(m-trifluoromethylphenyl)-acrylic acid was hydrogenated according to Filler and Novar[344] to give N-acetyl-m-trifluoromethylphenylalanine, 86%, m.p. 129-130° (lit.[344] 80%, m.p. 132-133°).

m-Trifluoromethylphenylalanine.

This amino acid was prepared from the N-acetyl compound by the method described for the preparation of m-methylphenylalanine. The yield was 53%. Recrystallisation from water gave the pure amino acid, m.p. 198-202° d (lit.[344] 219-222° d). Calc. for $C_{10}H_{10}NO_2F_3$: C, 51.50; H, 4.29; N, 6.01; F, 24.5%. Found: C, 51.83; H, 4.41; N, 5.71; F, 25.0%.

(e) PREPARATION OF THE AMINO ACIDS BY THE MALONIC ESTER METHOD.

o-FLUOROPHENYLALANINE.

o-Fluorobenzyl bromide.

The method used was essentially that of Olah et al.[489] o-Fluorotoluene (11.0 g, 0.1 mole) was dissolved in dry benzene (15 ml). The solution was heated under reflux, irradiated with a 200W incandescent light, and dry bromine (4.5 ml, 0.09 mole) added dropwise over 1 hour. After a further hour, heated under reflux, the solution was fractionated under reduced pressure to give o-fluorobenzyl bromide, 12.3 g (74%), b.p. 96-100°/25 mm (lit.[489] 195-202°).

Diethyl α-acetamido-α-(o-fluorobenzyl)-malonate.

Sodium (0.69 g, 0.03 mole) was dissolved in dry ethanol (50 ml), diethyl acetamidomalonate (6.51 g, 0.03 mole), and o-fluorobenzyl bromide (4.9 g, 0.026 mole) were added. The mixture was heated under reflux for 4 hours, filtered hot, and water (100 ml) added to the filtrate.

The diethyl α-acetamido-α-(o-fluorobenzyl)-malonate was filtered off after cooling, and was crystallised from aqueous ethanol. The yield of material, m.p. 111-112° (lit.[341] 107-108°) was 60%.

o-Fluorophenylalanine.

Diethyl α-acetamido-α-(o-fluorobenzyl)-malonate (3.25 g, 0.01 mole) and 48% hydrobromid acid (30 ml) were heated under reflux for 6 hours, the solution brought to pH 6 with concentrated ammonia, and concentrated in vacuo until crystallisation began. The amino acid was filtered off and washed well with water to remove ammonium bromide. The yield of acid, m.p. 212-214° d (lit.[490] 258.5-259° d) was 1.1 g (58%).

2,4-DIFLUOROPHENYLALANINE.

4-Amino-2-fluorotoluene.

2-Amino-4-nitrotoluene, m.p. 105-108° (lit.[491] 107° was prepared by nitration of o-toluidine according to Ullmann et al.[491] 2-Amino-4-nitrotoluene (152 g, 1 mole) was suspended in a mixture of concentrated hydrochloric acid (210 ml) and water (300 ml). The mixture was stirred vigorously, cooled to 0°C with a dry ice/acetone bath, and this temperature maintained while a solution of sodium nitrite (87 g) in water (220 ml) was added dropwise. The filtered diazonium solution was stirred at 0°C while an ice-cold solution of sodium fluoborate (138 g, 1.25 mole) in water (300 ml) was added over 10 minutes. After stirring for ½ hour, the diazonium fluoborate was filtered off, washed successively with a

little cold water, methanol, ether, and dried. The yield was 160 g (85%). The diazonium salt was decomposed in dry chlorobenzene according to Brown et al.[492] and the resulting 2-fluoro-4-nitrotoluene reduced in situ with iron and acetic acid[492] to give 4-amino-2-fluorotoluene, 78%, b.p. 94-97°/16 mm (lit.[492] 202-204°).

2,4-Difluorotoluene.

4-Amino-2-fluorotoluene was converted to the diazonium fluoborate in the manner described above for 2-amino-4-nitrotoluene. The yield was 79%. Thermal decomposition of the dry diazonium salt in the usual way[493] gave 2,4-difluorotoluene, 64%, b.p. 113-115° (lit.[492] 113-117°).

2,4-Difluorobenzyl bromide.

Free radical bromination of 2,4-difluorotoluene by the method of Olah et al.[489] gave 2,4-difluorobenzyl bromide, 70%, b.p. 74-76°/13 mm. A sample, b.p. 75°/13 mm was submitted for analysis. Calc. for $C_7H_5F_2Br$: C, 40.57; H, 2.42; F, 18.4. Found: C, 40.47; H, 2.41; F, 18.4%.

Diethyl α-acetamido-α-(2,4-difluorobenzyl)-malonate.

The reaction of 2,4-difluorobenzyl bromide (5.42 g, 0.026 mole), diethyl acetamidomalonate (6.51 g, 0.03 mole), and sodium (0.69 g, 0.03 mole) in dry ethanol (50 ml) under the conditions described in the preparation of o-fluorophenylalanine gave diethyl α-acetamido-α-(2,4-difluorobenzyl)-malonate, 6.6 g (74%), m.p. 133-134°. Calc. for

$C_{16}H_{19}NO_5F_2$: C, 55.98; H, 5.54; N, 4.08; F, 11.1. Found: C, 56.09; H, 5.65; N, 4.01; F, 11.3%.

2,4-Difluorophenylalanine.

The malonic ester was converted to the amino acid by the method described in the synthesis of o-fluorophenylalanine. The yield of amino acid, m.p. 215-220° d, was 76%. Recrystallisation from water raised the melting point to 230-234° d. Calc. for $C_9H_9NO_2F_2$: C, 53.73; H, 4.48; N, 6.97; F, 18.9. Found: C, 53.73; H, 4.35; N, 7.11; F, 18.9%.

N-Acetyl-2,4-difluorophenylalanine.

Diethyl α-acetamido-α-(2,4-difluorobenzyl)-malonate (1.72 g, 0.005 mole) was heated under reflux for 4 hours with 2.5N sodium hydroxide (25 ml), the pH adjusted to 2-3 with concentrated hydrochloric acid, and reflux continued for a further hour. On cooling the product crystallised. The yield was 0.78 g (65%), m.p. 148-151°. Calc. for $C_{11}H_{11}NO_3F_2$: C, 54.33; H, 4.53; N, 5.76; F, 15.6. Found: C, 53.96; H, 4.59; N, 5.70; F, 15.4%.

m-BROMOPHENYLALANINE.

m-Bromobenzyl bromide.

This compound was obtained in 75% yield by free-radical bromination of m-bromotoluene. It had b.p. 122-126°/12 mm, m.p. 35-38° (lit.[494] 40°).

Diethyl α-acetamido-α-(m-bromobenzyl)-malonate.

m-Bromobenzyl bromide (6.75 g, 0.027 mole), diethyl acetamido-malonate (6.51 g, 0.03 mole), and sodium (0.69 g, 0.03 mole) in dry ethanol (50 ml) were reacted as described in the preparation of o-fluorophenylalanine to give diethyl α-acetamido-α-(m-bromobenzyl)-malonate, 7.6 g (79%), m.p. 90-96°. Further recrystallisation from aqueous ethanol raised the melting point to 100-101°. Calc. for $C_{16}H_{20}NO_5Br$: C, 49.73; H, 5.18; N, 3.63; Br, 20.8%. Found: C, 50.06; H, 5.40; N, 3.52; Br, 20.6%.

m-Bromophenylalanine.

The malonic ester (5.8 g, 0.015 mole) and 48% hydrobromic acid (50 ml) were heated under reflux for 6 hours. The acid was removed under reduced pressure, water (50 ml) added, and that removed under reduced pressure. The residue was dissolved in hot water (30 ml) and the pH brought to 6 with concentrated ammonia. The amino acid was filtered off after cooling and crystallised from water, m.p. 215-220° d. The yield was 64%. Calc. for $C_9H_{10}NO_2Br$: C, 44.26; H, 4.10; N, 5.74; Br, 32.8. Found: C, 44.30; H, 4.11; N, 5.42; Br, 32.9%.

p-BROMOPHENYLALANINE.

p-Bromobenzyl bromide.

Free-radical bromination of p-bromotoluene by the method of Olah et al.[489] gave p-bromobenzyl bromide, 86%, b.p. 140-146°/22 mm, m.p. 55-60° (lit.[495] 61°).

Diethyl α-acetamido-α-(p-bromobenzyl)-malonate.

This compound was prepared from p-bromobenzyl bromide by the method described in the preparation of o-fluorophenylalanine. The yield was 75%, m.p. 125-132°. Recrystallisation from aqueous ethanol raised the melting point to 133-134°. Calc. for $C_{16}H_{20}NO_5Br$: C, 49.73; H, 5.18; N, 3.63; Br, 20.8. Found: C, 50.09; H, 5.41; N, 3.88; Br, 20.5%.

p-Bromophenylalanine.

Diethyl α-acetamido-α-(p-bromobenzyl)-malonate (19.3 g, 0.05 mole) was heated under reflux for 6 hours with 48% hydrobromic acid (75 ml). The pH was adjusted to 6 with concentrated ammonia, the acid filtered off, and washed with water; yield 12.2 g (quant.) m.p. 215-219° d (lit.[496] 258° d).

p-TRIFLUOROMETHYLPHENYLALANINE.

Ethyl p-trifluoromethylbenzoate.

p-Trifluoromethylbenzoyl chloride (10 g) was added to dry pyridine (10 g) and anhydrous ethanol (25 ml) cautiously added. The mixture was heated under reflux for 3 hours, poured into dilute hydrochloric acid and the ester extracted with ether. After drying the extracts with magnesium sulphate, the ether was removed under reduced pressure and the residue distilled in vacuo to give ethyl p-trifluoromethylbenzoate, 9.1 g (94%), b.p. 102-104°/20 mm (lit.[497] 80-80.5°/5.5 mm).

p-Trifluoromethylbenzyl alcohol.

The ester (9.1 g) in dry ether (50 ml) was added to a stirred suspension of lithium aluminium hydride (1.71 g) in dry ether (50 ml). After the addition, stirring was continued for 15 minutes and wet ether, water, and dilute sulphuric acid added successively. The ether layer was separated, the aqueous layer extracted with ether, and the extracts dried. Removal of the ether in vacuo followed by distillation in vacuo gave p-trifluoromethylbenzyl alcohol, 6.7 g (85%), b.p. 112-113°/24 mm (lit.[497] 78.5-80°/4 mm).

p-Trifluoromethylbenzyl bromide.

The alcohol (6.7 g, 0.038 mole), 48% hydrobromic acid (20 g, 0.12 mole), and concentrated sulphuric acid (3 ml) were heated under reflux for 5 hours. The mixture was poured into water and the benzyl bromide extracted with ether. The ether extracts were dried, the ether removed, and the residue distilled to give p-trifluoromethylbenzyl bromide, 8.4 g (90%), b.p. 96-97°/22 mm (lit.[497] 65-66°/5 mm).

Diethyl α-acetamido-α-(p-trifluoromethylbenzyl)-malonate.

p-Trifluoromethylbenzyl bromide, diethyl acetamidomalonate, and sodium ethoxide gave, in the usual way, the malonic ester, 64%, m.p. 129-131°. Recrystallisation from aqueous ethanol raised the melting point to 132-133°. Calc. for $C_{17}H_{20}NO_5F_3$: C, 54.40; H, 5.33; N, 3.73; F, 15.2. Found: C, 54.10; H, 5.60; N, 4.00; F, 15.1%.

p-Trifluoromethylphenylalanine.

p-Trifluoromethylphenylalanine was prepared from the above ester by the same method employed in the preparation of m-bromophenylalanine. The yield of recrystallised amino acid, m.p. 203-208° d, was 60%. Calc. for $C_{10}H_{10}NO_2F_3$: C, 51.50; H, 4.29; N, 6.01; F, 24.5. Found: C, 51.30; H, 4.34; N, 5.82; F, 24.2%.

(f) 5-FLUOROTRYPTOPHAN.

p-Fluorophenylhydrazine.

p-Fluoroaniline (22 g, 0.2 mole) was converted to the diazonium salt and reduced with sodium sulphite according to Schiemann and Winkelmüller[374] to give 19.3 g (77%) of p-fluorophenylhydrazine, b.p. 125-128°/17 mm (lit.[374] 133°/25 mm) which solidified on cooling.

o-Fluorophenylhydrazone of γ-acetamido-γ,γ-dicarbethoxybutyraldehyde.

The method of this and subsequent steps is essentially that of Rinderknecht and Niemann.[360] Diethyl acetamidomalonate (21.7 g, 0.1 mole) was suspended in benzene (35 ml) containing a catalytic amount of sodium methoxide [sodium (20 mg) in dry methanol (1 ml)]. The mixture was cooled to 10° and freshly distilled acrolein (6.9 ml, 5.6 g, 0.1 mole) in benzene (10 ml) added dropwise with stirring. The temperature rose to 40° and stirring was continued for 1 hour. The resulting solution was filtered and to the filtrate acetic acid (3 ml) and p-fluorophenylhydrazine (12.6 g, 0.1 mole) were added, the solution warmed to 50° and

then cooled in the refrigerator for 2 days. Contrary to the literature, no precipitate of the p-fluorophenylhydrazone was obtained. Removal of the solvent in vacuo gave a yellow gum.

Ethyl α-acetamido-β-(5-fluoro-3-indole)-propionate.

The yellow gum from the preceding step was suspended in water (200 ml) and concentrated sulphuric acid (10 ml), and the mixture heated under reflux, with stirring, for 3 hours. The resulting mixture was extracted with ether, the ether extracts dried, and the ether removed by distillation. A red gum was obtained which, after trituration with ethanol, partly crystallised. The solid was filtered off and recrystallised from aqueous ethanol to give the above compound, 7.1 g (20% based on p-fluorophenylhydrazine), m.p. 139-140° (lit.[360] 137-138°).

N-Acetyl-5-fluorotryptophan.

Ethyl α-acetamido-β-(5-fluoro-3-indole)-propionate (5.9 g) and 10% sodium hydroxide solution (45 ml) were heated under reflux for 4 hours, the solution cooled and acidified. The α-acetamido-α-carboxy-β-(5-fluoro-3-indole)-propionic acid which crystallised was collected and heated under reflux with water (60 ml) for 1 hour. On cooling N-acetyl-5-fluorotryptophan crystallised, 2.84 g (66%) of material, m.p. 193-196° (lit.[360] 189-192°) was obtained.

(g) 6-FLUOROTRYPTOPHAN:

(i) from 4-amino-2-nitrotoluene.

4-Fluoro-2-nitrotoluene.

4-Amino-2-nitrotoluene was converted to the above compound in an overall yield of 45% according to Steck and Fletcher.[498] The yield of the diazonium fluoborate was 80%, thermal decomposition of which gave 4-fluoro-2-nitrotoluene (56%), b.p. 102-103°/20 mm (lit.[498] 108-109°/23 mm).

Potassium salt of ethyl 4-fluoro-2-nitrophenylpyruvate.

Potassium (19.5 g, 0.5 mole) was cautiously dissolved in dry ethanol (125 ml) under nitrogen. To this solution of potassium ethoxide, dry ether (600 ml) was added, followed by redistilled ethyl oxalate (65.7 g, 0.45 mole) and 4-fluoro-2-nitrotoluene (77.5 g, 0.5 mole). The red potassium salt of ethyl 4-fluoro-2-nitrophenylpyruvate, 13.0 g (66%) was filtered off after 2 days.

6-Fluoroindole-2-carboxylic acid.

The method employed was essentially that of Allen et al.[381] The potassium salt from the preceding section (93 g) was suspended in 0.880 ammonia (630 ml) and water (770 ml), and a hot solution of ferrous sulphate (660 g) in water (750 ml) added. The mixture was heated under reflux for 3 hours and filtered. The filtrate was acidified, cooled, and the 6-fluoroindole-2-carboxylic acid filtered off. The solid material

from the ferrous sulphate-ammonia reduction was dried, powdered, placed in a soxhlet and extracted with ether for 24 hours. Removal of the ether by distillation gave crude ethyl 6-fluoroindole-2-carboxylate which was heated under reflux with 2N sodium hydroxide (300 ml) and ethanol (200 ml) for 1 hour. The resulting solution was cooled, acidified, and the 6-fluoroindole-2-carboxylic acid filtered off. The combined acid preparations were recrystallised from aqueous ethanol. 6-Fluoroindole-2-carboxylic acid, 29.4 g, (52%), m.p. 235-240° d (lit.[381] 245-246° d).

6-Fluoroindole.

(a) 6-Fluroindole-2-carboxylic acid (11.0 g) was heated strongly in a 250 ml flask fitted with a reflux condenser. Heating was continued until no more gaseous products were evolved and sublimation ceased. Steam distillation of the residue and the material which had sublimed gave 6-fluoroindole (4.3 g, 52%), m.p. 74.5-76° (lit.[381] 75°).

(b) 6-Fluoroindole-2-carboxylic acid (26.9 g, 0.15 mole), copper chromite (18.1 g, 0.075 mole), and freshly distilled quinoline (100 ml) were heated under reflux for 8 hours, and the hot solution poured onto a mixture of ice and hydrochloric acid. The mixture was filtered, the filtrate extracted with ether, the extracts dried, and the ether removed in vacuo. Steam distillation of the residue gave 6-fluoroindole, 1.35 g (7%), m.p. 74-75° (lit.[381] 75°).

6-Fluorogramine.

The method employed in this and subsequent steps is essentially that of Bergmann and Hoffmann.[383] With cooling and stirring, 6-fluoroindole (4 g) in dioxan (30 ml) was added to a mixture of dioxan (30 ml), acetic acid (30 ml), formalin solution (2.5 ml), and aqueous dimethylamine solution (55%, 3.5 ml) over 30 minutes. After standing for 12 hours, the 6-fluorogramine, 5.0 g (87%), m.p. 132-137° (lit.[383] 137°) was filtered off.

Ethyl α-acetamido-β-(6-fluoro-3-indole)-propionate.

A mixture of 6-fluorogramine (5.0 g), diethyl acetamidomalonate (5.6 g), sodium hydroxide (0.4 g), and toluene (50 ml) were heated under reflux, in an atmosphere of nitrogen, for 4 hours. On cooling ethyl α-acetamido-β-(6-fluoro-3-indole)-propionate crystallised. The crystals were collected and recrystallised from isopropanol to give this compound, 4.3 g (45%), m.p. 201-205° (lit.[383] 201°).

N-Acetyl-6-fluorotryptophan.

The above ester (4.0 g) and 10% sodium hydroxide solution (35 ml) were heated under reflux for 4 hours. The resulting solution was filtered, cooled, and acidified. α-Carboxy-β-(6-fluoro-3-indole)-propionic acid, 2.36 g (80%) was obtained. The acid was decarboxylated by heating under reflux with water (60 ml). The solution, on cooling, gave N-acetyl-6-fluorotryptophan, 1.74 g (86%), m.p. 192-184° (lit.[383] 178-179°).

(ii) from indoline.

6-Nitroindoline.

The method employed was essentially that described in the literature.[384,385] Indoline (40 g) was dissolved, with stirring, in concentrated sulphuric acid (330 ml), the temperature being kept below 5° with an ice salt bath. At the same temperature, a mixture of concentrated nitric (15 ml) and concentrated sulphuric acid (330 ml) was added dropwise. After stirring for a further hour, the mixture was poured onto ice (1.5 kg) and the solution obtained made alkaline with sodium hydroxide solution, the temperature being kept below 40°. Contrary to the report of Ikan et al.[385] the product was precipitated before the solution reached pH 6, and not in the range pH 6-8. The 6-nitroindoline obtained was recrystallised from X-60/ether; 43.9 g (80%) of material m.p. 68-69° (lit.[385] 66.5-67.5°) was obtained.

1-Acetyl-6-nitroindoline.

6-Nitroindoline (24.6 g, 0.15 mole) was cautiously added to refluxing acetic anhydride (100 ml). The mixture was heated under reflux for a further hour, cooled, and the solid which crystallised, filtered off. This was washed with water and dried to give 1-acetyl-6-nitroindoline, 25.0 g (81%), m.p. 159-160° (lit.[385] 154.5-155°).

1-Acetyl-6-aminoindoline.

1-Acetyl-6-nitroindoline (30.9 g, 0.15 mole) was suspended in absolute ethanol (180 ml), 5% palladium on carbon (4 g) added, and the

mixture hydrogenated for 2 hours while irradiated with an IR lamp. The reaction mixture was diluted to 400 ml with ethanol, heated to boiling, and filtered quickly through a hot Buchner funnel. 1-Acetyl-6-aminoindoline, 21.6 g (84%), m.p. 182-184° (lit.[385] 181°) was obtained.

1-Acetyl-6-fluoroindoline.

1-Acetyl-6-aminoindole (17.6 g, 0.1 mole) was converted to the diazonium fluoborate, 23.9 g (87%) in the usual way.[493] The diazonium fluoborate (23.9 g) was mixed with sand (50 g) and decomposed thermally.[493] Decomposition was extremely vigorous and the reaction was not further investigated.

(h) PREPARATION OF FLUORINATED CINNAMIC ACIDS.

Ethyl p-methylcinnamate.

The method employed was essentially that of Kucherov et al.[401] A solution of p-tolualdehyde (2.40 g, 0.02 mole) and carboethoxymethylidenetriphenylphosphorane (16.06 g, 0.046 mole) in benzene (200 ml) was heated under reflux, in an atmosphere of nitrogen for 6 hours. The benzene was removed under reduced pressure, the residue stirred well with X-4 and filtered. The solid triphenylphosphine oxide which was filtered off was washed well with X-4. Removal of the solvent from the combined filtrates gave the crude ester. Distillation under reduced pressure gave ethyl p-methylcinnamate, 3.32 g (87%), b.p. 166-168°/24 mm (lit.[499] 158-159°/17 mm).

p-Methylcinnamic acid.

(a) The ester (2.0 g) and 10% sodium hydroxide (30 ml) were heated under reflux until solution became complete. The solution was cooled, made acid, cooled in ice, and the acid filtered off. Recrystallisation from acetic acid/water gave p-methylcinnamic acid, 1.25 g (73%), m.p. 196-197° (lit.[500] 197°).

(b)[398] p-Tolualdehyde (2.40 g, 0.02 mole), malonic acid (2.18 g, 0.02 mole), pyridine (0.4 ml), and ethanol (10 ml) were heated under reflux for 3 days. Water (50 ml) was added, the solution cooled, the acid filtered off and recrystallised from acetic acid/water. p-Methylcinnamic acid, 1.48 g (45%), m.p. 197-199° (lit.[500] 197°) was obtained.

o-Fluorocinnamic acid.

Reaction of o-fluorobenzaldehyde (2.48 g, 0.02 mole) with the phosphorane as described in the preparation of ethyl p-methylcinnamate gave ethyl o-fluorocinnamate, 3.37 g (86%), b.p. 150-152°/25 mm (lit.[394] 140-141°/11 mm). Saponification of the ester (2.0 g) gave the acid, 1.43 g (83%), m.p. 179-183° (lit.[394] 175°).

m-Fluorocinnamic acid.

m-Fluorobenzaldehyde (2.48 g, 0.02 mole) was reacted with carbomethoxymethylidenetriphenylphosphorane (16.06 g, 0.046 mole) as described in the preparation of ethyl p-methylcinnamate. Ethyl m-fluorocinnamate, (84%) was obtained, b.p. 154-156°/26 mm (lit.[395] 137°/11 mm).

Saponification of the ester (2.0 g) gave the acid, 1.34 g (80%), m.p. 160-166° (lit.[395,501] 154-155°, 166.5°).

p-Fluorocinnamic acid.

p-Fluorobenzaldehyde (2.48 g, 0.02 mole) similarly gave ethyl p-fluorocinnamate, 3.31 g (83%), b.p. 136-138°/15 mm (lit.[394] 135-140°/11 mm). Saponification of the ester (2.0 g) gave p-fluorocinnamic acid, 1.40 g (82%), m.p. 204-206° (lit.[394,397] 208°, 202°).

2,4-Difluorocinnamic acid.

2,4-Difluorobenzaldehyde (3.10 g, 0.218 mole) was reacted as described previously to give ethyl 2,4-difluorocinnamate, 3.79 g (82%), b.p. 148-152°/24 mm which solidified on cooling, m.p. 35-40°. Recrystallisation from X-4 raised the melting point to 40-41°. Calc. for $C_{11}H_{10}O_2F_2$: C, 62.27; H, 4.72; F, 17.9. Found: C, 62.25; H, 4.61; F, 17.9%. Saponification of the ester (2 g) gave 2,4-difluorocinnamic acid, 1.40 g (81%), m.p. 205-206° (lit.[400] 206°).

m-Trifluoromethylcinnamic acid.

m-Trifluoromethylbenzaldehyde (3.48 g, 0.02 mole) similarly gave ethyl m-trifluoromethylcinnamate, 4.17 g (86%), b.p. 151-153°/22 mm which solidified on cooling, m.p. 38-40°. Recrystallisation from X-4 raised the melting point to 40-41°. Calc. for $C_{12}H_{11}O_2F_3$: C, 59.02; H, 4.51; F, 23.4. Found: C, 58.78; H, 4.60; F, 23.3%. Saponification

of the ester (2.0 g) gave the acid, 1.31 g (74%), m.p. 133-135° (lit.[399] 135.5-136.5°).

p-Fluorophenylpropionic acid.

p-Fluorocinnamic acid (1 g) in ethanol (50 ml) was hydrogenated at 4 atmospheres for 1 hour in the presence of 5% palladium on carbon (100 mg). The reaction mixture was filtered through celite, and the residue, after removal of the ethanol in vacuo, crystallised from X-4. p-Fluorophenylpropionic acid, 0.86 g (86%), m.p. 89-90° (lit.[406] 91°) was obtained.

α-Fluorocinnamic acid.

The method employed was essentially that of Bergmann and Shahak.[405] To a suspension of sodium hydride (50% dispersion in oil, 2.4 g, 0.05 mole) in dry xylene (50 ml) at 40-50°, a few drops of dry ethanol were added, followed by ethyl fluoroacetate (8.0 g, 0.055 mole) with stirring. At the same temperature and with stirring, a solution of ethyl fluoroacetate (5.3 g, 0.05 mole) was added dropwise. After ½ hour, the ethanol formed was distilled off; the reaction mixture being heated to the boiling point of xylene. A small quantity (∿ 5 ml) of xylene was distilled off and after ½ hour, benzaldehyde (5.3 g, 0.05 mole) in dry xylene (15 ml) was added. The mixture was heated under reflux for 15 minutes and poured into water (250 ml). The xylene layer was separated, washed successively with dilute sodium bicarbonate

solution and water, and dried. The xylene was removed in vacuo and the residue distilled. Ethyl α-fluorocinnamate, 6.7 g (65%), b.p. 158-160°/32 mm (lit.[405,403] 120-122°/20 mm, 132-134°/13 mm). The ester (5.8 g, 0.03 mole) was dissolved in ethanol (25 ml) and hot 25% potassium hydroxide solution (15 ml) added. After standing for ½ hour, the ethanol was removed in vacuo and concentrated hydrochloric acid (15 ml) added to the residue. The acid was filtered off and recrystallised from acetic acid/water; 4.5 g (75%) of acid, m.p. 157-160° (lit.[405] 157°) was obtained.

(i) PREPARATION OF 3-CARBOXY-7-FLUORODIHYDROISOCARBOSTYRIL.

2-Cyano-4-nitrotoluene.

Nitration of o-tolunitrile (117 g, 1 mole) using concentrated nitric acid (S.G. 1.42, 64 ml) and concentrated sulphuric acid (500 ml) according to Ruggli and Meyer[407] gave 2-cyano-4-nitrotoluene, 126 g (77%), m.p. 103-107° (lit.[407] 105°).

4-Amino-2-cyanotoluene.

2-Cyano-4-nitrotoluene (40.5 g, 0.25 mole) was dissolved in ethyl acetate (350 ml), 5% palladium on carbon (4 g) added, and the mixture hydrogenated at 4-5 atmospheres for 1½ hours while heated with an IR lamp. The catalyst was removed by filtration through Celite, the solvent removed in vacuo, and the residue crystallised from benzene/hexane. 4-Amino-2-cyanotoluene, 28.1 g (84%) was obtained, m.p. 89-91° (lit.[402] 92°).

2-Cyano-4-fluorotoluene.

4-Amino-2-cyanotoluene (79.2 g, 0.6 mole) was suspended in a mixture of concentrated hydrochloric acid (125 ml) and water (150 ml), and diazotised at 0^{o} with a solution of sodium nitrite (52.2 g) in water (150 ml). To the filtered diazonium solution at 0^{o}, a solution of sodium fluoborate (88 g) in water (180 ml) was added with stirring. After stirring for a further ½ hour, the diazonium fluoborate was filtered off, washed successively with water, ethanol and ether, and dried. The yield was 116 g (84%). Thermal decomposition in the usual way[493] gave 2-cyano-4-fluorotoluene (52%), b.p. 92-94^{o}/20 mm, which solidified on cooling. A sample was purified by recrystallisation from X-4, m.p. 44-45^{o}. Calc. for C_8H_6NF : C, 71.12; H, 4.45; N, 10.37; F, 14.1. Found: C, 71.30; H, 4.74; N, 10.61; F, 15.0%.

2-Cyano-4-fluorobenzyl bromide.

2-Cyano-4-fluorotoluene (28.9 g, 0.214 mole) was heated to 140^{o} and irradiated with a 200W globe. With stirring, bromine (35 g, 11.3 ml, 0.219 mole) was added over 2 hours. After the addition, the mixture was fractionated under reduced pressure to give 2-cyano-4-fluorobenzyl bromide, 29.8 g (65%), b.p. 132-136^{o}/12 mm. A sample b.p. 133^{o}/12 mm was submitted for analysis. Calc. for C_8H_5NBrF : C, 44.84; H, 2.34; N, 6.54; Br, 37.4. Found: C, 45.24; H, 2.36; N, 6.40; Br, 36.9%.

Diethyl α-acetamido-α-(2-cyano-4-fluorobenzyl)-malonate.

To a solution of sodium (3.45 g, 0.15 mole) in dry ethanol (120 ml), diethyl acetamidomalonate (32.6 g, 0.15 mole) was added and the mixture maintained at 50° for 1 hour. 2-Cyano-4-fluorobenzyl bromide (27.8 g, 0.13 mole) in the minimum amount of absolute ethanol was added dropwise and the solution maintained at 50° for 5 hours. The solution was then poured into water (450 ml). The product which crystallised on cooling was collected and recrystallised from chloroform/hexane; 34.6 g (76%) of diethyl α-acetamido-α-(2-cyano-4-fluorobenzyl)-malonate, m.p. 125-130° was obtained. Further recrystallisation from aqueous ethanol raised the melting point to 130-131°. Calc. for $C_{17}H_{19}N_2O_5F$: C, 58.28; H, 5.43; N, 8.00; F, 5.4. Found: C, 58.01; H, 5.43; N, 7.89; F, 5.1%.

3-Carboxy-7-fluorodihydroisocarbostyril.

The preceding compound (30 g) in 6N sodium hydroxide (50 ml) was heated under reflux for 50 hours. The pH was adjusted to 2 and reflux continued for a further 2 hours. The solution was then evaporated to dryness, 48% hydrobromic acid (70 ml) added, and the resulting solution heated to boiling. The hot solution was poured onto ice, the resulting solid collected and recrystallised from water with decolourising charcoal. 3-Carboxy-7-fluorodihydroisocarbostyril, 8.0 g (45%), m.p. 229-231° d was obtained. Calc. for $C_{10}H_8NO_3F$: C, 57.43; H, 3.83; N, 6.70; F, 9.1. Found: C, 57.32; H, 3.87; N, 6.81; F, 8.9%.

3-Carbomethoxy-7-fluorodihydroisocarbostyril.

The preceding acid (5 g) was added to a solution, prepared by the careful dropwise addition of thionyl chloride (2.2 ml) to ice cold absolute methanol (25 ml) at 0°. The resulting solution was then maintained at 40° for 1 hour, the excess methanol removed in vacuo and the residue dissolved in chloroform. The chloroform solution was washed successively with water, sodium bicarbonate solution, water, and dried. Removal of the chloroform in vacuo gave a crystalline residue which was recrystallised from chloroform/hexane to give 4.9 g (92%) of 3-carbomethoxy-7-fluorodihydroisocarbostyril, m.p. 106-108°. Further recrystallisation from chloroform/hexane raised the melting point to 107-108°. Calc. for $C_{11}H_{10}NO_3F$: C, 59.20; H, 4.48; N, 6.28; F, 8.5. Found: C, 59.36; H, 4.57; N, 6.01; F, 8.3%.

(j) MISCELLANEOUS.

Ethyl thioltrifluoroacetate.

Ethyl thioltrifluoroacetate, b.p. 90-91° (lit.[347] 90.5°) was prepared from ethane thiol and trifluoroacetic anhydride by the method of Hauptschein et al.[347] The yield was 59% (lit.[347] 84%).

Diethyl acetamidomalonate.

The method employed was that of Shaw and Nolan.[503] Diethyl acetamidomalonate, m.p. 94-96° (lit.[503] 96-97°) was obtained in an overall yield of 65%.

Aceturic acid.

Aceturic acid, m.p. 207-209° (lit.[504] 207-208°) was prepared by the method of Herbst and Shemin.[504] The yield was 90% (lit.[504] 89-92%).

Phenylglycine.

Phenylglycine was prepared in 30% yield (lit.[505] 33-37%) by the method of Steiger.[505]

Carbomethoxymethylidenetriphenylphosphorane.

This compound, m.p. 125-128° (lit.[506] 125-127.5°) was prepared in 90% overall yield from ethyl bromoacetate and triphenylphosphine according to Denny and Ross.[506]

2,4-Difluorobenzaldehyde.

The method employed was essentially that of Liebermann and Connor.[507] 2,4-Difluorotoluene (9.2 g, 0.072 mole), glacial acetic acid (114 ml), and acetic anhydride (113 ml) were cooled in an ice-salt bath. Concentrated sulphuric acid (17ml) was added dropwise, with stirring, followed by chromium trioxide (20 g), the temperature being maintained below 0°. Stirring was continued for 10 minutes and the reaction mixture poured onto crushed ice (800 g). The volume of the solution was made up to 1200 ml, the solid filtered off, and washed with water until the washings were colourless. After drying, 2,4-difluorobenzaldi-

acetate, 7.1 g (40%), was obtained. The crude diacetate (6.1 g, 0.025 mole), water (10 ml), ethanol (10 ml), and concentrated sulphuric acid (1 ml) were heated under reflux for 30 minutes and the mixture then steam distilled. The distillate was extracted with ether, the ether extracts dried, and the ether removed under reduced pressure. Distillation of the residue in vacuo under nitrogen gave 2,4-difluorobenzaldehyde, 3.10 g (87%), b.p. 68-71°/26 mm (lit.[400] 62-63°/11 mm).

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APPENDIX.

Graphs of I_o vs. $1/\delta$ for the racemic inhibitors studied. I_o is the concentration of the D enantiomer (mM) and δ is the separation of the resonances (in Hz at 56.4 MHz). The slope is $E_o\Delta$ and the intercept $-(K_D + E_o)$. Hence Δ = slope/E_o and K_D = intercept $-E_o$ (section 2). These data refer to the trifluoroacetyl ^{19}F nuclei except where otherwise stated.

Figure	Inhibitor, N-trifluoroacetate of	K_D(mM)	Δ(Hz)
1	phenylalanine, pH 5.3	4.4	-8.4
2	tryptophan, pH 5.3	*	-21
3	p-fluorophenylalanine, pH 5.3	1.2	-33
4	m-fluorophenylalanine, pH 5.3	3.4	-14
5	p-methylphenylalanine, pH 5.3	0.6	-19
6	m-methylphenylalanine, pH 5.3	2.4	-18
7	p-bromophenylalanine, pH 5.3	*	-33
8	m-bromophenylalanine, pH 5.3	2.2	-32
9	2,4-difluorophenylalanine, pH 5.3	3.9	-21
10	p-bromophenylalanine, pH 8.2	6.4	-23
11	p-fluorophenylalanine, pH 5.3, ring fluorine nuclei	*	-51
12	p-bromophenylalanine, pH 5.0	*	-31
13	p-bromophenylalanine, pH 6.0	*	-31

Figure	Inhibitor, N-trifluoroacetate of	K_D(mM)	Δ(Hz)
14	p-bromophenylalanine, pH 6.6	*	-31
15	p-bromophenylalanine, pH 7.6	3.0	-41
16	p-fluorophenylalanine, pH 7.7	2.5	-28
17	m-bromophenylalanine, pH 7.6	4.4	-36
18	p-fluorophenylalanine, pD 5.4 (in D_2O)	0.7	-37

* K_D determined as < 0, indicating weakness in approximations.

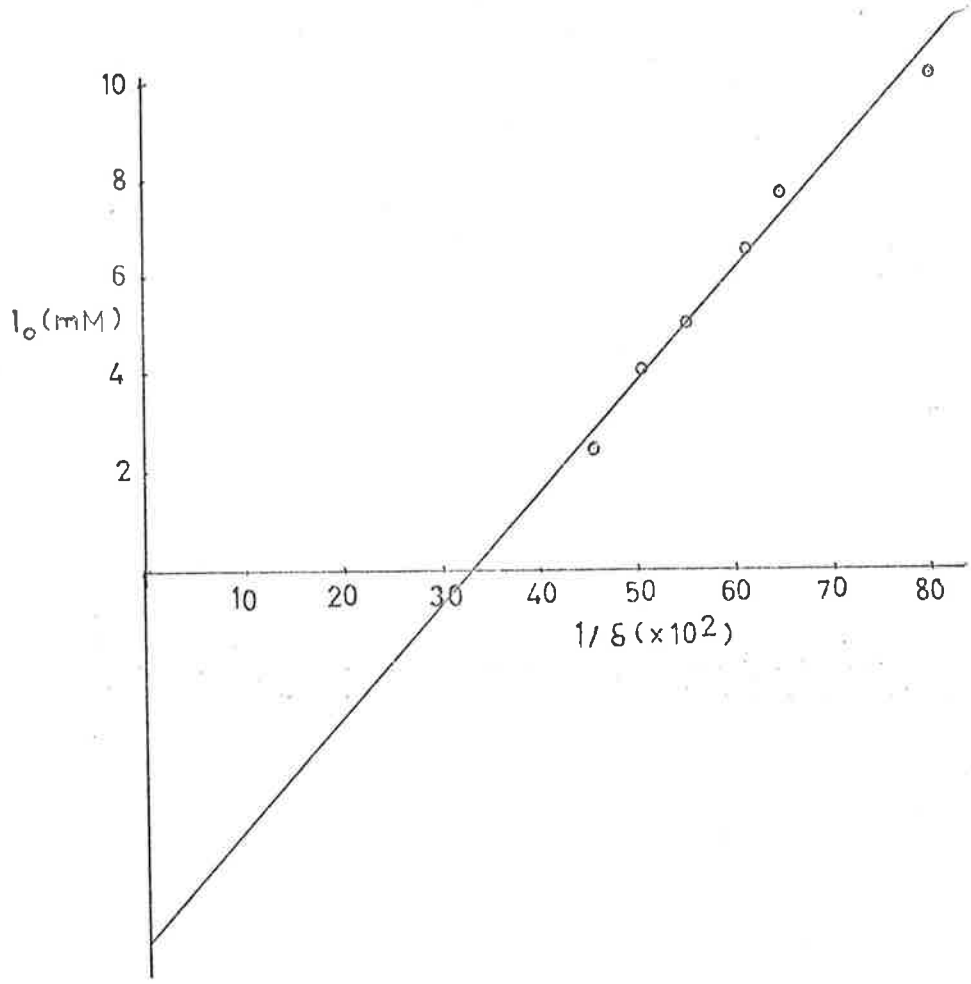
10
8
6
l_o(mM)
4
2
10
20
30
40
50
60
70
80
1/ δ (x10^2)

Fig. 1.

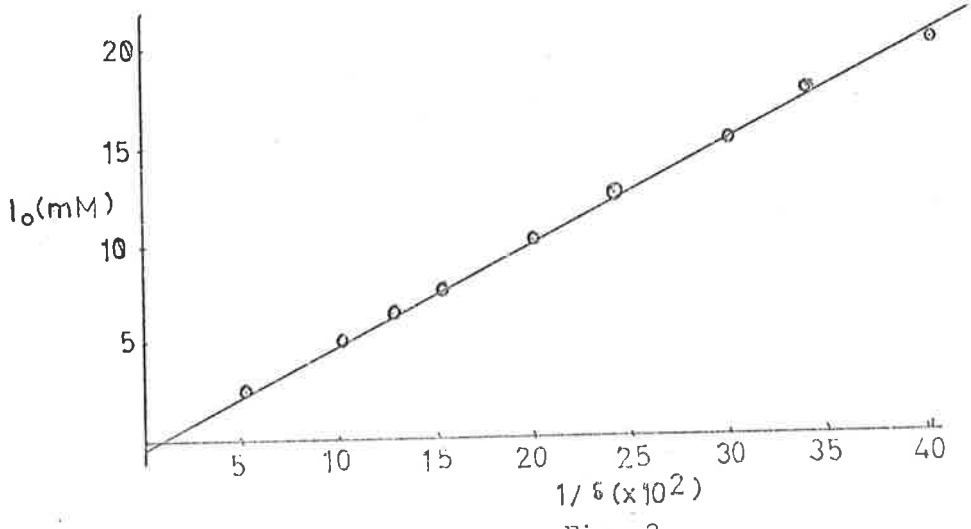
20
15
l_o(mM)
10
5
5
10
15
20
25
30
35
40
1/ δ (x10^2)

Fig. 2.

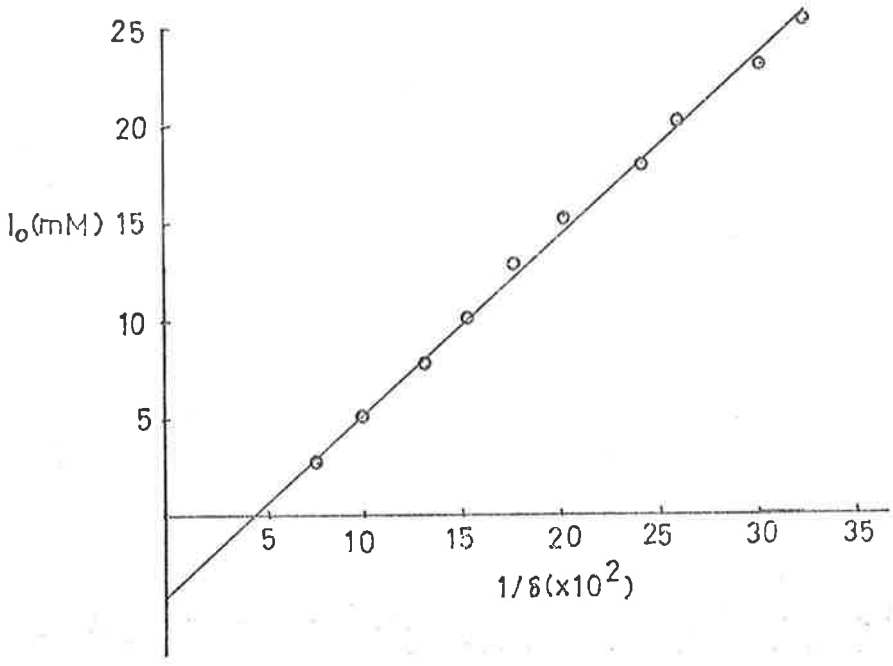
25
20
I_o(mM) 15
10
5
5
10
15
20
25
30
35
$1/\delta(x10^2)$

Fig. 3.

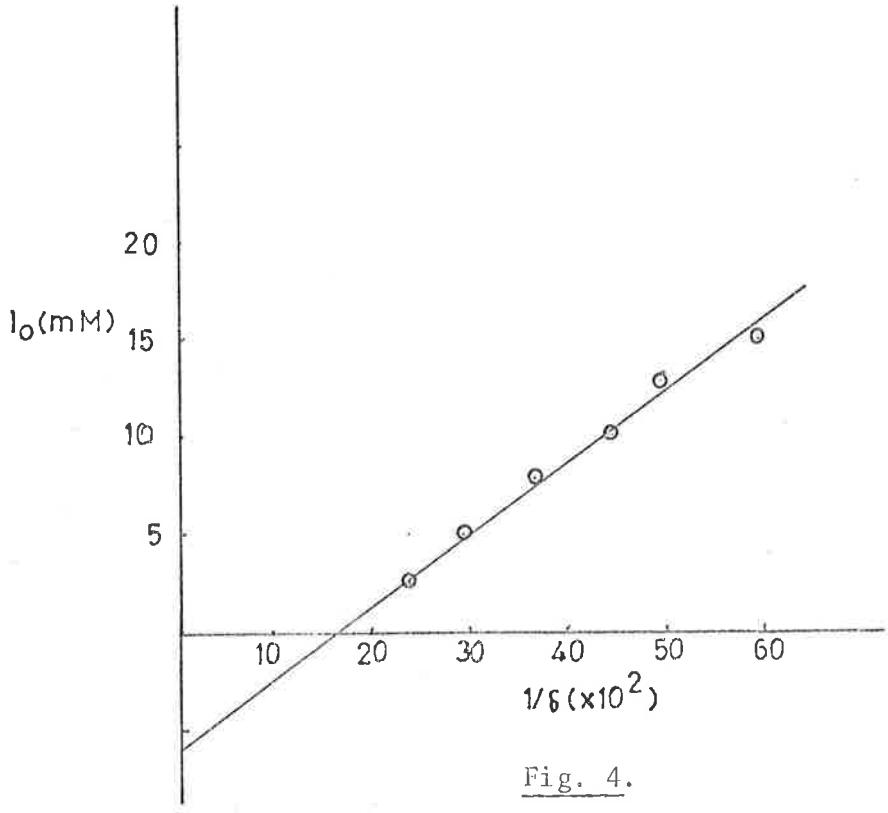
20
I_o(mM) 15
10
5
10
20
30
40
50
60
$1/\delta(x10^2)$

Fig. 4.

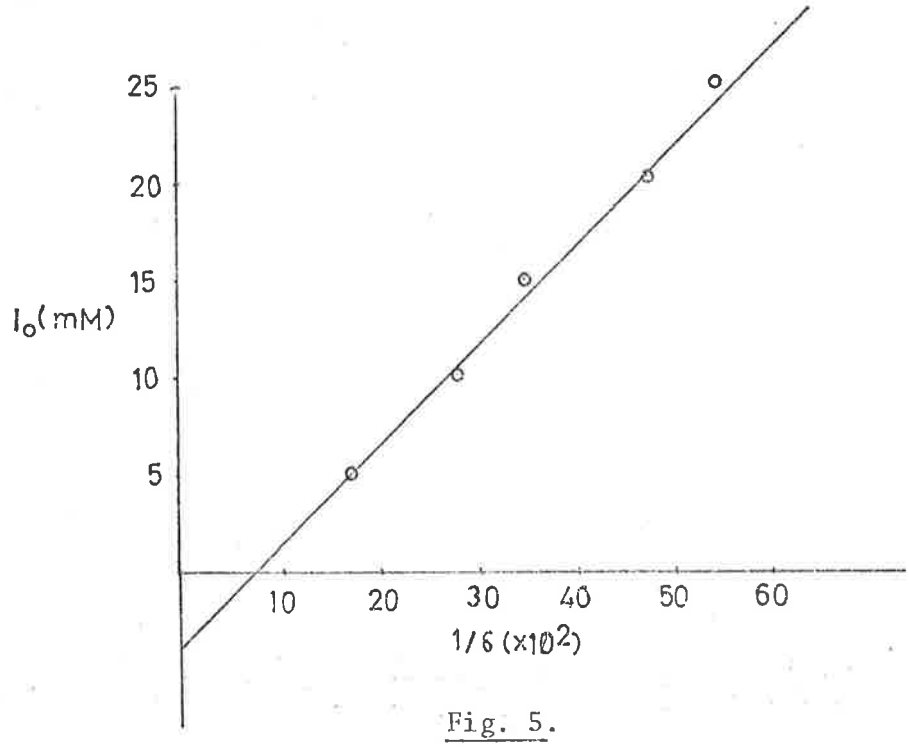
25
20
15
10
5
I_o(mM)
10
20
30
40
50
60
1/δ (x10²)

Fig. 5.

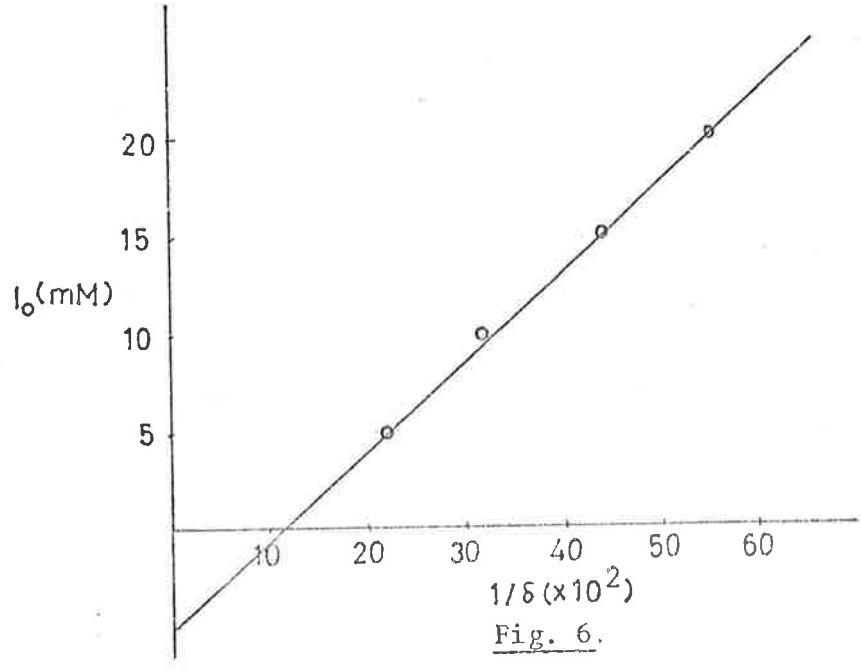
20
15
10
5
I_o(mM)
10
20
30
40
50
60
1/δ (x10²)

Fig. 6.

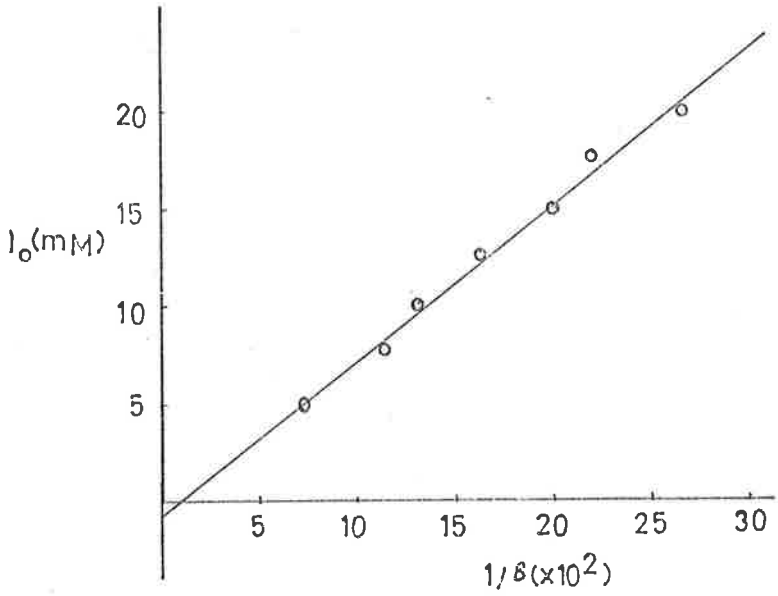
20
15
10
5
I_o(mM)
5
10
15
20
25
30
$1/\delta(\times 10^2)$

Fig. 7.

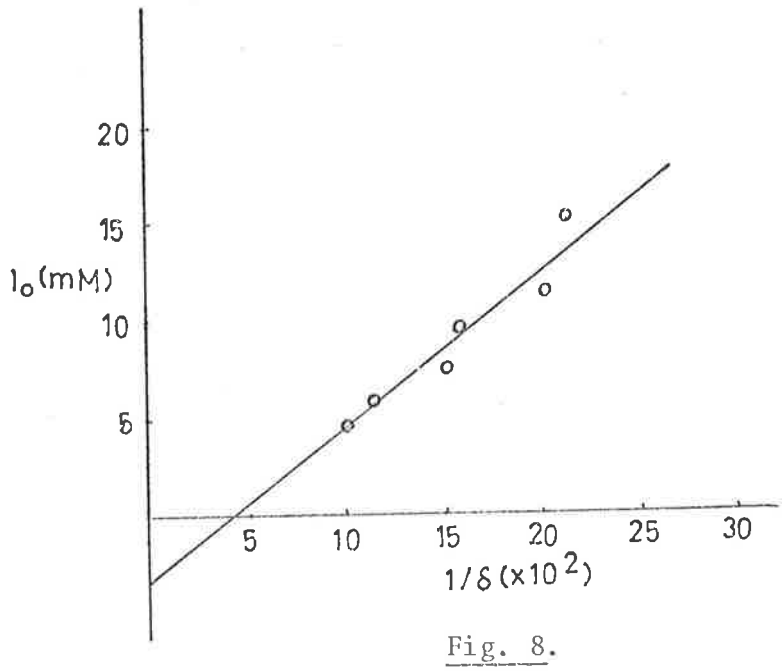
20
15
I_o(mM)
10
5
5
10
15
20
25
30
$1/\delta(\times 10^2)$

Fig. 8.

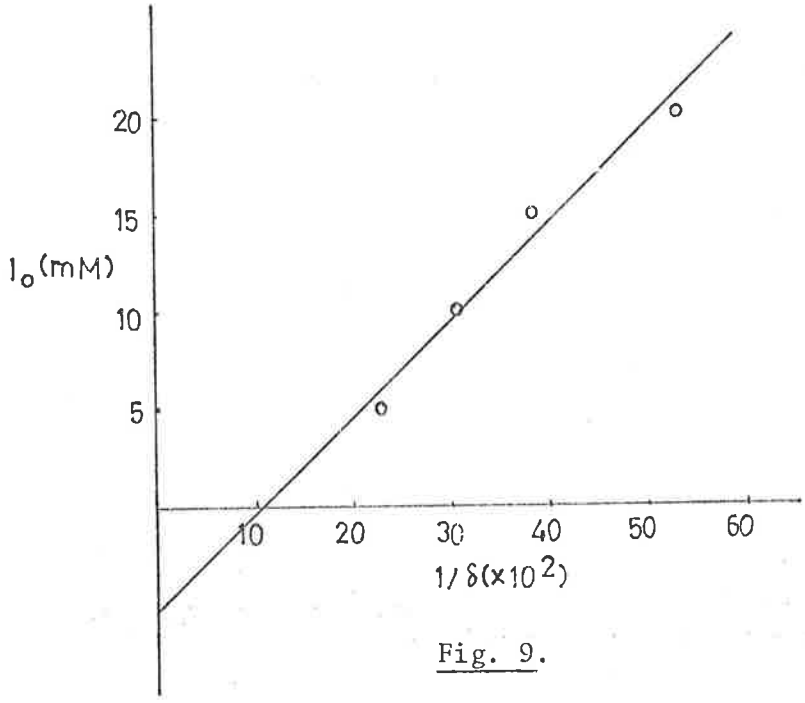

Fig. 9.

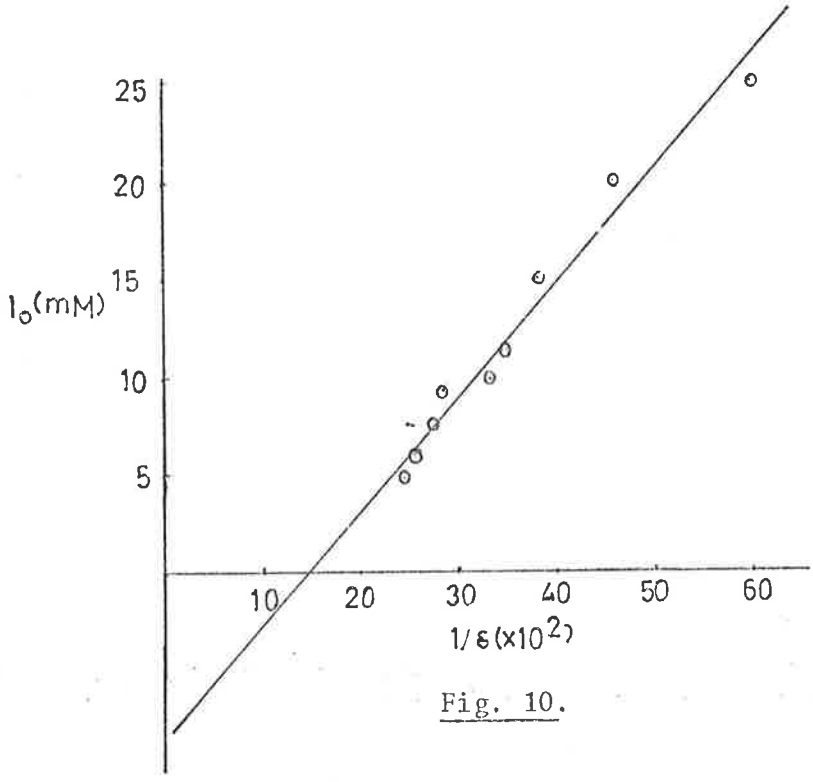
25
20
15
10
5
I_o(mM)
10
20
30
40
50
60
$1/\varepsilon (x10^2)$

Fig. 10.

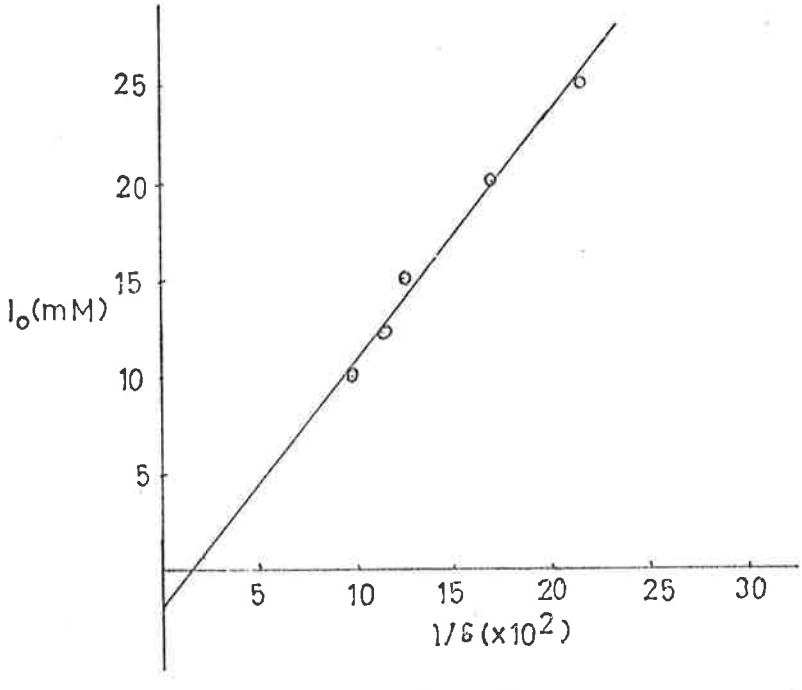
25
20
15
10
5
I_o(mM)
5
10
15
20
25
30
$1/\varepsilon (x10^2)$

Fig. 11.

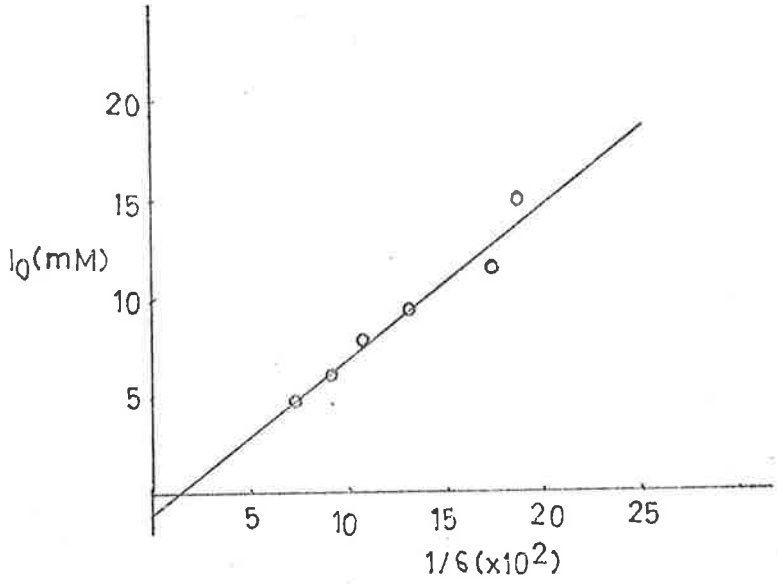
20
15
I_0(mM)
10
5
5
10
15
20
25
1/δ(x10^2)

Fig. 12.

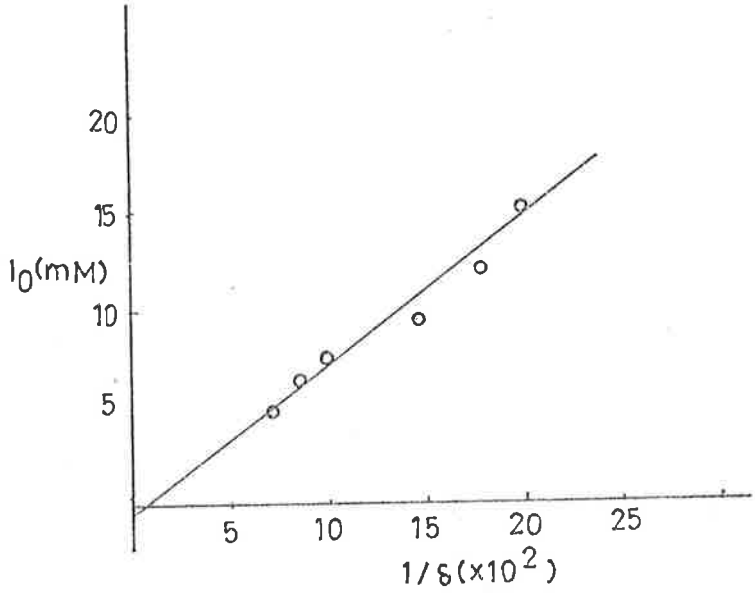
20
15
I_0(mM)
10
5
5
10
15
20
25
1/δ(x10^2)

Fig. 13.

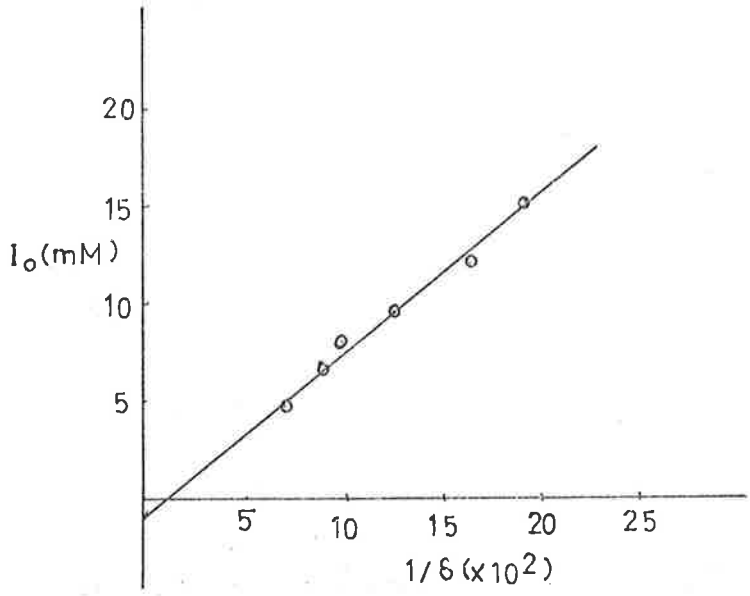
20
15
I_o(mM)
10
5
5
10
15
20
25
1/δ (x10^2)

Fig. 14.

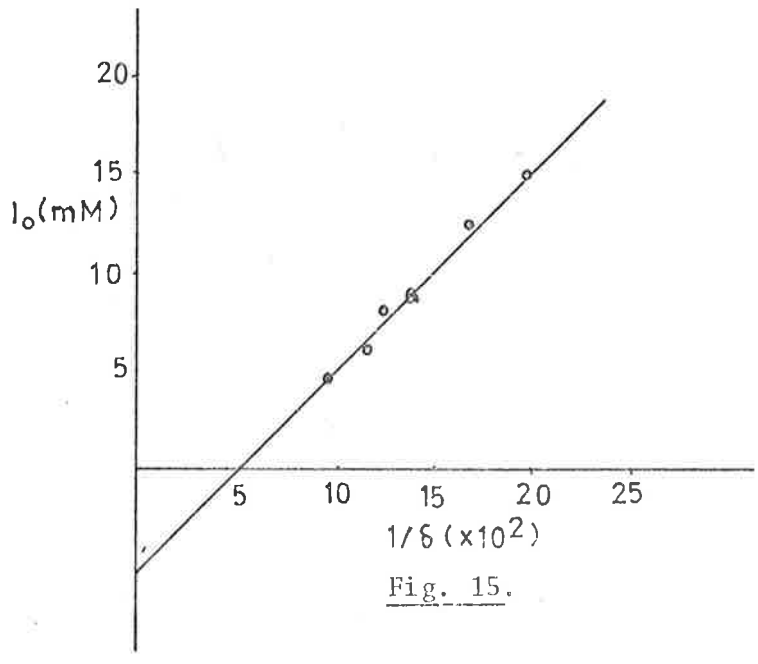
20
15
I_o(mM)
10
5
5
10
15
20
25
1/δ (x10^2)

Fig. 15.

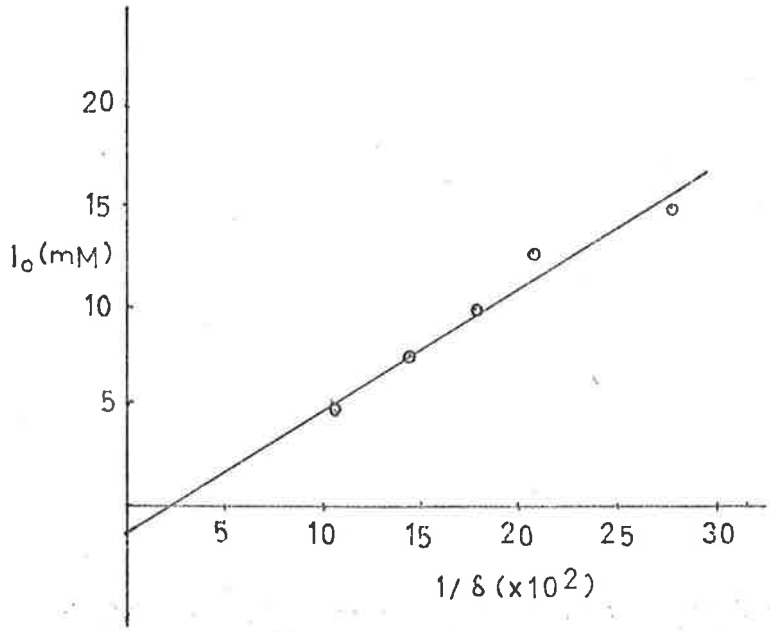
20
15
I_o(mM)
10
5
5
10
15
20
25
30
1/δ (x10^2)

Fig. 16.

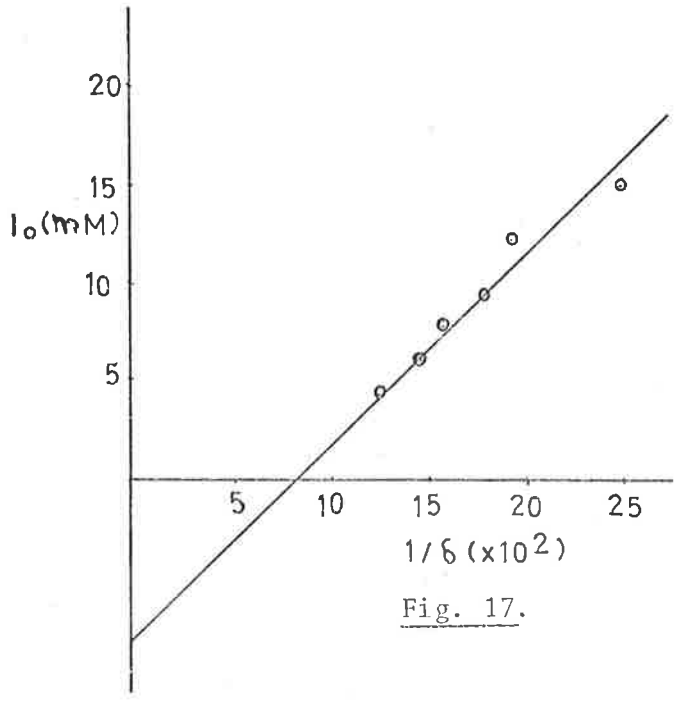
20
15
I_o(mM)
10
5
5
10
15
20
25
1/δ (x10^2)

Fig. 17.

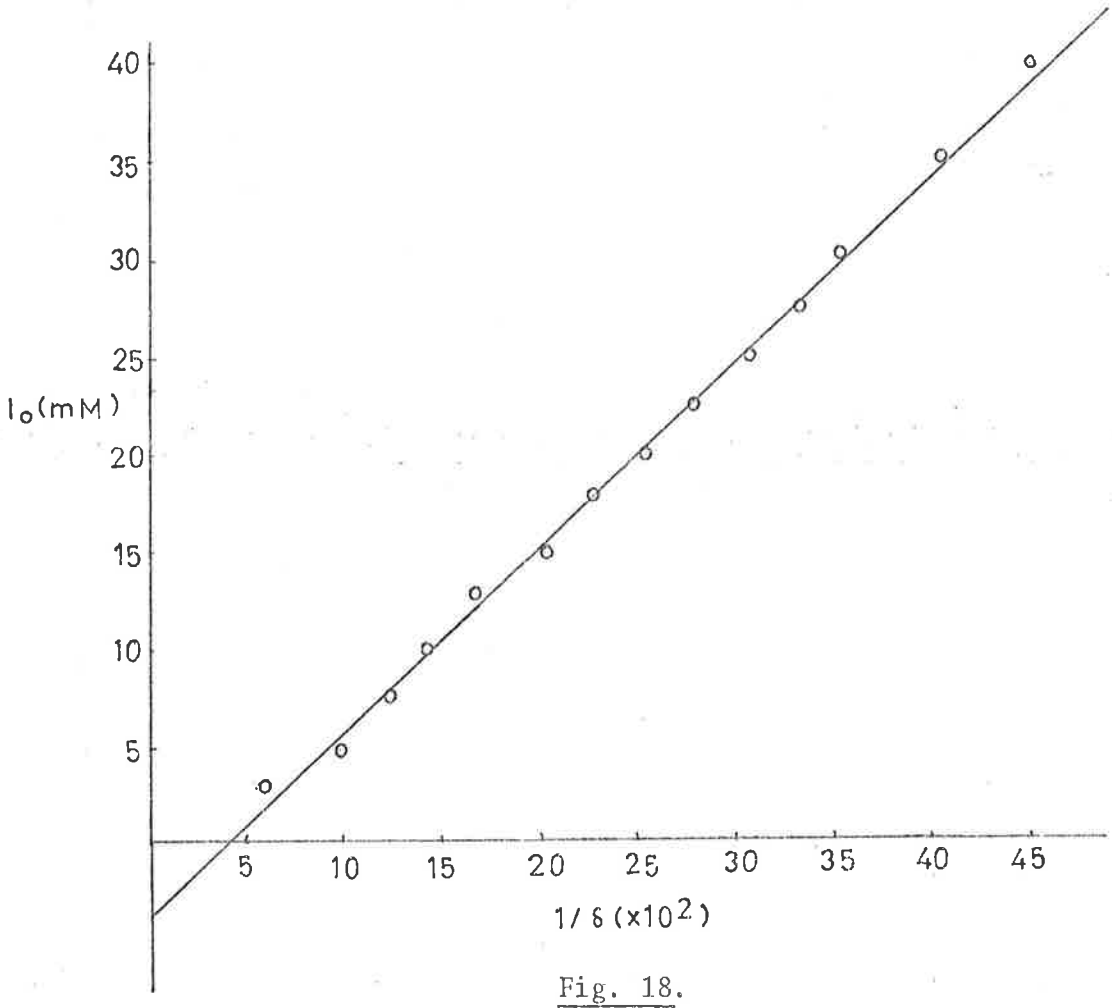

Fig. 18.

Nicholson, B. C. & Spotswood, T. M. (1973). Nuclear magnetic resonance studies of the interaction of inhibitors with chymotrypsin. *N*-trifluoroacetyl derivatives of phenylalanine. *Australian Journal of Chemistry 26*(1), 135-145.