387. *Peptides. Part III.* Selective Degradation from the Carboxyl End. The Use of Carbodi-imides.*

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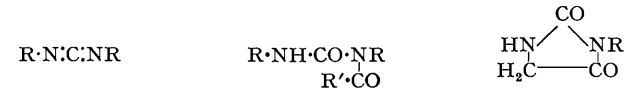
Reaction of carbodi-imides with *N*-acylpeptides and subsequent alkaline degradation of the resulting acylureas of the type (III) have been examined as a possible method for the selective degradation of peptides from the end bearing a free carboxyl group. Although effective in certain cases, the degraded peptides thus obtained tend to be contaminated with the starting materials regenerated from the acylureas by simple hydrolysis.

Mechanisms for the reaction between carbodi-imides and carboxylic acids and for the alkaline degradation of *N*-acylureas are discussed.

No completely satisfactory procedure exists for degradation of peptides from the end bearing the free carboxyl group although several approaches have been made (Bergmann and Zervas, *J. Biol. Chem.*, 1936, **113**, 341; Bettzieche and Menger, *Z. physiol. Chem.*, 1926, **161**, 37; Chibnall and Rees, *Biochem. J.*, 1951, **48**, xlvii; Fromageot, Jutisz, Meyer, and Pénasse, *Biochim. Biophys. Acta*, 1950, **6**, 283). All suffer from the disadvantages that the terminal amino-acid is not recovered as such and the conditions employed for degradation are often too severe. The last objection also applies to Schlack and Kumpf's method (*Z. physiol. Chem.*, 1926, **154**, 125; see also Watson and Waley, *J.*, 1951, 2394, and Tibbs, *Nature*, 1951, **168**, 911) which appears to be the best of those hitherto reported. The present paper deals with our own efforts to devise a practical method for the above-mentioned purpose.

* Part II, preceding paper.

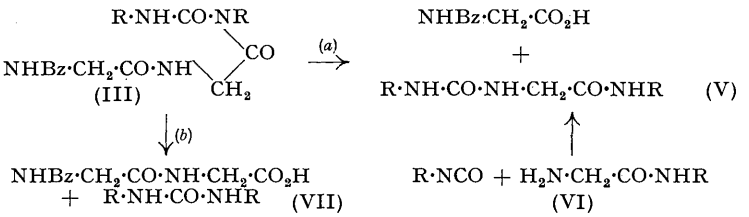
It seems clear that in principle two conditions outlined in the preceding paper must be satisfied. First, a reagent should be attached to the terminal carboxyl group under mild conditions and, secondly, in a subsequent distinct step, the terminal peptide bond should be selectively cleaved by interaction with the elements of the attached reagent. Aromatic carbodi-imides (I; R = *p*-tolyl, *p*-dimethylaminophenyl, etc.) appeared to be the reagents of promise. They react readily with carboxylic acids at room temperature, giving usually the acylureas (II) (Zetzsche *et al.*, *Ber.*, 1938, **71**, 1088, and many subsequent papers). It appeared possible that the similar addition compounds derived from peptides (*e.g.*, III; R = *p*-tolyl) might decompose in presence of alkali to remove the terminal amino-acid residue as a hydantoin (*e.g.*, IV; R = *p*-tolyl).



(I) (II) (IV)

The carboxyl group of peptides was set free for reaction with carbodi-imides by combination of the amino-group with a benzoyl or a 2 : 4-dinitrophenyl residue. Benzoylglycylglycine reacted in absolute ethanol with di-*p*-tolylcarbodi-imide at room temperature, to give an excellent yield (over 85%) of the acylurea (III; R = *p*-tolyl). Highly crystalline derivatives (VIII—XII) of several dipeptides (see Table) were similarly prepared. Their alkaline degradation was followed by paper chromatography of the acylamino-acids liberated.

The acylurea (III; R = *p*-tolyl) although quite stable to acid was rapidly and completely degraded at room temperature by 0·01N-sodium hydroxide in 75% aqueous ethanol to benzoylglycine and a crystalline insoluble neutral product, $C_{17}H_{19}O_2N_3$. Like the expected hydantoin, $C_{10}H_{10}O_2N_2$, this substance was hydrolysed by barium hydroxide to glycine and *p*-toluidine and was shown to be *N'*-*p*-tolylcarbamylglycine *p*-toluidide (V; R = *p*-tolyl) by direct comparison with a specimen prepared from *p*-tolyl *iso*cyanate and glycine *p*-toluidide (VI; R = *p*-tolyl). The water content of the solvent had an important effect on the reaction. In anhydrous ethanol containing one equivalent of sodium ethoxide simple ethanolysis took place at 0° producing di-*p*-tolylurea (VII) and the ethyl ester of the benzoyldipeptide. In ethanol containing less than 25% of water mixtures of products were obtained corresponding to simultaneous degradation (*a*) and hydrolysis (*b*).



The acylureas derived from benzoylglycyl-DL-valine and benzoylglycyl-DL-phenylalanine (VIII and IX respectively) were also degraded by 0·01N-sodium hydroxide in 75% aqueous ethanol to benzoylglycine, slightly contaminated with the original benzoyldipeptides. The terminal amino-acids (DL-valine and DL-phenylalanine) were recovered after alkaline hydrolysis of the respective neutral crystalline " cleavage products " (XIII) possibly contaminated with small amounts of by-products of type (XIV).

Acylureas, R'·NH·CHR''·CO·NH·CHR'''·CO·NR·CO·NHR.

	R	R'	R''	R'''		R	R'	R''	R'''
(VIII)	*p*-C_6H_4Me	Bz	H	Pr^i	(XV)	*p*-C_6H_4·NMe_2 ...	Bz	H	H
(IX)	,,	,,	H	CH_2Ph	(XVI)	,, ...	,,	Me	H
(X)	,,	,,	Me	H	(XVIII)	*cyclo*Hexyl	,,	H	H
(XI)	,,	,,	Bu^i	H	(XIX)	,, ...	,,	Me	H
(XII)	,,	DNP *	Me	H					

* DNP = 2 : 4-dinitrophenyl.

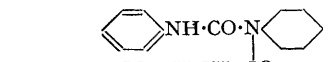
The competing hydrolytic reaction (*b*) was more prominent in degradation of the acylurea (X) derived from benzoyl-DL-alanylglycine. Treatment with alkali in 75% aqueous ethanol afforded approximately equimolar quantities of benzoyl-DL-alanine and the original dipeptide derivative. Even in 40% aqueous ethanol the ratio of these products was no better than 3 : 1 and this result could not be appreciably improved by other modifications in the reaction conditions. Similar behaviour was shown by the derivatives (XI and XII).

$NHBz{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CHR'{\cdot}CO{\cdot}NH{\cdot}C_6H_4Me$ (XIV)

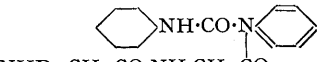
$NHR{\cdot}CO{\cdot}NH{\cdot}CHR'{\cdot}CO{\cdot}NHR$
(XIII; R = *p*-C_6H_4Me, R′ = Pr^i or CH_2Ph)
(XVII; R = *p*-$C_6H_4{\cdot}NMe$, R′ = H)

In further attempts to avoid simple hydrolysis, the basicity of the nitrogen atoms in the acylureas was increased through the use of bis-*p*-dimethylaminophenyl- and di*cyclo*hexyl-carbodi-imide. However, the acylureas (XV and XVI) prepared from the former reacted rather analogously to the corresponding tolyl compounds (III and X); the terminal glycine residue was cleaved, presumably as (XVII). The use of di*cyclo*hexylcarbodi-imide gave distinctly better results but this advantage was offset by the less convenient preparation of the adducts (XVIII and XIX); as recommended by Zetzsche and his co-workers (*Ber.*, 1939, **72**, 1735) this was carried out by the portionwise addition of the benzoylpeptides to a boiling pyridine solution of the carbodi-imide.

Finally, an unsymmetrical carbodi-imide was used to prepare an acylurea containing nitrogen atoms of unequal basicity. It seemed probable that a compound of type (XX)



(XX)



(XXI)

would be more likely to undergo degradation rather than simple hydrolysis. However, as shown below the adduct derived from *N-cyclo*hexyl-*N*′-phenylcarbodi-imide and benzoylglycylglycine proved to be the isomeric acylurea (XXI) which on alkaline treatment gave the expected mixture of benzoyl derivatives of glycine and glycylglycine.

Purely chemical interest, however, resides in the reaction of carboxylic acids with unsymmetrical carbodi-imides. The neutral fraction from the reaction mentioned above was a mixture of *N-cyclo*hexyl-*N*′-phenylurea and (*N-cyclo*hexylcarbamyl)glycine anilide, $C_6H_{11}{\cdot}NH{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO{\cdot}NHPh$, identical with a specimen prepared from *cyclo*hexyl *iso*cyanate and glycine anilide. This substance must have arisen from (XXI); the isomer (XX) would have given phenylcarbamylglycine *cyclo*hexylamide.

Di-*p*-tolylcarbodi-imide appeared from the sum of these results to be the reagent of choice. It was therefore applied to the benzoyl derivatives of the two accessible tripeptides, DL-leucylglycylglycine and glycylglycylglycine, which afforded the expected acylureas (XXII and XXIII) in excellent yields. (XXII) was degraded in *ca.* 40% aqueous ethanol

$NHBz{\cdot}CHR'{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO$ (NHR·CO·NR)
(XXII; R = *p*-C_6H_4Me, R′ = Bu^i)
(XXIII; R = *p*-C_6H_4Me, R′ = H)

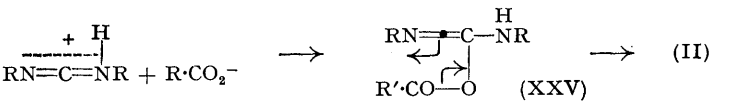
almost entirely to benzoyl-DL-leucylglycine and (V). However, (XXIII) and alkali gave, in addition to a trace of the original tripeptide, benzoylglycylglycine and benzoylglycine in approximately equal amounts. The insoluble neutral product of degradation proved to be mainly a mixture of (V) and a compound $C_{19}H_{22}O_3N_4$ which is assigned the structure (XXIV) (see scheme).

(XXIII) —(*a*) *ca.* 50%→ $NHBz{\cdot}CH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO_2H$ + (V)
(XXIII) —(*c*) *ca.* 45%→ $NHBz{\cdot}CH_2{\cdot}CO_2H + C_6H_4Me{\cdot}NH{\cdot}CO{\cdot}[NH{\cdot}CH_2{\cdot}CO]_2{\cdot}NH{\cdot}C_6H_4Me$ (XXIV)
(XXIII) —(*b*) trace→ $NHBz{\cdot}CH_2{\cdot}CO{\cdot}[NH{\cdot}CH_2{\cdot}CO]_2{\cdot}OH$ + (VII)

It is now possible to make some general comments. The procedure of degradation is simple and the conditions are mild. For stepwise degradation, contamination of the degraded

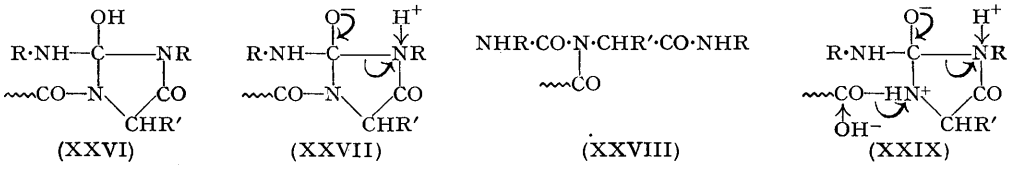
peptide with the starting material is the chief obstacle. However, it appears to be a convenient and useful way of identifying the terminal amino-acid with a free carboxyl group, since hydrolysis of the mixture of " cleavage product " and urea will liberate the terminal amino-acid only. All the experiments described have been carried out with the very simple peptides at present available in this laboratory; with more complex peptides complications might arise (cf. the two-way cleavage of benzoylglycylglycylglycine). The slight variations in the results of the alkaline cleavage of the acylureas studied show, in general, that the course of the degradation is influenced by the nature of the amino-acids forming the terminal peptide bond or bonds. Some modifications of the fundamental technique might be necessary in the case of peptides with varying solubility characteristics. Anhydrous pyridine appears to be a suitable solvent for dinitrophenyl and benzoylpeptides even though its use in the present cases has not been necessary. Incorporation of the maximum possible amount of water into the solvent employed for degradation is advisable. More sensitive terminal amino-acids might be recovered more efficiently from the " cleavage product " by acidic hydrolysis than by treatment with barium hydroxide, which causes some decomposition (Bremner, *Nature*, 1951, **168**, 518).

In the reaction of carboxylic acids with carbodi-imides the first step is probably attachment of a proton to one of the two nitrogen atoms and this is followed by the nucleophilic attack of the acid anion at the central carbon atom to form the intermediate (XXV) which would give after rearrangement the acylureas of type (II). In the particular case of



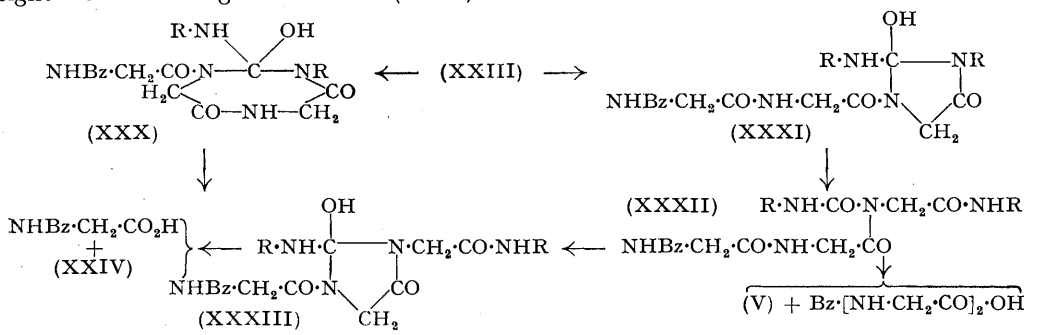
an asymmetrical carbodi-imide, addition of the first proton would occur on the more basic nitrogen atom and consequently in the final products the acyl group would be attached to the less basic nitrogen atom of the original carbodi-imide. This was so with *N-cyclo*hexyl-*N'*-phenylcarbodi-imide.

With regard to the alkaline cleavage of the acylureas, some interaction between the carbonyl group of the substituted ureas and the nitrogen atom of the terminal amino-acid must obviously be postulated although its exact nature is at present not clear. The " cyclol " structures advanced by Stoll *et al.* (*Helv. Chim. Acta*, 1951, **34**, 1544) for the peptide moieties of the ergot alkaloids may be involved as intermediates (XXVI) in the degradations of the acylureas and it may be reasonably supposed that the important role of water observed in these reactions consists in facilitating the ring formation. If this is so then the first step in the alkaline cleavage would be the removal of proton from the alcohol (XXVI) to give (XXVII), followed by the addition (this being aided by water) of a proton to the imide-nitrogen atom to give (XXVIII). The last would be comparable in lability under alkaline conditions with the original acylurea and would be hydrolysed to form the products obtained in the degradation. Alternatively, the structure (XXIX) may contribute considerably to the resonance hybrid of the acylureas, in which case the degradation reaction would proceed by the attack of hydroxyl ion on the carbonyl group of the terminal peptide bond accompanied by the electronic shifts shown; attachment of a proton on the



imide-nitrogen atom would complete the process. Water being a stronger acid than alcohol would thus be more effective in promoting the degradation reaction. The behaviour of the acylurea (XXIII) derived from benzoylglycylglycylglycine calls for special comment. The formation of (V) follows from the mechanism outlined above, whereas (XXIV) may arise through the subsequent transformations (XXXII, XXXIII) of the five-membered-

ring intermediate (XXXI); an alternative would be the simultaneous formation of an eight-membered ring-intermediate (XXX).



Experimental

M.p.s are uncorrected.

Preparation of Carbodi-imides.—Di-*p*-tolyl-, bis-*p*-dimethylaminophenyl-, and di*cyclo*hexylcarbodi-imide were prepared according to Zetzsche *et al.* (*Ber.*, 1938, **71**, 1512) and Schmidt and Seefelder (*Annalen*, 1950—51, **571**, 83). N-cyclo*Hexyl*-N'-*phenylcarbodi-imide*, made by the standard method from *N-cyclo*hexyl-*N'*-phenylthiourea, distilled at *ca.* 120°/0·5 mm. (Found : C, 78·3; H, 8·0; N, 14·2. $C_{13}H_{16}N_2$ requires C, 78·0; H, 8·1; N, 14·0%).

Preparation of Acylureas from Di-p-*tolylcarbodi-imide and Benzoylpeptides.*—N-(*Benzoylglycylglycyl*)-NN'-*di*-p-*tolylurea* (III; R = *p*-tolyl). To benzoylglycylglycine (236 mg.), dissolved in warm anhydrous ethanol (*ca.* 8 c.c.), was added di-*p*-tolylcarbodi-imide (244 mg., 1·1 mol.). N-(*Benzoylglycylglycyl*)-NN'-*di*-p-*tolylurea* began to separate within ½ hour at room temp. and was collected after 24 hours and washed with ether. The yield was 365 mg. (80%). The mother-liquors gave on concentration a further crop (30 mg.) of the compound, m. p. 248—249°. The product was recrystallised from ethanol and formed long fibrous needles (Found, in a sample dried at 80° in a high vacuum for 18 hours : C, 68·2; H, 5·5; N, 12·1. $C_{26}H_{26}O_4N_4$ requires C, 68·1; H, 5·7; N, 12·2%).

N-(*Benzoylglycyl*-DL-*valyl*)-NN'-*di*-p-*tolylurea* (VIII) was prepared by keeping an ethanol–ether solution of benzoylglycyl-DL-valine and di-*p*-tolycarbodi-imide for 24 hours (yield, in two crops, 87%); recrystallised from ethanol–ether it formed rosettes of silky needles, m. p. 248—250° with previous darkening (Found : C, 69·5; H, 6·4; N, 11·4. $C_{29}H_{32}O_4N_4$ requires C, 69·6; H, 6·4; N, 11·2%).

N-(*Benzoylglycyl*-DL-*phenylalanyl*)-NN'-*di*-p-*tolylurea* (IX), prepared in *ca.* 80% yield, crystallised from dioxan as clusters of fine needles, m. p. 174—175° (Found, in a sample dried for 18 hours at 80° in a high vacuum : C, 72·6; H, 6·2; N, 10·3. $C_{33}H_{32}O_4N_4$ requires C, 72·3; H, 5·9; N, 10·2%).

N-(*Benzoyl*-DL-*alanylglycyl*)-NN'-*di*-p-*tolylurea* (X).—To a solution of benzoyl-DL-alanylglycine (250 mg.) in anhydrous ethanol (3 c.c.) was added di-*p*-tolylcarbodi-imide (244 mg., 1·1 mol.). After 6 hours the reaction mixture was diluted with ether to complete crystallisation. The *acylurea* (X) was collected after 24 hours and washed with ether (yield, over 90%; m. p. 160—161° and 198—199° after resolidification) (Found : C, 68·9; H, 6·0; N, 11·9. $C_{27}H_{28}O_4N_4$ requires C, 68·6; H, 6·0; N, 11·9%).

N-(*Benzoyl*-DL-*leucylglycyl*)-NN'-*di*-p-*tolyurea* (XI), prepared in over 85% yield in two crops, crystallised from alcohol–ether as long fibrous needles, m. p. 149—150° followed by resolidification and a second m. p. 212—213° (Found, in a sample dried in a high vacuum at 75° for 18 hours : C, 69·6; H, 6·7; N, 10·6. $C_{30}H_{34}O_4N_4$ requires C, 70·0; H, 6·9; N, 10·9%).

N-(2 : 4-*Dinitrophenyl*-DL-*alanylglycyl*)-NN'-*di*-p-*tolylurea* (XII) (yield 82%) crystallised from ethanol as long yellow needles, m. p. 123—125° (Found, in a sample dried at 75° for 18 hours in a high vacuum : C, 57·9; H, 5·1; N, 15·9. $C_{26}H_{26}O_7N_6$ requires C, 58·4; H, 4·9; N, 15·7%).

N-(*Benzoyl*-DL-*leucylglycylglycyl*)-NN'-*di*-p-*tolylurea* (XXII), prepared from benzoyl-DL-leucylglycylglycine (350 mg.) and di-*p*-tolylcarbodi-imide (250 mg.) in the usual way in 85% yield (in two crops), crystallised from ethanol–ether as a mass of fine needles, m. p. 162—163° (Found, in a sample dried at 70° in a high vacuum : C, 67·5; H, 6·15; N, 12·6. $C_{32}H_{37}O_5N_5$ requires C, 67·2; H, 6·5; N, 12·3%).

N-(*Benzoylglycylglycylglycyl*)-NN′-*di*-p-*tolylurea* (XXIII).—Benzoylglycylglycylglycine (294 mg.) was dissolved in hot anhydrous ethanol (20 c.c.) and the solution rapidly cooled. Di-*p*-tolylcarbodi-imide (250 mg.) was then added and the clear solution kept for 6 hours. The solution was then concentrated to *ca.* 8 c.c. and set aside for a further 18 hours; addition of ether completed the crystallisation. The yield was 430 mg. (85%), and the mother-liquor gave more product. When recrystallised slowly from ethanol the *urea* had m. p. 122—123° followed by solidification and a second m. p. 255—260° (decomp.). Rapid crystallisation from aqueous ethanol afforded a sample which showed only the second m. p. (Found, in the latter sample after drying in a high vacuum at 80° : C, 65·0; H, 5·7; N, 13·7. $C_{28}H_{29}O_5N_5$ requires C, 65·2; H, 5·7; N, 13·6%).

Preparation of the Acylureas from Bis-p-*dimethylaminophenylcarbodi-imide.*—N-(*Benzoylglycylglycyl*)-NN′-*bis*-p-*dimethylaminophenylurea* (XV) was prepared as the corresponding urea (III), in 80% yield, a further crop being obtained from the mother-liquor. It had m. p. 180° and is much less soluble in hot ethanol than is (III) (Found, in a sample dried in a high vacuum at 75° : C, 64·8; H, 5·9; N, 16·25. $C_{28}H_{32}O_4N_6$ requires C, 65·1; H, 6·2; N, 16·3%).

N-(*Benzoyl*-DL-*alanylglycyl*)-NN′-*bis*-p-*dimethylaminophenylurea* (XVI), prepared by the standard method in good yield and recrystallised from ethanol, had m. p. 175—176° (Found : C, 65·4; H, 6·5; N, 16·0. $C_{29}H_{34}O_4N_6$ requires C, 65·6; H, 6·5; N, 15·9%)

Preparation of Acylureas of Dicyclohexylcarbodi-imide —N-(*Benzoylglycylglycyl*)-NN′-*di*-cyclo*hexylurea* (XVIII). When benzoylglycylglycine (236 mg.) was added portionwise to a hot ethanolic solution (10 c.c.) of di*cyclo*hexylcarbodi-imide (250 mg., 1·2 mol) during 2 hours, the product proved to be a mixture of the desired *urea* (XVIII) (*ca.* 300 mg.) and *NN′*-di*cyclo*hexylurea (65 mg.). They were separated by fractional crystallisation from hot ethanol, the latter compound being less soluble. The acylurea was, however, practically the entire product when pyridine was employed as the solvent and the addition of the benzoyl peptide to a boiling solution of the alicyclic carbodi-imide was completed in 45 minutes. After being kept at room temperature for 2 hours the clear solution was evaporated to a syrup. The product (377 mg., 86%), crystallised from alcohol–ether, had m. p. 155—157°. A sample was twice recrystallised from aqueous ethanol and formed long slender needles (Found : C, 65·25; H, 8·1; N, 12·7. $C_{24}H_{34}O_4N_4$ requires C, 65·15; H, 7·75; N, 12·7%).

N-(*Benzoyl*-DL-*alanylglycyl*)-NN′-*di*cyclo*hexylurea* (XIX).—This was prepared as described for the foregoing acylurea but was presumably contaminated with a small amount of *NN′*-di*cyclo*-hexylurea and could not be obtained analytically pure owing to its tendency to form a gel in various mixtures of solvents. A sample of the gel obtained from aqueous ethanol was dried in a high vacuum over phosphoric oxide (Found : C, 66·6; H, 7·8; N, 12·0. Calc. for $C_{25}H_{36}O_4N_4$: C, 65·8; H, 7·9; N, 12·3%). (The degradative experiments described below leave no doubt about the identity of the compound.)

N-(*Benzoylglycylglycyl*)-N′-cyclo*hexyl*-N-*phenylurea* (XXI). To a warm ethanolic solution of benzoylglycylglycine (118 mg.) was added *N-cyclo*hexyl-*N′*-phenylcarbodi-imide (115 mg., 1·1 mol) and the whole was set aside overnight. The solution was filtered from a trace of *N-cyclo*hexyl-*N′*-phenylurea (4 mg.), concentrated *in vacuo* below 35°, and diluted with ether. The *urea* (XXI) then separated (150 mg. in one crop) as a mass of needles. A further recrystallisation from alcohol–ether did not alter the m. p. (148°) (Found : C, 66·1; H, 6·5; N, 12·7. $C_{24}H_{28}O_4N_4$ requires C, 66·0; H, 6·5; N, 12·9%).

When higher temperatures (50—80°) were used for the preparation of the above acylurea, yields were much lower and isolation of the product was difficult. The unsymmetrical carbodi-imide appeared to undergo partial polymerisation.

Degradation of N-(*Benzoylglycylglycyl*)-NN′-*di*-p-*tolylurea* (III).—(a) *In acid.* An aqueous-alcoholic solution (20 c.c.) of (III) (50 mg.) was kept in N-hydrochloric acid (5 c.c.) for 2 hours at room temperature. The starting material was recovered unchanged. Similarly, no degradation occurred at 50° in dilute acid solution.

(b) *In alkali.* The acylurea (III) (57 mg.) was dissolved in aqueous ethanol (75% ; 20 c.c.) by warming and the solution cooled rapidly. 0·1N-Sodium hydroxide (2 c.c., *ca.* 1·5 mol.) was added under agitation and the solution set aside for 10 minutes during which crystals rapidly appeared. The contents were then neutralised with dilute hydrochloric acid to pH 6 and the solvent was removed under a vacuum. The residue was treated with a few c.c. of dilute sodium hydrogen carbonate solution, and the insoluble crystalline material collected. The clear filtrate after acidification and concentration *in vacuo* to a small volume (0·5 c.c.) deposited long needles (16 mg., 72%), m. p. 180—182° undepressed on admixture with authentic benzoylglycine (when mixed with benzoylglycylglycine, 164—168°).

The insoluble " cleavage product," p-*tolylcarbamylglycine* p-*toluidide* (V) (34 mg., *ca* 90%), recrystallised from absolute ethanol, had m. p. 239—240° (Found, in a sample dried at 120° in a high vacuum for 18 hours : C, 68·7, 68·7; H, 6·7, 6·3; N, 14·3. $C_{17}H_{19}O_2N_3$ requires C, 68·7; H, 6·5; N, 14·1%). When the compound (5 mg.) was heated in aqueous barium hydroxide (0·2N; 2 c.c.) at 150° for 6 hours and the mixture then saturated with carbon dioxide, boiled, and filtered from the insoluble matter, glycine was shown to be present in the clear filtrate by paper chromatography in the solvent systems (phenol–H_2O) and (*n*-butanol : acetic acid : water, 4 : 1 : 5).

In numerous experiments carried out before the above satisfactory procedure, the effects of temperature and alkali concentration, and the role of the solvent, were investigated. Higher temperature and excess of alkali caused coloration and unspecific decomposition of the acylurea. In experiments in absolute alcohol a variable mixture of benzoylglycine and the original peptide was obtained, and (V) was mixed with *NN'*-di-*p*-tolylurea.

(c) *In anhydrous ethanol with sodium ethoxide.* Sodium ethoxide (15 mg.) in anhydrous ethanol (2 c.c.) was added during 45 minutes to the acylurea (III) (57 mg.) in the same solvent (10 c.c.) at −10°. The clear solution was then diluted with water and neutralised with dilute hydrochloric acid. After removal of the solvent *in vacuo* the residue was diluted with water, and the insoluble product collected. It had m. p. 258—260° after recrystallisation from ethanol, undepressed on admixture with authentic *NN'*-di-*p*-tolylurea. The clear aqueous filtrate furnished practically pure benzolyglycylglycine after alkaline hydrolysis of the ethyl ester.

Degradation of N-(*Benzoylglycylglycyl*)-NN'-*bis*-p-*dimethylaminophenylurea* (XV).—This was carried out as described in (*b*) above. Benzoylglycine was obtained iu 70% yield and p-*dimethylaminophenylcarbamylglycine* p-*dimethylaminoanilide* (XVII) in practically quantitative yield. Recrystallisation from dilute alcohol afforded fine needles, m. p. 237—238° (Found : C, 64·0; H, 6·8; N, 19·9. $C_{19}H_{25}O_2N_5$ requires C, 64·0; H, 7·1; N, 19·7%).

Degradation of N-(*Benzoylglycylglycyl*)-NN'-*di*cyclo*hexylurea* (XVIII).—Degradation in 75% ethanol also afforded pure benzoylglycine and cyclo*hexylcarbamylglycine*cyclo*hexylamide*. A trace of *NN'*-di*cyclo*hexylurea was removed by washing with a small amount of cold acetone in which only it dissolved. The amide separated from hot acetone in long shining needles, m. p. 234° (Found : C, 64·2; H, 9·8; N, 14·75. $C_{15}H_{27}O_2N_3$ requires C, 64·0; H, 9·7; N, 14·9%).

Degradation of N-(*Benzoylglycylglycyl*)-N'-cyclo*hexyl*-N-*phenylurea* (XXI).—By the above procedure (*b*) an approximately equimolar mixture of benzoylglycine and benzoylglycylglycine was obtained. The mixture was resolved by paper chromatography (ascending technique) in *n*-butanol–ammonia, and the constituent acids were located by their purple fluorescence in ultraviolet radiation (benzoylglycine, R_F 0·225; benzoylglycylglycine, R_F 0·144). Authentic samples were used as markers. The insoluble neutral " cleavage product " was washed with a small amount of cold acetone. The insoluble portion, recrystallised from aqueous alcohol, had m. p. 208° undepressed on admixture with *cyclo*hexylcarbamylglycine anilide described below.

Degradation of N-(*Benzoylglycyl*-DL-*valyl*)- *and* N-(*Benzoylglycyl*-DL-*phenylalanyl*)-NN'-*di*-p-*tolylurea* (VIII and IX).—On treatment of these compounds with alkali in aqueous ethanol a faint yellow colour was formed. In both cases the acid obtained proved to be mostly benzoylglycine (R_F in *n*-butanol–ammonia, 0·192), contaminated with traces of benzoylglycyl-DL-valine (R_F 0·32) and benzoylglycyl-DL-phenylalanine (R_F 0·37). The " cleavage products," presumably (XIII; R = Pr^i and CH_2Ph), after recrystallisation from aqueous ethanol were hydrolysed with barium hydroxide (0·25N) at 150° for 6 hours and afforded DL-valine and DL-phenylalanine which were identified by paper chromatography. In both cases, a small amount of a ninhydrin-reacting substance (probably glycine) with lower R_F value was also detected on the chromatogram. Authentic DL-phenylalanine when heated with barium hydroxide under identical conditions also showed the formation of the slow-travelling substance.

Degradation of N-(*Benzoyl*-DL-*alanylglycyl*)*ureas*.—*N*-(Benzoyl-DL-alanylglycyl)-*NN'*-di-*p*-tolylurea (X) afforded a mixture of benzoyl-DL-alanylglycine and benzoyl-DL-alanine in approx. equal amounts, separated readily by paper chromatography (*n*-butanol–ammonia) (benzoyl-DL-alanylglycine, R_F 0·183; benzoyl-DL-alanine, R_F 0·282).

When the degradation was carried out in 40% aqueous ethanol, the ratio of benzoylalanine to the recovered dipeptide was approx. 3 : 1. The " cleavage product " (V) admixed with *NN'*-di-*p*-tolylurea gave on hydrolysis with barium hydroxide glycine which was identified by paper chromatography.

Degradation of *N*-(benzoyl-DL-alanylglycyl)-*NN'*-bis-*p*-dimethylaminophenylurea (XVI) gave a result similar to the above. The " cleavage product " was a mixture of (XVII) and *NN'*-bis-*p*-dimethylaminophenylurea.

Degradation of *N*-(benzoyl-DL-alanylglycyl)-*NN'*-di*cyclo*hexylurea (IX) in aqueous ethanol (40%) at 10° in the usual way yielded a mixture of the original peptide and benzoyl-DL-alanine, the latter being the main product (*ca.* 80%). Many variations of temperature and mode of the addition of alkali did not make it possible to eliminate completely the formation of benzoyl-DL-alanylglycine.

In one experiment the acylurea (60 mg.) was dissolved in phenol (1·5 g.) and N-sodium hydroxide (0·2 c.c.) was added. The clear solution was kept for 20 minutes and the phenol removed by "freeze-drying." The residue was diluted with water and filtered from the insoluble material which presumably contained some starting material. Paper chromatography of the clear aqueous solution after acidification and concentration revealed mainly the original dipeptide.

Degradation of *N*-(2 : 4-dinitrophenyl-DL-alanylglycyl)-*NN'*-di-*p*-tolylurea (XII) and *N*-(benzoyl-DL-leucylglycyl)-*NN'*-di-*p*-tolylurea (XI) in aqueous ethanol (40%) furnished, as above, mixtures of the respective original and degraded peptides.

Degradation of N-(*Benzoyl-DL-leucylglycylglycyl*)-NN'-*di*-p-*tolylurea* (XXII).—Treatment in aqueous ethanol (40%) with alkali afforded practically pure (V); the degraded acid (benzoyl-DL-leucylglycine; R_F in *n*-butanol–ammonia, 0·51) showed only a very slight contamination with the original peptide (R_F, 0·38).

Degradation of N-(*Benzoylglycylglycylglycyl*)-NN'-*di*-p-*tolylurea* (XXIII).—The usual method furnished mixed "acids" which were separated by paper chromatography (*n*-butanol–ammonia). Benzoylglycylglycylglycine (R_F, 0·077) was present only in traces, whereas benzoylglycylglycine (R_F, 0·124) and benzoylglycine (R_F, 0·192) were found to be in approximately equal amounts. The mixture of the insoluble "cleavage products" was treated with hot ethanol. The soluble portion contained mainly (V), and the insoluble fraction, p-*tolylcarbamylglycylglycine* p-*toluidide* (XXIV), was recrystallised from glacial acetic acid containing a little water to give a mass of fine needles, m. p. 248—250° (Found, in a sample dried at 100° in a high vacuum for 18 hours: C, 64·6; H, 6·2; N, 16·1. $C_{19}H_{22}O_3N_4$ requires C, 64·4; H, 6·3; N, 15·8%).

Synthesis of p-*Tolylcarbamylglycine* p-*Toluidide* (V).—Phthaloylglycine was converted into the acid chloride (Sheehan and Frank, *J. Amer. Chem. Soc.*, 1949, **71**, 1856) which was added in chloroform to a stirred solution in the same solvent of *p*-toluidine (2·5 mol.), giving a heavy precipitate. Chloroform was removed *in vacuo* and the residue washed with dilute hydrochloric acid. *Phthaloylglycine* p-*toluidide* crystallised from 2-ethoxyethanol as long needles, m. p. 262° (Found: C, 69·7; H, 4·7; N, 9·4. $C_{17}H_{14}O_3N_2$ requires C, 69·4; H, 4·8; N, 9·6%).

Glycine p-*Toluidide* (VI).—The foregoing compound (5·88 g.) was suspended in ethanol, and hydrazine hydrate (100% w/v; 1·1 c.c.) was added. The whole was heated under reflux for 2 hours and the solvent removed *in vacuo*. Treatment of the solid cake with warm dilute hydrochloric acid for ½ hour and removal of the insoluble phthalhydrazide afforded the hydrochloride from which *glycine* p-*toluidide* (VI) was liberated by ammonia. Recrystallisation from benzene–light petroleum (b. p. 40—60°) gave plates, m. p. 105—107° (Found, in a sample sublimed at 90°/10^{-3} mm: C, 65·4; H, 7·1; N, 17·2. $C_9H_{12}ON_2$ requires C, 65·85; H, 7·4; N, 17·0%).

When the benzene solutions containing equivalent amounts of freshly distilled *p*-tolyl *iso*cyanate and glycine *p*-toluidide were mixed, *p*-tolycarbamylglycine *p*-toluidide (V) separated in quantitative yield. Recrystallised from ethanol it had m. p. 240° undepressed by a sample obtained from the alkaline cleavage of *N*-(benzoylglycylglycyl)-*NN'*-di-*p*-tolylurea (III; R = *p*-tolyl).

cyclo*Hexylcarbamylglycine Anilide.*—*cyclo*Hexyl *iso*cyanate was made by addition of *cyclo*hexylamine to an excess of carbonyl chloride in dioxan, followed by removal of the solvent and distillation of the product (admixed with *NN'*-di*cyclo*hexylurea) over phosphoric oxide (cf. Skita, *Ber.*, 1920, **53**, 1242). Glycine anilide was prepared according to the directions of Sheehan and Frank (*J. Amer. Chem. Soc.*, 1949, **71**, 1856). On the addition of a benzene solution of glycine anilide (1·5 g.) to a solution in the same solvent of *cyclo*hexyl *iso*cyanate (1·25 g.), cyclo*hexylcarbamylglycine anilide* separated in practically quantitative yield. Recrystallised from ethanol it formed long shining needles, m. p. 208—209° (Found: C, 65·35; H, 7·6; N, 15·5. $C_{15}H_{21}O_2N_3$ requires C, 65·4; H, 7·7; N, 15·3%).

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