STUDIES IN PEPTIDE SYNTHESIS

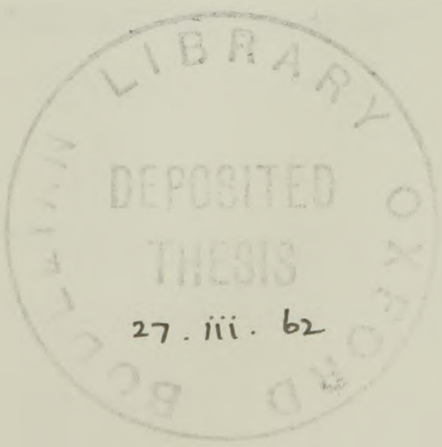
A thesis submitted for the degree of

Doctor of Philosophy

by

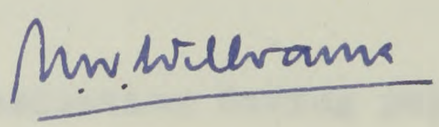
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I would like to express my sincere appreciation of the constant advice and patient encouragement given to me by my supervisor, Dr.G.T.Young. My thanks are also due to Dr.S.G.Waley and Dr.J.Rudinger for helpful discussions.



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INTRODUCTION.

General Introduction

The chemical synthesis of peptides serves to verify the structures which are deduced from degradative evidence. It also allows modifications of these structures to be made, which are essential to establish relationships between chemical structure and physiological activity. The discovery of an ever increasing number of physiologically important peptides and also the development of new chromatographic techniques of purification and analysis have recently led to great advances in the synthetic approach.

The condensation of amino-acids to form peptides involves the activation of the carboxyl group of one molecule towards the amino group of the molecule to which it is to be attached. All reactive centres in the molecules other than those involved in the coupling reaction must be rendered inactive under the conditions of that reaction by suitable protecting groups which can subsequently be removed without impairment of the peptide molecule.

The work desribed in the thesis involves an investigation of coupling procedures using the condensation of two protected amino-acids. It is therefore proposed to outline some of the major protecting groups in current use and the coupling

procedures used or investigated in the present work.

The most commonly used group for amino-protection is the benzyloxycarbonyl group, which can be removed under a variety of mild conditions, such as catalytic hydrogenation (1), treatment with hydrogen bromide in acetic acid (2), hydrogen chloride in ethanol (3), or liquid ammonia (4). Other groups which are sometimes employed are the phthaloyl (e.g.5), trityl (6), formyl (7), and trifluoroacetyl (8) groups. The carboxyl function is usually modified by esterification for the purpose of protection. Methyl, ethyl, benzyl and t-butyl esters (9) are in common use.

A great diversity of methods exists for the condensation of carboxyl and amino groups to form amide bonds. The acid azide method (10), although one of the earliest to be introduced, is still employed. The azide is formed by treatment of an acid hydrazide with sodium nitrite in aqueous acid solution, and it reacts smoothly with a primary amine to give an amide. The possible Curtius rearrangement to give an isocyanate is usually avoided by conducting the reaction at 0° (11).

Activation of the carboxyl group by formation of a mixed anhydride with a carboxylic acid has become an important general method. Mixed anhydrides with benzoic or isovaleric

acids were first proposed (12), but these have been superseded by mixed carbonic anhydrides (13) which cleave more specifi--cally in the required position on aminolysis. The comparative studies of Vaughan have led to the use of mixed anhydrides of isobutyl or ethyl hydrogen carbonate. These are made from the protected amino-acid or peptide and isobutyl or ethyl chloroformate, respectively in the presence of a base.

$$R.COO^{-}\ \overset{+}{N}HEt_3 + Cl.COOR' \longrightarrow R.CO.O.COOR' + \overset{+}{N}HEt_3\bar{C}l$$

Anderson has developed a series of related methods involv--ing the use of phosphorous esters. Diethyl, o-phenylene or ethylene phosphorochloridite react with carboxylate functions to give anhydrides which readily acylate primary or secondary amino groups (14). This is referred to as the "anhydride" procedure.

$$R.COO^{-}\ \overset{+}{N}HEt_3 + Cl.P(OEt)_2 \longrightarrow R.CO.O.P(OEt)_2 + \overset{+}{N}HEt_3\bar{C}l$$

$$\downarrow\ NH_2R'$$

$$R.CONHR' + (EtO)_2P(H)OH$$

Alternatively, the phosphorochloridite is allowed to react with the amino component to give a phosphoramidite which condenses with the carboxyl function; this is the"amide proce--dure".

$$(EtO)_2P.Cl + NH_2R' \longrightarrow \left[\ (EtO)_2P.NHR'\ \right]$$

$$\downarrow\ R.COOH$$

$$R.CONHR' + (EtO)_2P(H).OH$$

The same active intermediates can be made by using tetraethyl pyrophosphite. With this reagent, a "standard" procedure has also been described, in which the reagent is added to a solution of carboxyl and amino components (15). The use of diethyl phosphite as solvent has been recommended.

A group of coupling methods has been developed, based on the fact that the carbonyl group of the ester RCO.OR' is activated towards the nucleophilic primary amino component when R' is electron-attracting. Among this "activated ester" type is the p-nitrophenyl ester method. These esters were originally made from acid chlorides or anhydrides (16) and were consequently not of great synthetic value, but a consid--erable advance was made when two new syntheses of p-nitro--phenyl esters were published. One, due to Schwyzer, involves the use of diaryl sulphites or triaryl phosphites in the presence of pyridine (17). In the other, esterification is effected with p-nitrophenol in the presence of dicyclohexylca-rbodi-imide (18). Kenner has used tri-p-nitrophenyl phosphoro-trithioite for the preparation of p-nitrothiophenyl esters, and has found that the relative rates of acylation of alanine in aqueous dioxan at 21° by a p-nitrophenyl ester, thiophenyl ester and p-nitrothiophenyl ester are 16:1:140 (19).

As the result of an extensive survey of the reaction between benzylamine and various active esters of benzoylglycine, Schwyzer has recommended cyanomethyl esters, which acylate primary amines quantitively at room temperature in an inert solvent (20). These esters are highly crystalline, stable solids which are made from the acid and chloroacetonitrile in the presence of triethylamine.

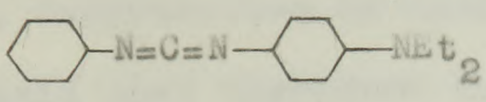
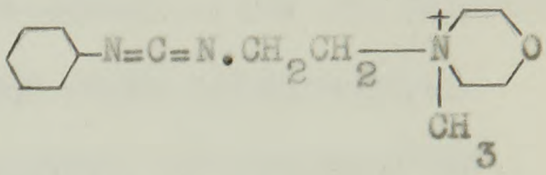
$$R.CO\bar{O}\ \overset{+}{N}HEt_3 + ClCH_2CN \longrightarrow R.CO.OCH_2CN + \overset{+}{N}HEt_3\bar{Cl}.$$

In another type of coupling method, the carboxyl component is activated by addition to an unsaturated compound. The intermediate is not isolated, but allowed to react with the amino component <u>in situ</u>. The important di-imide coupling method belongs to this type. In the presence of one mole of dicyclohexylcarbodi-imide, the primary amine salt of the carboxyl component is converted into the amide (21). Conditions must be chosen so as to avoid rearrangement of the activated complex to give an acylurea since this is often difficult to remove from the product (22).

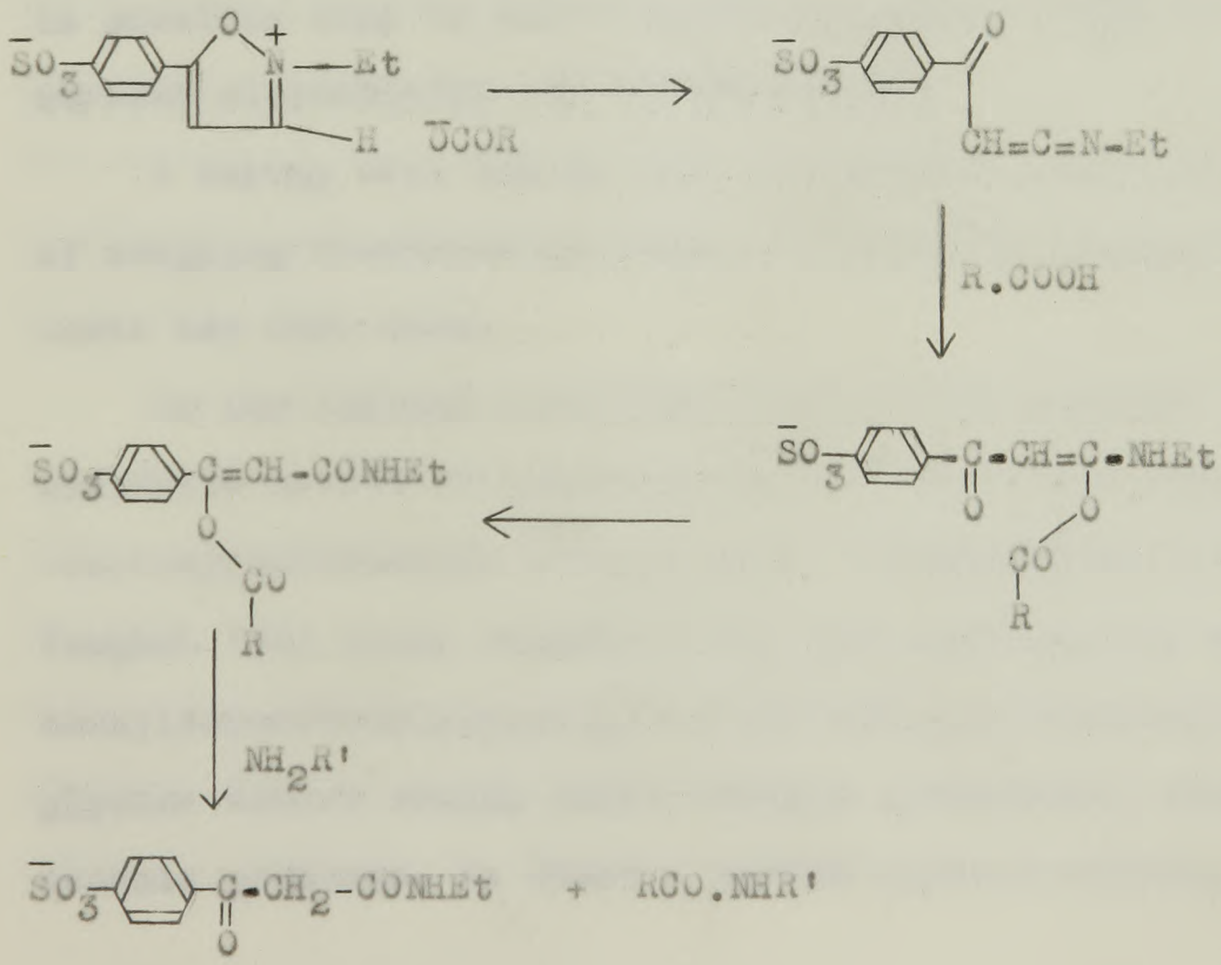
$$R.N{=}C{=}N.R \longrightarrow R.\overset{+}{N}H{=}C{=}N.R \xrightarrow{R'.CO\bar{O}} R.NH{-}C{=}N.R$$

$$R.NH.CO{-}N.R \quad (COR') \longleftarrow R.NH{-}C{=}N.R \quad (O.COR') \xrightarrow{NH_2R''} R.NH.CO.NHR + R'CONHR''$$

The difficulty of removing acyl-urea can be overcome by using a water-, or acid-soluble di-imide (23).

e.g.  and 

Woodward has recently described a new condensing agent in which the activated carboxyl component is analogous to that in the di-imide ease (24). This is the zwitterion N-ethyl-5-phenylisoxazolium-3'-sulphonate.



Racemisation during peptide synthesis. Most naturally occurring α-amino-acids are asymmetrically substituted at their α-carbon atoms. Condensation of optically active amino-acids in peptide synthesis must therefore take place with full preservation of this asymmetry in order to give a peptide identical with the natural product. Loss of activity during a coupling reaction limits the yield at that stage, but a more serious consequence is that the presence of even minute amounts of racemates or diastereoisomers in a peptide is often responsible for failure to crystallise that peptide (e.g.25), so that their removal is possible only by more lengthy procedures such as counter-current distribution and chromatography.

A survey will now be made of examples from the literature of coupling reactions in which a deliberate search for race-mate has been made.

In the initial investigations of the carbonic mixed anhydride method of coupling, it was shown that benzyloxy-carbonylamino-acids retain their configuration (13), but Vaughan (26) later reported that the condensation of benzyloxycarbonylglycyl-L-leucine and-L-phenylalanine with glycine esters could, under certain conditions, lead to racemic products. He found a marked solvent effect; in the

latter reaction for example, when a mixture of chloroform and toluene was used as solvent, racemisation was nearly complete, but with tetrahydrofuran as solvent no racemate was detected. Synthesis from benzyloxycarbonylglycine and L-phenylalanylglycine ethyl ester gave fully active material. Again, when the carbonic mixed anhydride of benzyloxycarbonyl--glycyl-L-phenylalanine was formed in anhydrous tetrahydrofuran and allowed to react with an aqueous solution of the sodium salt of glycine, partial racemisation occurred, although dibenzyloxycarbonyl-L-lysine coupled with L-valine under similar conditions to give a fully active product (27). These experiments show that the extent of racemisation depends upon the nature of the adjacent acyl group, and that the benzyloxy--carbonyl group hinders racemisation of the residue to which it is attached.

Kenner (28) has coupled benzyloxycarbonylglycyl-L-phenyl--alanine with glycine and benzyloxycarbonylglycyl-L-alanine with L-phenylalanylglycine using his dimethylformamide-sulphur trioxide complex as a condensing agent. In the latter case very small amounts of the diasteresisomer, formed by racemisation of the alanyl residue, could be detected by counter-current distribution. In aqueous solutions, the amount of

racemisation depended greatly upon the pH, becoming very slight at pH 6·8, and none was detectable (by crystallisation) when the corresponding esters were acylated in anhydrous dimethylformamide solution.

The realisation that a benzyloxycarbonyl group inhibits racemisation of the amino-acid which it protects, and that the coupling of a benzyloxycarbonylamino-acid therefore does not constitute a test for the preservation of activity during that reaction, led to a greater awareness of the problem and, in 1955, North and Young initiated a study of peptide coupling reactions from the point of view of racemisation, using the condensation of acetyl-L-leucine with glycine ethyl ester as a model reaction (29). The acid azide procedure gave fully active product, the acid chloride and phosphorazo methods gave peptides which were chiefly racemic, the latter demon-strating clearly that those methods which have been postulated to involve activation of the amino component may still cause racemisation of the carboxyl component. The tetraethyl pyrophosphite and mixed carbonic anhydride methods gave partially racemic peptide. These authors also demonstrated that benzyloxycarbonyl-L-leucine lost no optical activity when treated with acetic anhydride and sodium hydroxide-conditions

which cause rapid and complete racemisation of benzoyl-L-leucine.

Anderson and co-workers have used the coupling of benzyloxycarbonylglycyl-L-phenylalanine with glycine ethyl ester as a model for racemisation tests. In 1952, they reported that the degree of racemisation in the tetraethyl pyrophosphite method varied considerably with the conditions employed and concluded that "heating of the anhydride and the presence of hydrogen chloride may cause racemisation" (15). In a later report, discussing the use of the phosphorochloridites, they state that "racemisation has been observed only when reactions were performed in diethyl or trimethyl phosphite in the presence of hydrogen chloride, free or bound as the hydrochloride No racemisation is observed in the absence of hydrogen chloride, as in the pyrophosphite reactions or with chlorophos--phites when triethylamine in inert solvents like benzene or toluene is used. In these solvents the acid is effectively removed from the solution" (30). The 17% of racemate which was isolated by the use of tetraethyl pyrophosphite in the "anhydride" procedure with distilled ester, seems to be an exception to this generalisation (15). It is interesting to note that the employment of trialkyl phosphites as acceptors

of hydrogen chloride permits more racemisation than does the conventional triethylamine technique.

In 1958, Anderson and Callahan elaborated their racemisation test (31) and showed that isolation of racemate from benzyloxycarbonylglycylphenylalanylglycine ethyl ester was possible by fractional crystallisation from ethanol, even when less than 1% was present. They applied this test to the di-imide method of coupling and isolated 6·6 - 8·2% of racemate using conditions under which Sheehan (21) had previously obtained product whose rotation indicated full retention of activity. Sheehan has also reported (32) that the coupling of formyl-L-phenylalanine with glycine anilide (which gave racemic product by the carbonic mixed anhydride procedure) gave active material. Schwartz and Bumpus (33), using di-imide in dimethylformamide or tetrahydrofuran, have isolated diastereoisomer formed by racemisation of the acylating residue in the coupling of benzyloxycarbonyl-L-valyl-L-tyrosine with L-isoleucine methyl ester.

Condensation of benzyloxycarbonyl-L-phenylalanine with L-alanine methyl ester by the phosphorazo method has been shown to give a racemic product (34). Most of the recently introduced methods of coupling have been tested for racemisation

using the Anderson-Callahan model. Thus the use of ethoxyacetylene gave a fully active product (35), but the *in situ* liberation of the amino component from the hydrochloride by treatment with a tertiary amine, has led to racemisation during the coupling of several dipeptide acids (36).

The N,N'-carbonyldi-imidazole method gave a partially racemic product when tetrahydrofuran was used as solvent, but this could be almost completely avoided by using anhydrous dimethylformamide at -10° (37).

The use of the Anderson test in the Woodward method of coupling led to 3·6% of racemate when the solvent was acetonitrile but 6% in nitromethane. This was later attributed to racemic starting material (38) in spite of the apparent dependence of the degree of racemisation upon the solvent.

Condensation by the method involving the oxidation of a hydrazide with N-bromosuccinimide was accompanied by 1·1% racemisation (39).

Nefkens has synthesised benzyloxycarbonylglycyl-L-phenylalanylglycine ethyl ester using the hydroxyphthalimide ester method and has found no racemate on crystallising the product from ethanol (40), but this cannot be regarded as a valid test for racemisation because the synthesis involved the

condensation of a benzyloxycarbonylamino-acid activated ester at both stages.

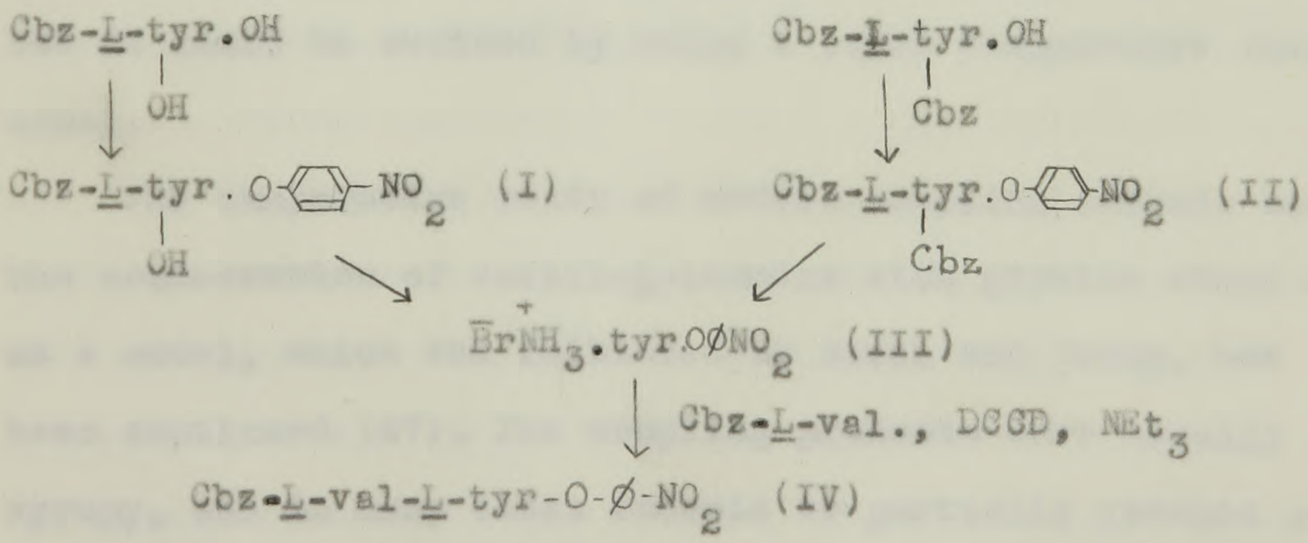
Zoaral and Arnold have employed the acetyl-L-leucine model to test their procedure using the dimethylformamide-phosgene complex, and have found the product to be almost completely racemic (41). They consider that the reaction proceeds, at least partly, via the acid chloride.

In the activated ester methods of coupling, loss of activity has often been found to accompany the preparation of the active intermediate. Thus the early syntheses of thiophenyl (42) and nitrophenyl (16) esters via the acid chlorides or mixed anhydrides gave completely racemic esters. In an attempt to overcome this, Kenner developed a preparation of thiol esters involving the use of tri-p-nitrophenyl phosphorotrithioite (19b). The synthesis of benzyloxycarbonylglycyl-L-phenylalanine thiol ester and the subsequent condensation with glycine gave the active peptide in good yield, but the condensation of benzyloxycarbonylglycyl-L-alanine with L-phenylalanylglycine by the same method gave about 30% of the racemic product.

Iselin and Schwyzer have made a study of the preservation of optical activity during the synthesis of dipeptide p-nitro-

-phenyl esters by three routes (43). They prepared benzyloxy-carbonyl-S-benzyl-L-cysteinyl-L-tyrosine p-nitrophenyl ester from the dipeptide acid by treatment with di-p-nitrophenyl sulphite in the presence of pyridine; the product was shown to be optically pure by regeneration of the acid by hydrolysis. On the other hand, treatment of benzyloxycarbonyl-L-valyl-L-tyrosine with sulphite under the same conditions gave a considerably racemised ester. The extreme susceptibility of the tyrosine residue in valyltyrosine has also been noted by Schwartz and Bumpus (44).

Racemic benzyloxycarbonyl-L-valyltyrosine p-nitrophenyl ester was also obtained by the Elliott and Russell di-imide procedure. The same peptide was then synthesised by the method of Goodman and Stueben (45).



The optical activity of (I) and (II) were demonstrated

by hydrolysis to the acids, and of (III) by saponification to L-tyrosine. The optical rotation of (IV) was higher than that obtained by the two previous methods, but saponification gave partially racemic dipeptide acid. The authors deduce that (IV) must be partially racemic on the basis of anology with benzyloxycarbonyl-L-valyl-L-tyrosine methyl and ethyl esters, hydrolysis of which under the same conditions gives optically active acids. Stueben, however, has found that saponification of benzyloxycarbonyl-glycyl-L-phenylalanine p-nitrophenyl ester leads to the racemic dipeptide acid (46) and that racemisation occurs prior to hydrolysis.

Only one case has been reported of loss of activity in the cyanomethyl ester method. This occurred during the esterification of benzyloxycarbonyl-S-benzyl-L-cysteine (20d), but it could be avoided by using a lower temperature than usual.

The comparative study of modern coupling methods using the condensation of acetyl-L-leucine with glycine ethyl ester as a model, which was initiated by North and Young, has been continued (47). The coupling products were usually syrupy, but in many cases racemic or partially racemic peptide crystallised out. The rotation of the fractions enabled a

semiquantitative estimate of the degree of racemisation to be made in each case.

No racemate was isolated from the product made by the azide method. The use of dicyclohexylcarbodi-imide in dichloromethane or anhydrous tetrahydrofuran, with free glycine ethyl ester, gave chiefly L-peptide, but the presence of racemate was confirmed by crystallisation of the first fractions. More racemisation occurred when dimethylformamide or aqueous tetrahydrofuran were used as solvent.

The carbonic mixed anhydride method, using ester hydrochloride in tetrahydrofuran or chloroform as solvent, led to extensive racemisation, but the use of free ester in tetrahydrofuran gave product of higher activity.

In the phosphorous ester methods, the degree of racemisation was found to be critically dependent upon the procedure employed. Using free ester, diethyl phosphorochloridite in the "anhydride procedure" gave some racemate, but the optical activity of the fractions was high. No racemate was isolated from the product of the "amide procedure" although the properties of the solid fraction indicated its presence. Tetraethyl pyrophosphite, both in "anhydride" and "amide procedures", gave products containing racemate. The use of

glycine ester hydrochloride and triethylamine led to increased racemisation in all the cases investigated.

Authentic acetyl-L-leucylglycine ethyl ester was synthesised for reference by a route which should not allow racemisation. This compound was crystalline in the pure state, but the presence of racemate impeded crystallisation greatly. A consequence of this was that racemic or largely racemic peptide was readily isolated from a coupling product by preferential crystallisation. The method is therefore of value for the rapid demonstration that a coupling method may be accompanied by loss of activity, but an accurate assessment of the racemic content based on specific rotation is rendered suspect by the possible inclusion of impurities in the syrups.

The work described in the first part of the thesis concerns the development of a new model for the rapid quantitative determination of racemisation during coupling, and this leads to an investigation of the effect of conditions on the extent of this loss of activity, in the hope of illuminating the mechanism of this phenomenon.

No work has been described on the mechanism of racemisation under the conditions employed in peptide coupling, but the nature of the racemisation of acylamino-acids has been the

subject of many investigations. A literature survey will now be made.

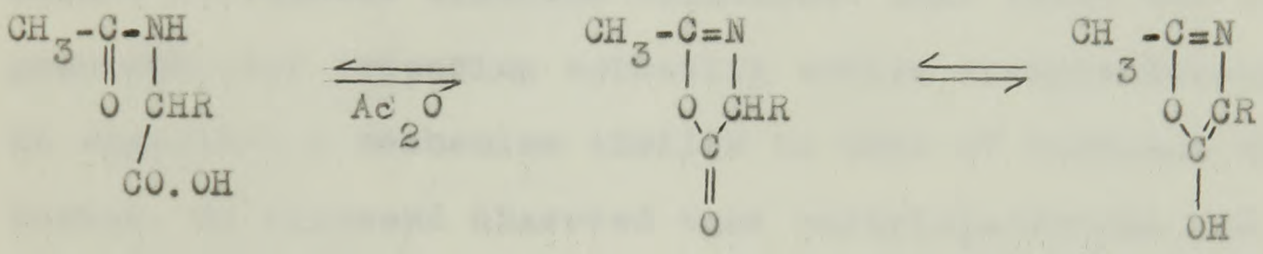
The mechanism of racemisation of acylamino-acids The first observations relevant to the elucidation of the mechanism of racemisation of acylamino-acids were made by Karrer and workers. In 1925, they treated "hippuryl chloride" with diazomethane (48) and obtained a substance which in 1941 was characterised as 2-phenyl-5-oxazolone (49). This reaction has been accounted for by the explanation that "hippuryl chloride" in fact exists as the azlactone hydrochloride (50). Much evidence has accumulated to support this belief. Thus the reaction of hippuric acid with phosphorus tribromide yields a crystalline salt which, in the presence of either diazomethane or sodium acetate under an organic solvent, gives 2-phenyl-5-oxazolone. Carter and Hinman (51), as a result of a study of the chemical and ultra-violet spectroscopic properties of the acid halides of benzoyl-, tosyl-, and benzyloxycarbonyl-p-methoxy-L-phenylalanine, concluded that the benzoyl derivatives are actually 2-phenyl-4-p-methoxybenzyl-5-oxazolone hydrohalides but the other two derivatives give normal acid halides of the generally accepted structures.

Some observations have been made, however, which are difficult to interpret on the above basis. For example the treatment of 2-phenyl-5-oxazolone with hydrogen chloride

gives an ether-insoluble salt, but hippuryl chloride is freely soluble in ether. It must therefore be assumed that α-acylamino-acid halides can exist in equilibrium with azlactone hydrohalides, but the positions of such equilibria depend upon individual circumstances. This conclusion, coupled with the observation that azlactones had never been isolated in optically active forms, seemed adequate to account for the loss of activity in reactions of α-acylamino-acid halides e.g. the coupling with a primary amine.

The bulk of the observations relating to the racemisation of acylamino-acids have been concerned with the action of acetic anhydride under various conditions. Bergmann, in 1926, found that acetylation of L-arginine by the action of cold excess acetic anhydride afforded the racemic product (52). Further observations were made by Bergmann and Zervas (53), who found that acetylation of an amino-acid with exactly one equivalent of acetic anhydride in glacial acetic acid solution at 100°, gave a fully active product, but in the presence of two or more moles of acetic anhydride, an isolable azlactone was formed which gave rise, in turn, to a racemic acetylamino-acid. No azlactone could be isolated when between one and two moles of anhydride was employed, but catalytic racemisation

occurred. Again an optically active acetylamino-acid was racemised by less than one mole of acetic anhydride in acetic acid solution. This catalytic racemisation has been attributed to the transitory formation of an azlactone.

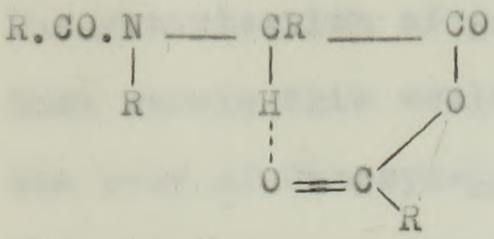


The mechanism of the catalytic racemisation of α-acylamino -acids in glaxial acetic acid was further elucidated by Carter and Stevens (54), who observed that benzoyl-p-methoxy-L-phenylalanine was racemised at an appreciably more rapid rate in the presence of catalytic amounts of azlactones, than in the presence of an equivalent amount of acetic anhydride. This was attributed to "trans-azlactonisation", a reaction which illustrates the structural similarity between an anhydride and an azlactone. The correctness of this assumption was later confirmed by the finding that benzoylphenylalanine, when warmed with one equivalent of 2-benzyl-4-methyl-5-oxazolone was converted to 2-phenyl-4-benzyl-5-oxazolone which could be isolated in good yield (50).

du Vigneaud found that the action of acetic anhydride on an aqueous solution of an optically active acylamino-acid caused loss of activity at acid pH values or in the presence of a base such as pyridine or sodium acetate (55). [No racemisation occurs in aqueous alkaline solutions- this being the standard procedure for preparing optically active acetylamino-acids]. He suggested a mechanism similar to that of Bergmann and Zervas. du Vigneaud observed that acetyl-L-proline and acetyl-methyl-L-phenylalanine could not be racemised by this procedure, but Carter and Stevens (54) later found that "certain acyl derivatives of L-proline and N-methyl-D-phenylalanine are rapidly racemised by the action of acetic anhydride in glacial acetic acid". Similar observations were apparently made by Greenstein (56).

Acylimino-acids cannot form azlactones of the conventional kind, so the racemisation of these by the action of acetic anhydride must clearly occur by a different mechanism. Various workers have suggested that the activating effect of a mixed anhydride group increases the lability on the proton at the asymmetric carbon atom of an acylamino-acid. (57,58). Neuberger (59) has suggested that the steric arrangement of the anhydride molecule allows hydrogen bond formation, thereby

promoting the ionisation of the proton at the α-carbon atom.



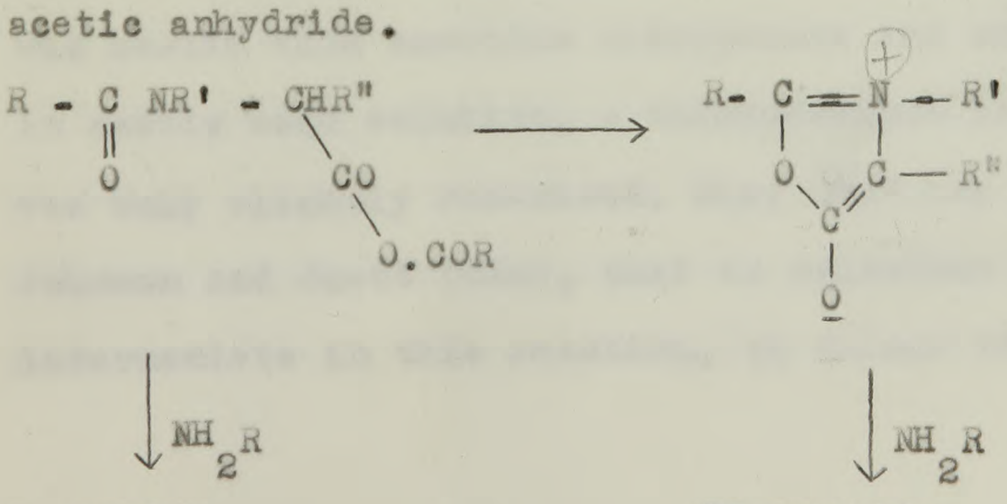
The increased lability of the asymmetrically-attached proton of an acylamino-acid mixed anhydride compared with, say, an ester can be interpreted in terms of the greater resonance stabilisation of the anion formed by loss of the proton.

If it is assumed that this "direct abstraction" mechanism accounts for the racemisation of acylimino-acids, which cannot form conventional azlactones, the question then arises whether this mechanism is operative in addition to the azlactone mechanism during the racemisation of an acylamino-acid when treated with acetic anhydride. In the case of benzoyl-L-leucine, this is not so; for it has been shown that, when this is dissolved in acetic anhydride, loss of optical rotation and formation of an azlactone occur concurrently (50), until conversion is complete, when the azlactone concentration remains virtually constant but the rotation decreases slowly, due to racemisation of the azlactone.

Here we have an apparent inconsistency, for if direct

proton abstraction from the asymmetric centre accounts for the racemisation of an acylimino-acid in acetic anhydride solution, then surely this would also be operative to some extent in the case of benzoyl-L-leucine. Two possibilities suggest themselves: that the loss of activity of an acylimino-acid when treated with acetic anhydride occurs very much more slowly than that of benzoyl-L-leucine under the same conditions, so that the azlactone mechanism is almost entirely responsible for the racemisation of the latter. Carter and Stevens, however, state that "certain acyl derivatives are rapidly racemised ---------". An alternative mechanism must therefore be considered.

An explanation has been suggested (59) which would account for all the above observations, even if the rates of racemisation of acylamino- and acylimino-acids in acetic anhydride solution are comparable. This is that the latter can cyclise to give a " zwitterionic azlactone" in the presence of acetic anhydride.



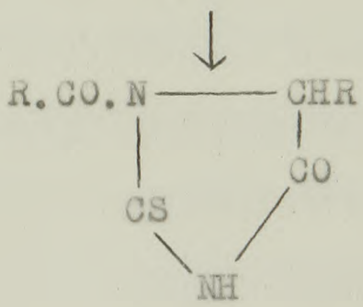
In the series of experiments correlating loss of optical activity and azlactone formation when benzoyl-L-leucine is dissolved in acetic anhydride (50), it was found that, when the acetic anhydride was diluted with an inert solvent, loss of activity occurred at a slower rate than azlactone formation, and this effect was most pronounced when the solvent was a mixture of equal parts of acetic anhydride and dioxan. This observation led to the isolation of an optically active azlactone, which racemised rapidly on keeping.

More recently, optically active 2-methyl-4-isobutyl-5-oxazolone has been prepared by treatment of acetyl-L-leucine with dicyclohexylcarbodi-imide in ethyl acetate solution(60). The product was an oil whose rotation decreased rapidly at room temperature. Hydrolysis gave chiefly racemic acetylleucine.

In 1932, Csonka and Nicolet (61) found that if D-alanine was heated with ammonium thiocyanate and acetic anhydride in acetic acid solution, a thiohydantoin is obtained which was only slightly racemised. They used the result of Johnson and Scott (62a), that an azlactone is formed as an intermediate in this reaction, to deduce that azlactones

possess full optical activity when they are formed. Although the subsequent isolation of optically active azlactones has verified this result, the conclusion of Johnson and Scott cannot now be accepted. In an earlier paper (62b), these authors had shown that a benzenesulphonylamino-acid, when treated with ammonium thiocyanate and acetic anhydride in acetic acid is converted into a thiohydantoin smoothly, in good yield. It is now known that benzenesulphonylamino-acids cannot cyclise in the presence of acetic anhydride to form azlactones, and therefore these cannot be formed as intermediates in the hydantoin-forming reaction. In this case it is likely, according to present knowledge, that the active intermediate in this reaction is a mixed anhydride.

$$R.CONH.CHR.CO.OCOCH_3 + N\overline{C}S \longrightarrow R.CONH.CHR.CO.N{=}C{=}S$$



The bulk of the evidence supports azlactone formation as being largely responsible for the racemisation of an acylamino-acid, when treated with acetic anhydride, and it is reasonable to suppose that this is also true for the

racemisation in mixed anhydride methods of peptide coupling. Clearly, the extent of such racemisation will depend upon the relative rates of azlactone formation, racemisation of the azlactone, and aminolysis of the anhydride and azlactone.

DESCRIPTIVE.

PART I. The coupling of benzoyl-L-leucine with glycine ethyl ester as a quantitative test for racemisation during peptide synthesis. It has been mentioned in the introduction that the coupling of acetyl-L-leucine with glycine ethyl ester has been used as a model reaction for the investigation of racemisation during various coupling procedures, but the syrupy nature of the product, however, did not allow an accurate assessment of the degree of racemisation. Therefore, with a view to developing a new model reaction suitable for quantitative studies, it was decided to investigate the crystallising properties of the optically active and racemic forms of benzoylleucylglycine ethyl ester.

Benzoyl-DL-leucine was prepared by benzoylating L-leucine with benzoyl chloride in aqueous sodium hydroxide and racemising the product by adding acetic anhydride to the neutral solution (55). It was obtained as a colourless crystalline compound, m.p. 141°, which compares with the literature value of 137-141°. This was coupled with glycine ethyl ester, using the tetraethyl pyrophosphite "standard" procedure in chloroform solution, to give benzoyl-DL-leucyl-glycine ethyl ester as a colourless crystalline solid, m.p. 146°. This is a new compound.

Advantage was taken of the ability to couple a benzyloxycarbonylamino-acid without fear of loss of activity in order to synthesise authentic benzoyl-L-leucylglycine ethyl ester. Thus benzyloxycarbonyl-L-leucine syrup was condensed with glycine ethyl ester by the di-imide procedure. The product was obtained as a colourless crystalline solid, m.p. 103-103·5°, $[\alpha]_D^{20}$ - 26·9° (in ethanol). This compares with values of, m.p. 104-105°, $[\alpha]_D^{24}$ - 25·6° (in ethanol), quoted in the literature for peptide made by the mixed carbonic anhydride procedure (63). Decarbobenzyloxylation with hydrogen bromide in acetic acid, followed by benzoylation with benzoyl chloride in the presence of sodium hydrogen carbonate in a two phase system, gave benzoyl-L-leucylglycine ethyl ester. This is a new compound and it is characterised by m.p. 157°, $[\alpha]_D^{20}$ -34·0° (in ethanol). It differs from the corresponding acetyl peptide in being highly crystalline.

Low rotation of a coupling product cannot of itself be regarded as sufficient evidence that racemisation has occurred, even if the analysis of that product is satisfactory, since the presence of an impurity of similar analysis cannot be sensitively detected by such a means. Confirmation must therefore be furnished by isolation and characterisation

of a racemate or a derivative thereof, obtained by a method which should not itself cause loss of activity.

Experiments were therefore conducted to investigate the ease of isolation of benzoyl-DL-leucylglycine ethyl ester from a mixture with the optical isomer. The first attempts involved fractional crystallisation. Separation was found to be possible only when the racemic content was high (~ 50%) ; crystallisation of peptide containing only 10% of racemate gave a first fraction of almost pure L-isomer.

It was then decided to investigate the ease of isolation of benzoyl-DL-leucylglycine from the partially racemic dipeptide acid ; for saponification of the ethyl ester should be possible without loss of activity or rupture of the peptide bond by using cold dilute sodium hydroxide in aqueous dioxan solution (64).

Saponification of the ethyl ester using this procedure easily afforded benzoyl-DL-leucylglycine as a highly crystalline compound, m.p. 165°. A similar approach to benzoyl-L-leucylglycine gave a syrup which was uncrystallisable, even after attempted purification by repeated recrystallisations of the dicyclohexylamine salt. The elementary analyes were satisfactory and the rotation, $[\alpha]_D$ - 25° (in ethanol).

The analogy with the crystallising properties of the acetyl peptides was evident and therefore isolation of racemate from the saponified coupling product suggested itself as a convenient general procedure. The method was tested by saponifying several synthetic mixtures of benzoyl-L- and DL-leucylglycine ethyl ester, varying in composition between 20% and 1% of racemate. In the former case, attempted crystallisation direct from the neutralised and diluted aqueous saponification medium yielded pure benzoyl-DL-leucylglycine, characterised by m.p. and zero rotation. Saponification of the mixture containing 1% racemate failed to give racemic dipeptide acid, but in all other cases, material was isolated, which, after further recrystallisation from water, gave the racemic or chiefly racemic peptide acid, having m.p. one or two degrees lower than the authentic material and specific rotation of -2 or -3°. In several of these cases, however, the first fraction consisted of crystalline material, in amounts sometimes considerably exceeding the quantities of racemate present, and melting at approximately 139°. This material was shown by infrared spectra of chloroform solutions to be a mixture of benzoyl-DL-leucine and benzoyl-DL-leucylglycine. The latter, however, could always be obtained as

the first fraction in a further recrystallisation of the mixture, so that this did not detract from the value of the method, especially since the amount of loss of racemic dipeptide by hydrolysis is proportional to the racemic content of the peptide ; the loss is therefore negligible in those cases where the racemic content is low and the greatest sensitivity of detection is required. In such cases, therefore, most of the benzoyl-DL-leucine would arise from hydrolysis of benzoyl-L-leucylglycine ethyl ester. The loss of activity might well occur during the recrystallisation procedure. The results are summarised in Fig.1.

Fig.1.

% DL	1st.fn.		Recrest. 1st. fn.		
	Wt.,gm.	m.p.	Wt.,gm.	m.p.	$[\alpha]_D$ in EtOH
20%	0·08	159-161	0·04	163·5-164·5	0
10%	0·07	146-150	0·014	163 - 164	-2·2
5%	0·047	137-139	0·019	162.5-163·5	-3·4
2%	0·13	138-139	0·009	162 - 163.5	-
1%	0	-	-	-	-
0%	0	-	-	-	-

c.f. Benzoyl - DL - leucine, m.p. 141°.

That peptide hydrolysis occurs to some extent in the above saponification procedure using this particular dipeptide, was further demonstrated by the observation that in one saponification of authentic benzoyl-L-leucylglycine ethyl ester, attempted crystallisation of the product from the neutralised aqueous mixture gave benzoyl-DL-leucine, m.p. 141°. Further careful crystallisation however gave no benzoyl-DL-leucylglycine, even with seeding.

It was considered, in the light of the above results, that the failure to crystallise the benzoyl-L-leucylglycine obtained by saponification of the esters could possibly be accounted for by the presence of benzoyl-DL-leucine as an impurity. A synthetic route which precluded this possibility was then attempted. Saponification of benzyloxycarbonyl-L-leucylglycine ethyl ester by the procedure described above gave the dipeptide acid as a crystalline product which was rigorously freed from any benzyloxycarbonylleucine which may have been present, by repeated recrystallisations. m.p. 116-117°, $[\alpha]_D^{22.5}$ -27·4° (in ethanol). Bergmann quotes, m.p. 115° (65). Treatment with hydrogen bromide in acetic acid gave L-leucylglycine hydrobromide as a hygroscopic crystalline compound, m.p. 195-6°. Benzoylation in aqueous sodium

carbonate solution gave benzoyl-L-leucylglycine as an uncrystallisable glass, $[\alpha]_D$ - 26° (in ethanol)

It is now apparent that isolation of racemate from a dipeptide by a method which involves crystallisation is rendered easier when the L-isomer is syrupy, as in the case of benzoyl-L-leucylglycine, or crystallises with difficulty (in the presence of racemate), such as acetyl-L-leucylglycine ethyl ester. The facile isolation of racemate in the acetyl model makes it particularly suitable for the rapid demonstration that racemisation can occur during a peptide-forming reaction, but the benzoyl model is complementary in the sense that the product is wholly crystalline, and conversion to a derivative is necessary for the isolation of racemate. The saponification procedure described, however, enables this to be done even when the racemic content of the coupling product is as low as 2%.

The high crystallinity of benzoylleucylglycine ethyl ester, whatever the racemic content, and the high specific rotation of the L-isomer make the coupling of benzoyl-L-leucine with glycine ethyl ester a very suitable model for quantitative investigations of racemisation.

Benzoyl-L-leucine was first prepared as a crystalline

solid by Fischer (66) by the direct benzoylation of L-leucine in aqueous sodium hydroxide solution. It crystallised as the semi-etherate, m.p. 60°, from ethereal solutions, but the unsolvated form, m.p. 105-7°, could be obtained by evacuation of the powered etherate. The specific rotation was recorded as $[\alpha]_D^{20}$ + 6·6° (in potassium hydroxide solution). It was obtained by Karrer (67) as an uncrystallisable oil by saponification of the methyl ester. A modification of Fischer's method has been found to give a crystalline product, m.p. 105-6°, $[\alpha]_D$ - 5·5° (in ethanol). Crystallisation of benzoyl-L-leucine was found to be easier after purification by recrystallisation of the cyclohexylamine salt. The optical purity of the product was demonstrated by conversion with diazomethane to the methyl ester, $[\alpha]_D$ - 24·3° (in ethanol), which compares with $[\alpha]_D$ - 23·7° (in ethanol) for material obtained by benzoylation of L-leucine methyl ester hydrochloride.

PART II. Investigation of coupling procedures using the condensation of benzoyl-L-leucine with glycine ethyl ester.

No racemisation has ever been reported to accompany the acid azide procedure for peptide coupling and it was therefore considered that it would be instructive to investigate this procedure using the coupling of benzoyl-L-leucine with glycine ester, especially since it was considered that this coupling might possibly be a particularly stringent test for racemisation.

Benzoyl-L-leucylhydrazide was obtained as a colourless crystalline solid, m.p. 155°, by the action of hydrazine hydrate on the methyl ester in methanolic solution. Coupling with glycine ester afforded benzoylleucylglycine ethyl ester, $[\alpha]_D$-34·0° (in ethanol), and in a second experiment, the rotation of the crude product, which gave an acceptable elementary analysis, was -29·8°. These figures compare with $[\alpha]_D$- 34·0° for the authentic active peptide. Saponification of the second product afforded no benzoyl-DL-leucylglycine, even after seeding, so it may be safely deduced that less than 2% of racemate was present.

The only case which has been reported of loss of activity in the cyanomethyl ester method of coupling is

that during the esterification of benzyloxycarbonyl-S-benzyl-L-cysteine, and Smart (68) has found that the coupling of acetyl-L-leucine with glycine ethyl ester by this procedure gave a syrupy product of high rotation which did not crystallise . Benzoyl-L-leucine has now been coupled with glycine ester by this method. In the initial experiments, the ester was made by refluxing the carboxyl component with chloroacetonitrile and triethylamine in ethyl acetate, and then coupled at room temperature in this solvent. The product possessed a high specific rotation (- 30·6), but was badly discoloured. Trituration with cold ethyl acetate, however, extracted the brown impurities, leaving a colourless crystalline solid, $[\alpha]_D$ - 33·8°. A small quantity of crystalline material was recovered from the ethyl acetate extract and saponified, but no evidence of racemisation was obtained. In a second experiment, a similarly discoloured product was obtained, $[\alpha]_D$ - 30.1°.

The esterification with chloroacetonitrile was then conducted at 0°, and coupling effected in ethyl acetate solution at room temperature. Peptide of rotation, $[\alpha]_D$ - 33·6° was obtained and no racemic dipeptide acid crystallised in the usual saponification procedure. In a second experiment,

a crystalline product, $[\alpha]_D$ - 32.3°, was obtained.

Coupling to free glycine ester by the mixed carbonic anhydride method, using isobutyl chloroformate in tetrahydrofuran solution —— conditions which had been found to be accompanied by the maximum retention of activity in the acetyl-L-leucine coupling — gave highly racemised products, whose rotations in the two experiments indicated 18 and 22% of optical isomer, in excess of that present in racemate. Benzoyl-DL-leucylglycine ethyl ester was isolated from these products by crystallisation from acetone-ether. It appears from this result that the stringency of the coupling of benzoyl-L-leucine as a test for racemisation is comparable with that of the coupling of acetyl-L-leucine with glycine ester, in which case the product was estimated to contain at least 70% racemate.

A reinvestigation was made of the tetraethyl pyrophosphite coupling method, using "standard" and "amide" procedures, but using chloroform as solvent, since the work of Smart has shown that coupling in diethyl phosphite tends to give discoloured products. Furthermore, it has been found that heating a carboxylic acid and a primary amine in this solvent results in some amide formation in the absence of added

coupling reagent (69). It was found in the present work that there is no significant difference in the degree of racemisation accompanying the "standard" and "amide" procedures, and it has been confirmed that the use of free glycine ester leads to less racemisation than the use of the hydrochloride in the presence of base.

The results obtained when benzoyl-L-leucine was coupled with glycine ester by the di-imide method indicate that the benzoyl model is a more sensitive test for racemisation than the acetyl model, at least in this case. When distilled glycine ester was used, in dichloromethane as solvent, the product obtained in two experiments contained 53 and 54% L-isomer. This compares with an estimated maximum of 30% racemate in the coupling of acetyl-L-leucine under the same conditions. Saponification of the benzoyldipeptide obtained in one experiment yielded racemic benzoylleucylglycine.

The coupling of benzoyl-L-leucine with glycine ethyl ester with N-ethyl-5-phenylisoxazolium-3'-sulphonate, using glycine ester hydrochloride and triethylamine in acetonitrile following the procedure of Woodward (24), gave an 81% yield of crystalline peptide, $[\alpha]_D$ -29·8°. The elementary analysis

of the crude product was good and saponification allowed the isolation of benzoyl-DL-leucylglycine, m.p. 159-161°. In a repeat experiment, the rotation of the product was $[\alpha]_D$ - 30·6°, and saponification again gave clear evidence that racemisation had occurred.

When the procedure was modified, using distilled glycine ester in place of the ester hydrochloride, a significant increase in the rotations of the coupling products was observed. Thus, in one experiment, a 77% yield of peptide, $[\alpha]_D$ - 32·8°, was obtained, and in another, a 75% yield of product, $[\alpha]_D$ - 32·5°. Furthermore, no racemic benzoylleucylglycine could be isolated from these products by the usual saponification procedure.

These results are summarised in Fig. 2. The percentage L-isomer, calculated from the specific rotations of the crude products, excludes that present in racemate. The elementary analyses of all the crude products are acceptable. + (r), + (s) denote that racemate has been isolated by recrystallisation and saponification, respectively. -(s) denotes that the product was saponified but no racemate found.

Fig. 2.

Method	Conditions	% L	Racemate found
Acid azide	Ether as solvent	100	
		88	-(s)
Cyanomethyl ester	Ester formed by refluxing in EtOAc	90	-(s)
		89	
	Ester formed at 0°	99	-(s)
		95	
Mixed carbonic anhydride	glyOEt, T.H.F.	18	+ (r)
		22	
Tetraethyl pyrophosphite	"Standard", $CHCl_3$, glyOEt	86	+(s)
		90	
	"Standard", $CHCl_3$, glyOEt. HCl + NEt_3	52	
		49	+(r)
	"Amide", $CHCl_3$ glyOEt	48	+(r)
	"Amide", $CHCl_3$ glyOEt. HCl + NEt_3	27	+(r)
Di-imide	glyOEt, CH_2Cl_2	53	+(s)
		54	
Woodward	glyOEt.HCl+NEt_3, CH_3CN	88	+(s)
		90	+(s)
	glyOEt, CH_3CN	97	-(s)
		96	-(s)

PART III.

Mechanism of racemisation during coupling. Part III (a). p-Nitrophenyl Ester Method. Activated ester coupling methods are particularly suitable for the investigation of the mechanism of racemisation because the activated intermediates are usually stable crystalline compounds. This enables the two stages of the coupling procedure, namely the formation of the activated carboxyl component and the condensation of this with the amino-component, to be studied separately.

Many investigators have shown that the esterification methods currently employed for the preparation of p-nitrophenyl esters may lead to racemic products, and the object of the present work was to investigate the extent of racemisation, if any, which accompanies the condensation of the ester with a primary amine, the dependence of this racemisation upon the conditions employed, whether azlactone formation can be held responsible for this loss of activity, and if so, to what extent.

It was decided to use the model reaction already developed, i.e. the coupling of benzoyl-L-leucine with glycine ethyl ester, for this investigation. The first requirement was therefore the synthesis of authentic benzoyl-L-leucine p-

nitrophenyl ester. This was achieved by decarbobenzoxylation of benzyloxycarbonyl-L-leucine p-nitrophenyl ester, followed by benzoylation of the ester hydrobromide with benzoyl chloride in the presence of sodium carbonate. Benzyloxycarbonyl-L-leucine p-nitrophenyl ester was made by the method of Elliott and Russell. It was obtained as a colourless crystalline compound, m.p. 95°, $[\alpha]_D^{20}$ - 33.6° (in dimethylformamide). The literature values are, m.p. 95°, $[\alpha]_D^{20}$ - 33.5° (in dimeth-ylformamide) (70). Treatment with hydrogen bromide in acetic acid gave L-leucine p-nitrophenyl ester hydrobromide as a colourless crystalline compound, m.p. 198-200°, $[\alpha]_D^{28}$ + 10.8° (in ethanol). Goodman and Stueben (71) quote, m.p. 198.5-199.5, $[\alpha]_D^{25}$ + 11.4° (in ethanol). Benzoylation of this compound in a two phase system in the presence of sodium carbonate gave benzoyl-L-leucine p-nitrophenyl ester as a highly crystalline compound, m.p. 95.5 - 96.5, $[\alpha]_D^{23}$ - 43.6° (in ethanol). It was found that removal of solvent at 60° gave a product whose infrared spectrum in chloroform solution showed that it contained some 2-phenyl-4-isobutyl-5-oxazolone, but this could be avoided by removing the solvent at room temperature.

The effect of conditions upon the amount of racemisation

which accompanies the aminolysis of this ester was investigated by allowing the coupling to proceed in the presence of varying amounts of N-methylpiperidine, using the amino component as the free ester or as the hydrochloride. The crude products were all crystalline and their essential purity was established by elementary analyses. The values of the specific rotations then gave the L-isomer content, in excess of that present as racemate. The results are shown in Fig. 3.

The following conclusions may be drawn from an analysis of these results:

Fig. 3.

Conditions	Solvent	% L-isomer
glyOEt (1)	EtOAc	100
glyOEt (5)	"	90
glyOEt (1)+NMP(0.5)	"	90
glyOEt (1)+NMP (1)	"	90
glyOEt.HCl+NMP (1)	"	80
glyOEt (1)	$CHCl_3$	96
glyOEt (1)+NMP (1)	"	73
glyOEt.HCl(1)+NMP(1)	"	75
glyOEt.HCl(1)+NMP(2)	"	54
glyOEt.HCl(1)+NMP(3)	"	51

glyOEt.HCl(1)+NMP(10)	"	49, 44
glyOEt.HCl(1)+NMP(1)	pyridine	90

(1). The use of one mole of distilled glycine ester in ethyl acetate or chloroform as solvent gives a product of high rotation from which no racemate could be isolated by the saponification procedure. This shows that the maximum possible racemic content is 2%, this being the limit of the sensitivity of the method.

(2) Racemisation can be induced by the presence of base, either as excess glycine ester or tertiary base such as triethylamine or N-methylpiperidine.

(3) The use of glycine ester hydrochloride in the presence of one mole of N-methylpiperidine led to 20% racemisation in ethyl acetate and 25% in chloroform. This increased racemisation when the amino component is liberated from the hydrochloride <u>in situ</u> with a tertiary base has been observed by other workers, using different coupling procedures, and has been discussed in the introduction.

Using chloroform as solvent, the racemisation induced in this way is as much as that produced when the free ester is coupled in the presence of one mole of N-methylpiperidine.

This may be attributed to several effects; firstly glycine ethyl ester hydrochloride is only partially soluble in chloroform and consequently the liberation of the free ester with a tertiary base is a heterogeneous process, and since in the coupling procedure the liberation is conducted in the presence of the p-nitrophenyl ester, it is possible that the base is responsible for some loss of activity of the active ester before it is completely converted into its salt.

Other possibilities are a specific racemising effect of the halide ion and catalysis of racemisation by the presence of hydrogen chloride. It has been mentioned that Anderson has attributed this increased racemisation, in the tetraethyl pyrophosphite procedure to "the presence of hydrogen chloride, free or combined" (15). This "chloride ion effect" is discussed at greater length in a later part of this chapter. It is interesting that, in ethyl acetate as solvent, the use of ester hydrochloride and tertiary base leads to more racemisation than the use of distilled ester and one mole of tertiary base ; in this case, therefore, the racemisation cannot be accounted for on the basis of "heterogeneous liberation" alone. (4). In chloroform solution, the maximum amount of racemisation which could be induced by the addition of tertiary base

was approximately 50%, using ester hydrochloride and 10 moles of N-methylpiperidine.

(5) The use of pyridine as solvent in the coupling of glycine ester hydrochloride in the presence of one mole of N-methylpiperidine led to only 10% racemisation, whereas in chloroform the figure is 25%. This is somewhat surprising in view of the fact that the racemisation has been found to be base-catalysed. It may be significant that glycine ester hydrochloride is more soluble in pyridine than in chloroform, so that the "heterogeneous liberation" effect is less important in pyridine. Further observations are made later, in the light of further results on chloride ion effect.

The above results then led to the investigation of the mechanism of this racemisation. In view of the work which has been done on the racemisation of acylamino-acids by treatment with acetic anhydride, the first possibility to be considered was the azlactone mechanism. For this purpose, the azlactone of benzoylleucine, i.e. 2-phenyl-4-isobutyl-5-oxazolone was synthesised by treatment of an acetic acid solution of benzoylleucine with acetic anhydride (72). It was obtained as a colourless crystalline solid, m.p. 57°. The literature value is 56-57°. An infrared spectrum of a

dilute chloroform solution showed two strong peaks in the carbonyl region of the spectrum. (Fig. 4b). These are assigned as follows: 1664cm.$^{-1}$ due to C = N conjugated with phenyl group, and 1832 cm.$^{-1}$ due to the γ -lactone carbonyl group. Rasmussen and Brattain (73) have reported that 2-phenyl oxazolones show a C = N absorption at 1641 - 1657 cm.$^{-1}$ Thompson (74) reports that saturated oxazolones have two intense bands between 1600 - 1830 cm.$^{-1}$ A comparison may be made with the values: approx. 1800 cm.$^{-1}$ for CO in $\beta\gamma$ -unsaturated lactones (75) and 1630 - 1660 cm.$^{-1}$ for the system Ar-C=N (76). The ultraviolet spectrum of 2-phenyl-4-isobutyl-5-oxazolone in dilute chloroform solution is shown in Fig. 5b. λ max 2450A, E max 15,000. The literature values are: λ max 2425 A, E max 11,500 for an ethanolic solution (77).

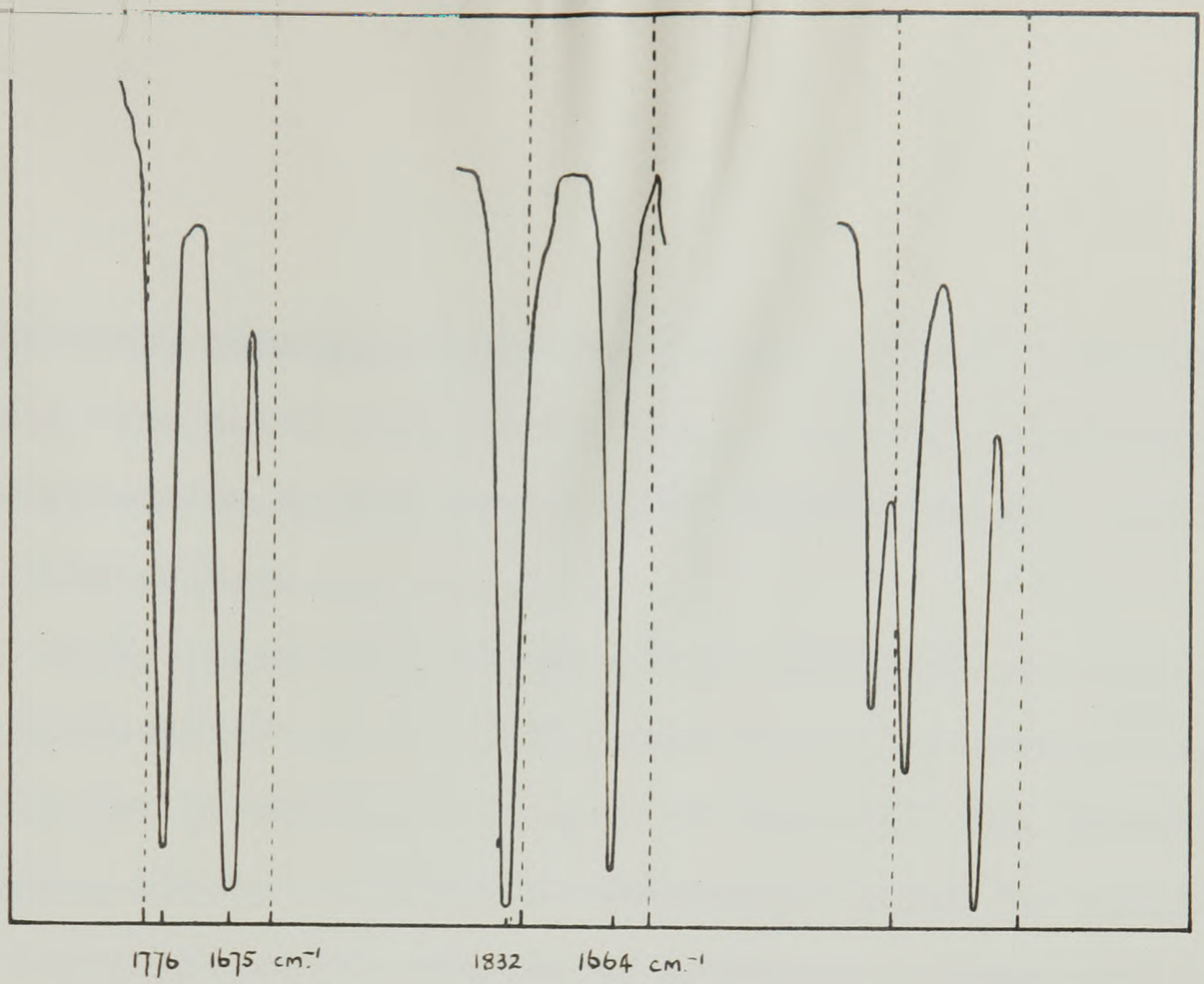
The infrared and ultraviolet spectra of benzoylleucine p-nitrophenyl ester in chloroform solution are shown in Figs. 4a and 5a respectively. In the infrared carbonyl region, there are two strong peaks; the ester absorption at 1776 cm.$^{-1}$ and the amide absorption at 1675 cm.$^{-1}$ Normal saturated esters absorb in the range 1735 - 1750 cm.$^{-1}$ (78), but aromatic esters absorb at higher frequencies. (e.g.79). All amides show a strong absorption near 1680cm.$^{-1}$ in dilute solution. In

Fig. 4a. I.R. spectrum of benzoylleucine p-nitrophenyl ester in chloroform solution (0.33 mole/lit.)

Fig. 4b. I.R. spectrum of 2-phenyl-4-isobutyl-5-oxazolone in chloroform solution (0.33 mole/lit.)

Fig. 4c. I.R. spectrum of solution of benzoylleucine p-nitrophenyl ester + N-methylpiperidine (1 mole) after leaving at room temperature for 20 min.

Figs 5 a,b. U.V. spectra of benzoylleucine p-nitrophenyl ester and 2-phenyl-4-isobutyl-5-oxazolone respectively, in chloroform solution.



Figs. 4a 4b 4c

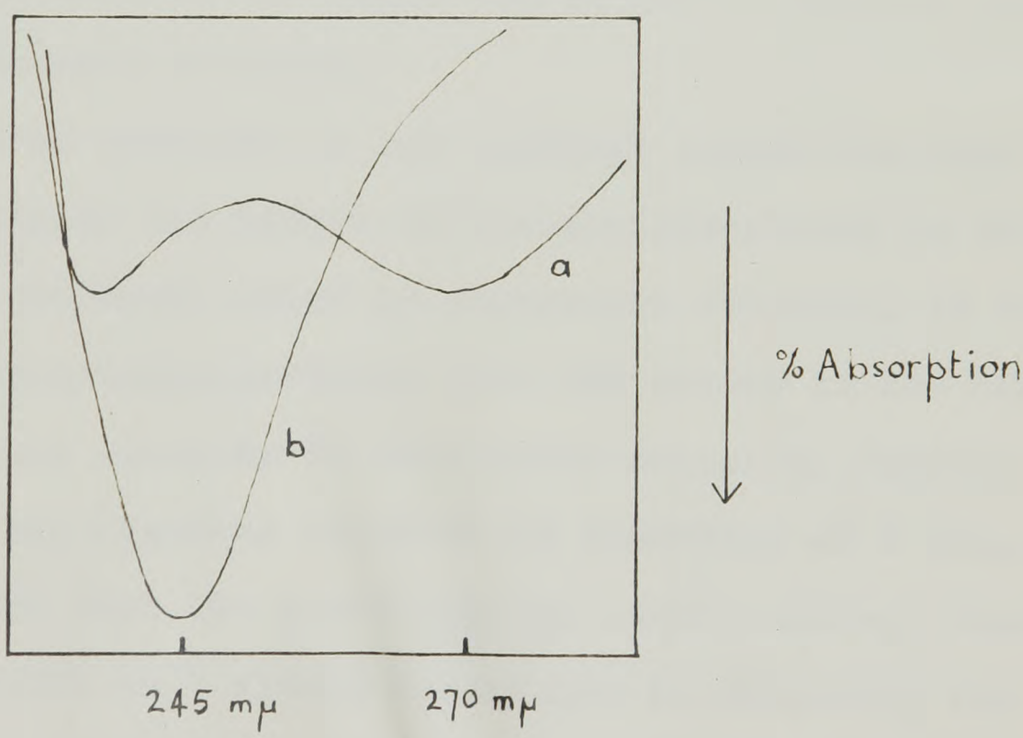


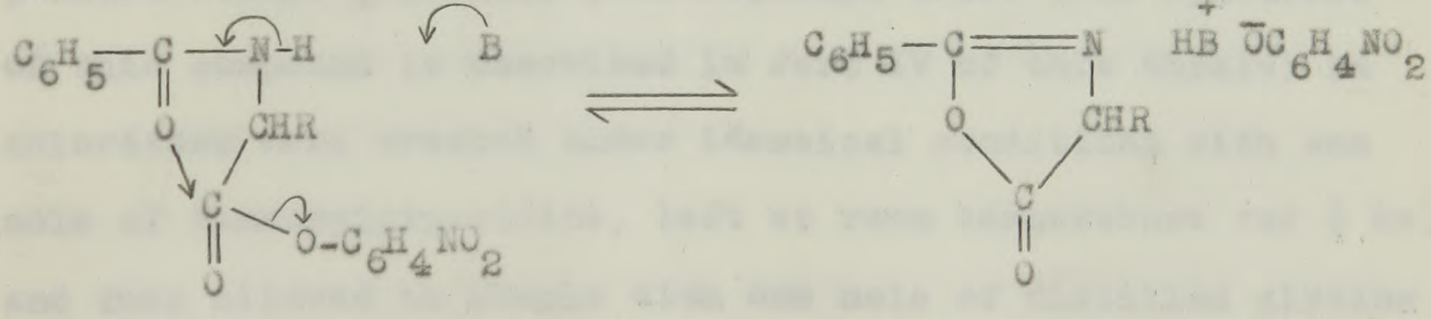
Fig. 5

particular, benzoylglycine ethyl ester absorbsat 1650 $cm.^{-1}$ This is the amide I absorption (80). In the ultraviolet, benzoylleucine p-nitrophenyl ester absorbs at 2375 and 2700 A; for both peaks E max = 11,000.

It is clear that the azlactone cannot be detected in the presence of the ester by means of its ultraviolet spectrum, but it can by the infrared peak at 1832 $cm.^{-1}$ The infrared spectrum should therefore be extremely useful for qualitative and semiquantitative studies of mixtures of these two compounds, but the ultraviolet absorption at 2700 A can be used for quantitative studies of the ester and at 2450 A for the azlactone in the absence of ester.

The infrared spectrum in the carbonyl region was then used to investigate the effect of N-methylpiperidine on benzoylleucine p-nitrophenyl ester in chloroform solution. It was verified that N-methylpiperidine does not absorb in the carbonyl region of the spectrum in chloroform solution. Spectra were taken of the reaction solution at intervals of 5 min., and it was found that the p-nitrophenyl ester carbonyl absorption peak at 1776 $cm.^{-1}$ slowly diminished in intensity and was replaced by the azlactone carbonyl peak at 1832 $cm.^{-1}$, until after 20 min., when the intensities of the two carbonyl

peaks were of comparable magnitude, no further change occured, indicating that an equilibrium had been attained. (Fig. 4c) A calculation showed that the ratio of ester to azlactone concentration at equilibrium was 1 : 0.62. In order to verify that the final spectrum did in fact represent an equilibrium, this was approached from the other side, i.e. equimolar quantities of azlactone, p-nitrophenol and N-methylpiperidine were dissolved in chloroform and spectra were again taken at intervals of 5 min. In this case, the 1832 $cm.^{-1}$ peak was gradually replaced by the 1776 $cm.^{-1}$ peak until no further change occurred after 1 hr. The relative concentrations of ester and azlactone were calculated to be 1 : 0.59. This demonstrates that when benzoylleucine p-nitrophenyl ester is treated with one mole of tertiary base the only reaction which occurs is azlactone formation.



Further evidence was obtained by isolating a sample of 2-phenyl-4-isobutyl-5-oxazolone from a solution of benzoyl-leucine p-nitrophenyl ester which had been treated with one

mole of N-methylpiperidine in ethereal solution. The product was characterised by m.p. , mixed m.p. , an infrared spectrum of a chloroform solution and elementary analysis.

It is now clear that benzoylleucine p-nitrophenyl ester can be converted into its azlactone under the conditions used in peptide coupling, i.e. in the presence of strong base, and since azlactones are known to racemise with great ease in solution (81), it follows that azlactonisation may be responsible for the loss of activity which has been observed in this method of coupling. The question then arises, to what extent is azlactone formation responsible for this loss of activity in the presence of a base ? The following experiments were conducted in order to attempt to clarify this matter.

(1). Solutions of benzoyl-L-leucine p-nitrophenyl ester and p-nitrobenzoyl-L-proline p-nitrophenyl ester (The synthesis of this compound is described in Part IV of this thesis) in chloroform were treated under identical conditions with one mole of N-methylpiperidine, left at room temperature for ½ hr. and then allowed to couple with one mole of distilled glycine ethyl ester. Benzoylleucylglycine ethyl ester was obtained whose specific rotation indicated 9% L-isomer content but the p-nitrobenzoylprolylglycine ethyl ester was 98% active.

Whereas it is realised that the difference in structure between leucine and proline make it impossible to draw a definite conclusion from this result, it is felt that it furnishes a good indication that in the former case the loss of activity is caused chiefly if not entirely by azlactonisation since, in the latter case, no azlactone can be formed. It should be mentioned that the proline peptide was obtained in a 96% yield, which eliminates the possibility of the p-nitrobenzoyl-L-proline p-nitrophenyl ester being converted into a racemic intermediate which does not couple to give a peptide.

(2). Benzoyl-L-leucine p-nitrophenyl ester was coupled with glycine ethyl ester in the presence of two moles of N-methyl-piperidine but the reaction was stopped when aminolysis was estimated to be about half complete. The products were separated by fractional crystallisation and the racemic content of each solid fraction estimated from its specific rotation. The result is shown in Fig. 6.

Fig. 6.

Fn.	Wt.,gm.	m. moles	$[\alpha]_D$	m.moles DL
(1) Pept.	0.30	0.94	-27.1°	0.19
(2) Azl.	0.102	0.50	-	0.50
(3) Ester	0.21	0.59	-37.2°	0.03

(4)	Ester	0.07	0.20	-42.1	0.00
(5)	Ester	0.21	0.59	-34.1	0.04

The reaction was conducted using 3.3 m. moles of benzoyl-L -leucine p-nitrophenyl ester and the five fractions separated from the product represent 2.8 m. moles. All of these fractions were crystalline and gave satisfactory elementary analyses.

It is reasonable to suppose that, had the reaction been allowed to proceed to completion, the azlactone would have given only racemic peptide, especially since the coupling occurred in the presence of a 2-molar excess of N-methylpiperidine. Since 0.5 m. moles of azlactone had been formed when the reaction was 2/3 complete, about 0.75 m. moles would have been formed if the reaction had been allowed to proceed to completion. This represents the minimum amount of racemate which would be formed by the azlactone mechanism. Any racemic ester present in the recovered ester would have been formed by direct proton abstraction. The maximum amount of racemate in fractions (3), (4) and (5) is 0.07 m. moles, so in the complete reaction the maximum amount of racemate which could be formed by this mechanism is(0.07 x 3/2) = 0.10 m. moles. Therefore, in the complete aminolysis, the maximum percentage

of the racemate which could be formed by direct proton abstraction is $\frac{0.1}{0.75 + 0.1} \times 100\% = 12\%$.

(3.) The extent to which the azlactone mechanism is responsible for the racemisation of benzoyl-L-leucine p-nitrophenyl ester in the presence of a tertiary base was then assessed by comparing the rate of loss of optical activity with the rate of conversion of the ester to the azlactone. Preliminary experiments showed that one mole of triethanolamine caused azlactone formation at a suitable rate in chloroform solution at room temperature. In view of the previous experiments on azlactonisation of benzoylleucine p-nitrophenyl ester with N-methylpiperidine, it was considered that azlactone formation would be the only reaction to occur under these conditions. It is known that alcoholysis of p-nitrophenyl esters occurs at extremely low rates. The reaction was followed using the ultraviolet absorption of the p-nitrophenyl ester at 2700 A in chloroform solution. The azlactone does not absorb at this wavelength. (Fig. 5) The decrease of concentration and optical activity with time are shown in Fig.7. It is clear that the loss of optical activity follows the rate of conversion of ester to azlactone very closely.

(4.) When benzoyl-L-leucine p-nitrophenyl ester was coupled

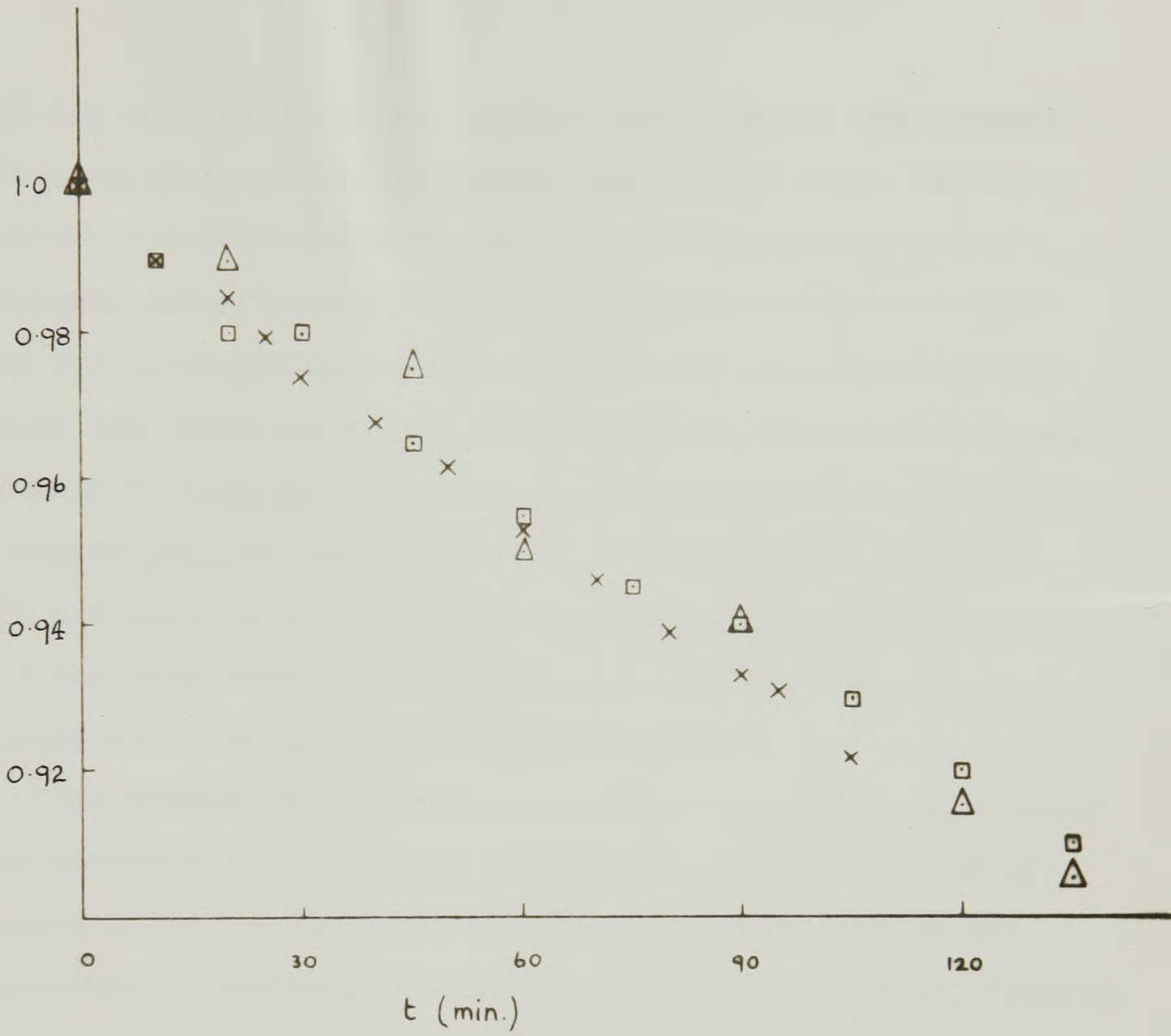


Fig. 7 Benzoyl-L-leucine p-nitrophenyl ester with triethanolamine in chloroform

⊡ , △ Ester concentration

× Optical rotation

with one mole of distilled glycine ethyl ester, the product contained at least 98% L-peptide. This means that, if the base-catalysed racemisation in this reaction is caused by azlactone formation only then the coupling of glycine ester with the p-nitrophenyl ester must proceed at a considerably faster rate than azlactone formation under these conditions. In order to test this, the rate of condensation of benzoyl-L-leucine p-nitrophenyl ester was determined and compared with the rate of azlactomisation in the presence of one mole of a tertiary base possessing the same basic strength as glycine ethyl ester in chloroform solution.

The problem then arose of finding a tertiary amine whose base strength in chloroform solution is similar to that of glycine ester. Little work seems to have been done on the dependence of basicity of amines on the solvent. Thus, Pearson and Vogelsong (82) report that "the base strengths of amines vary with solvent in a complex manner, depending upon dielectric constant and solvating properties of the solvent". They have developed a method of determining relative proton affinities of bases in organic solvents, depending upon measurement of the equilibrium constant of the equilibrium set up with 2 : 4-dinitrophenol, and have found that, in

general, primary amines tend to be stronger than tertiary in water and ethyl acetate, but the reverse is true for pure chloroform. Also, the addition of four mole % of ethanol to the chloroform (approx 2% ethanol by weight) has no effect upon the strength of tertiary amines, but greatly increases the strength of secondary and primary amines. Some equilibrium constants are shown in Fig. 8.

Fig. 8.

	$CHCl_3$	$CHCl_3$ + 2% EtOH
$MeNH_2$	35	3,500
Me_2NH	1,200	12,000
Me_3N	2,700	2,700

It appears therefore, that in chloroform containing about 2% ethanol by weight, methylamine and trimethylamine have approximately similar strengths. It was therefore decided to prepare dimethylglycine ester and compare its basic strength with that of glycine ester in chloroform solution.

Dimethylglycine methyl ester was prepared by the destructive distillation of betaine. This is possible since betaines isomerise to the methyl esters at their melting points (83). Two fractional distillations gave a small yield of colourless

liquid, b.p. 137°/758 mm., n_D^{25} 1.4110. c.f. lit., b.p. 135°. A satisfactory analysis was obtained.

The relative basic strengths of dimethylglycine methyl ester and glycine ethyl ester were determined in chloroform solution, containing about 1% by weight of ethanol. Since the dependence on the base strength upon the percentage of ethanol seems to be critical, the same sample of chloroform was used in all the determinations. The values for triethanolamine and benzyltrimethylammonium chloride were also determined, since these have been employed in this investigation. The basic strength of dimethylglycine methyl ester in aqueous solution was also determined by potentiometric titration against standard hydrochloric acid. The averaged results are shown in Fig. 9.

Fig. 9.

Base	K, $CHCl_3$	pKa, $CHCl_3$	pKa, H_2O
NH_2CH_2COOEt	8	0.8	7.8
$Me_2N.CH_2COOMe$	13	1.1	7.1
$N(CH_2CH_2OH)_3$	60	1.8	7.8
$BzNMe_3^+ \bar{Cl}$	17	1.2	-7

pKa in $CHCl_3$ refers to : $R_3\overset{+}{N}H + \bar{O}Ar \rightleftharpoons R_3N + HOAr$

pKa in H_2O refers to: $R_3\overset{+}{N}H + H_2O \rightleftharpoons R_3N + \overset{+}{H_3O}$

It is interesting that the base strength of the chloride ion in chloroform solution (containing ethanol) is about the same as that of dimethylglycine methyl ester, and slightly greater than that of glycine ethyl ester.

The rate of azlactonisation of benzoylleucine p-nitrophenyl ester in the presence of one mole of dimethylglycine methyl ester was determined at 25° by following the rate of decrease of the ester absorption at 2700 A. It was verified that dimethylglycine methyl ester does not absorb at this value. Since this reaction is reversible, only the initial stages were followed (5-10%). A straight line plot was obtained by assuming second order kinetics and the second order rate constant determined (Figs 32a +b) The aminolyses of ester and azlactone were followed using the ultraviolet absorption maxima at 2700 A and 2450 A in chloroform solution respectively. The resulting peptide does not absorb in these regions.

Assuming second order kinetics for the aminolysis of the azlactone, a plot of the appropriate concentration factor against time gave a straight line, but in the aminolysis of the p-nitrophenyl ester the same plot was found to give a curve (see Figs. 26a + b). This has also been observed for the aminolysis of methyl and ethyl esters (84). The reaction

has been found to assume second order kinetics only to a rough approximation, and the order w.r.t. amine lies between 1.0 and 1.5. Various mechanisms have been postulated for the catalysis of this reaction by the amino component, but a mechanism involving an intermediate complex is now favoured (84d). Now, in the aminolysis of benzoylleucine p-nitrophenyl ester with glycine ester, this deviation from second order kinetics might also be caused by the simultaneous azlactonisation of the p-nitrophenyl ester. However, an approximate value of the second order rate constant for the aminolysis was obtained from the slope of the tangent to this curve at the origin. The rate constants at 25° are summarised in Fig 10.

Fig.10.

Reaction	K(l.mol.$^{-1}$sec.$^{-1}$)	Fig.	Rel. k
pnp-ester $\longrightarrow$ azlactone	1.8×10^{-5}	32a	1
	1.2×10^{-5}	32b	
pnp-ester + glyOEt	5.8×10^{-4}	26a	40
	6.2×10^{-4}	26b	
azlactone + glyOEt	2.9×10^{-3}	29a	160
	2.2×10^{-3}	29b	

It is seen that the rate of aminolysis of the ester with glycine ester is greater than the rate of azlactonisation in the presence of a base slightly stronger than glycine ester by a factor of 40. Therefore, when benzoyl-L-leucine p-nitro-

phenyl ester is treated in chloroform solution with one mole of glycine ethyl ester the maximum amount of peptide which could be formed *via* the azlactone is approximately 2%. (The curve obtained on assuming second order kinetics for this reaction cannot therefore have been due to two reactions occurring concurrently). Furthermore, under these conditions, the rate of aminolysis of the azlactone is much greater than the rate at which it is formed, so it may well be that much of the azlactone couples to give peptide before it racemises. These kinetic results therefore show that, if azlactonisation is the only mechanism of racemisation under these conditions, the upper limit to the amount of racemate that can be formed is about 2%, and probably it is less than this.

This is indeed consistent with the result of coupling benzoyl-L-leucine p-nitrophenyl ester with one mole of glycine ethyl ester in chloroform solution, where failure to isolate racemate by the saponification method furnishes evidence that the peptide has a maximum racemic content of 2%, this being the limit of sensitivity of this procedure.

Four kinds of experiment have therefore been conducted, the result of each of which is consistent with azlactone intermediacy being chiefly or exclusively responsible for the

base-catalysed racemisation during the coupling of benzoyl-L-leucine p-nitrophenyl ester. Whereas each result, taken separately is an indication, taken together they form a convincing argument for the preponderence of this mechanism.

A further investigation was then made of the nature of the "chloride ion effect". To what extent is the increased racemisation which results from the use of the amino component as the hydrochloride in the presence of one mole of tertiary base caused by (1) slow heterogeneous liberation of the free ester, during which time the tertiary base catalyses racemisation of the p-nitrophenyl ester ; (2) catalysis of the aminolysis of the azlactone by the presence of amine hydrochloride ; and (3) a specific chloride ion effect?

Carter, Handler and Stevens (85) have reported that the aminolysis of 2-phenyl-4-benzyl-5-oxazolone with aniline is greatly catalysed by addition of a small quantity of aniline hydrochloride, pyridine hydrochloride or ammonium bromide. It seemed possible that, during the aminolysis of benzoyl-L-leucine p-nitrophenyl ester, such catalysis might upset the ester-azlactone equilibrium in such a way that a

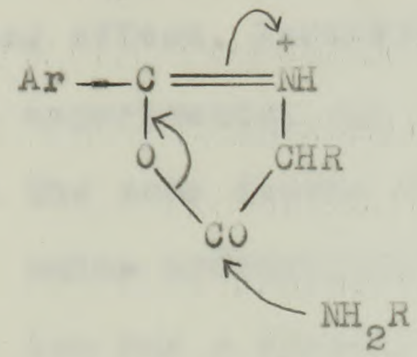
greater proportion of the product would be formed *via* the azlactone, thus resulting in a greater degree of racemisation. In view of this possibility, it was decided to make a semiquantitative investigation of this catalysis. The approximate rate of aminolysis of 2-phenyl-4-isobutyl-5-oxazolone with p-toluidine in chloroform solution was determined by taking infrared spectra of aliquots of the reaction solution at suitable intervals and calculating the concentrations of azlactone from the intensities of the 1832 $cm.^{-1}$ peak. This was repeated in the presence of 0.1 mole of various salts. The second order rate constants are shown in Fig.11.

Fig.11.

Solvent	Salt	Rel.k
$CHCl_3$	-	1
"	$Bz_3\overset{+}{N}H\ \bar{C}l$	100
"	$Bz_3\overset{+}{N}H\ S\bar{O}_3C_7H_7$	100
"	$Bz_3\overset{+}{N}H\ \bar{C}lO_4$	100
pyridine	$Bz_3\overset{+}{N}H\ \bar{C}l$	20

The presence of 0.1 mole of tribenzylamine cation therefore increases the rate 100 times in chloroform but only 20 times in pyridine, which is consistent with the belief that

this catalysis is caused by protonation of the azlactone nitrogen atom, and also



with the diminished "chloride ion effect" in pyridine as solvent (See Fig.3).

Further evidence for the nature of the "chloride ion effect was sought by coupling benzoyl-L-leucine p-nitrophenyl ester with glycine ester in the presence of a quaternary ammonium halide. The results, together with some which have already been described, are shown in Fig. 12.

Fig. 12.

Conditions	% DL
(i) glyOEt + $Et_4\overset{+}{N}\ \overline{Cl}$	14
(ii) glyOEt + $BzNMe_3\overset{+}{}\overline{Cl}$	8,18
(iii) glyOEt + NEP.HCl	12
c.f.(iv) glyOEt + NMP	27
(v) glyOEt.HCl + NMP	25

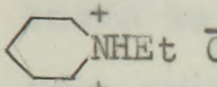
That an added quaternary ammonium chloride should cause

racemisation in this reaction was a most revealing result, indicating clearly that the chloride ion has a specific racemising effect. Furthermore, within the limits of accuracy of these experiments, one mole of quaternary ammonium chloride produces the same degree of racemisation as one mole of a tertiary amine hydrochloride, which is to be expected if the chloride ion has a specific effect. This means that it is unnecessary to postulate acid catalysis of the aminolysis of the azlactone to account for the increased racemisation in (v) and (iii), since it cannot occur in (i) and (ii).

A comparison of (iii) and (v) shows that heterogeneous liberation of the amino component also contributes to the racemisation in (v), since this is greater than that caused by one mole of chloride ion alone, and must therefore be caused by the N-methylpiperidine, which is more effective in causing racemisation (iv).

In order to throw light upon the mechanism of this specific chloride ion effect in the catalysis of racemisation, benzoylleucine p-nitrophenyl ester was treated, in solution, with various salts and the infrared spectrum in the carbonyl region examined for evidence of azlactone formation. The results are summarised in Fig. 13.

Fig. 13.

Salt	Solvent	Azlactone
	$CHCl_3$	+
$Bz_3\overset{+}{N}H\ \bar{C}l$	"	+
$Et_4\overset{+}{N}\ \bar{C}l$	"	+
$Pr_4\overset{+}{N}\ \bar{I}$	"	+
$Bz_3\overset{+}{N}H\ \bar{C}lO_4$	"	-
$BzNMe_3\overset{+}{\bar{C}}lO_4$	DEP	-
$BzNMe_3\overset{+}{\bar{O}}C_6H_4NO_2$	CH_2Cl_2	+

Diethyl phosphite was used with benzyltrimethylammonium perchlorate because this did not dissolve in chloroform. The quaternary ammonium p-nitrophenate was found to react with chloroform, on warming, with the formation of a green solution. It is seen that halide ions and the p-nitrophenate ion cause azlactone formation but the perchlorate ion does not. This is consistent with the observation that the chloride ion in chloroform solution possesses a basic strength comparable with that of dimethylglycine methyl ester.

It cannot be concluded, however, that it is only the basicity of the chloride ion which is responsible for the azlactonisation of benzoylleucine p-nitrophenyl ester, since

it is possible that nucleophilic displacement can occur at the carbonyl group to give the acid chloride, which is known to azlactonise very rapidly. It was demonstrated that such nucleophilic displacement can occur, by refluxing p-nitrophenyl phenylacetate with a quaternary ammonium chloride in dichloromethane solution. Liberation of the p-nitrophenate ion was detected by its ultraviolet absorption maximum at 4000 A, and it was estimated that, at equilibrium, 0.6% of the p-nitrophenyl ester had been converted to the acid chloride.

Benzyltrimethylammonium p-nitrophenate was found to cause very rapid azlactonisation of benzoylleucine p-nitrophenyl ester in methylene chloride solution. This result is interesting because nucleophilic displacement in this case would merely give the same ester and therefore not cause azlactone formation.

It is emphasised that the results which have been described above apply only to the compounds and model reactions which have been investigated, and that, whereas a knowledge of these results can usefully be extended to analagous cases for the purpose of making predictions, say, of the amount of racemisation which is likely to accompany certain

conditions in a coupling reaction, detailed results will, of course, be dependent upon the actual compounds and conditions employed.

An example of this was provided by the coupling of benzoyl-L-leucine p-nitrophenyl ester with one mole of benzylamine in chloroform solution. The product obtained was 14% racemised, which is to be contrasted with the virtual absence of racemisation when one mole of glycine ester is used under the same conditions. It can only be concluded that, in going from glycine ester to benzylamine the basicity has increased more than the nucleophilicity.

Authentic benzoyl-L-leucylbenzanilide was synthesised in the usual way which has been employed for the avoidance of racemisation, i.e. *via* the benzyloxycarbonyl peptide. The latter was synthesised by the p-nitrophenyl ester method, and was obtained as a colourless crystalline compound, m.p. 112-3°, $[\alpha]_D$ - 16.6° (in ethanol). Benzoyl-L-leucylbenzanilide is a colourless crystalline compound, m.p. 145°, $[\alpha]_D$ - 12.2° (in ethanol). Coupling of benzoyl-L-leucine p-nitrophenyl ester with one mole of benzylamine in chloroform solution gave a crystalline product, of good analysis, $[\alpha]_D$ - 10.5° (in ethanol).

Part III (b). The tetraethyl pyrophosphite coupling method.

An investigation of the dependence of the amount of racemisation upon the conditions employed in the tetraethyl pyrophosphite coupling procedure was made by condensing benzoyl-L-leucine with glycine ethyl ester using the "standard procedure" in the presence of a variety of salts, using chloroform as solvent. The results are shown in Fig.14.

Fig. 14.

Conditions	% L
(a) glyOEt	76,76
(b) glyOEt.HCl+NEP	44,43
(c) glyOEt+NEP.HCl	47,45
(d) glyOEt+NEP.tosylate	45,44
(e) glyOEt+$Et_4\overset{+}{N}\ \overline{Cl}$	43,43
(f) glyOEt+$Pr_4\overset{+}{N}\ \overline{I}$	67
(g) glyOEt+$BzNMe_3\ \overline{BF_4}$	78

The tetraethyl pyrophosphite used for these coupling reactions was a combination of the two fractions: b.p. 80-85°/0.3 mm. and 60-75°/0.2 mm. The refractive index was n_D^{26} 1.4306. Anderson quotes: b.p. 79-81°/0.15 mm., n_D^{26} 1.4313.

The coupling experiment which has been described in Part II of the thesis, using tetraethyl pyrophosphite of

b.p. 60 - 75°/0.2 mm., n_D^{25} 1.4301, gave a product containing an average of 88% L-isomer, but in this series, the same experiment gave a value of 76% L-isomer. It can only be concluded that the degree of racemisation in this procedure is dependent upon the boiling point of the reagent employed, even if the refractive indices are identical. In the preparation of the reagent, it has been observed that triethylamine hydrochloride has usually crystallised out of the low boiling fractions, but the absence of chloride ion in the fractions which have been employed in the coupling experiments has been verified by treatment with silver nitrate solution. This dependence of the degree of racemisation upon the fraction of reagent employed cannot therefore be ascribed to a chloride ion effect. Although the cause of this phenomenon is unknown, it is considered that it is reasonable to compare the results of coupling experiments in which the same sample of tetraethyl pyrophosphite was used, because the use of any so given sample of this reagent has always given reproducible results.

It is interesting that in this procedure, conditions (b) and (c) give rise to the same degree of racemisation, whereas in the p-nitrophenyl ester method, coupling in the

presence of tertiary base hydrochloride was found to be accompanied by less racemisation than when the amino component was liberated from the hydrochloride by the addition of one mole of tertiary base (see p. 63). This observation lends weight to the interpretation which has been given to the latter phenomenon, since, in the tetraethyl pyrophosphite "standard procedure", the free base is liberated from the hydrochloride before addition of the coupling reagent, so that the tertiary base is wholly neutralised before coupling occurs.

The presence of a quaternary ammonium chloride leads to as much racemisation as a tertiary base hydrochloride, an observation which has also been made in the p-nitrophenyl ester method, but a quaternary ammonium iodide is less effective in causing racemisation than the chloride, which is also consistent with the observation that the iodide ion causes azlactonisation of benzoylleucine p-nitrophenyl ester at a slower rate than the chloride ion.

The quaternary ammonium borofluoride was found to be only sparingly soluble in refluxing chloroform, so no conclusion may be drawn from this experiment concerning the effect of the $\overline{B}F_4$ ion on racemisation.

Evidence has been obtained for the ability of tetraethyl pyrophosphite to convert benzoylleucine into its azlactone under the conditions employed in coupling reactions. Thus, benzoylleucine was refluxed in chloroform solution for ten minutes in the presence of tetraethyl pyrophosphite. Removal of solvent gave the azlactone in an almost pure form.

Tetraethyl pyrophosphite condensations are usually conducted in diethyl phosphite as solvent, and it was considered that an investigation of the halide ion effect in this solvent would be of interest, especially since a comparison could be made with the effect of the perchlorate ion, it having been found that benzyltrimethylammonium perchlorate is sufficiently soluble in this solvent. The results of these experiments are shown if Fig. 15.

Fig.15.

Conditions	% L
glyOEt	53,48
glyOEt+$BzNMe_3^+Cl\bar{O}_4$	48,49
glyOEt+$Et_4N^+\bar{C}l$	22,21
glyOEt+NEP.HCl	30
glyOEt.HCl+NEP	~~31~~ 25

It is interesting that, in this solvent, a quaternary ammonium chloride causes more racemisation than a tertiary amine hydrochloride. The perchlorate ion does not increase the degree of racemisation, a result which is not surprising in view of the lack of basicity and nucleophilicity of this ion.

In order to test the generality of the chloride ion effect it was decided to investigate the coupling with a primary amine considerably weaker than glycine ester. This might also yield interesting results in view of the possibility that it may be the basicity of the chloride ion which leads to the increased racemisation. It was decided to use p-toluidine as the primary amino component. Authentic benzoyl-L-leucyl-p-toluidide was made in the usual way from the benzyloxycarbonyl compound, which, in turn, was made by condensing benzyloxycarbonyl-L-leucine p-nitrophenyl ester with p-toluidine. It was found that the usual conditions i.e. several hours at room temperature led to only partial aminolysis, but refluxing for 3 hours gave peptide, m.p. 161°, $[\alpha]_D^{21}$ - 44.5° (in chloroform). In order to check that no loss of activity had accompanied this aminolysis, the preparation was repeated using the tetraethyl pyrophosphite

procedure. The product was identical; m.p. 160°, $[\alpha]_D$ -44.4° (in chloroform). Conversion to the hydrobromide and benzoylation in the usual way gave benzoyl-L-leucyl-p-toluidide as a highly crystalline compound, m.p. 210°, $[\alpha]_D$ -61.3°. The corresponding racemate was synthesised by aminolysis of 2-phenyl-4-isobutyl-5-oxazolone with p-toluidine. It is a highly crystalline compound, m.p. 223-4°.

The results of the coupling experiments are shown in Fig.16.

Fig. 16.

Conditions	% L	TEPP
(i) $CH_3C_6H_4NH_2$	81	a
	64	b
(ii) $CH_3C_6H_4NH_2 + Et_4\overset{+}{N}\,\overline{Cl}$	48	a
(iii) $CH_3C_6H_4NH_2 + Bz_3\overset{+}{N}H\,\overline{Cl}$ (0.5 mole)	64	b
(iv) $CH_3C_6H_4\overset{+}{N}H_3\overline{Cl} + Bz_3N$	66	b

TEPP: Fn. (a), b.p. (80-85°/0.3 mm. + 60-75°/0.2 mm.), n_D^{26} 1.4306

Fn. (b), b.p. 85 - 95°/0.3 mm., n_D^{25} 1.4336.

Again, the dependence of the degree of racemisation upon

the fraction of reagent used is demonstrated, but if those experiments using the same reagent are compared, it is clear that, although tetraethylammonium chloride causes a considerable increase in racemisation, the use of the amino component as the hydrochloride in the presence of one mole of tertiary base does not increase the racemisation in this case.

An interpretation of this interesting result may be formulated in terms of the relative basic strengths of tribenzylamine and the chloride ion in chloroform solution. The proton affinity of the chloride ion relative to that of the 2:4-dinitrophenate ion has already been determined (Part IIIa) and a similar determination for tribenzylamine showed that this is a considerably weaker base than the chloride ion in chloroform solution (containing approx. 1% of ethanol by weight), the relative proton affinities being about 1 : 50. It follows that, in experiments (iii) and (iv), the chloride ion is protonated preferentially to the tribenzylamine, thus rendering it ineffective both as a base and a nucleophile. In experiment (ii), on the other hand, the chloride ion has no opportunity to become protonated and it therefore catalyses additional racemisation.

The di-imide coupling method. An investigation of the

chloride ion effect in this coupling method was made by condensing benzoyl-L-leucine with glycine ethyl ester in methylene dichloride solution, using glycine ester hydrochloride in the presence of triethylamine and using free glycine ester in the presence of tetraethylammonium chloride. The results, together with those obtained using free ester are summarised below.

Conditions	% L
glyOEt	53,54
glyOEt.HCl + NEt_3	16
glyOEt + $Et_4\overset{+}{N}\,\overline{Cl}$	15

It is concluded that there is a marked halide ion effect and tetraethylammonium chloride causes the same amount of racemisation as triethylamine hydrochloride.

The acid azide method. An attempt was made to induce racemisation to occur in this coupling procedure, by allowing the condensation to occur in the presence of an excess of N-methylpiperidine. In two experiments, the rotations of the products were $[\alpha]_D$ - 30.9°, - 31.0°. Satisfactory elementary analyses were obtained, and saponification of one of the products gave no benzoyl-DL-leucylglycine, after seeding and leaving for several weeks. This indicates that even in the presence of a

2-molar excess of tertiary base, less than 2% racemisation accompanies this condensation.

An attempt was then made to azlactonise benzoyl-L-leucyl-azide, by treating it with an excess of N-methylpiperidine in ethereal solution at 0°. No peak appeared at approximately 1832 $cm.^{-1}$ even after several weeks. It was verified that had an azlactone been formed in this solution, it could have been detected by this means.

A satisfactory correlation has therefore been obtained between the resistance of benzoyl-L-leucylazide to form an azlactone under forcing conditions and the absence of racemisation when this is coupled under these conditions.

The cyanomethyl ester coupling method. No racemisation has ever been reported to accompany this coupling procedure. Therefore, in view of the probability that this would occur by azlactone formation, it was of interest to attempt to find conditions under which benzoylleucine cyanomethyl ester would form an azlactone. Benzoyl-DL-leucine cyanomethyl ester was made as a highly crystalline compound, m.p. 76-77°, by treatment of benzoyl-DL-leucine with chloroacetonitrile in the presence of triethylamine. The infrared spectrum in

chloroform solution showed peaks at 1767 and 1672 $cm.^{-1}$ due to ester and amide groups respectively. This compound was refluxed in chloroform in the presence of a 2-molar excess of N-methylpiperidine. There was no change in the infrared spectrum of the carbonyl region after six hours. Refluxing in the presence of 2 moles of benzyltrimethylammonium chloride for six hours, however, caused the appearance of a very weak absorption at 1832 $cm.^{-1}$ This is probably due to the formation of a very small concentration of azlactone, in which case the mechanism would involve nucleophilic attack by the chloride ion, rather than the chloride ion acting as a base.

PART IV. Racemisation of acylproline derivatives. It has been mentioned in the introduction that Carter and Stevens have reported that "certain acyl derivatives of L-proline and N-methyl-D-phenylalanine are rapidly racemised when treated with acetic anhydride in glacial acetic acid solution". This observation is of interest because acylimino-acids cannot form azlactones of the conventional kind, and for this reason, in the planning of peptide syntheses, the possibility of racemisation is usually discounted when a peptide containing an imino-acid in the C-terminal position is to be coupled.

The present investigation was initiated by verifying the observation of Carter and Stevens. For this purpose, p-nitrobenzoyl-L-proline was made and characterised as its cyclohexylamine salt, m.p. 175-6°, $[\alpha]_D$ - 73°, since the free acid could not be induced to crystallise. This was treated with a five-molar excess of acetic anhydride both in acetic acid and chloroform solutions at room temperature for one day. The p-nitrobenzoyl-L-proline was recovered as its cyclohexylamine salt and purified by recrystallisation. The specific rotations of the products, which analysed satisfactorily for C, H and N, were, $[\alpha]_D$ - 41°, - 58° respectively, indicating that approximately 30 - 40% racemisation had occurred. This shows that p-nitrobenzoyl-L-proline can be racemised by acetic

anhydride at a rate comparable with that of benzoyl-L-leucine (50).

p-Nitrobenzoyl-L-proline was then coupled with glycine ethyl ester by several methods, in order that the relative losses of optical activity, if any, could be estimated from the specific rotations of the products. The results are shown in Fig. 17.

Fig. 17.

Coupling method	Rot.^{n} of product.
Cyanomethyl ester	-98°
Acid azide	-97°
Mixed carbonic anhydride	-98°
p-nitrophenyl ester	-96°
Di-imide	-95°

It is clear that the rotations of the above products agree to within the limits of experimental error, and it is reasonable to assume from this, that, since the conditions employed in the various procedures are so diverse, it would be improbable that the products should possess the same rotation unless no racemisation had occurred.

The p-nitrobenzoyl-L-proline p-nitrophenyl ester used

in the above experiment was obtained from benzyloxycarbonyl-L-proline p-nitrophenyl ester, which, in turn, was made by the Elliott and Russell procedure from benzyloxycarbonyl-L-proline. The rotation of the p-nitrobenzoylproline p-nitrophenyl ester obtained in this way was $[\alpha]_D$ - 64°, but when this compound was made directly from p-nitrobenzoyl-L-proline by the Elliott and Russell method, the rotation was considerably lower, $[\alpha]_D$ - 40°. Satisfactory elementary analyses were obtained with both products.

It appears, therefore, that although p-nitrobenzoyl-L-proline can be racemised by acetic anhydride, there is little danger of racemisation in the coupling procedures described above, except in the case where the Elliott and Russell procedure is employed to synthesise the p-nitrophenyl ester of the acylproline.

EXPERIMENTAL SECTION.

New compounds are denoted by asterisks.

PART I.

Benzyloxycarbonyl-L-leucine was obtained as a colourless syrup by acylating L-leucine with benzyl chloroformate, using the Schotten-Baumann procedure (86).

Benzyloxycarbonyl-L-leucylglycine ethyl ester was prepared by coupling benzyloxycarbonyl-L-leucine with glycine ethyl ester using dicyclohexylcarbodi-imide as a condensing agent. Recrystallisation from ethyl acetate-light petroleum gave peptide, m.p. 103-103·5°, $[\alpha]_D^{20}$ - 26·9 (c.2·76 in ethanol). c.f. Vaughan and Osato, using the mixed carbonic anhydride coupling procedure, obtained peptide, m.p. 104-105°, $[\alpha]_D^{24}$ - 25·6 (c. 5 in ethanol)

* Benzoyl-L-leucylglycine ethyl ester Benzyloxycarbonyl-L-leucylglycine ethyl ester (7·0g. ; o.o2 mole) was dissolved in ethanol (50ml.). Glacial acetic acid (2·3ml.; 0·04 mole) and 10% palladium-charcoal (7g.) were added and the suspension hydrogenated at room temperature and atmospheric pressure. After the uptake of hydrogen was complete (about 3hr.), the mixture was filtered and the filtrate evaporated under reduced pressure to give L-leucylglycine ethyl ester acetate as a colourless syrup. This was dissolved in water (20ml.).

Ether (50ml.) was added and the mixture stirred vigorously at the interface. Solid sodium bicarbonate (3·7g. ; 0.044 mole) was added portion wise, followed by benzoyl chloride (2·8ml. ; 0·024 mole) in ethyl acetate (40ml.) and 2N sodium bicarbonate solution (20ml. ; 0·04 mole). Stirring was continued at room temperature for 15 min. The ether-ethyl acetate layer was separated and washed with pyridine (5ml.), water (25ml.), dilute hydrochloric acid (2x25ml.) and sodium bicarbonate solution (25ml.). The solution was dried over magnesium sulphate and the solvent removed under reduced pressure, leaving a white crystalline solid (4·68g., 73%). Recrystallisation from ethyl acetate-light petroleum gave material, m.p. 156·5-157°, $[\alpha]_D^{20}$ -34·0 (c. 3·095 in EtOH). (Found: C, 63·7 ; H, 7·5 ; N, 8·8. $C_{17}H_{24}N_2O_4$ requires C, 63·7 ; H, 7·6 ; N, 8·8%).

Benzoyl-DL-leucine was prepared by benzoylating L-leucine by the Schotten-Baumann procedure and racemising the product with acetic anhydride (55). Recrystallisation from ethyl acetate-light petroleum gave material, m.p. 141° c.f. lit. (86), m.p. 137-141°.

* Benzoyl-DL-leucylglycine ethyl ester. Benzoyl-DL-leucine (4·7g. ; 0·02 mole) was dissolved in dry chloroform (25ml.). Glycine

ethyl ester (2·0ml. ; 0·02 mole) was added, followed by tetraethyl pyrophosphite (6ml.). The mixture was refluxed for ½ hr. After cooling, the solution was diluted with chloroform and washed with dil. hydrochloric acid (25ml.), sodium hydrogen carbonate solution (25ml.) and water (25ml.) After drying, the solvent was removed (the diethyl phosphite at 100° and 0·2 mm) to give a colourless crystalline solid. 6·3g. (98%). Recrystallisation from ethyl acetate-light petroleum gave material, m.p. 146°. (Found: C, 64·4 ; H, 7·0 ; N, 8·5. $C_{17}H_{24}N_2O_4$ requires: C, 63·7 ; H, 7·6 ; N, 8·8%).

* Benzoyl-DL-leucylglycine. Benzoyl-DL-leucylglycine ethyl ester (0·32g. ; 0·01 mole) was suspended in 1N aq. NaOH aqueous dioxan (3:1 , 3ml.). Solution was complete in 10 min., and it was left at room temperature for a further 50 min. After this time, it was acidified to Congo Red with dilute hydrochloric acid, heated to boiling and diluted with water sufficiently to dissolve the syrupy product which had separated out. On cooling, copious crystallisation of colourless needles occurred. 0·21g. (80%). Recrystallisation from water gave material, m.p. 165°. (Found: C, 61·4 ; H, 6·9 ; N, 9·8. $C_{15}H_{20}N_2O_4$ requires C, 61·6 ; H, 6·9 ; N, 9·6%).

* Benzoyl-L-leucylglycine.

(a) By saponification of the ethyl ester. Benzoyl-L-leucylglycine ethyl ester (1·60g. ; 0·005 mole) was shaken with 1N sodium hydroxide in aqueous dioxan (3:1), (5ml. ; 0·005 mole) at room temperature for 1 hr., after which time complete solution had occurred. The solution was acidified to Congo Red with dilute hydrochloric acid and extracted into ether (2 x 15 ml.). The ethereal solution was dried with $MgSO_4$ and the solvent removed, leaving a glass. All attempts to crystalline this glass failed. (Found : C, 61·2 ; H, 7·3 ; N, 9.3. $C_{15}H_{20}N_2O_4$ requires C, 61·6 ; H, 6·9 ; N, 9·6%.). $[\alpha]_D^{23}$ -24·8 (c. 4·12 in ethanol).

In a second experiment, using 0·48 g. (0·0015 mole) of peptide, crystallisation of the product was attempted from the acidified aqueous solution. After several weeks a small quantity of crystalline material had appeared. 0·04g., m.p. 137-139°. c.f. Benzoyl-DL-leucine m.p. 141°. The infra-red spectrum of a chloroform solution of the material obtained was identical with that of benzoyl-DL-leucine and differed from that of benzoyl-DL-leucylglycine.

The glassy solid obtained in the first experiment was taken up in ether and the dicyclohexylamine salt precipitated. (1·85g. ; 78%). Recrystallisation from methanol-ether gave

material, m.p. 184-5°. Found: C, 68·6 ; H, 9·1 ; N, 8·9. $C_{25}H_{40}N_2O_3$ requires C, 68·5 ; H, 9·2 ; N, 8·9%). The purified salt (0·70g.) was shaken with dilute sulphuric acid and ethyl acetate. The organic layer was dried with $MgSO_4$ and the solvent removed, to give a syrup which did not crystallise.

(b). From benzyloxycarbonyl-L-leucylglycine ethyl ester.

Benzyloxycarbonyl-L-leucylglycine ethyl ester (7.0g. ; 0·02 mole) was shaken with 1N sodium hydroxide in aqueous dioxan (3:1) (20 ml. ; 0·02 mole) at room temperature for 1 hr. The very small amount of undissolved solid was filtered off and the solute acidified to Congo Red with dilute hydrochloric acid. The oil which separated was extracted with ether, the solution dried and the solvent removed, leaving a colourless crystalline solid (5·5g. ; 85%). Recrystallisation from ethyl acetate-light petroleum gave material m.p. 116-117°. (Found: C, 59·4 ; H, 7·0 ; N, 8·8 Calc. for $C_{14}H_{19}NO_4$: C, 59·6 ; H, 6·8 ; N, 8·7%). $[\alpha]_D^{22·5}$ -27·4 (c. 1·92 in ethanol). c.f. Bergmann, Zervas + Fruton (65), m.p. 115°.

Benzyloxycarbonyl-L-leucylglycine (3·22g.; 0·01 mole) was dissolved in 33% hydrogen bromide in acetic acid (10ml.) and left at room temperature for 10 min. Pouring into ether caused precipitation of a colourless crystalline solid

which was washed with ether (2·30g. ; 85%). Recrystallisation from methanol-ether gave L-leucylglycine hydrobromide m.p. 195-6°

L-Leucylglycine hydrobromide (1·08g. ; 0·004 mole) was dissolved in water (10ml.) containing sodium carbonate (2·12g. ; 0·02 mole) and benzoyl chloride (0·46 ml. ; 0·04 mole) was added. The mixture was shaken at room temperature for ½ hr. when a crystalline solid separated out. Acidification to Congo Red with dilute hydrochloric acid, extraction into ethyl acetate, drying and removing solvent gave an uncrystallisable glass. (0·92g. ; 79%), $[\alpha]_D^{23}$ - 26·2 (c. 1.03 in ethanol).

Isolation of racemate from partially racemised benzoylleucylglycine ethyl ester.

(a) Recrystallisation from acetone-ether. A mixture of 0·1g. of L-peptide and racemate was recrystallised from acetone-ether. The first crystalline fraction was 0·06g., m.p. 146°, $[\alpha]_D$ zero. A mixture of 0·45g. L-peptide and 0·05g. racemate on recrystallisation gave a first fraction of 0·09g., m.p.156-7°, $[\alpha]_D$ -32° (c. 2·54 in ethanol).

(b) Saponification method. Six mixtures of L-peptide and racemate, each weighing 1g., and containing varying amounts of

racemate were saponified with 1N-sodium hydroxide in aqueous dioxan (3:1), (5ml.) at room temperature for 1 hr. Acidification to Congo Red with dilute hydrochloric acid was in each case followed by attempted crystallisation of the syrup formed, by addition of sufficient water to the hot mixture to cause complete solution, allowing to cool and seeding with a trace of benzoyl-DL-leucylglycine crystals. The results are tabulated.

% DL	1st. fn.		Recryst. 1st. fn.		
	wt.	m.p.	wt.	m.p.	$[\alpha]_D$ in EtOH
20%	0·08g.	159-161°	0·04g.	163.5-164.5°	0
10%	0·07g.	146-150°	0.014g.	163-4°	-2·2°
5%	0·047g.	137-139°	0·019g.	162·5-163·5°	-3·4°
2%	0·13g.	138-9°	0·009g.	162-163·5°	-
1%	0	-	-	-	-
0%	0	-	-	-	-

c.f. Benzoyl-DL-leucine m.p. 141°.

Benzoyl-L-leucine. L-Leucine (13·1g. ; 0·1 mole) was dissolved in 2N sodium hydroxide solution (50ml. ; 0·1 mole). The solution was stirred and cooled in ice. Benzoyl chloride (11.6 ml. ; 0·1 mole) was added portionwise, keeping the mixture always alkaline by addition of further 2N sodium hydroxide

solution (60ml. ; 0·12 mole). After addition was complete the solution was stirred for a further 1/4 hr. Ether (100ml.) was added, and the aqueous solution acidified to Congo Red with conc. hydrochloric acid, with stirring at the interface so that the benzoyl-L-leucine was immediately extracted into the ether. The aqueous layer was further extracted with ether (100ml.) and the combined ethereal extracts dried over magnesium sulphate.

Cyclohexylamine (10ml.) was added to precipitate the salt, which was filtered and well washed with ether. 26g. (78%). Recrystallisations from methanol-ether gave needles, m.p. 145-6°, $[\alpha]_D^{19}$ + 14·4 (c. 4·25 in ethanol). Benzoyl-L-leucine cyclohexylamine salt (12·3g.) was suspended in ethyl acetate (100ml.) and shaken with 2N hydrochloric acid (100ml.). After separation, the aqueous layer was further extracted with ethyl acetate (100ml.) and the combined extracts dried over magnesium sulphete. The solution was greatly concentrated by evaporation at room temperature and the product crystallised by addition of light petroleum. 7·6g. (88%), m.p. 105-6°. Recrystallisation from ethyl acetate -light petroleum or chloroform-light petroleum gave material m.p. 106°, $[\alpha]_D^{24}$ - 5·5 (c. 8·21 in ethanol). c.f. Fischer

quotes, m.p. 105-7°, $[\alpha]_D^{20}$ + 6·6 (in potassium hydroxide solution), (66).

Benzoyl-L-leucine methyl ester

(a) From benzoyl-L-leucine. Benzoyl-L-leucine (1·0g.) was dissolved in methanol (5ml.) and a solution of diazomethane added until the yellow colour persisted. After leaving the solution for ½ hr., the solvent was removed by distillation, leaving a colourless crystalline compound. m.p. 103-4°, $[\alpha]_D^{24}$ - 24·3 (c. 1·003 in ethanol.)

(b) From L-leucine methyl ester hydrochloride. L-leucine methyl ester hydrochloride (18·2g. ; 0·1 mole) was dissolved in water (10ml.). Ether (70ml.) was added and the mixture well stirred. Sodium hydrogen carbonate (9·2g. ; 0·11 mole) was added, followed by benzoyl chloride (14·4ml. ; 0·125 mole) in ethyl acetate (50ml.) and 2N sodium hydrogen carbonate solution (160ml. ; 0·32 mole). Stirring was continued for 1 hr., after which time the organic layer was washed with pyridine (10ml.), water (25ml.), dilute hydrochloric acid (2 x 25ml.), sodium hydrogen carbonate solution (25ml.) and then dried over magnesium sulphate. Removal of solvent gave a colourless crystalline solid. 24·2g. (97%). Recrystallisation from ethyl acetate-light petroleum gave needles, m.p.

104°, $[\alpha]_D^{24}$ -23·7 (c. 0·9686 in ethanol). c.f. lit. (67), $[\alpha]_D^{20}$ -21·1 (in ethanol).

PART II. The coupling of benzoyl-L-leucine with glycine ethyl ester.

Acid azide method.

* Benzoyl-L-leucine hydrazide. Benzoyl-L-leucine methyl ester (2·92g. ; 0·012 mole) was dissolved in ethanol (20ml.) and hydrazide hydrate (100%, 1·1ml.) added. After leaving overnight at room temperature the solvent was removed at reduced pressure leaving a colourless solid which was recrystallised from ethyl acetate-light petroleum. 2·55g. (87%), m.p. 155°. (Found : C, 62·7 ; H, 7·4 ; N, 16·7. $C_{13}H_{19}N_3O_2$ requires: C, 62·6 ; H, 7·7 ; N, 16·9%).

Benzoylleucylglycine ethyl ester. Benzoyl-L-leucine hydrazide (2·49g. ; 0·01 mole) was dissolved in a mixture of water (10ml.), concentrated hydrochloric acid (2ml.) and glacial acetic acid (2ml.). The solution was cooled to 0° and sodium nitrite (1·4g. ; 0·02 mole), dissolved in the minimum amount of water was added dropwise, with stirring. A colourless solid separated. Addition of ether (15ml.) caused immediate solution and the aqueous layer was extracted with more ether (3 x 15ml.). The combined ethereal extracts were dried over magnesium sulphate at 0° for 10 min. After filtering, glycine ethyl ester (2·0ml. ; 0·02 mole) was added dropwise to the azide

solution, cooled to 0° and stirred. A light brown oil separated, which partially solidified after stirring at room temperature overnight. Ethyl acetate (40ml.) and chloroform (25ml.) were added, and the solution washed with dilute hydrochloric acid (10ml.), sodium carbonate solution (10ml.) and water (15ml.). After drying over magnesium sulphate, the solvent was removed, leaving a cream-coloured crystalline solid. 2·77g. (87%), m.p. 153-155°, $[\alpha]_D^{20}$ - 34·0 (c. 3.13 in EtOH). (Found : C, 63·7 ; H, 7·6 ; N, 8·9. Calc. for $C_{17}H_{24}N_2O_4$: C, 63·7 ; H, 7·6 ; N, 8·8%).

In a second experiment, using half the above quantities, 1·18g. (74%) of material was obtained. m.p. 152-5°, $[\alpha]_D^{21}$ - 29·8 (c. 3·575 in EtOH). (Found: C, 63·7 ; H, 7·7 ; N, 8·7%) 1·0g. of this product was saponified with 5 ml. of IN sodium hydroxide in aqueous dioxan (3:1) at room temperature for 1 hr. The solution was acidified to Congo Red, water was added to the hot mixture until the syrupy peptide was just completely dissolved in the boiling solvent, and it was allowed to cool. No crystals of benzoyl-DL-leucylglycine appeared, even after seeding and leaving the solution for several weeks.

Cyanomethyl ester method. (a) Ester formed by refluxing in ethyl acetate. Benzoyl-L-leucine (2·35g. ; 0·01 mole),

chloroacetonitrile (1·27ml. ; 0·02 mole) and triethylamine (2·07ml. ; 0·015 mole) were dissolved in ethyl acetate (10ml.) and refluxed for 3 hr. After this time, the solution was diluted with ethyl acetate (40ml.), filtered, and washed with water (10ml.), dilute hydrochloric acid (15ml.) and water (10ml.) After drying over magnesium sulphate the solvent was removed under reduced pressure, leaving a colourless syrup which solidified to a white solid. 2·70g. (100%).

The cyanoester was dissolved in ethyl acetate (10ml.), glycine ethyl ester (1·1ml. ; 0·011 mole) was added and the solution left at room temperature overnight. After this time, long silky needles had separated out. Ethyl acetate (30ml.) was added to dissolve these and the solution was washed with dilute hydrochloric acid (10ml.), sodium bicarbonate solution (10ml.) and water (10ml.) It was dried over magnesium sulphate and the solvent removed, leaving a pale brown crystalline solid. 2·84g. (89%) , m.p. 133-137°, $[\alpha]_D^{20}$ - 30·6 (c. 6·445 in EtOH). Trituration of this product with cold ethyl acetate removed the brown colour, leaving a white crystalline solid. 1·12g. , m.p. 156-7° , $[\alpha]_D^{21}$ - 33·8 (c. 2·565 in EtOH). The brown solution was evaporated to dryness, leaving a brown sticky solid. Attempted crystallisation in ether caused

the separation of a small quantity of dark brown sticky solid. This was separated, leaving a pale yellow solution, evaporation of which gave a pale yellow solid. Saponification of this in the usual way gave no racemic dipeptide acid, even after seeding and leaving for 10 weeks.

In a repeat experiment, 2·87g. (90%) of brown solid was obtained, m.p. 127-134°, $[\alpha]_D^{18}$ -30.1 (c. 3·43 in EtOH). (Found: C, 63·5 ; H, 7·5 ; N, 9·0)

(b) Ester formed at 0° Benzoyl-L-leucine (1·18g. ; 0·005 mole) was added, with stirring and cooling to 0°, portionwise to a mixture of triethylamine (1·05ml. ; 0·0075 mole) and chloroacetonitrile (0·64ml. ; 0·01 mole) during 15 min. Stirring was continued at 0° for a further 30 min. and then at room temperature overnight. Ethyl acetate (20ml.) was added to the mixture, the triethylamine hydrochloride filtered off and the solution was washed with dilute hydrochloric acid (3ml.), sodium bicarbonate solution (3ml.) and water (3ml.) It was dried with magnesium sulphate, filtered and the volume reduced to about 5 ml. Glycine ethyl ester (0.5ml. ; 0·005 mole) was added to this and the mixture left at room temperature overnight. Some product separated. This was taken up in ethyl acetate (30ml.) and worked up in the usual way

to give a white crystalline solid. 1·12g. (70%) , m.p. 149-152°, $[\alpha]_D^{23}$ - 33·6 (c. 3·055 in EtOH). (Found: C, 64·1 ; H, 7·2 ; N, 9·1%). 0·8g. of the product was saponified with 4 ml. of 1N aqueous dioxan (3:1) in the usual way but no benzoyl-DL-leucylglycine was obtained, even after seeding.

In a second experiment, 1·19g. (74%) of white crystalline product was obtained. m.p. 153-155°, $[\alpha]_D^{23}$ - 32·3 (c. 3·41 in EtOH). (Found: C, 63·4 ; H, 7·6 ; N, 9·1%).

Mixed anhydride method: using isobutyl chloroformate in T.H.F.

Benzoyl-L-leucine (1·18g. ; 0·005 mole) was dissolved in dry T.H.F. (12ml.). The solution was cooled to-5° and triethylamine (0·70ml. ; 0·005 mole) added, followed by isobutyl chloroformate (0·66ml. ; 0·005 mole), dropwise and with stirring. After allowing a few minutes for anhydride formation, glycine ethyl ester (0·5ml. ; 0·005 mole) in T.H.F. (6ml.) was added slowly. The mixture was stirred and allowed to attain room temperature overnight. It was then filtered, the solid washed with ethyl acetate and the solvent removed under reduced pressure. The white crystalline residue was taken up in ethyl acetate (20ml.) and chloroform (5ml.) and the solution was washed with dilute hydrochloric acid (5ml.), sodium carbonate solution (5ml.) and water(5ml.). After

drying over magnesium sulphate, the solvent was removed, leaving a white crystalline product. 1·40g. (88%), m.p. 126-130°, $[\alpha]_D^{24}$ - 6·1 (c. 3·625 in EtOH). (Found : C, 63·8 ; H, 7·3 ; N, 9·0%). 0·5g. of the product was recrystallised from acetone-ether. The first fraction consisted of white needles. 0·32g. , m.p. 144·5-145·5° , $[\alpha]_D^{22}$ - 2·7 (c. 3·34 in EtOH).

In a repeat experiment, 1·36g. (85%) of product was obtained. m.p. 126-131°. , $[\alpha]_D$- 7·6 (c. 3·535 in EtOH). (Found: C, 63·1 ; H, 7·4 ; N, 8·9%).

Tetraethyl pyrophosphite method. "Standard" procedure, using distilled glycine ester in chloroform. Benzoyl-L-leucine (2·35g. ; 0·01 mole) and glycine ethyl ester (1·0ml. ; 0·01 mole) were dissolved in dry chloroform (10ml.) and tetraethyl pyrophosphite (3ml.) added. The solution was refluxed for ½ hr. After cooling, it was diluted with chloroform (30ml.) and washed with dil. hydrochloric acid (10ml.), sodium hydrogen carbonate solution (10ml.) and water (10ml.) After drying over magnesium sulphate, the solvent was removed, the last traces of diethyl phosphite at 100°/0.3mm., leaving a colourless crystalline solid. 2·61g. (82%). $[\alpha]_D^{20}$ -29.2 (c. 2·73 in ethanol), m.p. 146-150°. (Found : C, 63·3 ;

H, 7·6 ; N, 8·7%).

Recrystallisation of the product from acetone-ether gave a first fraction of 1·32g., $[\alpha]_D^{21}$ - 29·2. Evaporation of the mother liquor gave 1·00g., $[\alpha]_D^{21}$ - 29·3. This fraction was saponified with 10ml. of N-sodium hydroxide in aqueous dioxan (3:1) at room temperature as usual. A crystalline solid was obtained. 0·07g., m.p. 154-156°. Recrystallisation from water raised the m.p. to 162-163° $[\alpha]_D^{22}$ - 5·8 (c. 1·36 in ethanol).

In a second experiment, 2·68g. (84%) of peptide, $[\alpha]_D^{22}$ -30·6 (c. 2·84 in ethanol), was obtained. (Found: C, 63·2 ; H, 7·6 ; N, 9·0%).

"Standard" procedure, using ester hydrochloride and base in chloroform. Benzoyl-L-leucine (2·35g. ; 0·01 mole), triethylamine (1·38ml. ; 0·01 mole) and glycine ethyl ester hydrochloride (1·40g. ; 0·01 mole) were dissolved in chloroform (10ml.). Tetraethyl pyrophosphite (3ml.) was added and the solution refluxed for ½ hr. After cooling, the product was worked up in the usual way. 2·65g. (83%), m.p. 139-143°, $[\alpha]_D^{23}$ - 17·5 (c. 3·09 in ethanol). Found: C, 63·5 ; H, 7·6%)

In a second experiment, 2·56g. (80%) of a colourless crystalline product was obtained. m.p. 141-144°, $[\alpha]_D^{23}$ -

16·7 (c. 4·26 in ethanol). (Found: C, 63·7 ; H, 7·6 ; N, 8·7%). 0·5g. of this product was recrystallised with much loss from acetone-ether. The first fraction consisted of white needles. 0·41g., m.p. 145-145·5°, $[\alpha]_D^{22}$ zero (c.3.22 in ethanol).

"Amide" procedure, using glycine ester in chloroform. Glycine ethyl ester (0·5ml. ; 0·005 mole) was dissolved in dry chloroform (5ml.). Tetraethyl pyrophosphite (1·5ml.) was added and the solution refluxed for a few min. Benzoyl-L-leucine (1·18g. ; 0·005 mole) was then added and the solution refluxed for 30 min., and then diluted with chloroform (15ml.) and washed in the usual way. The solvent was removed, leaving a colourless crystalline solid. 1·55g. (97%), m.p. 138-141°, $[\alpha]_D^{22}$ - 16·6 (c. 4·075 in ethanol). (Found: C, 63.3 ; H, 7·6 ; N, 8·8%). Recrystallisation of 0·5g. from acetone-ether gave a first fraction of 0·16g., m.p. 144-5°, $[\alpha]_D^{20}$ - 2·2 (c. 1·795 in ethanol.).

"Amide" procedure, using ester hydrochloride and base in chloroform. Glycine ethyl ester hydrochloride (0·70g. ; 0·005 mole) was suspended in dry chloroform (5ml.). Triethylamine (0·70ml. ; 0·005 mole), followed by tetraethyl pyrophosphite (1·5ml.) were added and the solution refluxed for a few min. Benzoyl-L-leucine (1·18g. ; 0·005 mole) was then added and

refluxing continued for 30 min. The usual workup procedure gave 1.25g. (79%), m.p. 141-4°, $[\alpha]_D^{23}$ - 9.3 (c. 3.34 in ethanol). (Found : C, 63·2 ; H, 7·5 ; N, 8.8%) Recrystallisation of 0·5g. from acetone-ether gave a first fraction of 0·20g., m.p. 145·5-146°, $[\alpha]_D^{21}$ - 3·5 (c. 2·58 in ethanol).

Tetraethyl pyrophosphite. This was prepared by the method of Maclaren (87), using triethyl phosphite (83g. ; 0·5 mole) and phosphorus trichloride (22ml. ; 0·25 mole). The following fractions were collected at 0·3 mm.

Fn.	1.	2.	3.	4.
b.p.	34-80	80-85	85-95	95-100
Wt., gm.	67.7	17·2	24·3	5·6
n_D^{25}	-	1·4300	1·4336	1·4340

Fractions 3 and 4 were combined and redistilled at 0·2 mm.

Fn.	1 (a)	2 (a)	3 (a)
b.p.	60-75	75-85	85-102
n_D^{25}	1·4301	1·4336	1·4332

The presence of $\bar{C}l$ in fractions 1 (a) and 2 (a) was tested by addition of a few drops to aqueous silver nitrate solution. The solutions remained clear, but became dark brown after a few minutes. Solutions containing $\bar{C}l$ give an immediate

precipitate with aqueous silver nitrate. Fraction 1 (a) was used in the above coupling experiments. Anderson (15) quotes b.p. 79-81°/0·15 mm., n_D^{26} 1·4313 for pure teraethyl pyrophosphite.

Dicyclohexylcarbodi-imide method. Using glycine ethyl ester in methylene chloride. Benzoyl-L-leucine (1·18g. ; 0·005 mole) and glycine ethyl ester (0·5ml. ; 0·005 mole) were dissolved in dry methylene chloride (15ml.) and dicyclohexylcarbodi-imide (1·03g. ; 0·005 mole), dissolved in methylene chloride (10ml.), was added slowly with stirring. Stirring was continued at room temperature for 23 hr. A few drops of glacial acetic acid were added and, after stirring for a further ½ hr., the dicyclohexylurea was filtered off. The solvent was removed by distillation in vacuo and the residue extracted into cold ethyl acetate (50ml.). The solution was washed with dilute hydrochloric acid (15ml.), sodium carbonate solution (15ml.) and water (15ml.), dried and the solvent removed, leaving a colourless crystalline solid. 1.34g. (84%), m.p. 131-4°, $[\alpha]_D^{21}$ -18·1° (c. 3·83 in ethanol). (Found: C, 63·8 ; H, 7·14 ; N, 8·8.)

0.5g. of the product was recrystallised with much loss from acetone-ether. The first fraction consisted of 0.08g. ,

m.p. 143-4°, $[\alpha]_D^{20}$ -4·0 (c. 2·16 in ethanol). c.f. Benzoyl-DL-leucylglycine ethyl ester. m.p. 146°.

In a repeat experiment, 1·18g. (74%) of crystalline product was obtained. m.p. 130-134°, $[\alpha]_D^{21}$ - 18·3 (c. 2·545 in ethanol). (Found : C, 64·1 ; H, 7·7 ; N, 8·4).

Woodward method. (1). N-Ethyl-5-phenylisoxazolium-3'-sulphonate (1·012g. ; 0·004 mole) was stirred magnetically at 0° with distilled acetonitrile (10ml.). A solution of benzoyl-L-leucine (0·94g. ; 0·004 mole) and triethylamine (0·004 mole) in acetonitrile (8ml.) was added and the mixture was stirred at 0° for 1 hr. After this time, glycine ethyl ester hydrochloride (0·56g. ; 0·004 mole) was added, followed by triethylamine (0·004 mole) in acetonitrile (8ml.). Stirring was continued at room temperature for 22 hr., when solution was almost complete. The solvent was removed by distillation in vacuo and the residue taken up in ethyl acetate (80ml.) and water (20ml.). The organic layer was further washed in turn with sodium hydrogen carbonate solution (2 x 20ml.), 2N hydrochloric acid (20ml.), water (20ml.) and then dried over magnesium sulphate. Removal of solvent gave a colourless crystalline residue. 1·04g. (81%), m.p. 145-8°, $[\alpha]_D^{22}$ - 29·8 (c. 3·305 in ethanol). (Found : C, 63·9 ; H, 7·6 ; N,

8·4%).

0·80g. of the product was saponified with 3·5ml. of 1N sodium hydroxide in aqueous dioxan (3:1) at room temperature for 1 hr. Acidification to Congo Red caused the separation of a syrup. Sufficient water was added to this mixture to dissolve the syrup in the boiling solvent. Cooling and seeding with a small crystal of benzoyl-DL-leucylglycine resulted in copious crystallisation after 2 days. The mixture was left at room temperature for 4 weeks, when the solid was filtered. 0·22g., m.p. 137-9°. One recrystallisation from water gave a first fraction of 0·022g., m.p. 159-161°, $[\alpha]_D^{20}$ - 4.5 (c. 2·02 in ethanol). No depression was observed in a mixed m.p. with authentic benzoyl-DL-leucylglycine (m.p. 165°)

In a repeat experiment, 0·99g. (75%) of peptide was obtained, m.p. 150-153°, $[\alpha]_D^{21}$ - 30·6 (c. 1·93 in ethanol). (Found: C, 63·7; H, 7·4 ; N, 8·9%). Saponification of 0·95g. as desribed above gave 0·023g. of material m.p. 162-3°, $[\alpha]_D^{20}$ -4·1 (c. 2·12 in ethanol). There was no depression in a mixed m.p. with authentic benzoyl-DL-leucylglycine.

(2). Repeat, using free glycine ester. The above procedure was repeated on the same scale, but using distilled glycine ester instead of the hydrochloride and triethylamine. The product

was obtained as a white crystalline solid. 0·98g. (77%), m.p. 155-7°, $[\alpha]_D^{24}$ - 32·8 (c. 2·90 in ethanol). (Found: C, 63·8 ; H, 7·7 ; N, 8·6%).

0·90g. of the product was saponified with 5ml. of 1N sodium hydroxide. Crystallisation in the usual way gave 0·178g. of colourless prisms. m.p. 138·5-139°. Further recrystallisation from water gave no benzoyl-DL-leucylglycine, even after seeding with authentic material.

In a repeat experiment, 0·96g. (75%) of benzoyl-leucylglycine ethyl ester was obtained. m.p. 156-7°, $[\alpha]_D^{22}$ -32·5 (c. 3·27 in ethanol). (Found : C, 63·6 ; H, 7·6 ; N, 8·8%).

Saponification of the product gave no benzoyl-DL-leucylglycine.

Part III (a)

Benzyloxycarbonyl-L-leucine p-nitrophenyl ester. This was made by the esterification of benzyloxycarbonyl-L-leucine with p-nitrophenol in the presence of dicyclohexylcarbodiimide (18), in 97% yield. Recrystallisation from ethyl acetate gave material, m.p. 95°, $[\alpha]_D^{20}$ - 33·6 (c. 2·75 in dimethylformamide) c.f. Bodanszky and du Vigneand (70), m.p. 95°, $[\alpha]_D^{20}$ - 33·5 (c. 2 in dimethylformamide).

L-Leucine p-nitrophenyl ester hydrobromide was obtained by treating benzyloxycarbonyl-L-leucine p-nitrophenyl ester with a solution of hydrogen bromide in acetic acid, as described by Goodman and Stueben. Recrystallisation from acetone-ether gave material, m.p. 198-200°, $[\alpha]_D^{28}$ + 10·8 (c. 2·475 in ethanol). (Found : C, 43·1 ; H, 5.0 ; N, 8·7. Calc for $C_{12}H_{17}N_2O_4Br$: C, 43·2 ; H, 4·9, $[\alpha]_D^{25}$ + 11·4 (c. 2·20 in ethanol).

* Benzoyl-L-leucine p-nitrophenyl ester. L-leucine p-nitrophenyl ester hydrobromide (3·33g. ; 0·01 mole) was suspended in ethyl acetate (25ml.) containing benzoyl chloride (1·16ml. ; 0·01 mole). The suspension was stirred vigorously and a solution of sodium carbonate (2·65g. ; 0·05 mole) in water (15ml.) added. The mixture was stirred at the interface for ½ hr., and the organic layer separated and dried. Removal of

solvent under reduced pressure at 60° gave a colourless syrup which quickly crystallised. The infra-red spectrum of this showed a weak absorption at 1832 $cm.^{-1}$ in chloroform solution indicating that the presence of azlactone. Recrystallisation from ethyl acetate-light petroleum gave azlactone-free material. 3·0g. (84%), m.p. 96-7°, $[\alpha]_D^{23}$ - 43·2 (c. 3.39 in ethanol). (Found: C, 63·9 ; H, 5·5 ; N, 7·9. $C_{19}H_{20}N_2O_5$ requires., C, 64.0 ; H, 5·6 ; N, 7·9%.). When the solvent was removed in the final stage at room temperature under reduced pressure the infra-red spectrum showed that the crude product contained no azlactone. Recrystallisation from ether-light petroleum gave material m.p. 95·5-96.5, $[\alpha]_D^{23}$ - 43·6 (c. 3·06 in ethanol).

Effect of added base on racemisation during coupling of benzoyl-L-leucine p-nitrophenyl ester with glycine ethyl ester.

Benzoyl-L-leucine p-nitrophenyl ester (1·19g. ; 0.0034 mole) was dissolved in a solvent (ethyl acetate or chloroform) (3ml.) and glycine ethyl ester added, either as the free base or as the hydrochloride followed by N-methylpiperidine. Reaction was allowed to proceed at room temperature overnight and the product was worked up in each case by washing the diluted solution with dilute hydrochloric acid (3 x 10ml.), 2N sodium

carbonate solution (3 x 15ml.) and water (15ml.). After drying, the solvent was removed, leaving a colourless crystalline compound in each case. The essential purity of the products was demonstrated by C,H and N analysis, and the L-isomer content, in excess of that contained in any racemate, deduced from the specific rotations. The results are tabulated in Fig 18. The peptides obtained by using one mole of distilled glycine ester in both ethyl acetate and chloroform were saponified. No evidence of racemisation was obtained.

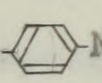
Fig. 18

Conditions	Solvent	$[\alpha]_D$	%L-isomer	Analysis of products C	H	N
glyOEt (1)	EtOAc	-34.3	100	63.1	7.3	9.1
glyOEt (5)	EtOAc	-30.7	90	63.1	7.5	9.2
glyOEt (1)+NMP($\frac{1}{2}$)	EtOAc	-30.7	90	63.6	7.4	8.8
glyOEt(1)+NMP(1)	EtOAc	-30.2	90	63.6	7.3	8.8
glyOEt.HCl(1)+NMP(1)	EtOAc	-26.8	80	63.5	7.3	8.8
glyOEt(1)	$CHCl_3$	-32.6	96	63.7	7.7	8.5
glyOEt(1)+NMP(1)	$CHCl_3$	-24.7	73	63.4	7.4	8.6
glyOEt.HCl(1)+NMP(1)	$CHCl_3$	-25.6	75	63.9	7.4	9.5
glyOEt.HCl(1)+NMP(2)	$CHCl_3$	-18.7	54	64.0	7.5	8.4
glyOEt.HCl(1)+NMP(3)	$CHCl_3$	-17.5	51	64.7	7.5	8.5
glyOEt.HCl(1)+NMP(10)	$CHCl_3$	-16.9	49	63.7	7.5	8.7
		-15.0	44	63.7	7.5	8.9

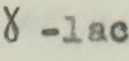
glyOEt.HCl(1)+NMP(1)	pyridine	-30·8	90	63·6 (63·7)	7·4 (7·6)	9·5 (8·8)

(calc. values)

The figures in parentheses denote the number of moles used. N.M.P. = N-methylpiperidine.

* Benzoyl-DL-leucine p-nitrophenyl ester. This was made from benzoyl-DL-leucine by the method of Elliott and Russell (18). It was obtained in 87% yield. Recrystallisation from ether-light petroleum gave material, m.p. 94-5°. (Found: C, 63·9 ; H, 5·5 ; N, 7·9. $C_{19}H_{20}N_2O_5$ requires : C, 64·0 ; H, 5·6 ; N, 7·9%). The infrared spectral peaks in the carbonyl region of a chloroform solution are assigned as follows: -CO.O- 1776 cm^{-1} ; -CO.NH- , 1675 cm^{-1} (amide I absorption). There are also two medium strong peaks at 1630 cm^{-1} and 1605 cm^{-1}. The ultraviolet absorptions in chloroform solution are: λ max 240mμ , E max 11,000 ; λ max 270mμ , E max 11,000.

2-Phenyl-4-isobutyl-5-oxazolone. This was prepared by the action of acetic anhydride on benzoyl-DL-leucine (72) in 81% yield. Recrystallisation from n-hexane or light petroleum gave material, m.p. 57°. cf. lit. 56-7°. The infrared spectral peaks in the carbonyl region of a dilute chloroform solution

are assigned as follows: γ-lactone CO, 1832 cm^{-1}. ; , 1664 cm^{-1} The ultraviolet peak in chloroform solution is at λ max 245mμ , E max 15,000.

Azlactonisation of benzoyl-DL-leucine p-nitrophenyl ester.

Benzoyl-DL-leucine p-nitrophenyl ester (0·356g. ; 0·001 mole) was dissolved in chloroform (3ml.), containing N-methylpiperidine (0.001 mole) at room temperature. The infrared spectrum of samples of this solution was taken at intervals of 5 min. (It was previously established that N-methylpiperidine does not absorb in the carbonyl region of the infrared spectrum). After 5 min., a small peak had appeared at 1832 cm^{-1} (azlactone CO). This grew in intensity and was accompanied by a corresponding decrease in intensity of the ester carbonyl peak at 1776 cm^{-1} After 20 min., no further change occurred, the two peaks now possessing comparable intensities. The relative concentrations of ester and azlactone at equilibrium were caculated as follows.

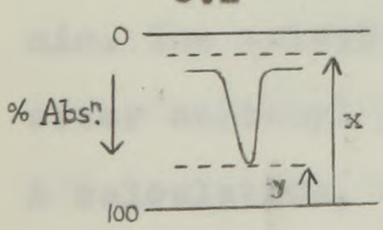
$E = \frac{M}{c.l} . \log \frac{x}{y}$ where

E = Molecular extinction coeff.

M = molecular weight.

c = concentration , moles/litre.

l = length of cell , cm.



Let l = 1 for path length of 0.1 mm.

c = 1 for a concentration of 0.001 mole in 3ml. solution.

Let Ea, Ee, Ma, Me, Ca, Ce be the extinction coefficients, molecular weights and concentrations of azlactone and ester respectively.

x = 88

When Ce = 1, y_e = 8. $\quad Ee = Me . \log \frac{88}{8}$

When Ca = 1, y_a = 4 $\quad Ea = Ma . \log \frac{88}{4}$

$$\frac{Ee}{Ea} = \frac{Me}{Ma} \cdot \frac{\log 88 - \log 8}{\log 88 - \log 4} \quad \text{——} \quad 1$$

At equilibrium, x = 96, y_e = 17, y_a = 24.

$$\text{Then} \quad \frac{Ee}{Ea} = \frac{Me\ Ca}{Ma\ Ce} \cdot \frac{\log 96 - \log 17}{\log 96 - \log 24} \quad \text{——} \quad 2$$

Equating 1 and 2, $\frac{Ca}{Ce} = 0.62$ at equilibrium.

<u>Reverse reaction</u>. 2-phenyl-4-isobutyl-5-oxazolone (0.217g. ; 0.001 mole) and p-nitrophenol (0.139g. ; 0.001 mole) were dissolved in chloroform (3ml.) containing N-methylpiperidine (0.001 mole) and the spectrum taken after 5, 15, 60 and 120 min. The azlactone carbonyl peak was slowly replaced by the ester carbonyl peak until no further change took place (1 hr.). A calculation, similar to the above, showed that the relative concentrations of azlactone to ester at equilibrium was 0.59 : 1.

Characterisation of azlactone obtained from ester by treatment with base. Benzoyl-DL-leucine p-nitrophenyl ester (0·712g. ; 0·002 mole) was refluxed in ether (6ml.) containing N-methyl-piperidine (0·002 mole). Complete solution occurred in 10 min. After refluxing for a further ½ hr. , the ether was boiled off and completely replaced by dropwise addition of n-hexane. This caused the deposition of an oil. The clear hexane solution was decanted. An infra-red spectrum of this solution showed a strong azlactone carbonyl peak but no ester carbonyl peak. The solution was diluted with ether and washed with dilute hydrochloric acid and then water. It was dried over magnesium sulphate and the solvent was removed, leaving a colourless crystalline compound. 0·12g. (28%). Recrystallisation from n-hexane gave material melting at 54·5 - 55°. ν max 1832 cm^{-1}, 1664 cm^{-1} in chloroform solution. There was no depression in a mixed melting point with authentic 2-phenyl-4-isobutyl-5-oxazolone. (Found : C, 71·9 ; H, 7·1 ; N, 6·3. Calc. for $C_{13}H_{15}NO_2$: C, 71·8 ; H, 7·0 ; N, 6·5%).

Expts. to investigate whether, during coupling of benzoyl-L-leucine p-nitrophenyl ester, base-catalysed racemisation is due to azlactone formation only.

(1). (a). Benzoyl-L- leucine p-nitrophenyl ester (0·356g. ;

0·001 mole) was dissolved in dry chloroform (1ml.) containing N-methylpiperidine (0·001 mole) and the solution left at room temperature for ½ hr. Glycine ethyl ester (0·1ml. ; 0·001 mole) was added and the solution left at room temperature overnight. By working up as usual, benzoyl-leucylglycine was obtained as a colourless crystalline product. 0.27g. (85%), m.p. 140-142° , $[\alpha]_D^{23}$ -3·1 (c. 2·96 in ethanol). (Found : C, 64·1 ; H, 7·9 ; N, 8·6%). % L-isomer, 9%.

(b). p-Nitrobenzoyl-L-proline p-nitrophenyl ester (see Part IV) (0·128g. ; 0·00034 mole) was dissolved in chloroform (0·34ml.) containing N-methylpiperidine (0·00034 mole) and the solution left at room temperature for ½ hr. Then glycine ethyl ester (0·034ml. ; 0·00034 mole) was added and the mixture left at room temperature overnight. The usual workup procedure gave p-nitrobenzoylprolylglycine ethyl ester as a colourless crystalline compound. 0·112g. (96%), m.p. 112-113·5°, $[\alpha]_D^{25}$ - 96·3 (c. 2.47 in ethanol). % L-isomer 98%, taking the value $[\alpha]_D$- 98 for pure p-nitrobenzoylprolylglycine ethyl ester (q.v.).

2. Recovery of unreacted ester. Benzoyl-L-leucine p-nitrophenyl ester (1·19g. ; 0·0034 mole) and glycine ethyl ester hydrochloride (0·47g. ; 0·0034 mole), in chloroform (5ml.),

were treated with N-methylpiperidine (0·80ml. ; 0·0068 mole). The mixture was shaken at room temperature for 2 min. and then diluted with ethyl acetate (40ml.) and washed with dilute hydrochloric acid (3 x 30ml.), 2N sodium carbonate solution (3 x 30ml.) and water (30ml.). The solution was dried and the solvent removed, by vacuum distillation at room temperature, leaving a syrup which readily crystallised. The unreacted ester was separated from the peptide by fractional crystallisation from ethyl acetate-light petroleum.

Fn.(1). 0.34g., m.p. 151-156°. Recrystallisation gave 0.30g., m.p. 155-157°, $[\alpha]_D^{23}$ -27·1 (c. 1·97 in ethanol). (Found : C, 63·9 ; H, 7·5 ; N, 8·5 . Calc. for $C_{17}H_{24}N_2O_4$: C, 63·7 ; H, 7·6 ; N, 8·8%). There was no yellow colour formation when a portion was heated with aqueous methanolic sodium hydroxide solution.

Fn. (2). Evaporation of the mother liquor gave a syrup which was triturated with hot petroleum (60-80). This was decanted and the solvent removed. There remained 0·102g. of colourless needles. Recrystallisation from n-hexane gave colourless needles 0·005g., m.p. 54-56°. ν max 1832 $cm.^{-1}$ in chloroform solution. c.f. 2-Phenyl-4-isobutyl-5-oxazolone, m.p. 57°, ν max 1832 $cm.^{-1}$ ($CHCl_3$).

Fn. (3). The above syrup, after the petroleum extraction, was crystallised from ethyl acetate-petroleum. Silky needles, 0·31g., m.p. 94-6°. Further recrystallisation gave two fractions : (i) 0·21g., m.p. 95-6° , $[\alpha]_D^{24}$ - 37·2 (c. 0·5 in ethanol). (Found : C, 64.0 ; H, 6·3 ; N, 7·8. Calc. for $C_{19}H_{21}N_2O_5$: C, 64·0 ; H, 5·6 ; N, 7·9%). (ii). 0·07g., m.p. 93-5°, $[\alpha]_D^{26.5}$ - 42·1 (c. 0·2078 in ethanol). (Found : C, 63·9 ; H, 6·0 ; N, 8·1%).

Fn. (4). 0·21g., m.p. 89-95°, $[\alpha]_D^{26.5}$ - 34·1 (c. 0·2008 in ethanol) (Found : C, 64·0 ; H, 5.9 ; N, 7·9%).

Rate of azlactonisation of benzoyl-L-leucine p-nitrophenyl ester in the presence of triethanolamine (1 mole). The reaction is followed by the rate of decrease of the ester absorption at 270 mμ in chloroform solution.

Benzoyl-L-leucine p-nitrophenyl ester (0·1424g. ; 0·0004 mole) was weighed in a 2ml. flask and made up with a solution of triethanolamine in chloroform (containing 0·0004 mole in 2ml. ; i.e. 0·2 mole/litre) at 25°. U.V. determinations were made on 400 x diluted solutions. The results are tabulated in Fig.19 .

Fig.19 .

t (hrs.)	Absorption	C ester(=a-x)	x	$\frac{x}{a(a-x)}$
0	[1.15]	0·2	0	0

0·34	1·14	0·198	0·002	0·0505
0·75	1·12	0·195	0·005	0·108
1·0	1·09	0·190	0·010	0·263
1·5	1·08	0·188	0·012	0·319
2·0	1·05	0·183	0·017	0·464
2·25	1·04	0·181	0·019	0·525
2·5	1·03	0·179	0·021	0·587
2·75	1·03	0·179	0·021	0·587
3·0	1·00	0·174	0·026	0·747
3·25	1·00	0·174	0·026	0·747
3·5	0·98	0·170	0·030	0·882
4·0	0·96	0·167	0·033	0·988
4·5	0·94	0·1635	0·0365	1·116

From graph λ (Fig. 21a), $k = 6{\cdot}7 \times 10^{-5}$ l. mol.$^{-1}$ sec.$^{-1}$, ($E = 11{,}500$)

Repeat experiment. Fig. 20.

t (hrs.)	Absorption	C_{ester} (=a-x)	x	$\frac{x}{a(a-x)}$
0	[1·14]	0·2	0	0
0.167	1·13	0·198	0·002	0·0505
0·34	1·115	0·196	0·004	0·102
0·50	1·115	0·196	0·004	0·102
0·75	1·10	0·193	0·007	0·181

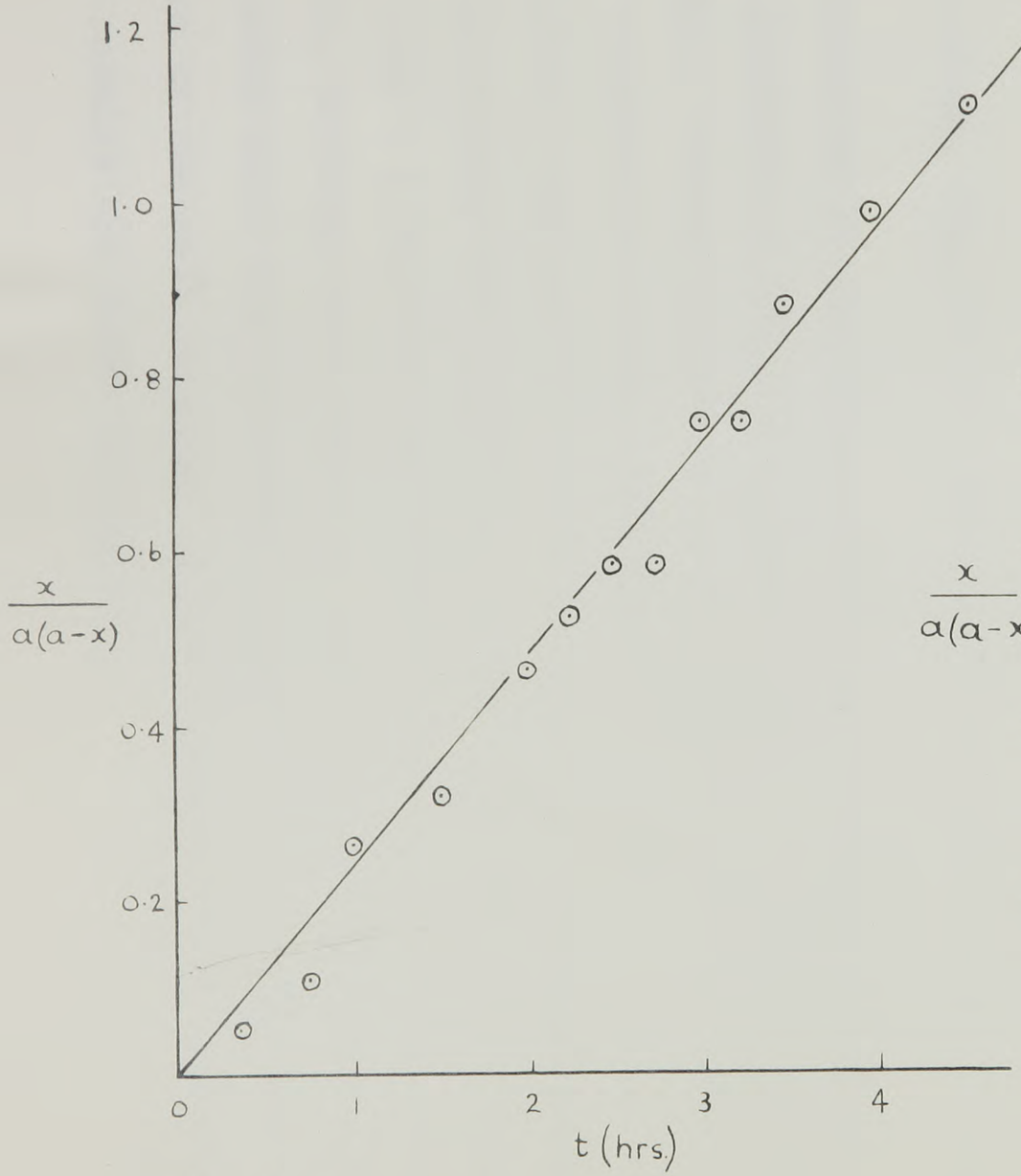


Fig 21 a

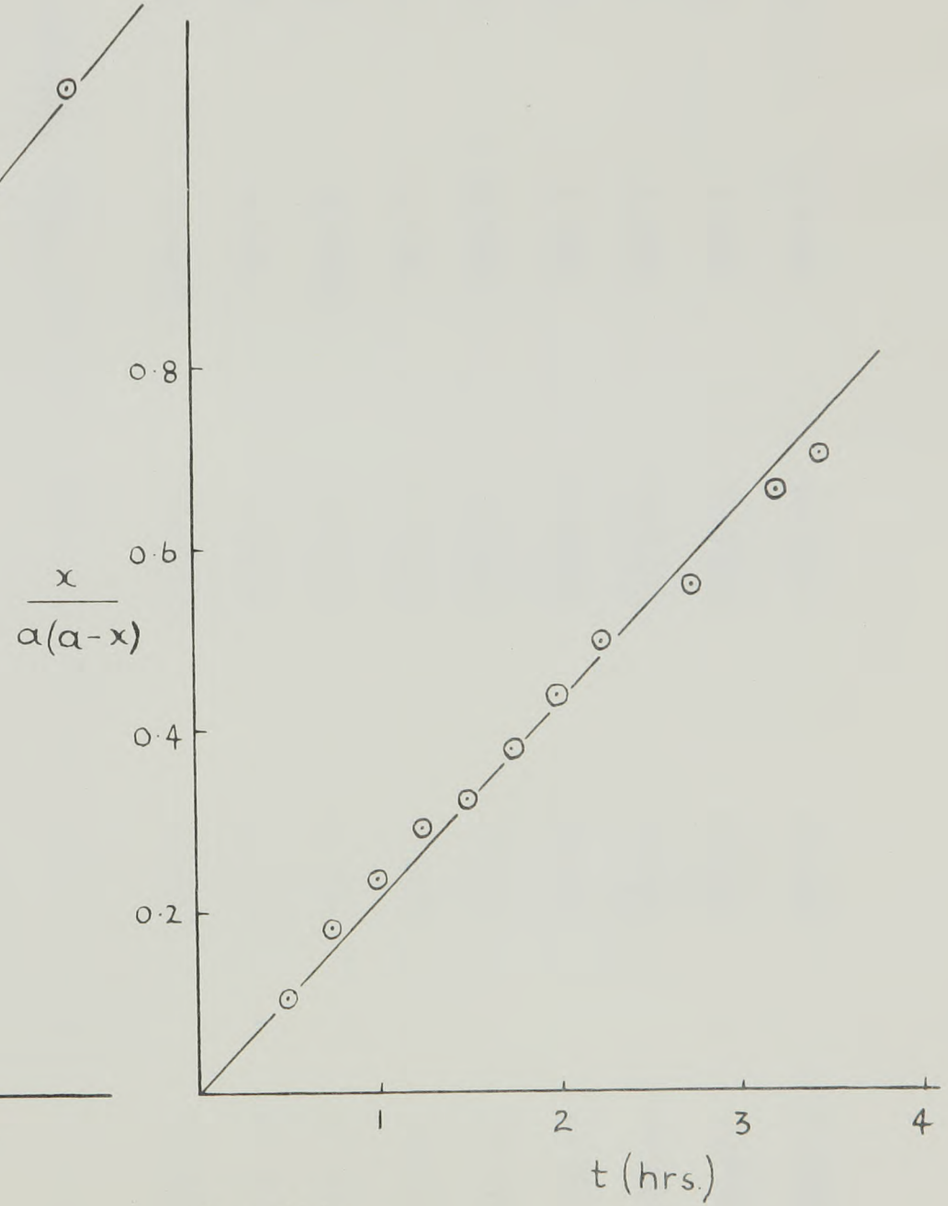


Fig 21 b

Azlactonisation of benzoyl-L-leucine p-nitrophenyl ester with triethanolamine

1·00	1·09	0·191	0·009	0·236
1·25	1·08	0·189	0·011	0·291
1·50	1·07	0·188	0·012	0·319
1·75	1·06	0·186	0·014	0·376
2·00	1·05	0·184	0·016	0·435
2·25	1·04	0·182	0·018	0·495
2·75	1·025	0·180	0·020	0·556
3·25	1·01	0·177	0·023	0·650
3·50	1·005	0·176	0·024	0·682

From graph, Fig.21b, $k = 6{\cdot}0 \times 10^{-5}$ l. $mol.^{-1}$ $sec.^{-1}$, E = 11,400.

Racemisation of benzoyl-L-leucine p-nitrophenyl ester in the presence of triethanolamine (1 mole) in chloroform solution at 24°. Benzoyl-L-leucine p-nitrophenyl ester (0·1424g.) was weighed into a 2ml. standard flask (conc. of ester = 0·2 mole/lit.) and made up with a solution of triethanolamine in chloroform (0·2 mole/lit.) at 24°. The solution was inserted into a 1 cm. cell and placed in an NPL automatic polarimeter. Readings were taken at intervals of 5 min. The values recorded below represent the average of 5 readings. The temperature of the cell remained at 24° throughout the determination. The results are shown in Fig.22.

Fig. 22.

time (min.)	α	t	α
10	0·1240	60	0·1196
20	0·1232	65	0·1189
25	0·1229	70	0·1184
30	0·1217	75	0·1177
35	0·1216	80	0·1173
40	0·1211	85	0·1170
45	0·1205	90	0·1166
50	0·1202	95	0·1163
55	0·1198	105	0·1153

A plot of α against t in this initial period of the reaction is virtually a straight line, extrapolation of which to zero time gives an initial rotation of 0·125. This value was used to caculate the % racemised at each time ; similarly, the % of the ester which had been converted into azlactone was calculated from the previous results. Fig. 7 is a plot of these results.

N,N-Dimethylglycine methyl ester. This was prepared by the destructive distillation of betaine, as described by Willstatter (83). The product was distilled and the fraction b.p. 120-150°

collected. This was twice fractionated and the liquid b.p. 137°/758 mm., collected as N,N-dimethylglycine methyl ester. n_D^{25} 1·4110. (Found : C, 51·1 ; H, 9·5 ; N, 12·1. Calc. for $C_5H_{11}NO_2$: C, 51·3 ; H, 9·4 ; N, 12·0%).

Determination of pK value of N,N-dimethylglycine methyl ester in water. This was determined by a potentiometric titration of a dilute solution of the base in water against 1N hydrochloric acid. The pH changes were followed on a Cambridge pH meter using a glass electrode in conjunction with a saturated calomel electrode for reference . The results are tabulated. It is clear from the graphs (Fig.24c) that the pH values at half neutralisation are 7·15 and 7·05 respectively. ∴ pK=7·1.

Expt. 1	Vol. HCl	pH		
t = 21°	0	8·8	0·09	6·95
	0·01	8·3	0·11	6·65
	0·02	7·85	0·13	6·20
	0·03	7·65	0·14	5·40
	0·04	7·50	0·142	4·60
	0·05	7·40	0·144	4·15
	0·06	7·25	0·146	3·90
	0·07	7·15	0·148	3·75
	0·08	7·05	0·152	3·55

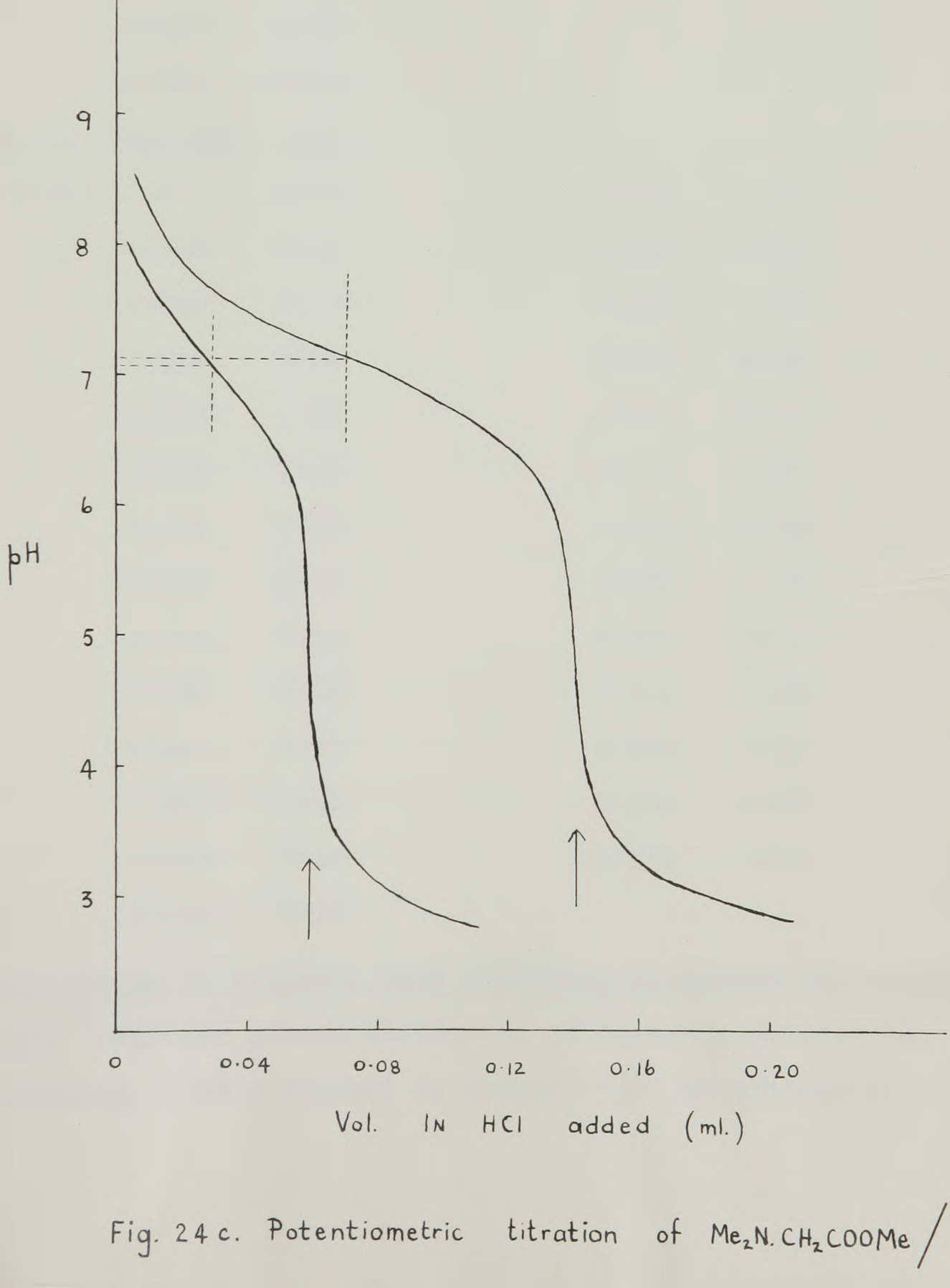


Fig. 24 c. Potentiometric titration of $Me_2N.CH_2COOMe$ / IN HCl

	0·162	3·30		
	0·170	3·15		
	0·180	3·05		
	0·200	2·90		

Expt. 2.	Vol. HCl	pH.		
t = 23·5°	0	8·30	0·057	5·80
	0·005	7·91	0·058	5·40
	0·010	7·70	0·059	4·80
	0·015	7·54	0·060	4·36
	0·020	7·38	0·062	4·02
	0·022	7·24	0·064	3·82
	0·024	7·26	0·066	3·58
	0·026	7·18	0·068	3·40
	0·028	7·10	0·070	3·42
	0·030	7·04	0·075	3·25
	0·040	6·83	0·080	3·10
	0·045	6·60	0·090	2·97
	0·050	6·42	0·100	2·86
	0·055	6·10		

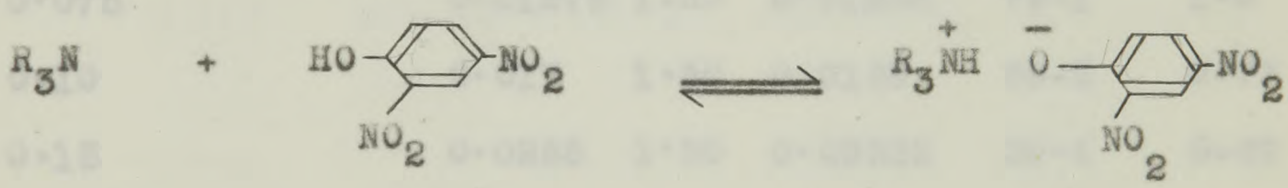
Determination of relative base strengths in chloroform solution.

Method. Relative proton affinities of bases in chloroform (containing ~ 1 % ethanol by weight) were determined by

measuring the equilibrium constants of the bases with 2:4-dinitrophenol in chloroform solution at 25°, as described by Pearson and Vogelsong (82).

A stock solution containing 0.0005 mole/lit. of 2:4-dinitrophenol was made. Aliquots of base (either in chloroform solution or measured directly by volume) were added and the solutions made up to a known volume. The concentrations of 2:4-dinitrophenate ion in these solutions were determined from the absorbancy at 400 mμ at 25°. The undissociated phenol does not absorb at this wavelength.

Calculation of equilibrium constants.



Let c = initial concentration of acid.

x = conc. of ion-pair. (= A/E).

A = absorbancy of solutions.

E = molecular extinction coeff of ion-pair.

b = base concentration (= c - x)

b_i = initial base concentration.

(All concentrations expressed in mol./lit.)

$$K = \frac{x}{b\,(c - x)} = \frac{A}{E\,b\,(c - A/E).}$$

$$\frac{1}{A} = \frac{1}{b.cEK} + \frac{1}{cE}$$

A plot of 1/A against 1/b gives a st. line, gradient $\frac{1}{cEK}$.

Intercept on 1/A axis = 1/cE. pKa =+log K

Results.

(a). Triethanolamine. Base stock solution. 0·34 M. Phenol stock solution. 0·0005 M. 1ml. of the phenol solution was measured into 2ml. standard flasks and aliquots of the base solution added.

Vol. base sol^{n}., ml.	b_i	A	$b(=b_i - A/E)$	1/b	1/A
0·075	0·01275	1·00	0·01265	79·1	1·0
0·10	0·017	1·30	0·0169	59·2	0·77
0·15	0·0255	1·50	0·02535	39·4	0.67
0·20	0·034	1·65	0·0338	29·6	0·61
0·30	0·051	1·80	0·0508	19·7	0·56

The gradient of the plot of 1/A against 1/b (Fig.23a) gives K = 70 , pKa = 1·9.

Repeat experiment, using base stock solution of strength 0·2M.

Vol.base soln., ml.	b_i	A	b	1/b	1/A
0·2	0·02	1·30	0·02	50	0·77
0·25	0·025	1·55	0·025	40	0·65
0·3	0·030	1·65	0·03	33·3	0·61

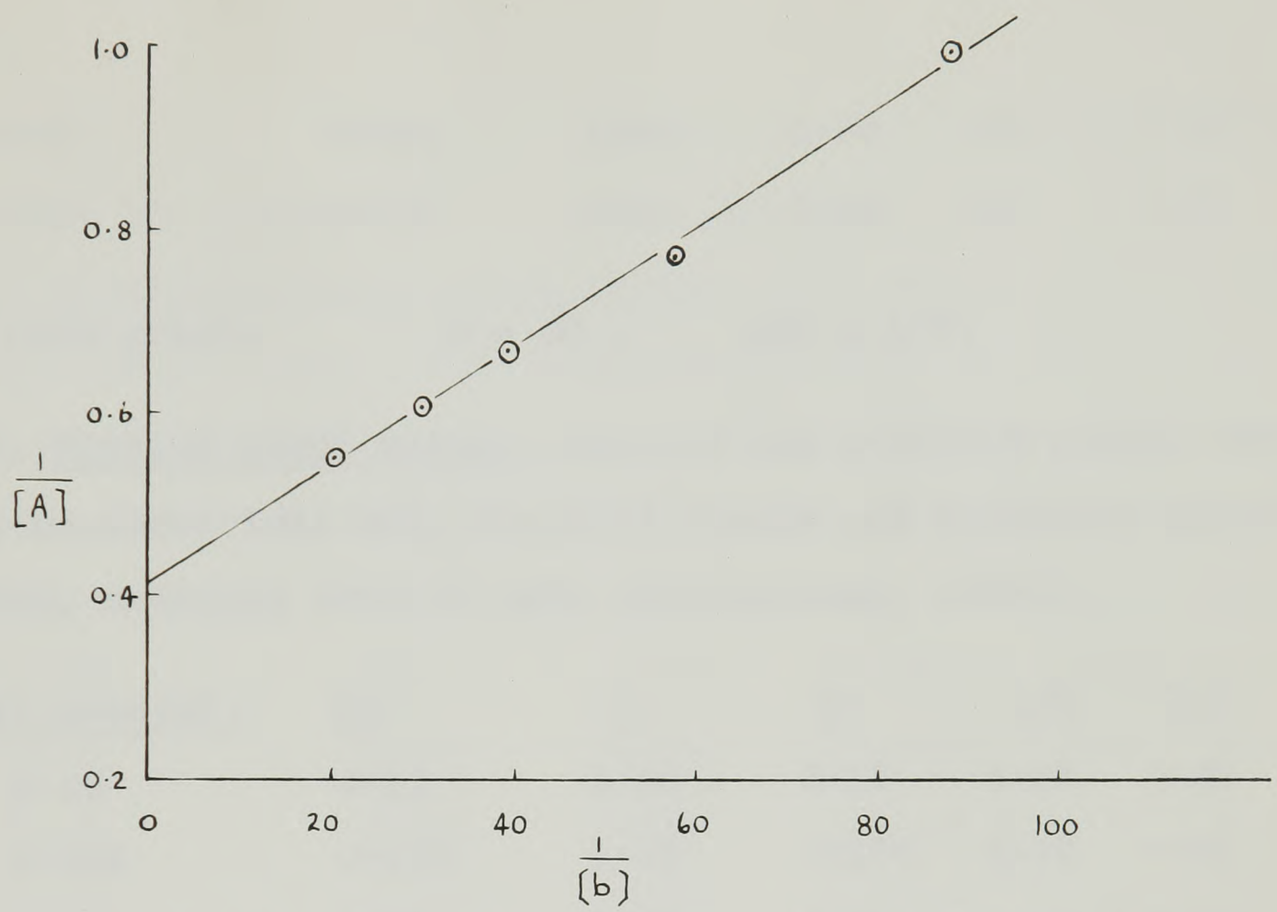


Fig. 23 a Base strength of $N(CH_2CH_2OH)_3$ in $CHCl_3$

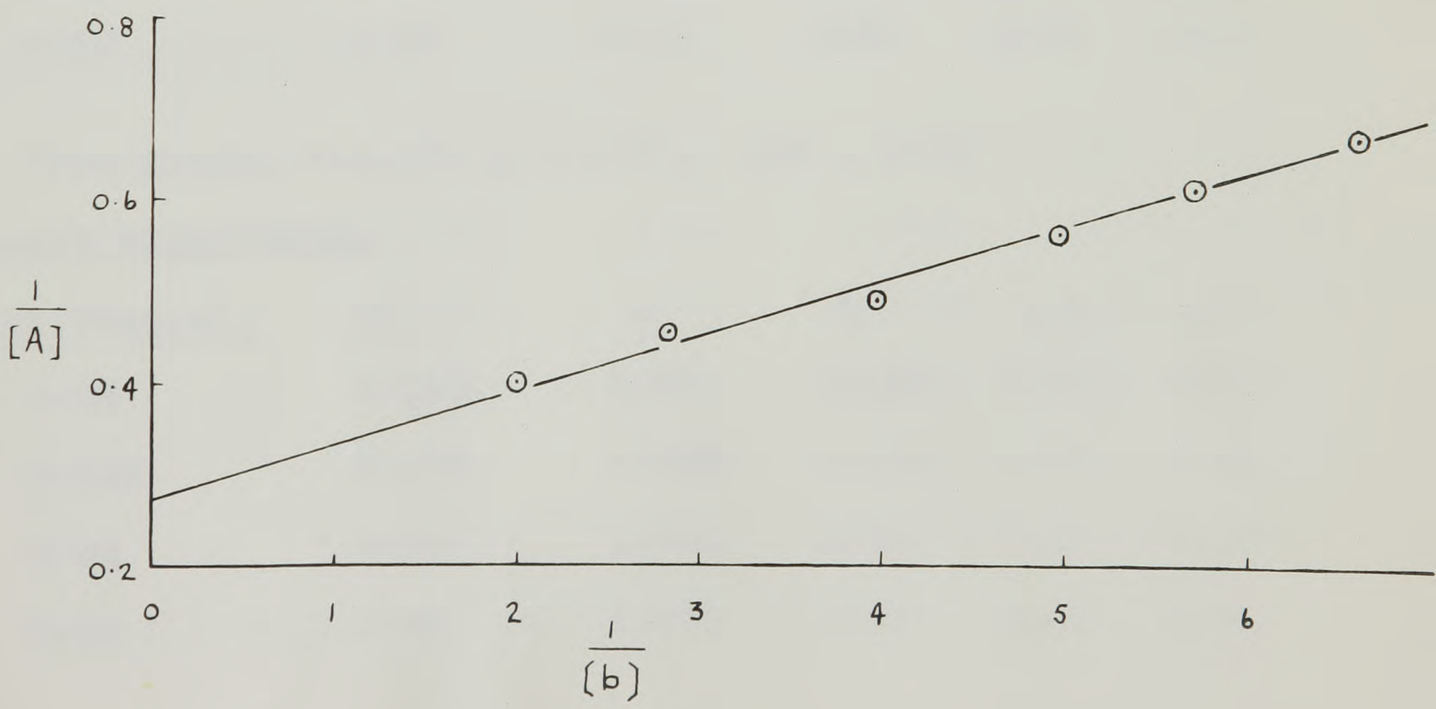


Fig. 23 b Base strength of glyOEt in $CHCl_3$

0·4	0·040	1·85	0·04	25	0·54
0·5	0·050	2·00	0·05	20	0·50

From graph, K = 50 , pKa = 1·7.

(b). Glycine ethyl ester. 1ml. of the 0·0005 M phenol solution was measured into 2ml. standard flasks and distilled glycine ester, measured from an Agla microsyringe, added.

Vol.base,ml.	bi	A	b	1/b	1/A
0·03	0·15	1·50	0·15	6·67	0·67
0·035	0·175	1·65	0·175	5·72	0·61
0·04	0·20	1·82	0·20	5·00	0·56
0·05	0·25	2·025	0·25	4·00	0·49
0·07	0·35	2·20	0·35	2·86	0·46
0·10	0·50	2·50	0·50	2·00	0·40

From graph, Fig.23b , K = 7 , pKa = 0·85

Repeat experiment.

Vol.base,ml.	bi	A	b	1/b	1/A
0·03	0·150	1·40	0·150	6.67	0·71
0·035	0·175	1·625	0·175	5.71	0·61
0·04	0·20	1·750	0·20	5·00	0·57
0·05	0·25	1·975	0·25	4·00	0·51

0·07	0·35	2·325	0·35	2·86	0·43

From the graph, K = 6, pKa = 0·8

(c). Dimethylglycine methyl ester. 1ml. of the phenol solution of strength 0·0005 M was measured into 2ml. standard flasks and dimethylglycine methyl ester, measured from a microsyringe, added. The density of the ester was found to be 0·96 at 24°.

Vol.base,ml.	bi	A	b	1/b	1/A
0·02	0·0821	1·45	0·0820	12·2	0·69
0·03	0·1231	1·80	0·1229	8·1	0·56
0·04	0·1642	2·05	0·1640	6·1	0·49
0·05	0·2052	2·15	0·2050	4·9	0·46
0·06	0·2462	2·25	0·2460	4·1	0·44

From the graph, K = 13, pKa = 1·1

Repeat experiment.

Vol.base,ml.	bi	A	b	1/b	1/A
0·02	0·0821	1·45	0·0820	12·2	0·69
0·03	0·1231	1·80	0.1229	8·1	0·56
0·04	0·1642	1·98	0·1640	6·1	0·51
0·05	0·2052	2·125	0·2050	4·9	0·47
0·06	0·2462	2·20	0·2460	4·1	0·41

From the graph, Fig.24a , K = 13, pKa = 1·1.

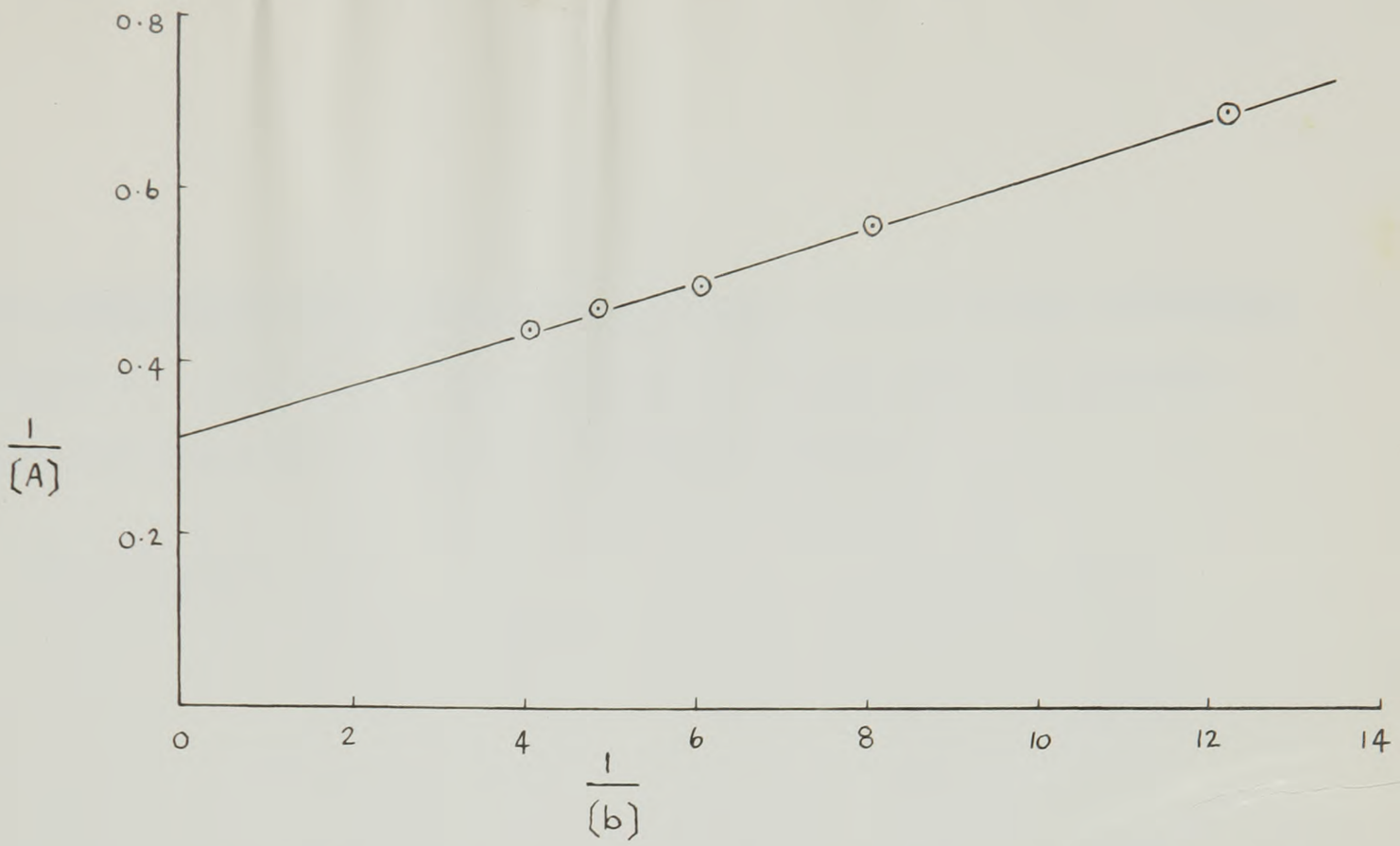


Fig. 24 a Base strength of $Me_2N.CH_2COOMe$ in $CHCl_3$

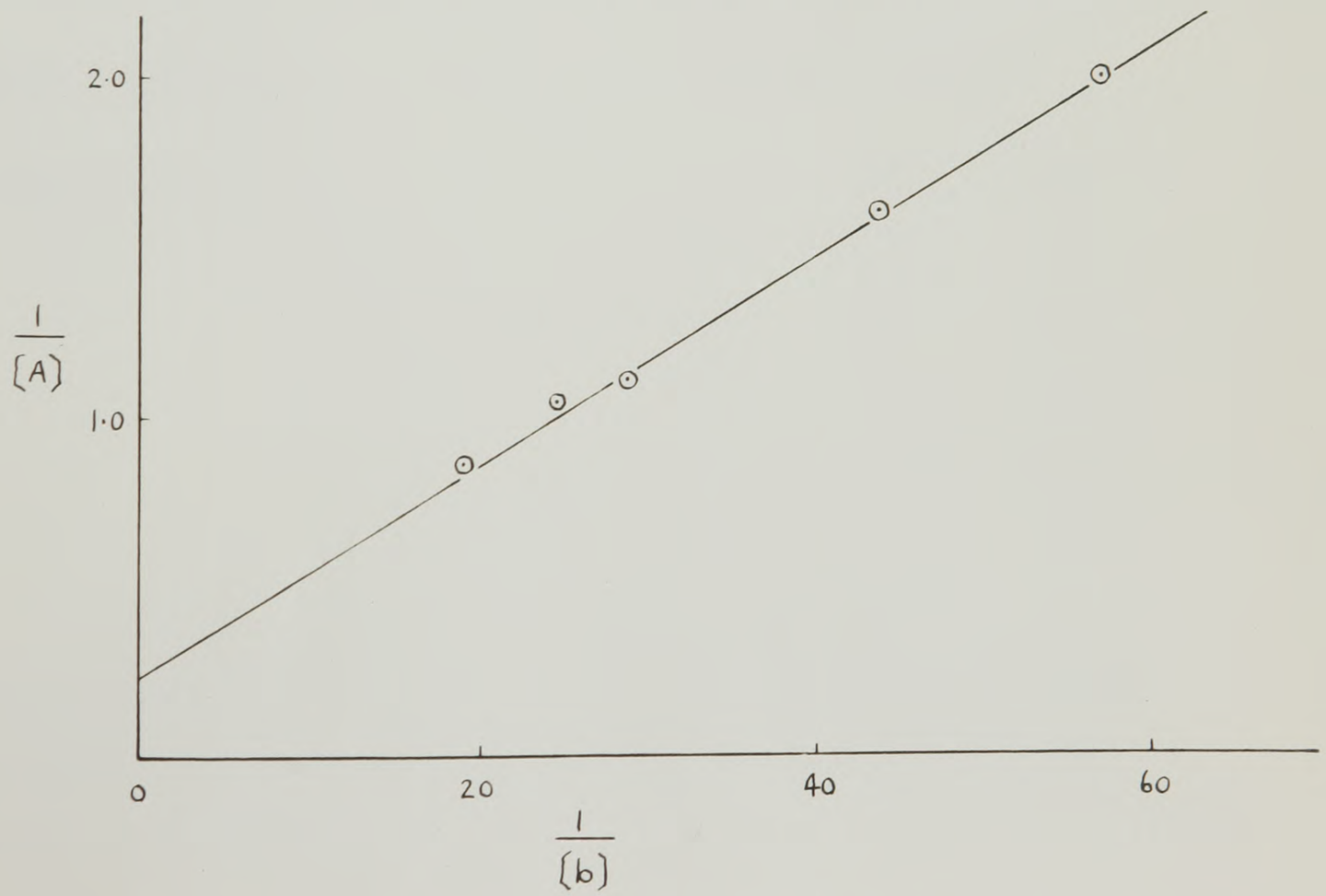


Fig. 24 b Base strength of $C_6H_5CH_2\overset{+}{N}Me_3\ \bar{Cl}$ in $CHCl_3$

(d). Benzyltrimethylammonium chloride. Phenol stock solution, 0·0005 M. Chloride stock solution 0·06 M. 1ml. of phenol solution measured into 2ml. standard flasks.

Vol. Cl^- soln.	bi	A	b	1/b	1/A
0·3	0·009	0.50	0·009	111	2·0
0·5	0·015	0·55	0·015	66·7	1·82
0·7	0·021	0·675	0·021	47·6	1·48
0·9	0·027	0·827	0·027	37·0	1·21

From graph, K = 20, pKa = 1·3.

Repeat experiment. Base stock solution 0·1162 M.

Vol. Cl^- soln.	bi	A	b	1/b	1/A
0·3	0·0174	0·50	0·0174	57·5	2·0
0·4	0·0233	0·625	0·0232	43·1	1·60
0·6	0·0349	0·90	0·0348	28·7	1·11
0·7	0·0407	0·95	0·0406	24·6	1·05
0·9	0·0523	1·175	0·0522	19.2	0·85

From graph, Fig. 24b, K = 13 , pKa = 1·1

Determination of rate of aminolysis of benzoyl-L-leucine p-nitrophenyl ester with glycine ethyl ester (1 mole).

Method. The rate of disappearance of the p-nitrophenyl ester

is followed by the decrease in the absorption at 270 mμ in chloroform solution. Glycine ester and benzoylleucylglycine ethyl ester were found not to absorb in this region of the ultraviolet spectrum. It is assumed coupling occurs at a considerably faster rate than catalytic azlactonisation. Benzoyl-L-leucine p-nitrophenyl ester (0·0712g. ; 0·00002 mole) was weighed into a 2ml. flask and made up with a solution of glycine ethyl ester (containing 0·00002 mole in 2ml.) at 25°, The solution was diluted 200x for the ultraviolet absorption determinations. The results are tabulated in Fig.25 .

Fig.25 .

t (min.)	Abs.^{n} at 270mμ	C_{ester} (=a-x)	x	$\frac{x}{a(a-x)}$
0	[1·12]	0·1	0	0
7	1·12	0·1	0	0
16	1·07	0·096	0·004	0·42
26	1·00	0·089	0·011	1·24
37	0·98	0·088	0·012	1·36
45	0·98	0·088	0·012	1·36
60	0·92	0·082	0·018	2·19
75	0·92	0·082	0·018	2·19
85	0·89	0·079	0·021	2·66
95	0·89	0·079	0·021	2·66

105	0·87	0·078	0·022	2·82
120	0·83	0·074	0·026	3·51
135	0·83	0·074	0·026	3·51
150	0·81	0·072	0·028	3·89
165	0·80	0·071	0·029	4·08
180	0·78	0·070	0·030	4·29

C_{ester} = concentration of ester., moles/litre.

a = initial concentration of ester

a - x = concentration of ester at time t

A graph of $\frac{x}{a(a-x)}$ /t (Fig.26a) gives an initial gradient k = $5{\cdot}8 \times 10^{-4}$ l. $mol.^{-1}$ $sec.^{-1}$ Also, extrapolation to zero time gives E = 11,200.

A repeat experiment, using 0·1068g. of ester in 2ml. solution, i.e. 0·15 moles/litre gave the following results, (Fig.27): (U.V. determinations were made on 300 x diluted samples.)

Fig.27

t (min.)	Absorption	C_{ester} (=a-x)	x	$\frac{x}{a(a-x)}$
0	[1·10]	0·150	0	0
5	1·05	0·143	0·007	0·33
15	1·025	0·140	0·010	0·48
25	0·97	0·132	0·018	0·91

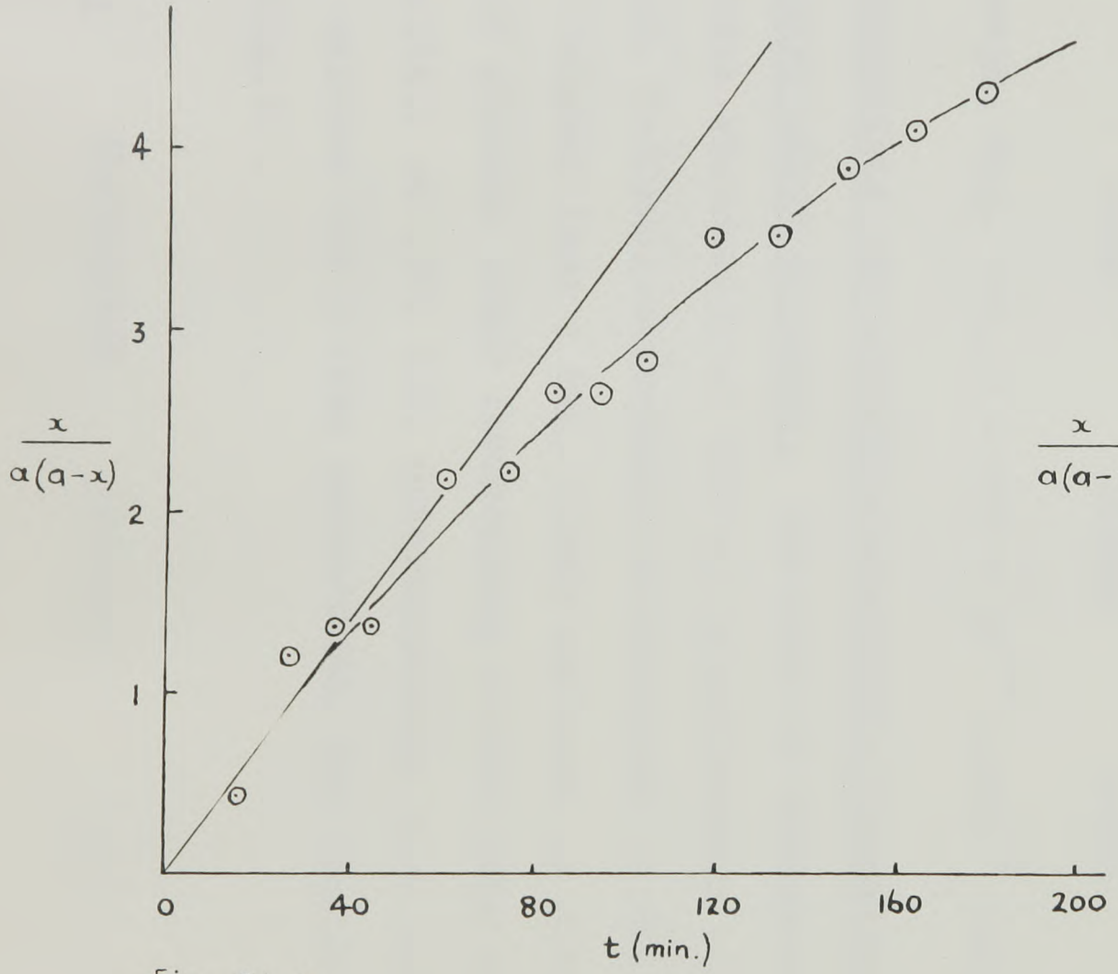


Fig. 2b a

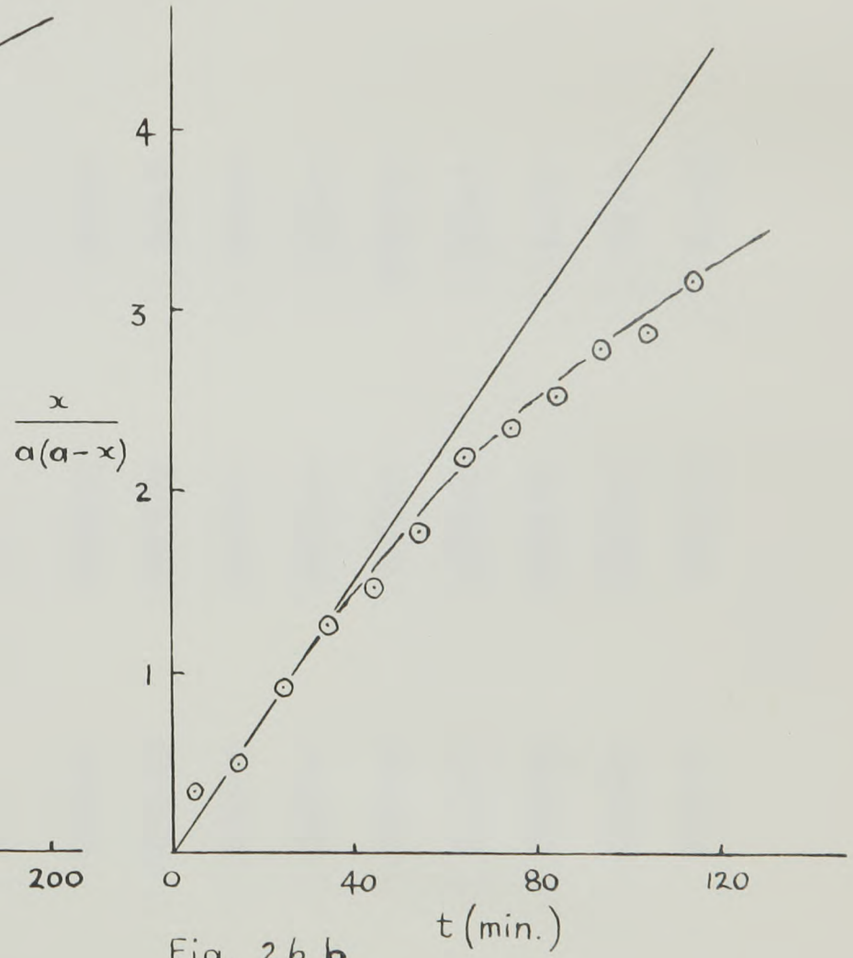


Fig. 2b b

Aminolysis of benzoyl-L-leucine p-nitrophenyl ester with glycine ethyl ester

35	0·93	0·127	0·023	1·24
45	0·88	0·123	0·027	1·46
55	0·87	0.119	0·031	1·74
65	0·83	0·113	0·037	2·18
75	0·815	0·111	0·039	2·34
85	0·80	0·109	0·041	2·51
95	0·78	0·106	0·044	2·77
105	0·77	0·105	0·045	2·86
115	0·75	0·102	0·048	3·14

From graph, Fig. 26b, $k = 6{\cdot}2 \times 10^{-4}$ l.mol.$^{-1}$ sec.$^{-1}$ (E = 11,000)

Rate of Aminolysis of 2-phenyl-4-isobutyl-5-oxazolone with glycine ethyl ester (1 mole). The rate of disappearance of the azlactone absorption at 245 mμ in chloroform solution is followed. 2-Phenyl-4-isobutyl-5-oxazolone (0·0217g. ; 0·0001 mole) was weighed into a 2ml. flask and made up with a solution of glycine ester (containing 0·0001 mole in 2ml. i.e. 0·05 mole/lit.) at 25°. U.V. determinations were made on solutions diluted 334 x with chloroform. The results are shown in Fig.28 .

Fig.28.

t (min.)	Absorption	Ca(=a-x)	x	$\frac{x}{a(a-x)}$

0	[0·45]	0·05	0	0
3	0·435	0·048	0·002	0·83
10	0·415	0·046	0·004	1·74
20	0·385	0·043	0·007	3·26
25	0·375	0·042	0·008	3·81
30	0·360	0·040	0·010	5·00
40	0·335	0·037	0·013	7·03
50	0·315	0·035	0·015	8·57
60	0·300	0·033	0·017	10·30
65	0·290	0·032	0·018	11·26
70	0·280	0·031	0·019	12·26
75	0·275	0·0305	0·0195	12·78
80	0·270	0·030	0·020	13·33

From graph, Fig.29a. $k = 2{\cdot}9 \times 10^{-3}$ l. $mol.^{-1}$ $sec.^{-1}$ ($E = 15{,}000$).

Repeat experiment, Fig.30 .

Fig. 30 .

t (min.)	Absorption	Ca(=a-x)	x	$\frac{x}{a(a-x)}$
0	[0·455]	0·050	0	0
2	0·45	0·0495	0·0005	0·20
10	0·43	0·047	0·0027	1·14

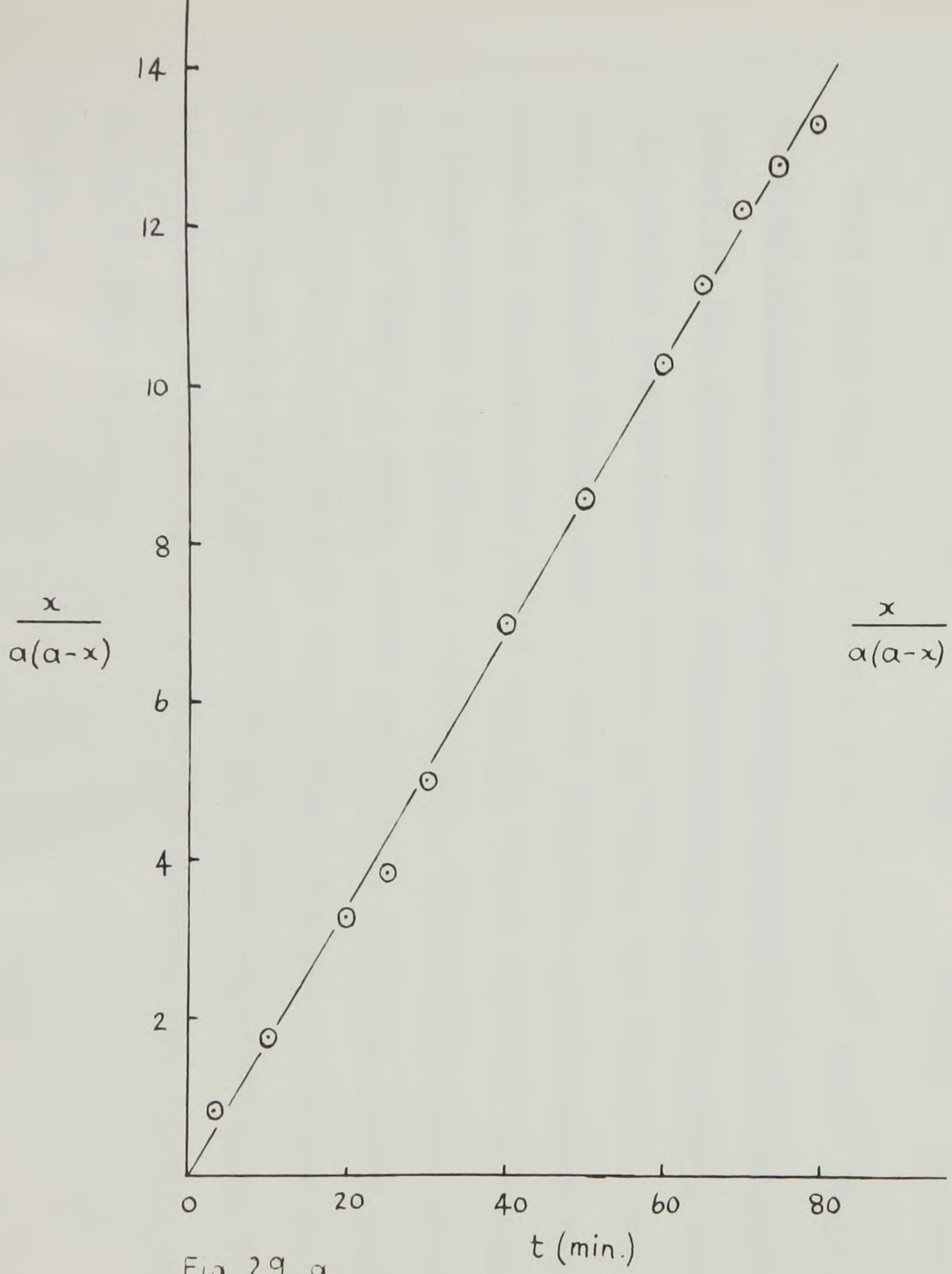


Fig. 29 a

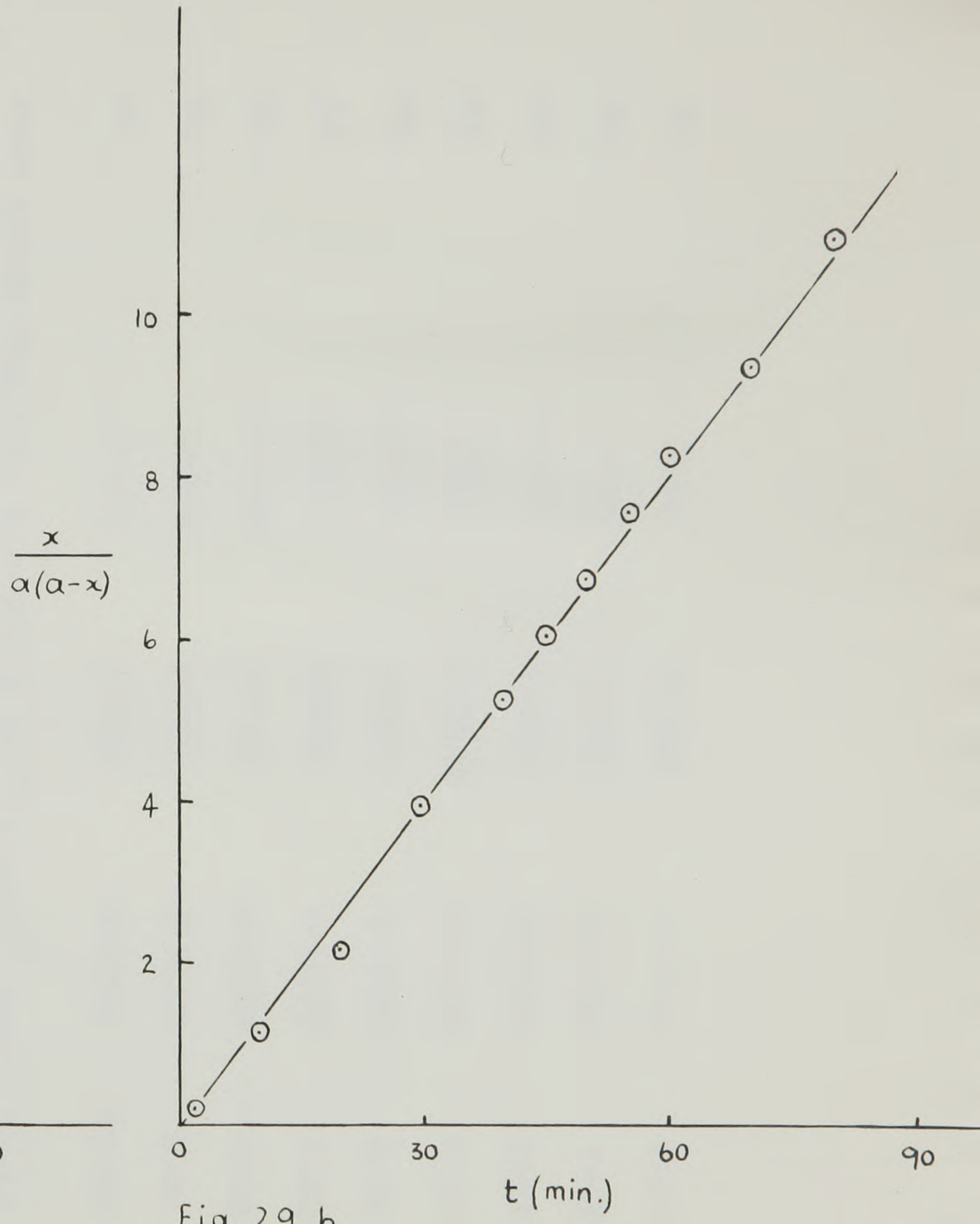


Fig. 29 b

Aminolysis of 2-phenyl-4-isobutyl-5-oxazolone with glycine ethyl ester

20	0·41	0·045	0·0049	2·17
30	0·38	0·042	0·0082	3·92
40	0·36	0·040	0·0104	5·25
45	0·35	0·0385	0·0115	6·03
50	0·34	0·037	0·0126	6·74
55	0·33	0·036	0·0137	7·55
60	0·322	0·035	0·0146	8·25
70	0·310	0·034	0·0159	9·31
80	0·295	0·032	0·0176	10·86

From graph Fig.29b. $k = 2{\cdot}2 \times 10^{-3}$ $1.mol.^{-1}$ $sec.^{-1}$ (E=15,200)

Rate of azlactonisation of benzoyl-L-leucine p-nitrophenyl ester in the presence of dimethylglycine methyl ester. Benzoyl -L-leucine p-nitrophenol ester (0·727g.) was weighed into a 2ml. standard flask (conc. 1·027 mole/lit.), chloroform was added to dissolve the solid and the flask allowed to attain a temperature of 25° in a water bath. Dimethylglycine methyl ester (0·25ml. ; conc. 1·027 mole/lit.) was then added and the solution made up with chloroform. The reaction was followed by the decrease of intensity of the ester absorbtion at 270mμ of aliquots diluted 2,500 x with chloroform. The results are shown in Fig.31 .

Fig.31 .

t (hrs.)	A	c(=a-x)	x	$\frac{x}{a(a-x)}$

0	[0·98]	1·027	0	0
0·083	0·97	1·016	0·011	0·0105
0·25	0·96	1·006	0·021	0·0203
0·50	0·95	0·995	0·032	0·0313
0·75	0·93	0·975	0·052	0·0519
1·00	0·92	0·964	0·063	0·0636
1·25	0·90	0·943	0·084	0·0868
1·50	0·89	0·933	0·094	0·0981
1·75	0·89	0·933	0·094	0·0981
2·00	0·88	0·922	0·105	0·111

From graph, Fig.32a, $k = 1{\cdot}8 \times 10^{-5}$ l. mol.$^{-1}$ sec.$^{-1}$

Repeat experiment, using 0·716 mole/lit. of ester and base.

Fig. 33.

t (hrs.)	A	c(=a-x)	x	$\frac{x}{a(a-x)}$
0	[0·67]	0·716	0	0
0·083	0·67	0·716	0	0
0·25	0·665	0·711	0·005	0·0098
0·50	0·66	0·705	0·011	0·0218
0·75	0·655	0·700	0·016	0·0329
1·00	0·65	0·694	0·022	0·0443
1·25	0·645	0·689	0·027	0·0547
1·50	0·640	0·684	0·032	0·0653

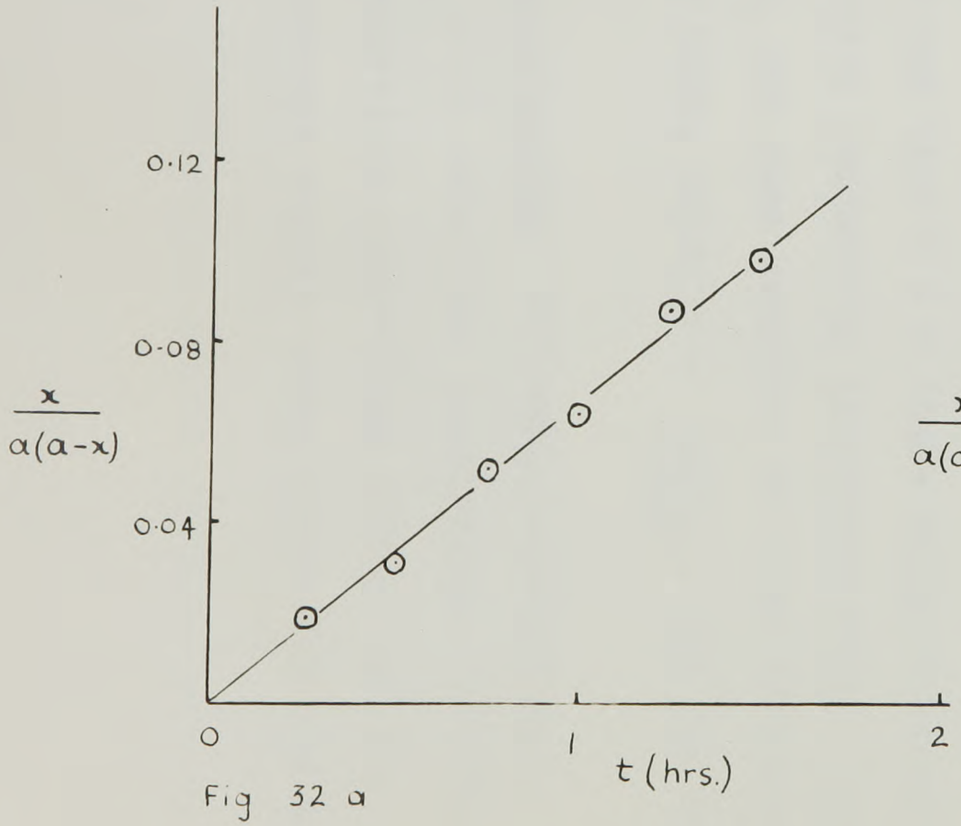


Fig 32 a

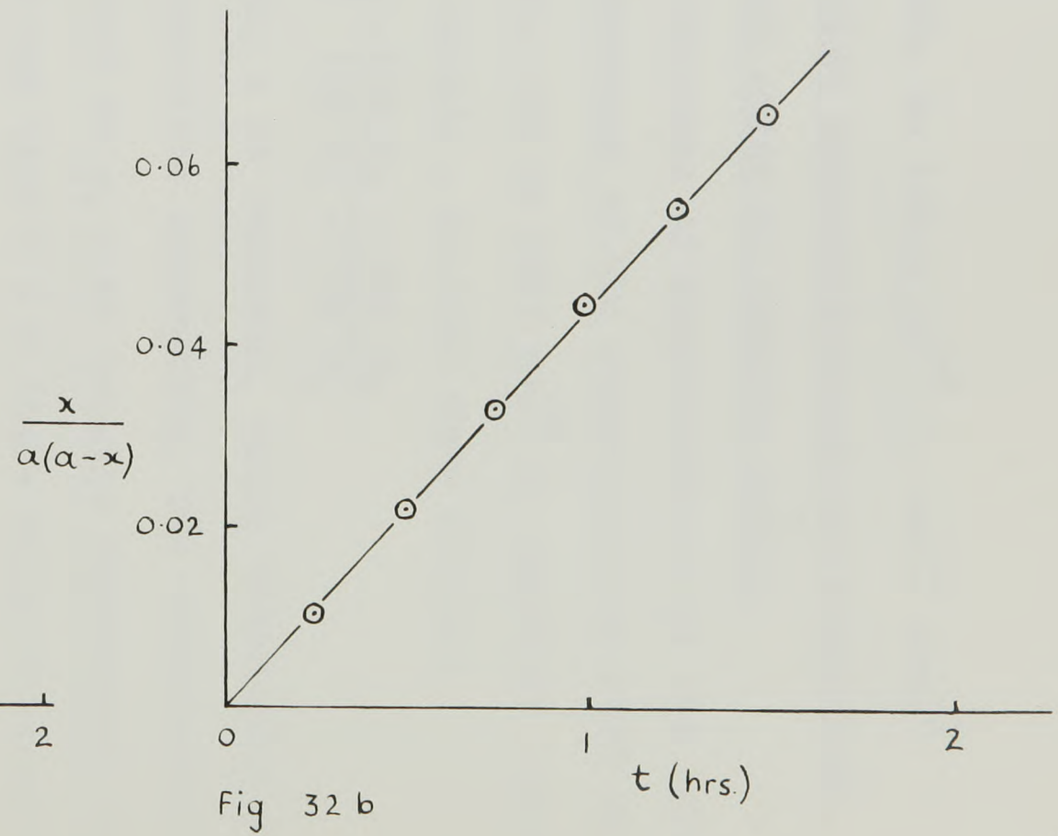


Fig 32 b

Azlactonisation of benzoyl-L-leucine p-nitrophenyl ester with $Me_2N.CH_2COOMe$

From graph, Fig. 32b, $k = 1{\cdot}2 \times 10^{-5}$ l. mol.$^{-1}$ sec.$^{-1}$

Catalysis of the reaction between 2-phenyl-4-isobutyl-5-oxazolone and a primary amine by various salts.

Method. The rate of decrease of concentration of the azlactone was followed by the decrease of the absorption at 1832 cm.$^{-1}$ in chloroform solution, and at 1815 cm.$^{-1}$ in pyridine solution. The formula $E = \frac{M}{cl} \cdot \log x/y$, in this case becomes $\log x/y \propto c$, and $\frac{c_o}{c} = \frac{\log x_o - \log y_o}{\log x_1 - \log y_1}$

For a given experiment, x is constant, being determined by the position of the base-line absorption. The concentration is then directly related to y. y is usually determined after two intervals of time and also t = o (this being determined accurately using solution containing azlactone only). Assuming second order kinetics, the rate constant is then determined for each value of the time, using $k = \frac{c_o - c}{t \cdot c_o c}$, and the average value taken.

1. Amminolysis with p.toluidine 2-Phenyl-4-isobutyl-5-oxazolone (0·217g. ; 0·001 mole) and p-toluidine (0·107g. ; 0·001 mole) were dissolved in chloroform (3ml.) and the infra-red spectrum of the carbonyl region taken after 275 and 405 mins.

t (sec.)	y	c (mole/lit.)	k
0	4	0·334	-
16,500	36·5	0·097	$4·4 \times 10^{-4}$
24,300	48	0·067	$4·9 \times 10^{-4}$

$(x = 90)$ Average $k = 4.6 \times 10^{-4}$ $l.mol.^{-1}sec.^{-1}$

2. <u>p.toluidine in the presence of 0·1 equivalent of tribenzylamine hydrochloride at 20°.</u> Tribenzylamine hydrochloride (0·0162g. ; 0·0005 mole) was dissolved in chloroform (1·5ml.) and the azlactone (0·1085g. ; 0·0005 mole) and p-toluidine (0·0535g. ; 0·0005 mole.) added. Spectra were taken after 5 and 10 mins.

$(x = 84.)$

t (sec.)	y	c(mole/lit.)	k
0	3	0·334	-
300	42	0·069	$3·8 \times 10^{-2}$
600	57·5	0·038	$3·9 \times 10^{-2}$

$k = 4 \times 10.^{-2}$ $l. mol.^{-1} sec.^{-1}$

3. <u>p.toluidine in the presence of 0·1 equivalent of tribenzylamine toluene-p-sulphonate at 20°.</u> Tribenzylamine toluene-p-sulphonate (0·0229g. ; 0·0005 mole) was dissolved in chloroform (1·5ml.) and the azlactone (0·1085g. ; 0·0005 mole) and p-toluidine (0·0535g. ; 0·0005 mole) added. Spectra were taken

after 5 and 10 mins.

$(x = 84)$

t (sec.)	y	c(mole/lit.)	k
0	3	0·334	-
300	48	0·057	$4·8 \times 10^{-2}$
600	60	0·034	$4·5 \times 10^{-2}$

$k = 4·5 \times 10^{-2}$ l. $mol.^{-1}$ $sec.^{-1}$

4.

<u>p-toluidine in the presence of 0·1 equivalent of tribenzylamine perchlorate at 20°</u>. Tribenzylamine perchlorate (0·0194g. ; 0·0005 mole) was dissolved in chloroform (1·5ml.) and the azlactone (0·1085g. ; 0·0005 mole) and p-toluidine (0·0535g. ; 0·0005 mole) added. Spectra were taken after 5 and 10 minutes.

$(x = 84)$

t (sec.)	y	c(mole/lit.)	k
0	3	0·334	-
300	46·5	0·060	$4·6 \times 10^{-2}$
600	65	0·031	$4·9 \times 10^{-2}$

$k = 5 \times 10^{-2}$ l. $mol.^{-1}$ $sec.^{-1}$

5.

<u>Aminolysis with p-toluidine in pyridine solution at 20°</u>

$(x = 93)$

t(sec.)	y	c(mole/lit.)	k
0	1	0·334	-
24,900	23·5	0·101	$2·8 \times 10^{-4}$

$k = 2·8 \times 10^{-4}$ l. $mol.^{-1}$ $sec.^{-1}$

Aminolysis with p-toluidine in the presence of 0·1 equivalent of tribenzylamine hydrochloride in pyridine solution at 20°.

$(x = 91)$

t (sec.)	y	c(mole/lit.)	k
0	1	0·334	-
7,200	66	0·023	$5·6 \times 10^{-3}$

$k = 5·6 \times 10^{-3}$ l. $mol.^{-1}$ $sec.^{-1}$

Effect of halide ion on racemisation during coupling of benzoyl-L-leucine p-nitrophenyl ester with glycine ethyl ester.

(a). Coupling in the presence of tetraethylammonium chloride. Benzoyl-L-leucine p-nitrophenyl ester (0·89g. ; 0·0025 mole) was coupled with glycine ethyl ester (0·25ml. ; 0·0025 mole) in dry chloroform (3ml.) in which was dissolved anhydrous tetraethylammonium chloride (0·41g. ; 0·0025 mole). A crystalline product was obtained. 0·67g. (84%), m.p.148-151; $[\alpha]_D^{21}$ - 29.1 (c. 4.025 in ethanol). (Found: C, 63.7 ; H, 7.7 ; N, 8.8%).

L-isomer 86%.

(b). Coupling in the presence of benzyltrimethylammonium chloride. (i) Coupling on a 0·004 molar scale, in the presence of benzyltrimethylammonium chloride (0·76g. ; 0.004 mole) gave 1·02g. (80%) of crystalline product. m.p.150-1°; $[\alpha]_D^{23}$ - 27·1 (c. 3·18 in ethanol). (Found : C, 63·8 ; H, 7·5 ; N, 8·8%).

% L-isomer. 82%. 0·93g. of the product was saponified with 2·9ml. of 1N sodium hydroxide solution (1 equivalent) in aqueous dioxan (3:1) at room temperature for 1 hr. Acidification to Congo Red, followed by recrystallisation from the aqueous solution gave a crystalline solid. 0·33g., m.p. 137-139°. Recrystallisation from water gave 0·06g., m.p. 163-4°.

In a repeat experiment, on a 0·001 molar scale, a 72% yield of peptide, m.p.150-152°, $[\alpha]_D^{24}$ - 31·5 (c. 2·52 in ethanol) was obtained. (Found: C, 63·8 ; H, 7·2 ; N, 8·8%). % L-isomer, 92%.

(ii). Benzoyl-L-leucine p-nitrophenyl ester (0·178g. ; 0·0005 mole) and benzyltrimethylammonium chloride (0·371g. ; 0·002 mole)were dissolved in chloroform (1ml.) and the solution left at room temperature for 2 hr. Glycine ethyl ester (0·05ml. ; 0.0005 mole) was added and the solution left overnight.

By the usual workup procedure, 0·148g. (92%) of crystalline product was obtained. m.p.150-3°; $[\alpha]_D^{23}$ - 23.2 (c. 2·495 in ethanol). (Found : C, 64·2 ; H, 7·3 ; N, 8.5%). % L-isomer, 32%.

(c). Coupling in the presence of N-ethylpiperidine hydrochloride. Benzoyl-L-leucine p-nitrophenyl ester (0·89g. ; 0.0025 mole) and N-ethylpiperidine hydrochloride (0·37g. ; 0·0025 mole) were dissolved in chloroform (3ml.). Glycine ethyl ester (0·25ml. ; 0·0025 mole) was added and the solution left overnight. The chloroform was removed under reduced pressure and replaced with ethyl acetate. The solution was washed as usual and the product worked up. 0.63g. (79%)..m.p.151-4°; $[\alpha]_D^{21}$ - 29·8 (c. 2.825 in ethanol). (Found : C, 63.6 ; H, 7·7 ; N, 8.6%). % L-isomer, 88%.

Catalysis of azlactomisation of benzoyl-L-leucine p-nitrophenyl ester in solution by various salts. Benzoyl-L-leucine p-nitrophenyl ester (0·178g. ; 0·0005 mole) and the salt (usually 0·001 mole) were dissolved in the solvent (1·5ml.) and the solution refluxed for 0·5 hr. (heated at 100° in the case of diethyl phosphite). The solutions were then examined by infrared spectroscopy for the presence of the characteristic azlactone carbonyl absorption at 1832 $cm.^{-1}$ It was verified in

each case that solution of the salts in the solvents used did not absorb in the carbonyl region of the spectrum. The results are tabulated in Fig. 34.

Fig. 34.

Salt	Molar quantity	Solvent	Presence of azlactone
	2	$CHCl_3$	+
$Bz_3\overset{+}{N}H\ \bar{C}l$	1	$CHCl_3$	+
$Et_4\overset{+}{N}\ \bar{C}l$	2	$CHCl_3$	+
$(n.Pr)_4\overset{+}{N}\ \bar{I}$	2	$CHCl_3$	+
$Bz_3\overset{+}{N}H\ \bar{C}lO_4$	2	$CHCl_3$	-
$Bz\overset{+}{N}Me_3\ \bar{C}lO_4$	2	D.E.P.	-
$Bz\overset{+}{N}Me_3\ \bar{O}C_6H_4NO_2$	1	CH_2Cl_2	+

Diethyl phosphite was used in one case because benzyltrimethylammonium perchlorate did not dissolve in chloroform, and methylene chloride because benzyltrimethylammonium p-nitrophenate reacts rapidly with boiling chloroform with the formation of a green colour. Azlactone formation was very rapid when this salt was used. The quaternary ammonium iodide caused less rapid azlactonisation than the chloride. When chloroform was used as solvent, a small carbonyl peak appeared at λ^{1754} cm.$^{-1}$ in those cases in which azlactone formation was not

very rapid. This is the ethyl ester carbonyl absorption, the ester being formed by reaction between the azlactone and the ethanol which is added to stabilise the chloroform. This peak did not appear when ethanol-free chloroform was used.

* Benzyltrimethylammonium p-nitrophenate. p-Nitrophenol (1·25g. ; 0·01 mole) was dissolved in a solution of benzyltrimethylammonium hydroxide (4·2g. of 40% solution in methanol ; 0·01 mole), and then the solvent wasremoved by distillation under reduced pressure, leaving a yellow syrup which crystallised from water. Yellow needles, m.p. 60-62° (Found : C, 56·1 ; H, 7·6 ; N, 8·2. $C_6H_5CH_2\overset{+}{N}(CH_3)_3\ \bar{O}C_6H_4NO_2.3H_2O$ requires C, 56·7 ; H, 7·6 ; N, 8·3%).

Nucleophilic reaction between chloride ion and p-nitrophenyl esters. The E value of $C_6H_5CH_2\overset{+}{N}(CH_3)_3\ \bar{O}C_6H_4NO_2.3H_2O$ in CH_2Cl_2 solution. was found to be 21,000 approx. p-Nitrophenyl phenylacetate (0·257g. ; 0·001 mole) and tetraethylammonium chloride (0·1655g. ; 0·001 mole) were refluxed together in methylene chloride solution for 1 hr. The solution was then made up to 200ml. with methylene chloride and the U.V. absorption at 420mμ determined.

This was + 0·64.

Let concentration of $\bar{O}C_6H_4NO_2$ be x gm.ion/200ml.

1 gm.ion $\bar{O}C_6H_4NO_2$ in 1000ml. gives abn. 21,000

x gm.ion $\bar{O}C_6H_4NO_2$ in 1000ml. gives abn. $\frac{0.64}{5}$

$$x = \frac{0.64}{5 \times 21,000} \text{ gm.ion}$$

But the 200ml. solution originally contained 0·001 gm.ion ester.

$$\frac{[\bar{O}C_6H_4NO_2]}{[\bar{}C_6H_5CH_2CO.OC_6H_4NO_2]} = \frac{0.64}{5 \times 21,000 \times 0.001} = 0.6\%$$

* Benzoyl-L-leucylbenzanilide. L-leucylbenzanilide hydrobromide syrup was dissolved in ethyl acetate (15ml.). Benzoyl chloride (0·23ml. ; 0·002 mole) was added, and the mixture shaken with a solution of sodium carbonate (1·1g. ; 0·01 mole) in water (10ml.). The two-phase mixture was stirred for 30 min. and then the organic layer separated and dried. Removal of solvent gave a glassy solid which was recrystallised from ethyl acetate-light petroleum. Colourless silky needles. m.p. 145°, $[\alpha]_D^{24}$ - 12·2 (c. 3·55 in ethanol). (Found : C, 74·1 ; H, 7·5 ; N, 8·5. $C_{20}H_{24}NO_2$ requires : C, 74·1 ; H, 7·4 ; N, 8·6%).

Coupling of benzoyl-L-leucine p-nitrophenyl ester with benzylamine. Benzoyl-L-leucine p-nitrophenyl ester (0·356g. ; 0·001 mole) was dissolved in ethyl acetate (3ml.) containing

benzylamine (0·109ml. ; 0·001 mole) and left at room temperature overnight. The usual working-up procedure gave a colourless solid. 0·31g. (96%) $[\alpha]_D^{23}$ - 9·7 (c. 1·935 in ethanol). (Found : C, 73·9 ; H, 7·4 ; N, 7·9%).

The experiment was repeated in chloroform solution. An 80% yield was obtained of product, $[\alpha]_D^{25}$ - 10·5 (c. 3.025 in ethanol.)

* Benzyloxycarbonyl-L-leucylbenzanilide. Benzyloxycarbonyl-L-leucine p-nitrophenyl ester (1·93g. ; 0·005 mole) was dissolved in ethyl acetate (5ml.). Benzylamine (0·55ml. ; 0·005 mole) was added and the solution left at room temperature overnight. It was then diluted with ethyl acetate and washed with dil. hydrochloric acid (30ml.), sodium carbonate solution (3 x 30ml.) and water (30ml.). After drying over magnesium sulphate, the solvent was removed, leaving a pale yellow solid. Recrystallisation from ethyl acetate-light petroleum gave colourless silky needles. 1·26g. (71%), m.p. 112-113°, $[\alpha]_D^{25}$ - 16·6 (c. 1·24 in ethanol) (Found : C, 71·1 ; H, 7·3 ; N, 7·4. $C_{21}H_{26}N_2O_3$ requires : C, 71·2 ; H, 7·4 ; N, 7·9%).

L-leucylbenzanilide hydrobromide. Benzyloxycarbonyl-L-leucylbenzanilide (0·71g. ; 0·002 mole) was dissolved in 14% hydrogen bromide in acetic acid (10ml.) and left at room

temperature for 1 hr. The solvent was removed by vac. distillation and the product obtained as a syrup by precipitation with ether.

Part III (b). The tetraethyl pyrophosphite coupling method. Benzoyl-L-leucine was coupled with glycine ethyl ester in chloroform solution in the presence of added salts. The tetraethyl pyrophosphite employed was a combination of two fractions, b.p. 80-85/0·3mm. and 60-75°/0·2mm. The refractive index of the combined fractions was n_D^{26} 1·4306. The products were worked up as described in Part II of the thesis. The % L-isomer was determined from the specific rotations of the products, which gave satisfactory elementary analyses. The results are tabulated in Fig. 35.

Fig. 35.

Conditions	Yield, %	$[\alpha]_D$-	% L	C	H	N
glyOEt	90	-25·9	76	63·6	7·8	8·6
	88	-25·8	76	63·8	7·8	8·6
glyOEt.HCl+NEP	80	-14·9	44	63·4	7·6	9·0
	85	-14·7	43	63·3	7·6	8·7
glyOEt+NEP.HCl	88	-15·9	47	64·0	7·8	8·6
	79	-15·4	45	63·6	7·7	8·6
glyOEt+NEP.tosylate	85	-15.3	45	63.1	7.8	8.2

	74	-15·0	44	63.7	7.8	8.5
glyOEt+$Et_4\overset{+}{N}\ \bar{Cl}$	83	-14.6	43	63.6	7.7	8.6
	83	-14.6	43	63.3	7.7	8.7
glyOEt+$Pr_4\overset{+}{N}\ \bar{I}$	68	-22.8	67	63.5	7.5	8.4
glyOEt+$BzNMe_3\overset{+}{}\bar{B}F_4$	75	-26.7	78	63.2	7.6	8.6

A solubility determination showed that the benzyltrimethyl borofluoride was almost insoluble in refluxing chloroform.

Action of tetraethyl pyrophosphite on benzoylleucine. Benzoyl-L-leucine (0·47g. ; 0·002 mole) was dissolved in dry chloroform (1ml.) and tetraethyl pyrophosphite (1ml., n_D^{25} 1·4250) added. The solution was refluxed for 10 min., and the solvent removed, the last traces of diethyl phosphite at 100°/$^{10}\lambda$cm. A colourless syrup remained. This was taken up in chloroform and the solution washed with sodium bicarbonate solution and dried. Removal of solvent gave a syrup which crystallised on scratching. 0·17g. (40%). Recrystallisation from light petroleum gave colourless needles, m.p. 55-6°. λ max. at 1664, 1832 $cm.^{-1}$ in chloroform solution.

Coupling in diethyl phosphite as solvent. The reagents were heated at 100° for 0.5 hr. in diethyl phosphite solution. The products were worked up by removing the solvent by distillation, taking up in ethyl acetate solution and washing in the usual way. The results are tabulated in Fig. 36. The coupling reagent used was the same as that used in the previous experiments.

Fig. 36.

Conditions	Yield,%	$[\alpha]_D$	%L	C	H	N
glyOEt	74	-18.1	53	63.0	7.5	8.9
	75	-16.2	48			
glyOEt+$BzNMe_3^+\bar{C}lO_4$	70	-16.3	48	63.3	7.4	9.0
	80	-16.8	49			
glyOEt+$Et_4^+\bar{N}Cl$	81	-7.6	22	63.5	7.6	8.5
	77	-7.0	21	63.5	7.5	8.4
glyOEt+NEP.HCl	78	-10.2	30	63.8	7.6	8.7
glyOEt.HCl+NEP	75	-8.4	25	63.4	7.5	8.7

* Benzyloxycarbonyl-L-leucyl-p-toluidide.

(a) p-Nitrophenyl ester method. Benzyloxycarbonyl-L-leucine p-nitrophenyl ester (3.86g. ; 0.01 mole) and p-toluidine (1.61g. ; 0.015 mole) were dissolved in ethyl acetate (5ml.) and the solution left at room temperature for 2 days. The

solution was diluted with chloroform (50ml.) and washed with dilute hydrochloric acid, sodium carbonate solution (2 x 5 ml.) water and dried. Removal of solvent gave a pale yellow crystalline solid. 3.3g. Recrystallisation from ethyl acetate-light-petroleum gave colourless crystals, 3.2g. ; m.p. 94-105°. c.f. benzyloxycarbonyl-L-leucine p-nitrophenyl ester, m.p. 95-6°. The product was taken up in ethyl acetate (5ml.), p-toluidine (1.61g.) was added and the solution refluxed for 3 hr. Working up as usual gave a colourless crystalline product, m.p. 161°, $[\alpha]_D^{21}$ - 44.5 (c.2.24 in chloroform. (Found : C, 71.2 ; H, 7.2 ; N, 7.9. $C_{21}H_{26}N_2O_3$ requires: C, 71.2 ; H, 7.4 ; N, 7.9%).

(b). Tetraethyl pyrophosphite method. Benzyloxycarbonyl-L-leucine (2.92g. ; 0.011 mole) and p-toluidine (1.18g. ; 0.011 mole) were refluxed in chloroform solution in the presence of tetraethyl pyrophosphite (3ml. ; n_D^{26} 1.4391) for 0.75 hr. The product was obtained as a colourless crystalline solid. Recrystallisation from ethyl acetate-light petroleum gave 1.94g. (50%), m.p. 159-160°, $[\alpha]_D^{21}$ - 44.4 (c. 2.93 in chloroform).

* Benzoyl-L-leucyl-toluidide. Benzyloxycarbonyl-L-leucine-p-

toluidide 1.77g. ; 0.005 mole) was dissolved in 35% hydrogen bromide in acetic acid (6ml.) and left at room temperature for 15 min. L-leucyl-p-toluidide hydrobromide was precipitated by addition of ether. 1.33g. (0.0044 mole ; 87%). This was dissolved in ethyl acetate (20ml.). Benzoyl chloride (0.51ml. ; 0.0044 mole) was added, followed by a solution of sodium carbonate (2.12g. ; 0.02 mole) in water (10ml.). The mixture was stirred vigorously at the interface for 0.5 hr., after which time the organic layer was separated, dried and the solvent removed, leaving a colourless crystalline solid. Recrystallisation from ethyl acetate-light petroleum gave 1.16g. (81%), m.p. 212°, $[\alpha]_D^{21}$ - 61.3 (c. 1.875 in chloroform). (Found : C, 74.1 ; 4, 7.7 ; N, 8.5. $C_{20}H_{24}N_2O_2$ requires: C, 74.1 ; H, 7.4 ; N, 8.6%).

* Benzoyl-DL-leucyl-p-toluidide. 2-Phenyl-4-isobutyl-5-oxazolone (1.09g. ; 0.005 mole) and p-toluidine (0.54g. ; 0.005 mole) were dissolved in ethyl acetate (2ml.) and left at room temperature overnight. The solid mass so obtained was transferred to the filter with light petroleum. 1.50g. (93%). Recrystallisation from aqueous ethanol gave material, m.p. 223-4°. (Found : C, 74.4 ; H, 7.3 ; N, 8.5. $C_{20}H_{24}N_2O_2$ requires :

C, 74.1 ; H, 7.4 ; N, 8.6%).

Coupling experiments. Benzoyl-L-leucine was coupled with p-toluidine in chloroform solution, using tetraethyl pyrophosphite in the "standard procedure". The results are tabulated in Fig. 37.

Fig.37.

Conditions	Yield,%	$[\alpha]_D$	%L	C	H	N	TEPP
(i) Tol.NH_2	63	-48.6	81	73.7	7.7	8.8	a
	95	-39.1	64	74.2	7.5	8.5	b
(ii) Tol.NH_2+$Et_4\overset{+}{N}\overline{Cl}$	70	-28.8	48	73.8	7.7	8.5	a
(iii) Tol.NH_2+$Bz_3\overset{+}{N}H\overline{Cl}$ (0.5mole)	100	-38.2	64	74.8	7.5	8.1	b
(N) Tol.$\overset{+}{N}H_3\overline{Cl}$+$Bz_3N$	91	-39.4	66	75.1	7.4	8.2	b

TEPP : Fn. (a), b.p. 80-85°/0.3mm. + 60-75°/0.2mm.), n_D^{26} 1.4306.

Fn. (b), b.p. 85-95°/0.3mm., n_D^{25} 1.4336.

In experiments (iii) and (iv), it was not possible to remove the tribenzylamine from the product by washing with dilute hydrochloric acid in the usual way, so the following procedure was adopted. The chloroform solution of the crude product was washed with sodium carbonate and then water. Removal of solvent gave a solid from which the tribenzylamine was extracted by trituration with light petroleum (50ml.). The

solid was filtered and washed with light petroleum until the washings no longer gave a ppt. when shaken with dilute hydrochloric acid. In this way it was possible to remove all the tribenzylamine. The absence of chloride ion in the product was demonstrated by treating an acetone solution of a portion of the product with aqueous silver nitrate. A white precipitate was formed, which dissolved on addition of further acetone. Silver chloride is insoluble in acetone. It was also verified that the peptide is virtually insoluble in cold light petroleum.

Basic strength of tribenzylamine in chloroform. Solutions of 2:4-dinitrophenol (0.0467 M) and tribenzylamine (0.2734 M) were made up in chloroform. 1 ml. portions of the phenol solution were measured into 2ml. flasks, measured volumes of the base solution added, and, after making up to the mark, the absorption at 4000 A was measured. The results are tabulated in Fig.38.

Fig.38.

Vol. base	A	bi = b	1/b	1/A
0.2ml.	2.50	0.0547	18.3	0.4
0.3ml.	3.05	0.0820	12.2	0.33
0.4ml.	3.65	0.1094	9.2	0.27
0.5ml.	4.25	0.1367	7.4	0.24

A plot of 1/A against 1/b gave a st. line. From the gradient, K= 0.27, pKa = -0.6.

The di-imide coupling method. Benzoyl-L-leucine was coupled with glycine ethyl ester using dicyclohexylcarbodi-imide in methylene dichloride as solvent, following the same procedure as described in Part II, but using ester hydrochloride in the presence of triethylamine, and free ester in the presence of tetraethylammonium chloride. The results are summarised below.

Conditions	Yield,%	$[\alpha]_D$	% L	C	H	N
(i) glyOEt.HCl+NEt_3	70	-5.5	16	64.5	7.3	8.5
(ii) glyOEt+$Et_4\overset{+}{N}\overline{Cl}$	57	-5.2	15	64.2	7.8	8.6

0.5g. of the product obtained in (i) was recrystallised from acetone-ether. The first fraction consisted of 0.24g., m.p. 145-145.5°, $[\alpha]_D^{21}$ - 1.0 (c. 2.145 in ethanol).

The acid azide coupling method. Benzoyl-L-leucine was coupled with glycine ethyl ester as described in Part II, but N-methyl-piperidine (2 equivalents) was added to the ethereal azide solution before the addition of the glycine ethyl ester. The results are summarised in the table below.

	Yield, %	$[\alpha]_D$	% L	C	H	N
(i)	67	-30.9	91	63.5	7.6	9.1
(ii)	76	-31.0	91	63.4	7.6	9.0

The product obtained in (i) was saponified in the usual way. No benzoyl-DL-leucylglycine crystallised, after seeding and leaving at room temperature for several weeks.

Attempted azlactonisation of benzoyl-L-leucylazide. A solution of benzoyl-L-leucylazide in dry ether was treated with N-methylpiperidine (2 equivalents) and kept at 0°. The infrared spectrum was taken at intervals. No peak appeared at 1830cm.$^{-1}$ even after several weeks. It was verified that the azlactone has a strong peak in this position.

The cyanomethyl ester coupling method. (a) Benzoyl-DL-leucine cyanomethyl ester was refluxed in chloroform solution in the presence of N-methylpiperidine (2 equivalents) for 6 hr. The infrared spectrum remained unchanged. i.e. ν max. 1767, 1672 cm.$^{-1}$ for ester and amide peaks respectively. (b). Benzoyl-DL-leucine cyanomethyl ester was refluxed in chloroform solution in the presence of benzyltrimethylammonium chloride (4 equivalents) for 6 hr. The infrared spectrum showed a very weak absorption at 1832 cm.$^{-1}$

PART IV.

* p-Nitrobenzoyl-L-proline. L-proline (2.30g. ; 0.02 mole) was dissolved in 2N sodium hydroxide solution (20ml. ; 0.04 mole). Solutions of p-nitrobenzoyl chloride (3.72g. ; 0.02 mole) and 2N sodium hydroxide (25ml. ; 0.05 mole) were added with rapid stirring at the interface. After 15 min. , the aqueous layer was neutralised to Congo Red with dilute hydrochloric acid. A syrup separated, which was dissolved by addition of more ether. The ethereal solution was dried over magnesium sulphate and cyclohexylamine was added dropwise to precipitate the salt. 6.95g. (96%). Recrystallisation from methanol-ether gave material, m.p. 175-6°, $[\alpha]_D^{20}$ = 73.3 (c. 2.89 in ethanol). (Found : C, 59.4 ; H, 6.8 ; N, 11.5. $C_{18}H_{25}N_3O_5$ requires : C, 59.5 ; H, 6.9 ; N, 11.6%). Liberation of the free acid always gave a syrup which could not be induced to crystallise.

Racemisation of p-nitrobenzoyl-L-proline. (a). With acetic anhydride in acetic acid. p-Nitrobenzoyl-L-proline cyclohexylamine salt (1.82g. ; 0.005 mole) was shaken with dilute hydrochloric acid (10ml.) and ether (20ml.). The aqueous solution was further extracted into ether (20ml.) The combined ethereal extracts were dried over magnesium sulphate and the

solvent removed, leaving a syrup, 0.98g. (74%). This was dissolved in glacial acetic acid (10ml.) and acetic anhydride (2.4ml. ; 0.025 mole) added. The solution was left at room temperature for 24 hr. and then the acetic acid and anhydride removed at 60°, the pressure being gradually reduced to 0.6mm. The syrupy residue was taken up in ether and the cyclohexylamine salt precipitated. This was recrystallised twice from methanol-ether. m.p. 173-6°, $[\alpha]_D^{20}$ - 41.4 (c. 1.67 in ethanol). (Found : C, 59.6 ; H, 6.8 ; N, 11.6%).

(b). With acetic anhydride in chloroform. p-Nitrobenzoyl-L-proline cyclohexylamine salt (1.14g. ; 0.0034 mole) was dissolved in dilute hydrochloric acid (10ml.) and extracted into chloroform (2 x 10ml.). The solution was dried, and freshly distilled acetic anhydride (1.60ml. ; 0.017 mole) was added. The solution was left at room temperature for 29 hr. and then the solvent was removed by distillation under reduced pressure. The residue was taken up in ether and the cyclohexylamine salt precipitated. This was recrystallised from methanol-ether. m.p. 174-6°, $[\alpha]_D^{21}$ - 53.8 (c. 1.645 in ethanol). (Found : C, 59.5 ; H, 7.2 ; N, 12.1%).

* p-Nitrobenzoyl-L-prolylglycine ethyl ester.

(i) Cyanomethyl ester method. p-Nitrobenzoyl-L-proline was

obtained from the cyclohexylamine salt (1.82g. ; 0.005 mole) as usual and dissolved in ethyl acetate (10ml.). Chloroacetonitrile (0.64ml. ; 0.01 mole) and triethylamine (1.04ml. ; 0.0075 mole) were added, and the mixture refluxed for 3 hrs. After cooling, ethyl acetate (30ml.) was added, and the solution washed with water (10ml.), dil. HCl (10ml.) and water (10ml.). It was dried over $MgSO_4$ and the solvent was removed, leaving the cyanomethyl ester as a syrup. This was dissolved in ethyl acetate (5ml.) and glycine ethyl ester (0.5ml. ; 0.005 mole) was added. After leaving at room temperature overnight, the solution was diluted with ethyl acetate (25ml.) and washed with dil.HCl (15ml.), Na_2Co_3 solution (15ml.) and water 15ml.). The solution was dried and solvent removed, leaving a colourless syrup which rapidly crystallised on rubbing under light petroleum. 0.95g. (55%). Recrystallisation from ethyl acetate-light petroleum gave white needles. m.p. 113-113.5°. $[\alpha]_D^{19}$ - 97.8 (c. 2.465 in ethanol.) (Found : C, 55.1 ; H, 5.5 ; N, 12.6. $C_{16}H_{19}N_3O_6$ requires C, 55.0 ; H, 5.5 ; N, 12.0%).

(ii). Acid azide method.

L-Proline methyl ester toluene-p-sulphonate. L-proline (4.60g. ; 0.04 mole) and anhydrous toluene-p-sulphonic acid (7.6g. ;

0.044 mole) were heated in dimethyl sulphite solution on a boiling water-bath for 6 hr. The clear solution was then poured into ether. A colourless crystalline product separated, which was recrystallised from ethyl acetate. 10.1g. (82%), m.p. 95.5-96°. c.f. J.M.Mabbott (88), m.p. 94.5-96°.

* p-Nitrobenzoyl-L-proline methyl ester. L-proline methyl ester toluene-p-sulphonate (3.01g. ; 0.01 mole) was added to an ethyl acetate solution of p-nitrobenzoyl chloride (1.86g. ; 0.01 mole). The suspension was stirred vigorously and a solution of sodium carbonate (5.4g. ; 0.05 mole) in water (30ml.) was added. Stirring was continued for 30 min. The ethyl acetate layer was separated and dried, and removal of solvent gave a colourless crystalline solid, 2.60g. (94%). Recrystallisation from ethyl acetate-light petroleum gave needles m.p. 108°, $[\alpha]_D^{22}$ - 83.0 (c. 1.235 in ethanol). (Found : C, 56.1 ; H, 5.0 ; N, 10.1%).

* p-Nitrobenzoyl-L-prolylhydrazide. p-Nitrobenzoyl-L-proline methyl ester (2.30g. ; 0.0083 mole) was dissolved in methanol (20ml.) and hydrazine hydrate (100% ; 1.5ml.) added. The solution was left at room temperature overnight and the solvent removed, leaving a syrup which rapidly crystallised, 2.3g. (100%). Recrystallisation from ethyl acetate-light petroleum

gave colourless needles, m.p. 150°, $[\alpha]_D^{23}$ - 82.7 (c. 1.61 in ethanol). (Found : C, 51.8 ; H, 4.8 ; N, 20.4. $C_{12}H_{14}N_4O_4$ requires : C, 51.8 ; H, 5.0 ; N, 20.2%).

p-Nitrobenzoyl-L-prolylglycine ethyl ester. p-Nitrobenzoyl-L prolylhydrazide (0.93g. ; 0.0034 mole) was dissolved in a mixture of water (4ml.), conc. HCl. (0.6ml.) and glacial acetic acid (0.6ml.). The solution was cooled to 0° and a solution of sodium nitrite (0.46g. ; 0.0066 mole) in water (2ml.) was added dropwise with stirring. A syrup separated. This was taken up in ether (10ml.) and stirring continued. The aqueous layer was further extracted with ether (2 x 5ml.). The combined extracts were dried over $MgSo_4$ at 0° for a few minutes, filtered, and glycine ethyl ester (0.67ml. ; 0.0067 mole) added dropwise with stirring at 0°. A pale yellow oil separated. The mixture was left in the refrigerator overnight when solidification occurred. The solid was dissolved by addition of chloroform and the solution washed with conc. HCl (10ml.), sodium bicarbonate solution (10ml.) and water (10ml.) After drying, the solvent was removed leaving a syrup, 0.75g. (56%) which rapidly crystallised on rubbing. Recrystallisation from ethyl acetate-light petroleum gave white needles. m.p. 113.5 - 114°, $[\alpha]_D^{23}$ - 97.0 (c. 3.285 in ethanol).

(iii). Mixed carbonic anhydride method. p-Nitrobenzoyl-L-proline (0.88g. ; 0.0034 mole) and triethylamine (0.46ml. ; 0.0034 mole) were dissolved in dry chloroform (8ml.), cooled to -5° and stirred. Ethyl chloroformate (0.32ml. ; 0.0034 mole) was added dropwise and stirring continued at - 5° for 10 min. Then glycine ethyl ester (0.34ml. ; 0.0034 mole) was added dropwise with stirring. The solution was allowed to attain room temperature. After few minutes the solution developed a bright scarlet colour.[1] After leaving at room temperature overnight the solution was washed with dil. HCl (10ml. ; immediate disappearance of red colour), $NaHCO_3$ solution (10ml.) and water (10ml.). The solution was dried over $MgSO_4$ and the solvent removed to give a reddish-coloured syrup (1.16 g. ; 100%). Recrystallisation from ethyl acetate-light petroleum gave white needles. m.p. 113 - 113.5°. $[\alpha]_D^{23}$ - 98.2 (c. 2.34 in ethanol).

* p-Nitrobenzoyl-L-proline p-nitrophenyl ester.

(a) Method of Elliott and Russell. p-Nitrobenzoyl-L-proline cyclohexylamine salt (0.91g. ; 0.0025 mole) was shaken with ethyl acetate (30ml.) and dil. hydrochloric acid(30ml.). The aqueous layer was further extracted with ethyl acetate (30ml.), the combined extracts dried over magnesium sulphate, and the

volume of the solution reduced to about 15ml. p-Nitrophenol (0.52g. ; 0.0037 mole) and dicyclohexylcarbodi-imide (0.51g. ; 0.0037 mole) were added. The solution immediately developed a vivid scarlet colour on addition of the di-imide[1]. This slowly faded, and the solution was left at room temperature overnight. The urea was filtered off and the solution washed with aqueous sodium carbonate solution (3 x 20ml.). Removal of solvent gave a pale yellow syrup which crystallised on rubbing. 0.70g. (73%). Recrystallisation from methanol gave material, m.p. 138.5 - 140°, $[\alpha]_D^{22}$ - 40.1 (c. 2.295 in chloroform). (Found : C, 56.1 ; H, 3.9 ; N, 11.0. $C_{18}H_{15}N_3O_7$ requires : C, 56.1 ; H, 3.9 ; N, 10.9%).

(b). From benzyloxycarbonyl-L-proline p-nitrophenyl ester.

Benzyloxycarbonyl-L-proline p-nitrophenyl ester was converted to L-proline p-nitrophenyl ester hydrobromide by treatment with hydrogen bromide in acetic acid. Recrystallisation from methanol-ether gave material, m.p. 197-9°. c.f. Goodman and Stueben, m.p. 198-9°.

L-proline p-nitrophenyl ester hydrobromide (0.63g. ; 0.002 mole) was suspended in ethyl acetate (10ml.) containing p-nitrobenzoyl chloride (0.372g. ; 0.002 mole) and a solution of sodium carbonate (1.06g. ; 0.01 mole) in water,

added. The mixture was stirred vigorously at the interface for ½ hr. and then the organic layer was separated and dried Removal of solvent gave a syrup which crystallised. Recrystallisation from ethyl acetate-light petroleum gave 0.72g. (94%) of crystalline material, m.p. 142-143.5°, $[\alpha]_D^{24}$ - 64.0 (c. 2.70 in chloroform). (Found : C, 56.4 ; H, 4.0 ; N, 11.3%).

p-Nitrobenzoylprolylglycine ethyl ester-p-nitrophenyl ester method.

p-Nitrobenzoyl-L-proline p-nitrophenyl ester obtained from benzyloxycarbonyl-L-proline p-nitrophenyl ester was coupled with glycine ethyl ester. This is described in Part III (a). The product had rotation, $[\alpha]_D^{25}$ - 96.3 (c. 2.47 in ethanol).

p-Nitrobenzoylprolylglycine ethyl ester. Di-imide method.

p-Nitrobenzoyl-L-proline was liberated from the cyclohexylamine salt (0.36g. ; 0.001 mole) by shaking with methylene dichloride (10ml.) and dilute sulphuric acid (5ml.). The organic layer was dried and glycine ethyl ester (0.1ml. ; 0.001 mole), followed by dicyclohexylcarbodi-imide (0.21g. ; 0.001 mole) were added. After leaving overnight at room temperature, the product was worked up as usual, and obtained as colourless crystals, 0.34g. (97%). Recrystallisation from ethyl acetate-

light petroleum gave material, m.p. 112-113°, $[\alpha]_D^{22}$ - 95.3 (c. 1.915 in ethanol). (Found : C, 55.6 ; H, 5.7 ; N, 11.9%).

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ABSTRACT.

One of the most important considerations in peptide synthesis is the avoidance of loss of optical activity at the asymmetric centres of the amino-acids when these are condensed together. This racemisation is undesireable not only because it limits the yield of required product but also because the presence of even traces of racemates or diastereoisomers may inhibit crystallisation of a peptide, thus rendering purification more difficult.

The most commonly employed amino protecting group, the benzyloxycarbonyl group, confers a remarkable optical stability upon the amino-acid which it protects, and the observation that a benzyloxycarbonylamino-acid may be coupled without danger of racemisation under conditions which may cause considerable loss of activity when the benzyloxycarbonyldipeptide, so formed, is coupled further (1), led to a much greater awareness of the problem. Thus, in 1955, North and Young initiated a study of coupling reactions from the point of view of racemisation, using the condensation of acetyl-L-leucine with glycine ester as a model reaction (2). They found that the azide procedure was remarkably free from the

danger of racemisation but other procedures, including those which have been postulated to involve amino-activation, may result in considerable loss of activity.

In 1952, Anderson used the condensation of benzyloxycarbonylglycyl-L-phenylalanine with glycine ester to investigate the variation of the extent of racemisation with the conditions employed in the tetraethyl pyrophosphite method of coupling (3), and in 1958, reported a procedure for isolating racemic peptide in this model reaction even when less than 1% is present (4). Many investigators have employed this test when reporting new coupling procedures, none of which has been found to be free from the danger of racemisation. The investigation using the model of North and Young has been continued (5). The acetylleucylglycine formed was usually syrupy, especially when partial racemisation had occurred, but racemic peptide crystallised out preferentially on standing. The rotations of the solid fractions so obtained enabled a rough assignment of coupling procedures to be made into categories, depending upon the extent of racemisation.

It is clear, however, that in order to make a more

detailed study of the dependence of racemisation upon conditions, it is desirable to use a model consensation reaction whose product is wholly crystalline so that an accurate assessment of the loss of activity can be made. The first part of the thesis desribes the development of such a model reaction. The condensation of benzoyl-L-leucine with glycine ethyl ester has always given a highly crystalline peptide, irrespective of the racemic content, and this could then be estimated from the specific rotation of the crude reaction product, if it could be shown to be essentially pure by analysis. Authentic benzoyl-L-leucylglycine ethyl ester was synthesised *via* the benzyloxycarbonyldipeptide, which can be made without danger of racemisation. It is characterised by m.p. 157°, $[\alpha]_D$ - 34°. The full optical activity of benzoyl-L-leucine was verified by conversion to the methyl ester, whose rotation was identical with that obtained by benzoylating L-leucine methyl ester hydrochloride.

Isolation of racemic peptide from the product was not possible by recrystallisation when the racemic content was low, but it was found that saponification of the peptide under mild conditions led to the benzoyldipeptide and when this was partially racemic, crystallisation from the saponification

medium afforded almost pure benzoyl-DL-leucylglycine, even when only 2% of racemate was present.

The second part of the thesis concerns the application of this model condensation in a reinvestigation of some coupling procedures. Of particular interest were the azide method, in which no racemisation has ever been reported, the cyanomethyl ester method for which no evidence could be obtained for racemisation using the condensation of acetyl-L-leucine with glycine ester, and the recent Woodward method, in which the racemate found using the Anderson model has been ascribed to racemic starting material (6), in spite of the not inconsiderable variation of the extent of racemisation with conditions which was previously reported (7).

The results of the coupling experiments are shown in Fig. 1..

In the condensation of benzoyl-L-leucine with glycine ethyl ester by the azide and cyanomethyl ester methods, therefore, the high rotations of the products and failure to isolate racemic peptide by the saponification procedure, furnish evidence that less than 2% racemisation has occurred, although the considerable racemisation in the mixed carbonic anhydride and di-imide procedures indicate that this is a

particularly stringent test.

Fig. 1.

Method	Conditions	%L	Racemate found
Acid azide	Ether as solvent	100 88	-(s)
Cyanomethyl ester	Ester formed by refluxing in EtOAc	90 89	-(s)
	Ester formed at 0°	99 95	-(s)
Mixed carbonic anhydride	glyOEt, T.H.F.	18 22	+(r)
Tetraethyl pyrophosphite	"Standard", $CHCl_3$, glyOEt	86 90	+(s)
	"Standard", $CHCl_3$, glyOEt.HCl $+NEt_3$	52 49	+(r)
	"Amide", $CHCl_3$ glyOEt	48	+(r)
	"Amide", $CHCl_3$ glyOEt.HCl+NEt_3	27	+(r)
Di-imide	glyOEt, CH_2Cl_2	53	+(s)
Woodward	glyOEt.HCl+NEt_3, CH_3CN	88 90	+(s) +(s)
	glyOEt, CH_3CN	97 96	-(s) -(s)

The percentage L-isomer, calculated from the specific rotations of the crude products, excludes that present in racemate. The elementary analyses of all the crude products are acceptable. +(r), +(s) denote that racemate has been isolated by recrystallisation and saponification, respectively. -(s) denotes that the product was saponified but no racemate found.

Positive evidence has been obtained for racemisation in the Woodward method, using the recommended procedure in acetonitrile as solvent, but the product possessed a significantly higher rotation when free glycine ester was employed. Furthermore no evidence for the presence of racemate in this product was furnished by saponification.

The third part of the thesis describes an investigation of the mechanism of racemisation during peptide synthesis. No work has been reported on the mechanism of racemisation under the conditions of a coupling reaction, but the racemisation of acylamino-acids in the presence of acetic anhydride has been the subject of much study (8). A review of this work up to the present time appears in the introduction. The bulk of the evidence indicates that the intermediate formation of an azlactone may be chiefly responsible for the loss of activity, but the observation that acylimino-acids, which cannot form azlactones of the conventional kind, can also be racemised in the presence of acetic anhydride suggests that some other mechanism may also be operative.

It was decided to investigate the coupling of p-nitrophenyl esters, since these esters are reported to be stable crystalline compounds (9). The initial approach involved a

study of the dependence of the loss of activity in this coupling procedure with the conditions employed, in the hope that a correlation could be made with the factors which cause azlactone formation from the active ester. Authentic benzoyl-L-leucine p-nitrophenyl ester was synthesised *via* the benzyloxycarbonyl compound. It is a colourless, highly crystalline compound, m.p. 97° , $[\alpha]_D$ - 43°. When this was condensed with one mole of glycine ethyl ester in chloroform or ethyl acetate, a fully optically active peptide was obtained, but no racemisation could be induced by conducting the condensation in the presence of excess glycine ester or a strong tertiary base. The liberation of the free ester from the hydrochloride *in situ* with N-methylpiperidine results in 25% racemisation, which exceeds that caused by coupling with free ester in the presence of one mole of N-methylpiperidine. Three possible causes are suggested.

It was found that benzoylleucine p-nitrophenyl ester, when treated with one mole of N-methylpiperidine in chloroform solution at room temperature, exists in equilibrium with the corresponding azlactone, and this equilibrium can be conveniently studied by the carbonyl region of the infrared spectrum. Experiments were then designed to investigate

the extent to which such azlactone formation is responsible for the loss of activity during a coupling reaction.

(1). Benzoyl-L-leucine p-nitrophenyl ester and p-nitrobenzoyl-L-proline p-nitrophenyl ester were treated with N-methylpiperidine and then allowed to couple with glycine ester under identical conditions. The peptides obtained possessed 9% and 98% respectively of their full optical activity.

(2). The condensation of benzoyl-L-leucine with glycine ethyl ester was stopped when half complete, and the components of the mixture so obtained, separated. It was estimated, from the amount of azlactone isolated that, had the reaction been allowed to proceed to completion, azlactone formation would have accounted for at least 88% of the loss of activity.

(3). Benzoyl-L-leucine p-nitrophenyl ester was treated with one mole of triethanolamine in chloroform solution. The rate of racemisation was found to be the same as the rate of disappearance of ester which was followed by its characteristic ultraviolet absorption spectrum.

(4). The rate of condensation of benzoylleucine p-nitrophenyl ester with glycine ester was found to be approximately 40 times greater than the rate of conversion of the ester to its azlactone by a tertiary amine whose base strength in

chloroform solution was slightly greater than that of glycine ester. Therefore, in the condensation of benzoyl-L-leucine p-nitrophenyl ester with one mole of glycine ester, the maximum amount of peptide which can be formed *via* the azlactone is approximately 2%, which means that if azlactonisation is the only mechanism of racemisation under these conditions, the maximum amount of racemisation which can accompany the reaction is about 2%. This is consistent with the failure to isolate racemate from a peptide formed under these conditions, the limit of the sensitivity of this method also being 2%.

These results, taken together, provide convincing evidence that the base-catalysed racemisation during the coupling of benzoyl-L-leucine p-nitrophenyl ester is chiefly, if not entirely, caused by the intermediate formation of an azlactone.

In a further investigation of the halide ion effect, it was found that a quaternary ammonium chloride induces the same degree of racemisation in the p-nitrophenyl ester procedure as a tertiary amine hydrochloride. Furthermore, azlactonisation of the ester is effectively catalysed by these salts in chloroform solution. This clearly indicates

a specific chloride ion effect in causing racemisation. The base strength of the chloride ion relative to the 2:4-dinitrophenate anion in chloroform solution was determined and found to be twice as great as that of glycine ester. It seems likely, therefore, that the chloride ion catalyses racemisation owing to its basicity in an aprotic or weakly protic solvent. It was demonstrated that nucleophilic attack by the chloride ion at the ester carbonyl group is also possible, and in the case of benzoylleucine p-nitrophenyl ester the resulting acid chloride would rapidly azlactonise (10). Both mechanisms are therefore likely to be operative.

A correlation between conditions which cause racemisation during coupling and azlactonisation of an activated carboxyl component was then attempted using other coupling procedures. Benzoylleucine azide and cyanomethyl ester could not be induced to azlactonise in the presence of N-methylpiperidine, which may be correlated with the absence of base-catalysed racemisation in these coupling procedures. In the tetraethyl pyrophosphite procedure, it was found that the degree of racemisation is dependent upon the b.p. of the reagent used, even if the refractive indices are similar. The presence of a quaternary ammonium chloride increases the degree of

racemisation to the same extent as a tertiary amine hydrochloride, but a perchlorate has no effect on racemisation. It was demonstrated by isolation that benzoylleucine is converted into its azlactone in the presence of tetraethyl pyrophosphite under the conditions employed in coupling.

Further evidence for the specific effect of the chloride ion in causing racemisation was furnished by coupling benzoyl-L-leucine with p-toluidine by the tetraethyl pyrophosphite method. A quaternary ammonium chloride causes a considerable increase in racemisation, but tribenzylamine hydrochloride has no effect. Tribenzylamine was found to be a 50 times weaker base than the chloride ion in chloroform solution; the latter is therefore protonated in preference to the former and is thus rendered ineffective as a base and nucleophile.

The fourth part of the thesis concerns an investigation of the danger of racemisation during the coupling of a proline derivative as the carboxyl component. The early report that an acylproline is rapidly racemised by acetic anhydride (11) has been verified in the case of p-nitrobenzoyl-L-proline. When benzyloxycarbonyl-L-proline p-nitrophenyl ester, made by the Elliott and Russell di-imide procedure (12), is converted into p-nitrobenzoyl-L-proline p-nitrophenylester, the product has specific rotation, $[\alpha]_D$-64°, and this condenses with glycine

ester to give peptide having the same rotation as that made by the azide, cyanomethyl ester, mixed carbonic anhydride and di-imide methods. All of these reactions are therefore deduced to proceed without loss of activity. When p-nitrobenzoylproline p-nitrophenyl ester was made from p-nitrobenzoyl-L-proline by the Elliott and Russell procedure, however, the specific rotation, $[\alpha]_D$ - 40°, indicated approximately 30% loss of activity. It is clear, therefore, that the danger of racemisation must also be borne in mind when a peptide containing an imino-acid residue in its C-terminal position is coupled to an amino-component.

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