715. *Amino-acids and Peptides. Part XIX.*[1] *The Mechanism of Racemisation during Peptide Synthesis. "The Chloride Effect."*

By M. W. Williams and G. T. Young.

The condensation of benzoyl-L-leucine *p*-nitrophenyl ester with glycine ethyl ester in the presence of tertiary amine gives partly racemised product, and it has been shown that this racemisation occurs mainly if not exclusively through the intermediate formation of 4-isobutyl-2-phenyloxazolone. The increased racemisation commonly found when coupling reactions are carried out with the amino-ester hydrochloride with an equivalent of tertiary amine (rather than with free amino-ester) is shown to be due, in the cases examined, to the chloride ion; other anions have a similar effect, which is attributed to their basicity in organic solvents. The condensation of *p*-nitrobenzoyl-L-proline with glycine ethyl ester gives peptide of similar optical activity by the acid azide, cyanomethyl ester, *p*-nitrophenyl ester, dicyclohexylcarbodi-imide, and carbonic mixed anhydride routes, but conversion of the acylproline into the *p*-nitrophenyl ester by the action of dicyclohexylcarbodi-imide resulted in partial racemisation.

Many observations suggest that when racemisation occurs during peptide synthesis it is due to the intermediate formation, racemisation, and coupling of an oxazolone. Neuberger [2] has reviewed the evidence that the racemisation of *N*-acetyl- and *N*-benzoyl-amino-acids by acetic anhydride, or through their acid chlorides, proceeds through such oxazolones, and the fact that benzyloxycarbonyl-, phthaloyl-, and toluene-*p*-sulphonyl-amino-acids neither form oxazolones nor (except in special cases) racemise when coupled supports the common assumption that the same intermediates are responsible for the racemisation which, as shown in Parts XV [3] and XVI [4] of this series, severely limits the usefulness of some common coupling methods. A recent investigation suggests that oxazolone formation is also responsible for the racemisation occurring during the hydrolysis of benzyloxycarbonyl-glycyl-L-phenylalanine *p*-nitrophenyl ester.[5] Nevertheless, racemisation by direct exchange of the hydrogen at the dissymmetric centre of the reactive acid derivative [2] may well obtain in certain cases (for example, in the partial racemisation of *p*-nitrophenyl esters of benzyloxycarbonyl-amino-acids [6]), and this alternative deserves serious consideration.

The condensation of benzoyl-L-leucine *p*-nitrophenyl ester with glycine ethyl ester has

[1] Part XVIII, *J.*, 1963, 5807.
[2] Neuberger, *Adv. Protein Chem.*, 1948, **4**, 356.
[3] Part XV, Smart, Young, and Williams, *J.*, 1960, 3902.
[4] Part XVI, Williams and Young, *J.*, 1963, 881.
[5] Goodman and Stueben, *J. Org. Chem.*, 1962, **27**, 3409.
[6] Bodanszky and Birkhimer, *Chimia*, 1960, **14**, 368; Liberek, *Tetrahedron Letters*, 1963, 925.

proved to be convenient for the study of this problem.[7] Although under normal conditions this reaction gives fully active peptide, the presence of tertiary amine or of a considerable excess of glycine ester has been found to result in some racemisation [see Table 1 (i)—(vi)].

Condensation of benzoyl-L-leucine with glycine ethyl ester.

1. *p*-Nitrophenyl ester method.

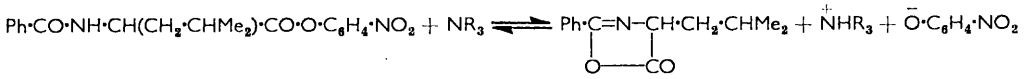
		Crude product			Found (%) [d]		
Conditions [a]	Solvent	Yield (%)	$[\alpha]_D$ [b]	L-Isomer (%) [c]	C	H	N
(i) A	EtOAc	95	−34·3°	100	Standard [e]		
(ii) A; NMP (0·5)	,,	90	−30·7	90	63·6	7·4	8·8
(iii) A; NMP	,,	92	−30·2	90	63·6	7·3	8·8
(iv) A; 5 equiv. of Gly-OEt	,,	95	−30·7	90	63·1	7·5	9·2
(v) A	$CHCl_3$	86	−32·6	96	Standard [e]		
(vi) A; NMP	,,	80	−24·7	73	63·4	7·4	8·6
(vii) A; NMP [f]	,,	85	−3·1	9	64·1	7·9	8·6
(viii) B; NMP (2·0)	,,	85	−18·7	54	64·0	7·5	8·4
(ix) B; NMP (3·0)	,,	—	−17·5	51	64·7	7·5	8·5
(x) B; NMP (10·0)	,,	80	−16·9	49	63·7	7·5	8·7
(xi) B; NMP	Py	75	−30·8	90	63·6	7·4	9·5
(xii) A; NEP,HCl	$CHCl_3$	79	−29·8	88	63·6	7·7	8·6
(xiii) A; $NEt_4^+Cl^-$	,,	84	−29·1	86	63·6	7·7	8·8
(xiv) A; $PhCH_2{\cdot}NMe_3^+Cl^-$	,,	80	−27·1	82	63·8	7·5	8·8
(xv) A; NEP tosylate	,,	72	−26·0	77	63·7	7·3	—
(xvi) A; $PhCH_2{\cdot}NMe_3^+,C_7H_7SO_3^-$	,,	75	−26·6	78	64·2	7·5	8·9
(xvii) A; $PhCH_2{\cdot}NMe_3^+Cl^-$ [g]	,,	92	−23·2	32	64·2	7·3	8·5
2. Tetraethyl pyrophosphite method (" Standard procedure ").							
(i) A [h]	$CHCl_3$ (a)	90	−25·9	76	63·6	7·8	8·6
	(b)	88	−25·8	76	63·8	7·8	8·6
(ii) A; NEP,HCl	,, (a)	88	−15·9	47	64·0	7·8	8·6
	(b)	79	−15·4	45	63·6	7·7	8·6
(iii) A; NEP tosylate	,, (a)	85	−15·3	45	63·1	7·8	8·2
	(b)	74	−15·0	44	63·7	7·8	8·5
(iv) A; $Et_4N^+Cl^-$	,, (a)	83	−14·6	43	63·6	7·7	8·6
	(b)	83	−14·6	43	63·3	7·7	8·7
(v) A; $Pr_4N^+I^-$	,,	68	−22·8	67	63·5	7·5	8·4
(vi) A	Et_2PHO_3 (a)	74	−18·1	53	63·0	7·5	8·4
	(b)	75	−16·2	48	63·2	7·6	8·9
(vii) A; NEP,HCl	,,	78	−10·2	30	63·8	7·6	8·7
(viii) A; $Et_4N^+Cl^-$	,, (a)	81	−7·6	22	63·5	7·6	8·5
	(b)	77	−7·0	21	63·5	7·5	8·4
(ix) A; $PhCH_2{\cdot}NMe_3^+ClO_4^-$	,, (a)	70	−16·3	48	63·3	7·4	9·0
	(b)	80	−16·8	49	—	—	—
3. *Dicyclohexylcarbodi-imide method.*							
(i) A	CH_2Cl_2	84	−18·1	53	Standard		
(ii) A; $NEt_4^+Cl^-$	,,	57	−5·2	15	64·2	7·8	8·6

[a] Conditions: A, Distilled glycine ester used. B, Ester hydrochloride used, with the named tertiary amine. (NMP = 1-methylpiperidine; NEP = 1-ethylpiperidine). The molar proportion of the added amine or salt is 1·0 (with respect to the acid component), except when other proportions are given in brackets. [b] Optical rotations were measured at 18—25° (*c* 2—4 in EtOH) in a 1-dm. tube. [c] Excluding L-isomer present as racemate. [d] Calc. for benzoyl-leucylglycine ethyl ester, $C_{17}H_{24}N_2O_4$: C, 63·7; H, 7·55; N, 8·75%. [e] Reported in Part XVI; included here for comparison. [f] The tertiary amine was allowed to react with the *p*-nitrophenyl ester for 30 min. before addition of the glycine ester. [g] The salt was allowed to react with the *p*-nitrophenyl ester for 2 hr. before addition of the glycine ester. [h] The tetraethyl pyrophosphite had n_D^{25} 1·4306 and was free from chloride. It will be seen that the racemisation found in experiments 2 (i) (a) and (b) is greater than in the comparable experiments reported in Part XVI [4] [5 (iv) and (v) in the Table there], but the same batch of pyrophosphite was used for the experiments reported here, and the results are therefore comparable among themselves.

Addition of 1-methylpiperidine to a solution of the *p*-nitrophenyl ester in chloroform resulted in a rapid diminution of the infrared absorption at 1776 cm.$^{-1}$ and the appearance

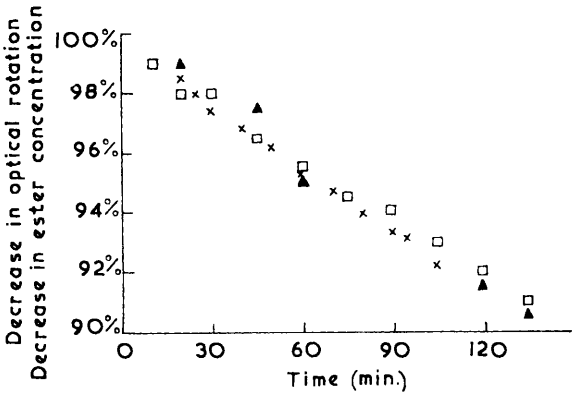
[7] Preliminary communication: Williams and Young, " Peptides: Proc. Fifth European Peptide Symp., Oxford, 1962," ed. G. T. Young, Pergamon Press, Oxford, 1963, p. 119.

of a new peak at 1832 cm.$^{-1}$, due to 4-isobutyl-2-phenyloxazolone. It has been shown that an equilibrium is established:



The oxazolone was isolated from the products of this reaction.

Three lines of evidence show that in this particular case racemisation proceeds mainly, if not entirely, through the oxazolone: (*a*) The optical rotation of a solution of benzoyl-L-leucine *p*-nitrophenyl ester in chloroform containing an equimolar amount of triethanolamine decreased at the same rate as did the ultraviolet absorption (due to *p*-nitrophenyl ester) at 270 mμ (see Figure). (*b*) The condensation of the *p*-nitrophenyl ester with glycine ethyl ester hydrochloride in the presence of excess of 1-methylpiperidine was interrupted; fractional crystallisation gave peptide and oxazolone which (since the latter gives racemic peptide) together corresponded to *ca.* 42% of racemisation in the products, while the recovered ester could not have contained more than 5% of racemate. (*c*) Solutions of benzoyl-L-leucine *p*-nitrophenyl ester and of *p*-nitrobenzoyl-L-proline *p*-nitrophenyl ester in chloroform were treated with 1-methylpiperidine and then with glycine ethyl ester. In



The action of triethanolamine on benzoyl-L-leucine *p*-nitrophenyl ester; the fall in optical rotation related to the decrease in ester concentration.

× Observed optical rotation as a percentage of the initial concentration.

(□, ▲) Concentration of ester as a percentage of the initial concentration.

the first case [1 (vii)] the isolated peptide was nearly inactive, and in the second case, fully active.

From these observations we conclude that the racemisation found when benzoyl-L-leucine *p*-nitrophenyl ester couples with glycine ethyl ester in the presence of tertiary amine proceeds mainly, if not exclusively, through the oxazolone. Clearly, the extent of racemisation during coupling in the absence of added base will depend on the relative *C*- and *H*-nucleophilicities of the amino-component. We have measured the initial rate of reaction of equimolar amounts of the *p*-nitrophenyl ester with glycine ethyl ester in chloroform solution, by the decrease in ultraviolet absorption (due to *p*-nitrophenyl ester) at 270 mμ; the calculation assumes that oxazolone formation is negligible under these conditions, but this is probably justified since no detectable racemisation occurs; we find $k_{25} = 6 \times 10^{-4}$ l. mole.$^{-1}$ sec.$^{-1}$. The rate of formation of oxazolone from the *p*-nitrophenyl ester by the action of tertiary amines has been measured similarly; with triethanolamine, $k_{25} = 6 \times 10^{-5}$ l. mole^{-1} sec.$^{-1}$ and with dimethylglycine methyl ester, $k_{25} = 1{\cdot}5 \times 10^{-5}$ l. mole^{-1} sec.$^{-1}$. The rate of reaction of the oxazolone with glycine ethyl ester was measured by the decrease in the ultraviolet absorption (due to the oxazolone) at 245 mμ: $k_{25} = 2{\cdot}5 \times 10^{-3}$ l. mole^{-1} sec.$^{-1}$. It is seen that, during the reaction of benzoyl-L-leucine *p*-nitrophenyl ester with glycine ethyl ester (without additional base), any oxazolone formed would react with glycine ester more rapidly than would the *p*-nitrophenyl ester. It cannot be assumed that the rate of oxazolone formation from the *p*-nitrophenyl ester is directly related to the

basicity of the amine present (although this appears to be so for triethanolamine and dimethylglycine methyl ester) but if the rate of oxazolone formation by the action of glycine ethyl ester is no greater than that caused by dimethylglycine methyl ester (a base of comparable strength in chloroform) then no detectable racemisation would be expected in this coupling reaction. An indication of the effect of changing the amino-component is shown by the reaction of the *p*-nitrophenyl ester with benzylamine (a stronger base), which under similar conditions gave product with 86% of the full optical activity when chloroform was solvent, and 79% when ethyl acetate was solvent; apparently in changing from glycine ester to benzylamine the *H*-nucleophilicity has increased more than has the *C*-nucleophilicity.

"*The Chloride Effect.*"—Throughout our work on coupling procedures [3,4] we have noted the significant increase in racemisation which often occurs when the free amino-ester is replaced by the ester hydrochloride with an equivalent of tertiary amine—an observation first made by R. W. Young, Wood, Joyce, and Anderson [8] in their work on phosphite reagents. Further evidence of this effect during the coupling of benzoyl-L-leucine with glycine ethyl ester by the *p*-nitrophenyl ester, tetraethyl pyrophosphite, and dicyclohexylcarbodi-imide methods is given in the Table. The fact that quaternary ammonium chlorides cause comparable racemisation shows that the chloride ion is responsible; when the *p*-nitrophenyl ester was allowed to react with benzyltrimethylammonium chloride before the addition of glycine ester, the peptide so formed contained only 32% of L-isomer [1 (xvii)]. Toluene-*p*-sulphonates also cause racemisation; iodide is less effective, and perchlorates have no action. When solutions of benzoyl-L-leucine *p*-nitrophenyl ester were heated briefly with tertiary amine hydrochloride or quaternary ammonium chloride, iodide, or *p*-nitrophenoxide, the characteristic oxazolone absorption appeared at 1832 cm.$^{-1}$; the action of the corresponding perchlorates caused no such absorption. We thought it possible that the oxazolone might result from nucleophilic attack by the anion on the active ester, to give acid halide and hence oxazolone; some *p*-nitrophenoxide anion was detected spectrophotometrically when *p*-nitrophenyl phenylacetate was heated with tetraethylammonium chloride in dichloromethane, but the amount was very small. *p*-Nitrophenoxide anion causes rapid formation of the oxazolone from benzoyl-L-leucine *p*-nitrophenyl ester, and since nucleophilic attack would cause no change, in this case the action is clearly that of a base. We believe this to be the reason for the effect of chloride ion, and the use of 2,4-dinitrophenol as indicator (by measurement of the absorption in the ultraviolet) [9] has shown that chloride ion is more basic than is glycine ethyl ester in chloroform; [7] an investigation of the strengths of anions and amines relevant to this work is in progress.[10]

The coupling of formyl-L-leucine with glycine ethyl ester, which was the subject of Part XVIII [1] of this series, is a case of special interest. It was reported there [2 (iv) in the Table in that paper] that when formyl-L-leucine *p*-nitrophenyl ester in ethyl acetate was treated with triethylamine, and the glycine ethyl ester was added next day, nearly inactive peptide resulted; when the *p*-nitrophenyl ester in dichloromethane was similarly treated with triethylamine, racemic ester could be recovered—and yet the solution showed no oxazolone absorption in the infrared. However, it was found that the absorption of 4-isobutyloxazolone at 1832 cm.$^{-1}$ is extinguished by the addition of tertiary amines; this observation has led us to examine the reaction of tertiary amines with a number of oxazolones, and the results will be separately reported. It was noted also in Part XVIII [1] that (in contrast to the benzoyl and acetyl analogues) the coupling of formyl-L-leucine by the carbonic mixed anhydride method is not significantly affected by chloride ion. When formyl-L-leucine *p*-nitrophenyl ester was heated with benzyltrimethylammonium chloride in chloroform, no change occurred in the infrared absorption, and fully active ester was

[8] R. W. Young, Wood, Joyce, and Anderson, *J. Amer. Chem. Soc.*, 1956, **78**, 2126.
[9] Pearson and Vogelsong, *J. Amer. Chem. Soc.*, 1958, **80**, 1038.
[10] Williams and Young, unpublished work.

recovered. Since the infrared absorption of the oxazolone is not altered by the addition of chloride, we conclude that chloride ion is insufficiently basic to cause formation of this oxazolone, and hence no racemisation results.

The Case of Proline.—It is usually assumed that acylprolines can be coupled without racemisation, and although Carter and Stevens [11] reported that " certain acyl derivatives " of L-proline are rapidly racemised by acetic anhydride in acetic acid, Jackson and Cahill [12] found no racemisation when L-proline was treated with keten under conditions which racemised other amino-acids, and (in contrast to the analogue containing *N*-methylphenylalanine), benzoyloxycarbonylglycyl-L-proline gives fully active *p*-nitrophenyl ester by the tris-*p*-nitrophenyl phosphite method.[5] We have not yet extended our mechanistic studies to such cases, but we find that *p*-nitrobenzoyl-L-proline can be partially racemised by acetic anhydride in acetic acid or in chloroform solution. We have also condensed this compound with glycine ethyl ester by the acid azide, cyanomethyl ester, *p*-nitrophenyl ester, dicyclohexylcarbodi-imide, and carbonic mixed anhydride routes, and find peptide of similar optical activity in each case; even when the *p*-nitrophenyl ester was first treated with 1-methylpiperidine, a high yield of fully active peptide was obtained. The common assumption that proline may be coupled without racemisation has, to this extent, been confirmed. However, conversion of *p*-nitrobenzoyl-L-proline into the *p*-nitrophenyl ester by the action of dicyclohexylcarbodi-imide gave product of considerably lower rotation than that obtained by *p*-nitrobenzoylation of L-proline *p*-nitrophenyl ester. It is well therefore to bear in mind the possibility of the formation of oxazolonium salts [13] from acylimino-acids (including acylproline), with consequent racemisation.

Experimental

Melting points were taken on a Kofler hot-stage apparatus. Infrared spectra were recorded on a Perkin-Elmer model 21 spectrophotometer. Solutions in organic solvents were dried over magnesium sulphate; evaporation was on the water-pump unless otherwise stated; tertiary amines were dried over sodium and redistilled.

The Coupling of Benzoyl-L-leucine with Glycine Ethyl Ester.—The basic coupling procedures are described in Part XVI [4] of this series; the scale was usually 0·003—0·01 molar, with respect to the acid and amino-components. The Table gives any variation in the conditions used, and the results obtained.

4-Isobutyl-2-phenyl-5-oxazolone from Benzoyl-DL-leucine p-Nitrophenyl Ester. Establishment of Equilibrium.—The *p*-nitrophenyl ester [4] (0·001 mole) was dissolved in chloroform (3 ml.) containing 1-methylpiperidine (0·001 mole). After 20 min. at room temperature the intensity of the infrared absorption at 1832 cm.$^{-1}$ (oxazolone CO) and at 1776 cm.$^{-1}$ (ester CO) was constant. A solution of the oxazolone (0·001 mole), *p*-nitrophenol (0·001 mole) and 1-methylpiperidine (0·001 mole) in chloroform (3 ml.) gave the same infrared absorption after 1 hr. From the change in intensity of the peaks, the approximate ratio of [oxazolone] : [ester] at equilibrium was calculated to be 0·62 and 0·59, respectively, in these two experiments.

The oxazolone was isolated from an analogous reaction using boiling ether as solvent; after 30 min., the ether was evaporated, with simultaneous addition of n-hexane. The solution of oxazolone was decanted from the *p*-nitrophenyl ester which separated, and was then diluted with ether and washed with dilute hydrochloric acid and then with water, and dried. Evaporation left the crystalline oxazolone (28% yield), which after recrystallisation from n-hexane had m. p. and mixed m. p. with authentic material [14] 54·5—55°; $\nu_{max.}$ 1832 and 1664 cm.$^{-1}$ in $CHCl_3$ (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%).

The Action of Triethanolamine on Benzoyl-L-leucine p-Nitrophenyl Ester. Change in Optical Rotation and in Ester Concentration.—*p*-Nitrophenyl ester (0·1424 g.; 0·0004 mole) was dissolved

[11] Carter and Stevens, *J. Biol. Chem.*, 1940, **133**, 117.
[12] Jackson and Cahill, *J. Biol. Chem.*, 1938, **126**, 37.
[13] Cornforth and Elliott, *Science*, 1950, **112**, 534; O'Brien and Nieman, *J. Amer. Chem. Soc.*, 1957, **79**, 80.
[14] Squibb Institute for Medical Research, quoted by Cornforth in " The Chemistry of Penicillin," Princeton Univ. Press, 1949, p. 775.

in chloroform (2 ml.) containing triethanolamine (redistilled, 0·60 g.; 0·0004 mole). The solution was kept at 25° and at intervals samples were removed and diluted 1 : 400; the optical density at 270 mμ was measured in a 2-mm. cell in a Unicam S.P. 500 spectrophotometer; ε was 11,450. The second-order plot $[x/a(a - x)$ (where x = decrease in ester concentration after time t and a = initial concentration of ester) against $t]$ for two sets of such readings showed straight lines which gave $k_{25} = 6{\cdot}7 \times 10^{-5}$ and $6{\cdot}0 \times 10^{-5}$ l. mole^{-1} sec.$^{-1}$.

A precisely similar solution was placed in the cell (1 cm. length) of an Ericsson automatic polarimeter and the optical rotation was measured at intervals; the temperature remained at 24° throughout. Extrapolation of the line obtained by plotting the observed rotation against time gave an initial observed rotation of 0·125°; benzoyl-L-leucine *p*-nitrophenyl ester has $[\alpha]_D^{22}$ −16·5° (*c* 5 in $CHCl_3$). The results of these experiments are compared in the Figure.

Interrupted Coupling of Benzoyl-L-leucine p-*Nitrophenyl Ester.*—The *p*-nitrophenyl ester (1·19 g.; 0·0034 mole), glycine ethyl ester hydrochloride (0·47 g.; 0·0034 mole), and 1-methylpiperidine (0·80 ml.; 0·0068 mole) were dissolved in chloroform (5 ml.). After 2 min. at room temperature, ethyl acetate (40 ml.) was added and the solution was washed with dilute hydrochloric acid, 2N-sodium carbonate, and water, and then dried. The solvent was evaporated, leaving a syrup which crystallised. Fractional crystallisation from ethyl acetate–light petroleum gave fraction (1), 0·30 g., m. p. 155—157° $[\alpha]_D^{23}$ −27° (*c* 1·97 in EtOH) (Found: C, 63·9; H, 7·5; N, 8·5. Calc. for benzoyl-leucylglycine ethyl ester $C_{17}H_{24}N_2O_4$: C, 63·7; H, 7·6; N, 8·8%). Evaporation of the mother-liquors gave a syrup (*A*) which was extracted with hot light petroleum; evaporation of the extract gave a crystalline fraction (2), 0·102 g., which by recrystallisation from n-hexane gave 0·005 g. of 4-isobutyl-2-phenyl-5-oxazolone m. p. 54—56°, $\nu_{max.}$ 1832 cm.$^{-1}$ in $CHCl_3$. The syrup remaining from (*A*) crystallised from ethyl acetate–light petroleum, giving (*a*) 0·31 g. of unchanged aryl ester m. p. 94—96°, which on recrystallisation gave fraction (3), 0·21 g., m. p. 95—96°, $[\alpha]_D^{24}$ −37° (*c* 0·5 in EtOH) (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%), and fraction (4), 0·07 g., m. p. 93—95°, $[\alpha]_D^{26·5}$ −42·1° (*c* 0·2 in EtOH) (Found: C, 63·9; H, 6·0; N, 8·1%); and (*b*) fraction (5), 0·21 g., m. p. 89—95°, $[\alpha]_D^{26·5}$ −34° (*c* 0·2 in EtOH) (Found: C, 64·0; H, 5·9; N, 7·9%). Hence 3·4 mmoles of benzoyl-L-leucine *p*-nitrophenyl ester gave 0·94 mmoles of peptide (containing 0·19 mmoles of racemate), 0·50 mmoles of the oxazolone (which yields racemic peptide), and 1·38 mmoles of recovered ester (containing at most 0·07 mmoles of racemate).

The Rate of Condensation of Benzoyl-L-leucine p-*Nitrophenyl Ester with Glycine Ethyl Ester.*—A solution in chloroform 0·15M with respect to each reactant was kept at 25° and at intervals samples were removed and diluted 1 : 300, and the absorption at 270 mμ was determined in a Unicam S.P. 500 spectrophotometer with a 2-mm. cell. The extinction is given in parentheses after the time (in min.): 0 (1·10 by extrapolation), 5 (1·05), 15 (1·025), 25 (0·97), 35 (0·93), 45 (0·88), 55 (0·87), 65 (0·83), 75 (0·815), 85 (0·80), 95 (0·78), 105 (0·77), 115 (0·75). The second-order plot from these figures gave a curve which from the initial slope gave $k_{25} = 6{\cdot}2 \times 10^{-4}$ l. mole^{-1} sec.$^{-1}$. A second experiment using 0·10M concentrations gave $k_{25} = 5{\cdot}8 \times 10^{-4}$ l. mole^{-1} sec.$^{-1}$. The ammonolysis and aminolysis of esters usually deviate from second-order kinetics.[15]

The Rate of Formation of 4-Isobutyl-2-phenyl-5-oxazolone by the Action of Dimethylglycine Methyl Ester on Benzoyl-L-leucine p-*Nitrophenyl Ester.*—The procedure has been described for the reaction with triethanolamine. The solution in chloroform was 1·027M with respect to each reactant, and was diluted 1 : 2500 for the determination of the absorption at 270 mμ (2 mm. cell). The extinction is given in parentheses after the time (in hr.): 0 (0·98 by extrapolation), 0·083 (0·97), 0·25 (0·96), 0·50 (0·95), 0·75 (0·93), 1·00 (0·92), 1·25 (0·90), 1·50 (0·89), 1·75 (0·89), 2·00 (0·88). The second-order plot from these figures gave a straight line from which $k_{25} = 1{\cdot}8 \times 10^{-5}$ l. mole^{-1} sec.$^{-1}$. A second experiment using 0·716M concentrations gave $k_{25} = 1{\cdot}2 \times 10^{-5}$ l. mole^{-1} sec.$^{-1}$.

The Rate of Reaction of 4-Isobutyl-2-phenyl-5-oxazolone with Glycine Ethyl Ester.—A solution in chloroform, 0·05M with respect to each reactant, was kept at 25°, samples were removed at intervals and diluted 1 : 334, and the absorption at 245 mμ was measured in a Unicam S.P. 500 spectrophotometer (2 mm. cell). The extinction is given in parentheses after the time (in min.): 0 (0·45 by extrapolation), 3 (0·435), 10 (0·415), 20 (0·385), 25 (0·375), 30 (0·360), 40 (0·335), 50 (0·315), 60 (0·300), 65 (0·290), 70 (0·280), 75 (0·275), 80 (0·270). The second-order plot gave a straight line from which $k_{25} = 2{\cdot}9 \times 10^{-3}$ l. mole^{-1} sec.$^{-1}$. A second experiment gave k_{25} =

[15] Bunnett, The Kekulé Symposium, Butterworths, 1959, p. 153.

$2{\cdot}2 \times 10^{-3}$ l. mole^{-1} sec.$^{-1}$. It is interesting to note that this aminolysis, in contrast to the aminolysis of esters,[15] follows second-order kinetics.

Benzyloxycarbonyl-L-leucine N-*Benzylamide*.—The reaction of benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester [16] (1·93 g.) in ethyl acetate (5 ml.) with benzylamine (0·55 ml.) gave the *amide* which was recrystallised from ethyl acetate–light petroleum and then had m. p. 112—113°, $[\alpha]_D^{25}$ −16·6° (*c* 1·2 in EtOH) (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%).

Benzoyl-L-leucine N-*Benzylamide*.—Benzyloxycarbonyl-L-leucine *N*-benzylamide (0·71 g.) was dissolved in a solution of hydrogen bromide in acetic acid (14%; 10 ml.). After 1 hr., the solvent was evaporated and ether was added. The resulting syrup was taken up in ethyl acetate and benzoylated by benzoyl chloride (0·2 ml.) with aqueous sodium carbonate. The *amide* was recrystallised from ethyl acetate–light petroleum, giving needles, m. p. 145°, $[\alpha]_D^{24}$ −12·2° (*c* 3·5 in EtOH) (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%).

Condensation of Benzoyl-L-leucine p-*Nitrophenyl Ester with Benzylamine*.—The normal procedure, with ethyl acetate as solvent, gave benzoyl-leucine *N*-benzylamide (96% yield), m. p. 143—144°, $[\alpha]_D^{23}$ −9·7° (*c* 1·9 in EtOH), corresponding to the presence of 79% of L-isomer (Found: C, 73·9; H, 7·4; N, 7·9%). When chloroform was the solvent for the reaction, the amide had $[\alpha]_D^{25}$ −10·5° (*c* 3 in EtOH), corresponding to the presence of 86% of L-isomer.

The Action of Salts on Benzoyl-L-leucine p-*Nitrophenyl Ester*.—The *p*-nitrophenyl ester and the salts were heated in the stated solvent at the b. p. (but at 100° for diethyl hydrogen phosphite) for 30 min. In each experiment except those with perchlorates, strong absorption was then found at 1832 cm.$^{-1}$. The salts and solvents used were as follows (the molar ratio to ester is given in brackets): (*a*) In chloroform: 1-ethylpiperidine hydrochloride (2); tribenzylamine hydrochloride (1); tetraethylammonium chloride (2); tetra-n-propylammonium iodide (2); (*b*) in diethyl hydrogen phosphite: tribenzylammonium perchlorate (2); (*c*) in dichloromethane: benzyltrimethylammonium *p*-nitrophenoxide trihydrate (1).

Benzyltrimethylammonium p-*nitrophenoxide trihydrate*. This was obtained directly in methanol solution and was crystallised from water, giving the *salt* as yellow needles, m. p. 60—62° (Found: C, 56·1; H, 7·6; N, 8·2. $C_{16}H_{20}N_2O_3,3H_2O$ requires C, 56·7; H, 7·6; N, 8·3%).

The Action of Tetraethylammonium Chloride on p-*Nitrophenyl Phenylacetate*.—*p*-Nitrophenyl phenylacetate (0·257 g.) and tetraethylammonium chloride (0·001 g.) in dichloromethane (10 ml.) were heated under reflux for 1 hr. The volume was made up to 200 ml. with dichloromethane and the absorption at 420 mμ determined in a 2 mm. cell. The extinction was 0·64. For benzyltrimethylammonium *p*-nitrophenoxide in dichloromethane $\varepsilon = ca.$ 21,000; the *p*-nitrophenoxide concentration corresponds to 0·6% conversion from the ester.

The Action of (a) *Triethylamine*, (b) *Benzyltrimethylammonium Chloride, on Formyl-L-leucine* p-*Nitrophenyl Ester*. (With A. L. Heard.)—(*a*) Triethylamine (0·243 ml.) was added to formyl-L-leucine *p*-nitrophenyl ester [17] (0·50 g.) in dichloromethane. Next day, the ester was recovered (75% yield), having m. p. 65—66°, $[\alpha]_D^{20}$ 0° (*c* 2·2 in EtOH) (Found: C, 55·6; H, 5·6; N, 9·8. $C_{13}H_{16}N_2O_5$ requires C, 55·7; H, 5·75; N, 10·0%).

(*b*) The ester (0·354 g.) was heated under reflux with a solution of benzyltrimethylammonium chloride (0·216 g.) in chloroform. After 2¼ hr. (the infrared absorption was still unchanged) the ester was recovered, giving a gum (0·32 g.) which under light petroleum containing ether gave a solid, m. p. 60·5°, $[\alpha]_D^{21}$ −87° (*c* 1·6 in EtOH) (Found: C, 55·7; H, 5·8; N, 9·75%). Authentic L-isomer [17] has m. p. 60—61°, $[\alpha]_D^{21}$ −90°.

p-*Nitrobenzoyl-L-proline Cyclohexylammonium Salt*.—To a solution of L-proline (2·30 g.) in 2N-sodium hydroxide (20 ml.) were added *p*-nitrobenzoyl chloride (3·72 g.) in ether (20 ml.) and 2N-sodium hydroxide (25 ml.) with rapid stirring. After 15 min., hydrochloric acid and more ether were added; the ether extract was dried; cyclohexylamine was added to precipitate the *salt* (6·95 g., 96%), which after recrystallisation from methanol–ether had m. p. 175—176°, $[\alpha]_D^{20}$ −73° (*c* 2·9 in EtOH) (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%). The free acid could not be crystallised, and was liberated when required by shaking the salt with dilute hydrochloric acid and ether; the ether extract was dried and evaporated.

[16] Bodanszky and du Vigneaud, *J. Amer. Chem. Soc.*, 1959, **81**, 5688.
[17] Heard and Young, *J.*, 1963, 5807.

Racemisation of p-*Nitrobenzoyl-*L-*proline*.—The free acid (syrup, 0·94 g.) was dissolved in glacial acetic acid (10 ml.), and acetic anhydride (2·4 ml.) was added. After 24 hr. the solution was evaporated at 60°; the residual syrup was taken up in ether and the cyclohexylammonium salt was precipitated. After recrystallisation from methanol–ether it had m. p. 173—176°, $[\alpha]_D^{20}$ −41° (*c* 1·7 in EtOH) (Found: C, 59·6; H, 6·8; N, 11·6%). A similar experiment, with acetic anhydride in chloroform, gave product m. p. 174—176°, $[\alpha]_D^{21}$ −54° (*c* 1·7 in EtOH) (Found: C, 59·5; H, 7·2; N, 12·1%).

p-*Nitrobenzoyl-*L-*proline* p-*Nitrophenyl Ester*.—(*a*) L-Proline *p*-nitrophenyl ester hydrobromide [18] (0·63 g.) was suspended in ethyl acetate (10 ml.) containing *p*-nitrobenzoyl chloride (0·372 g.), and sodium carbonate (1·06 g.) in water (6 ml.) was added. Stirring was continued for 30 min.; the usual procedure gave a syrup which crystallised; recrystallisation from ethyl acetate–light petroleum gave ester (0·72 g., 94%), m. p. 142—143·5°, $[\alpha]_D^{24}$ −64° (*c* 2·7 in $CHCl_3$) (Found: C, 56·4; H, 4·0; N, 11·3. $C_{18}H_{15}N_3O_7$ requires C, 56·1; H, 3·9; N, 10·9%).

(*b*) *p*-Nitrobenzoyl-L-proline cyclohexylammonium salt (0·91 g.) was shaken with ethyl acetate (30 ml.) and dilute hydrochloric acid (30 ml.); the dried ethyl acetate extract was concentrated to 15 ml., and *p*-nitrophenol (0·52 g.) and dicyclohexylcarbodi-imide (0·51 g.) were then added. A scarlet colour developed immediately and slowly faded. Next day, the urea was filtered off, the solution was washed with aqueous sodium carbonate (3 × 20 ml.), and the solvent was removed, leaving a syrup which crystallised when rubbed (0·70 g., 73%). Recrystallisation from methanol gave ester, m. p. 138·5—140°, $[\alpha]_D^{22}$ −40° (*c* 2·3 in $CHCl_3$).

Attempted Racemisation and Coupling of p-*Nitrobenzoyl-*L-*proline* p-*Nitrophenyl Ester*.—The *p*-nitrophenyl ester (0·128 g.) was dissolved in chloroform (0·34 ml.) containing 1-methylpiperidine (0·034 g.). After 30 min., glycine ethyl ester (0·034 ml.) was added. Next day, the *p*-nitrobenzoyl-L-prolylglycine ethyl ester was isolated as usual, giving 0·112 g. (96%) of product, m. p. 112—113·5°, $[\alpha]_D^{25}$ −96° (*c* 2·5 in EtOH) (authentic peptide is described below).

p-*Nitrobenzoyl-*L-*prolylhydrazide*.—To *p*-nitrobenzoyl-L-proline methyl ester [19] (2·30 g.) in methanol (20 ml.) was added hydrazine hydrate (100%; 1·5 ml.). Next day, the solvent was removed, leaving a syrup which crystallised (2·3 g., 100%). Recrystallisation from ethyl acetate–light petroleum gave the *hydrazide* as needles, m. p. 150°, $[\alpha]_D^{23}$ −83° (*c* 1·6 in EtOH) (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%).

The Coupling of p-*Nitrobenzoyl-*L-*proline with Glycine Ethyl Ester*.—(a) *Cyanomethyl ester method*. *p*-Nitrobenzoyl-L-proline (from 0·005 mole of the cyclohexylamine salt) was converted into the cyanomethyl ester (in boiling ethyl acetate) and the ester was condensed with glycine ethyl ester in the usual fashion [coupling (2) (a) in Part XVI [4]]. After recrystallisation from ethyl acetate–light petroleum the p-*nitrobenzoyl-*L-*prolylglycine ethyl ester* (55% yield) had m. p. 113—113·5°, $[\alpha]_D^{19}$ −98° (*c* 2·5 in EtOH) (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%).

(b) *Acid azide method*. *p*-Nitrobenzoyl-L-prolylhydrazide (0·93 g.) was converted into the azide, which was coupled with glycine ethyl ester in the usual way [4] (56% yield); after recrystallisation, the peptide had m. p. 113·5—114°, $[\alpha]_D^{23}$ −97° (*c* 3·3 in EtOH).

(c) *Carbonic mixed anhydride method*. *p*-Nitrobenzoyl-L-proline in chloroform was converted into the mixed anhydride with ethyl chloroformate; addition of the glycine ester gave a scarlet colour. The normal procedure [see coupling (6) in Part XVI [4]] gave peptide, m. p. 113—113·5°, $[\alpha]_D^{23}$ −98° (*c* 2·3 in EtOH).

(d) *Carbodi-imide method*. The normal procedure [see coupling (4) in Part XVI [4]], with dichloromethane as solvent, gave solid product (97%), which on recrystallisation gave peptide, m. p. 112—113°, $[\alpha]_D^{22}$ −95° (*c* 1·9 in EtOH). A transient scarlet colour was again noted during the coupling.

We are grateful to the D.S.I.R. for a Research Studentship (M. W. W.) and for a Grant for a Special Research.

THE DYSON PERRINS LABORATORY, OXFORD UNIVERSITY. [*Received, October 3rd*, 1963.]

[18] Goodman and Stueben, *J. Amer. Chem. Soc.*, 1959, **81**, 3980.
[19] Theobald, Williams, and Young, *J.*, 1963, 1927.