Formation and Degradation of Urea Derivatives in the Azide Method of Peptide Synthesis. Part 1. The Curtius Rearrangement and Urea Formation †

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The azide method of peptide synthesis ($R^1CON_3 + R^2NH_2 \longrightarrow R^1CO{\cdot}NHR^2$) has been investigated with respect to the effect of reaction conditions especially on the formation of urea derivatives ($R^1NH{\cdot}CO{\cdot}NHR^2$) as side products. Z-Gly-Phe-N_3 and H-Gly-OBu^t were used as model compounds for R^1CON_3 and R^2NH_2, respectively, and the rate of formation of peptide was compared with that of urea under various conditions. Peptide formation was estimated from the consumption of R^2NH_2 and urea formation by the extent of the rate-determining Curtius rearrangement ($R^1CON_3 \longrightarrow R^1NCO + N_2$). Data have also been obtained for other azides and amines. The results show that the conditions currently used in azide couplings (*ca.* 0.1 M-solutions and 0—5 °C) are generally adequate for minimising the side reaction. On the other hand, some urea derivatives, including Boc-Gly-Tyr-Ser-NH·CH(CH_2·CH_2·SMe)·NH·CO-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH (6) and Z-Lys(Boc)-Pro-Val-Gly-NH·CH-([CH_2]$_4$·NH-Boc)·NH·CO-Lys(Boc)-Arg-Arg-NH_2 (5) can be synthesised from R^1NCO and R^2NH_2. These ureas have been compared with the corresponding peptides in terms of chromatographic behaviour; the results suggest that when R^1 and R^2 are fairly large and complex as in (5) and (6) the separation of peptide from urea will present considerable difficulties.

THE azide method of peptide synthesis, introduced by Curtius over 70 years ago,[1] is still one of the major coupling procedures in current use. One reason for its continued use is that it permits coupling with minimum protection on amino-acid side chains. This is especially advantageous for the synthesis of long-chain peptides. Another reason, probably the most important, is that the method carries the least risk of racemisation.[2,‡] Because of this the azide method has been employed rather routinely for peptide–petide couplings, in spite of the well known fact that various kinds of undesirable

† The results described in this paper were presented at the 11th Symposium on Peptide Chemistry, Kanazawa, Japan, October 1973 (Proceedings of the 11th Symposium on Peptide Chemistry, ed. H. Kotake, Protein Research Foundation, Osaka, Japan, 1974, p. 30).

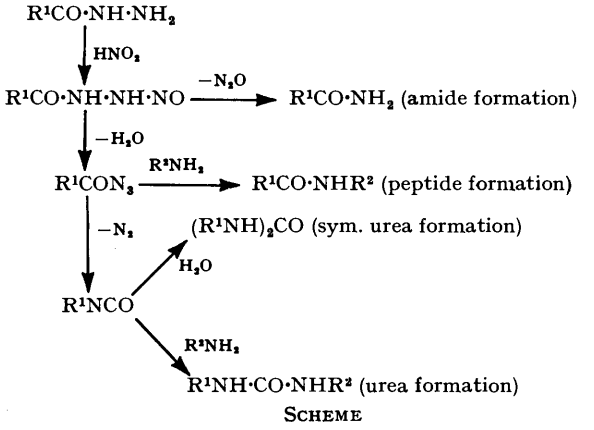
All the amino-acid residues mentioned are of the L-configuration. Abbreviations used are those recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (*Biochem. J.*, 1967, **102**, 23; 1967, **104**, 17; 1972, **126**, 733); Z = benzyloxycarbonyl, Boc = t-butoxycarbonyl, DCC = dicyclohexylcarbodi-imide, HOSu = *N*-hydroxysuccinimide, HOBt: 1-hydroxybenzotriazole, TFA = trifluoroacetic acid, DMF = *NN*-dimethylformamide, ACTH = adrenocorticotrophic hormone (corticotrophin).

‡ Since Weygand *et al.*[3] found that azides do give racemic products in the presence of an excess of base, racemisation in azide couplings has been observed in a number of instances.[4] However, the risk of racemisation seems to be generally lower than with other coupling methods, including the DCC–HOBt procedure,[5] as Young has noted recently.[6]

[1] T. Curtius, *Ber.*, 1902, **35**, 3226.
[2] N. A. Smart, G. T. Young, and M. W. Williams, *J. Chem. Soc.*, 1960, 3902.
[3] F. Weygand, A. Prox, and W. König, *Chem. Ber.*, 1966, **99**, 1451.
[4] G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, 1966, **88**, 1338; P. Sieber, M. Brugger, and W. Rittel, in ' Peptides 1969,' Proceedings of the 10th European Peptide Symposium, ed. E. Scoffone, North-Holland, Amsterdam, 1971, p. 60; P. Sieber, B. Riniker, M. Brugger, B. Kamber, and W. Rittel, *Helv. Chim. Acta*, 1970, **53**, 2135; D. S. Kemp, Z. Bernstein, and J. Rebek, jun., *J. Amer. Chem. Soc.*, 1970, **92**, 4756; D. S. Kemp, S. W. Wang, G. Busby III, and G. Hugel, *ibid.*, p. 1043; M. Dzieduszycka, M. Smulkowski, and E. Taschner, in ' Peptides 1972,' Proceedings of the 12th European Peptide Symposium, eds. H. Hanson and H. D. Jakubke, North-Holland, Amsterdam, 1973, p. 103.
[5] W. König and R. Geiger, *Chem. Ber.*, 1970, **103**, 788.
[6] G. T. Young, in ' Peptides 1972,' Proceedings of the 12th European Peptide Symposium, ed. H. Hanson and H. D. Jakubke, North-Holland, Amsterdam, 1973, p. 132.

side reactions [7] tend to occur during the course of the coupling procedure.

The important side reactions associated with the azide method are shown in the Scheme. Among them



SCHEME

the formation of amide, $R^1CO{\cdot}NH_2$, first observed by Prelog and Wieland [8] has been reported most frequently. Honzl and Rudinger found that amide formation is largely suppressed when the preparation of the azide from the hydrazide is performed in an anhydrous and homogeneous solution at low temperatures and low acidity with an alkyl nitrite (*e.g.* t-butyl nitrite) as nitrosating agent.[9] It is generally accepted that the amide may not be a product from the azide,[7,9,10] whereas the ureas are derived from isocyanate, R^1NCO, which is formed from azide, R^1CON_3, by the Curtius rearrangement.[7,11] The isocyanate produces a urea, $R^1NH{\cdot}CO{\cdot}NHR^2$, when it reacts with an amine component, R^2NH_2. The isocyanate can also produce a symmetrical urea $(R^1NH)_2CO$ on hydrolysis followed by reaction with unhydrolysed isocyanate. We will now consider the formation of the urea $R^1NH{\cdot}CO{\cdot}NHR^2$, which structurally resembles the desired peptide $R^1CO{\cdot}NHR^2$ most closely. The degree of resemblance increases with increasing size and complexity of the groups R^1 and R^2. For fairly large peptides, it could become extremely difficult to detect the presence of such a closely related by-product and to separate it from the desired compound. Thus urea formation is a serious problem in azide couplings, even though its occurrence has been reported less frequently.[12] In a search for a solution of this problem we have investigated the rate of formation of urea in relation to the rate of formation of the peptide bond and have compared the resulting ureas with the related peptides in terms of some chemical and physicochemical properties. These studies were performed with Z-Gly-Phe-$NH{\cdot}NH_2$ and Z-Gly-Tyr-$NH{\cdot}NH_2$ as model compounds and then with some peptide hydrazides related to an active fragment of corticotrophin, [Gly[1]]-ACTH(1—18)-NH_2.[13]

The conversion of hydrazide into azide was carried out in aqueous tetrahydrofuran in the presence of 2—3 equiv. of hydrochloric acid. The azide formed upon addition of sodium nitrite was extracted at pH 9 with ethyl acetate. The resulting azide solution was quickly dried over magnesium sulphate prior to immediate use and, if necessary, the solvent was removed *in vacuo*. Throughout these operations the temperature was maintained at 0 °C.

Preparation of Ureas.—The azide once formed spontaneously undergoes the Curtius rearrangement to produce the isocyanate. The azide and the isocyanate are characterised by i.r. bands at 4.75 and 4.5 μm, respectively.[14] Therefore, if an amine component is added when the azide band has disappeared, urea formation takes place rapidly, with concomitant disappearance of the isocyanate band. For example. when Z-Gly-Tyr-N_3 in ethyl acetate was kept at 25 °C overnight and H-Gly-OBu^t was then aded, prompt formation of the urea Z-Gly-$NH{\cdot}CH(CH_2{\cdot}C_6H_4{\cdot}OH){\cdot}NH{\cdot}CO$-Gly-$OBu^t$ (1) resulted. Compound (1) was isolated crystalline after chromatography on silica gel. Amino-acid analysis revealed the absence of tyrosine, as expected,* In this manner, some other urea derivatives were synthesised. These ureas and the corresponding peptides are shown in Table 1, which also includes a symmetrical urea (3) which may be compared with the amide Z-Gly-Phe-NH_2. Compound (3) was formed when a solution of Z-Gly-Phe-N_3 in wet ethyl acetate was kept at room temperature for a few days. Compounds (4) and (5) are related to the amino-acid sequence 11—18 of corticotrophin (ACTH) [15] and (6) to the sequence 1—10 of the hormone.[16] The ureas (1), (2), and (4) were clearly distinguished from the corresponding peptides by t.l.c., whereas (5) and (6) showed no separation from the peptides. These facts suggest that when the molecule is relatively small [as in (1) and (2)] or when polar side chains are fully

* The urea undergoes acidic hydrolysis in the following manner:[7]

$$R^1CO{\cdot}NH{\cdot}CHR^2{\cdot}NH{\cdot}CO{\cdot}NHR^3 \xrightarrow{3H_2O} R^1CO_2H + R^2CHO + R^3NH_2 + 2NH_3 + CO_2$$

In the case of compound (1), however, the amounts of glycine and ammonia released upon hydrolysis (6M-HCl; 110 °C; 24 h) were much smaller than the theoretical values. This probably indicates the liberation of hydantoic acid ($NH_2{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO_2H$) as a stable intermediate (see also following paper).

[7] E. Schnabel, *Annalen*, 1962, **659**, 168.

[8] V. Prelog and P. Wieland, *Helv. Chim. Acta*, 1946, **29**, 1128; for other observations see references cited in ref. 9.

[9] J. Honzl and J. Rudinger, *Coll. Czech. Chem. Comm.*, 1961, **26**, 2333.

[10] Y. S. Klausner and M. Bodanszky, *Synthesis*, 1974, 549.

[11] T. Curtius, *J. prakt. Chem.*, 1904, **70**, 57; T. Curtius and H. Curtius, *ibid.*, p. 158.

[12] (*a*) M. A. Nyman and R. M. Herbst, *J. Org. Chem.*, 1950, **15**, 108; (*b*) J. M. Hinman, E. L. Caron, and H. N. Christensen, *J. Amer. Chem. Soc.*, 1950, **72**, 1620; (*c*) K. C. Hooper, H. N. Rydon, J. A. Schofield, and G. S. Heaton, *J. Chem. Soc.*, 1956, 3148; (*d*) G. L. Tritsch and D. M. Woolley, *J. Amer. Chem. Soc.*, 1960, **82**, 2787; (*e*) K. Hofmann, T. A. Thompson, H. Yajima, E. T. Schwartz, and H. Inouye, *ibid.*, p. 3715.

[13] H. Otsuka, M. Shin, Y. Kinomura, and K. Inouye, *Bull. Chem. Soc. Japan*, 1970, **43**, 196.

[14] R. Schwyzer and H. Kappeler, *Helv. Chim. Acta*, 1961, **44**, 1991.

[15] H. Otsuka, K. Inouye, M. Kanayama, and F. Shinozaki, *Bull. Chem. Soc. Japan*, 1966, **39**, 882.

[16] H. Otsuka, K. Inouye, F. Shinozaki, and M. Kanayama, *Bull. Chem. Soc. Japan*, 1966, **39**, 1171.

blocked [as in (4)] the separation of urea from peptide may be possible,* whereas it must be difficult in more general cases such as (5) and (6). For all these urea derivatives, recovery of the amino-acid whose α-amino-group had been involved in the formation of the urea bond was generally low: Gly 20% [in compound (1)], 30% (2); Lys 70% (4), 80% (5); Glu 70% (6). This is probably because a relatively stable carbamoylamino-acid, $NH_2{\cdot}CO{\cdot}NH{\cdot}CHR{\cdot}CO_2H$, is formed as an intermediate upon hydrolysis of the urea.

TABLE 1

Some urea derivatives and the corresponding peptides

{Urea: Z-Gly-NH·CH(CH_2·C_6H_4·OH)·NH·CO-Gly-OBu^t (1)
{Peptide: Z-Gly-Tyr-Gly-OBu^t
{Urea: Z-Gly-NH·CH(CH_2Ph)·NH·CO-Gly-OBu^t (2)
{Peptide: Z-Gly-Phe-Gly-OBu^t
{Urea (sym): [Z-Gly-NH·CH(CH_2·Ph)·NH]$_2$CO (3)
{Peptide: Z-Gly-Phe-NH_2
{Urea: Z-Lys(Boc)-Pro-Val-Gly-NH·CH([CH_2]$_4$·NH-Boc)·NH·CO-Lys(Boc)-Lys(Boc)-Lys(Boc)-NH_2 (4)
{Peptide: Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-NH_2
{Urea: Z-Lys(Boc)-Pro-Val-Gly-NH·CH([CH_2]$_4$·NH-Boc)·NH·CO-Lys(Boc)-Arg-Arg-NH_2 (5)
{Peptide: Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-Lys(Boc)-Arg-Arg-NH_2 [15]
{Urea: Boc-Gly-Tyr-Ser-NH·CH(CH_2·CH_2·SMe)·NH·CO-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH (6)
{Peptide: Boc-Gly-Tyr-Ser-Met-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH [16]

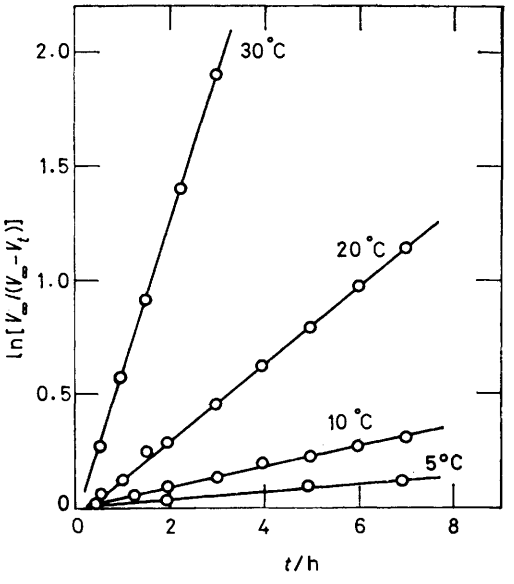


FIGURE 1 Curtius rearrangement of Z-Gly-Phe-N_3 in ethyl acetate: V_t = volume of evolved nitrogen at time t; V_∞ = total volume collected

Comparison of Peptide and Urea in Terms of Rate of Formation and Kinetics.—Attempts were made to compare the rates of formation of urea and peptide under various conditions. The formation of urea was estimated by the extent of the Curtius rearrangement, which determines the rate of urea formation. The rate of the Curtius rearrangement (decomposition of azide into isocyanate and nitrogen) was determined by measuring the volume of nitrogen evolved.[17] The rate of peptide formation was determined by measuring the consumption of amine component by the ninhydrin method.[18]

The results obtained from the Curtius rearrangement of the model compound Z-Gly-Phe-N_3 are shown in Figure 1 and Tables 2 and 3. Figure 1 demonstrates that the decomposition of the azide follows first-order kinetics. The reaction should, therefore, be independent of concentration, but it depends on conditions such as temperature and solvent. The remarkable temperature dependence of rate constant k_1 and half-life $t_{\frac{1}{2}}$ of this first-order reaction can be seen in Table 2. Table 3 shows values of k_1 measured in five different solvents, indicating that the nature of the solvent has much less effect on the reaction rate than the temperature. Rates of decomposition were also measured for a variety of acyl azides: the effects of changing the acyl groups were much less marked than that of temperature (Table 4).

TABLE 2

Rates of Curtius rearrangement of Z-Gly-Phe-N_3 in ethyl acetate

Temp. (°C)	10^4k_1/min^{-1}	$t_{\frac{1}{2}}$/min
5	2.9	2 400
10	7.5	930
20	27	260
30	100	69

TABLE 3

Rates of Curtius rearrangement of Z-Gly-Phe-N_3 in various solvents

Solvent	Temp. (°C)	10^3k_1/min^{-1}
Tetrahydrofuran	20	1.9
	30	7.2
Ethyl acetate	20	2.7
	30	10
Dimethylformamide	20	3.5
	30	10
Benzene	30	11
Chloroform	30	17

The aminolysis of Z-Gly-Phe-N_3 with an equimolar

* The reaction of an azide–isocyanate mixture, prepared from Z-Gly-Phe-N_3, with H-Gly-OBu^t led to Z-Gly-Phe-Gly-OBu^t and the urea (2) as the two major products. The peptide and the urea could be separated by chromatography on a silica gel column with chloroform–methanol as solvent. The combined yield was *ca.* 60%. In a similar manner, pure samples of Z-Gly-Tyr-Gly-OBu^t and the urea (1) were obtained from a mixture in which these compounds were the two major components. For details see Experimental section.

[17] Y. Yukawa and Y. Tsuno, *J. Amer. Chem. Soc.*, 1957, **79**, 5530; 1959, **81**, 2007.
[18] W. H. Stein and S. Moore, *J. Biol. Chem.*, 1948, **176**, 367.

amount of H-Gly-OBu^t in ethyl acetate was studied as a model system for peptide formation. The results obtained are shown in Figure 2 and Table 5. Figure 2

TABLE 4

Rates of Curtius rearrangement of acyl-peptide azides in ethyl acetate

Azide	Temp. (°C)	10^3k_1/min^{-1}
Z-Gly-Phe-N_3	20	2.7
	30	10
Z-Gly-Tyr-N_3	20	2.5
	30	9.2
Boc Boc Z-Lys-Pro-Val-Gly-Lys-N_3	20	2.4
	30	11
Boc-Ser-N_3 [16]	30	6.1
Boc-Ser-Tyr-N_3 [16]	30	7.8
Boc-Gly-Tyr-Ser-Met-N_3	30	5.3

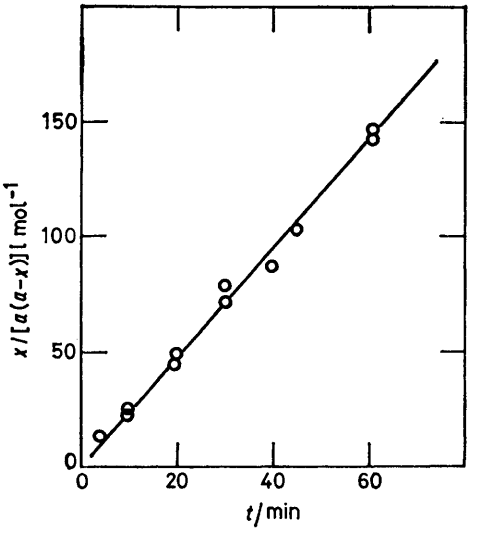


FIGURE 2 Aminolysis of Z-Gly-Phe-N_3 with H-Gly-OBu^t in ethyl acetate at 5 °C; a = initial concentration; x = concentration of H-Gly-OBu^t at time t; a = 10 mM for both reactants

TABLE 5

Rates of aminolysis of Z-Gly-Phe-N_3 with H-Gly-OBu^t in ethyl acetate

Initial conc. (mM)	Temp. (°C)	k_2/l mol^{-1} min^{-1}	$t_{\frac{1}{2}}$/min
10	5		42
16.7	5		22
33.3	5	2.8	10
66.7	5		4.7
16.7	5	2.8	22
16.7	10	3.4	18
16.7	20	4.1	15
16.7	30	5.9	10

is a plot of $x/[a(a - x)]$ against time (where a and x are concentrations of amine component at time 0 and time t, respectively). The satisfactory linearity confirms that the reaction follows the second-order law. Table 5 shows values of the rate constant k_2 and the half life $t_{\frac{1}{2}}$ of this second-order reaction under various conditions. These data indicate that the rate depends very much on the initial concentrations and to a relatively small extent on the temperature. In this respect the peptide formation offers a striking contrast to the first-order decomposition of azide. Table 6 summarises the results

TABLE 6

Rates of aminolysis of Z-Gly-Phe-N_3 with various amino-acid t-butyl esters in ethyl acetate

Ester	Conc. (mM)	Temp. (°C)	k_2/l mol^{-1} min^{-1}	$t_{\frac{1}{2}}$/min
H-Gly-OBu^t	33.3	5	2.8	10
H-Val-OBu^t	33.3	5	0.37	81
H-Glu(OBu^t)-OBu^t	33.3	5	0.23	134
H-Phe-OBu^t	33.3	5	0.15	200

obtained in the aminolysis of Z-Gly-Phe-N_3 with various amino-acid t-butyl esters under the same conditions. The reaction rate varies over a wide range depending on the nature of the amine component; *e.g.* k_2 for H-Phe-OBu^t is *ca.* 20 times smaller than that for the glycine ester.

The half-life values $t_{\frac{1}{2}}$ in Tables 2 and 5 indicate that for 16.7mM-solution and at 20 °C the aminolysis of Z-Gly-Phe-N_3 with H-Gly-OBu^t is only 17 times faster than the decomposition of the azide, whereas at 66.7mM and 5 °C the aminolysis proceeds 500 times faster than the decomposition. The conditions in the latter case are similar to those of azide couplings currently used in peptide synthesis, in which the initial concentration of reactants is usually *ca.* 0.1M and the temperature 0—5 °C. These conditions may generally be adequate for minimising the Curtius rearrangement. However, aminolysis by some amino-acid esters is not so fast as that by H-Gly-OBu^t (Table 6). In addition, the rate may also depend on the nature of the azide; when the azide is sterically hindered (*e.g.* valine azide [12a,b]) aminolysis may be much slower. In such cases the side reaction is likely to occur to a considerable extent. Since the Curtius rearrangement is a unimolecular reaction and the urea formation is thus inevitable, it should be borne in mind that peptide prepared by the azide method may be contaminated with urea if this side product is closely similar to the peptide in chemical and physicochemical properties.

EXPERIMENTAL

T.l.c. was performed on precoated silica-gel plates (Merck Kieselgel 60F_{254}) with the following solvent systems (ratios by volume): A, chloroform–methanol–acetic acid (95 : 5 : 3); B, chloroform–methanol–acetic acid (90 : 10 : 3); C, chloroform–methanol–acetic acid (80 : 15 : 10); D, ethyl acetate–acetic acid–water (3 : 1 : 1); E, butan-1-ol–acetic acid–water (4 : 1 : 2). For detection the plate was first sprayed with hydrobromic acid (sp. gr. 1.48), then heated at 150 °C for a few minutes (to dryness), and finally sprayed with ninhydrin (HBr–ninhydrin test). Kieselgel 60 (Merck) was used for silica gel column chromatography throughout. Samples for amino-acid analysis were hydrolysed with constant-boiling hydrochloric acid in evacuated sealed tubes at 110 °C for 24 h.

Z-Gly-Tyr-NH·NH_2.—Z-Gly-OH (9.62 g, 46 mmol) and H-Tyr-OMe (free base; 8.97 g, 46 mmol) were coupled with

DCC (9.48 g, 46 mmol) in DMF–ethyl acetate (1 : 5; 240 ml) to give Z-Gly-Tyr-OMe as an amorphous solid. This was then treated with hydrazine hydrate (5.75 ml, 115 mmol) in ethanol (100 ml) at 25 °C overnight to afford the crystalline *hydrazide* (16.1 g, 93%), m.p. 202—204° (decomp.), $[\alpha]_D^{23.5}$ +10.9 ± 0.5° (*c* 1 in M-HCl) (Found: C, 59.3; H, 5.7; N, 14.45. $C_{19}H_{22}N_4O_5$ requires C, 59.05; H, 5.75; N, 14.5%).

Z-Phe-NH·NH-Boc.—Z-Phe-OH (2.99 g, 10 mmol) and t-butyl carbazate (1.32 g, 10 mmol) were coupled with DCC (2.06 g, 10 mmol) in ethyl acetate (10 ml) at 4 °C overnight. The crude product was recrystallised from ether–petroleum to give the desired *compound* (3.92 g, 95%), m.p. 105—110°, $[\alpha]_D^{26}$ −27.0 ± 0.7° (*c* 1.0 in MeOH) (Found: C, 63.9; H, 6.6; N, 10.15. $C_{22}H_{27}N_3O_5$ requires C, 63.9; H, 6.6; N, 10.15%); homogeneous (HBr–ninhydrin) by t.l.c. in system B.

Z-Gly-Phe-NH·NH_2,HCl.—Z-Phe-NH·NH-Boc (8.27 g, 20 mmol) was hydrogenolysed over palladium in methanol and the product was coupled with Z-Gly-OH (4.18 g, 20 mmol) by the DCC method in the usual manner to give Z-Gly-Phe-NH·NH-Boc as an oil. This was then treated with M-hydrogen chloride in acetic acid (70 ml) at 25 °C for 60 min. The crystalline precipitate was filtered off, washed with acetic acid and ether, and dried *in vacuo* (5.7 g). Concentration of the filtrate gave more of the desired *product* (2.1 g). The two crops were combined and recrystallised from ethanol; yield 6.46 g (79%); m.p. 152—154° (decomp.); $[\alpha]_D^{24}$ +3.5 ± 0.3° (*c* 2.0 in 0.5M-HCl) (Found: C, 55.9; H, 6.0; Cl, 8.95; N, 13.7. $C_{19}H_{22}N_4O_4$,HCl requires C, 56.1; H, 5.7; Cl, 8.7; N, 13.75%).

Preparation of Azides (*General Procedure*).—The azides were prepared from the corresponding hydrazides immediately before use; the temperature was maintained at 0 °C throughout and the reagents had previously been chilled in an ice-bath.

To a solution of the hydrazide (1 mmol) in 1 : 1 water–tetrahydrofuran (4 ml) were added M-hydrochloric acid (2.5 ml) and 2M-sodium nitrite (0.55 ml), successively, and the mixture was stirred for 4 min. After addition of 50% potassium carbonate (3 ml) the mixture was extracted with ethyl acetate (7 ml × 2). The organic extracts were combined, dried ($MgSO_4$), and evaporated *in vacuo* to leave the azide.

Rate Determinations.—(a) *Curtius rearrangement.* The rate was determined by measuring the volume of nitrogen evolved.[17] The apparatus consisted of a round-bottomed flask and a gas burette with a three-way stop-cock and a drying tube in the line connecting the flask and the burette. The reaction mixture was not stirred, but a piece of sintered glass was added to prevent supersaturation by the nitrogen evolved.

The flask containing the azide, prepared from the corresponding hydrazide (1 mmol) as described above, was immersed in the bath at the appropriate temperature (±0.1 °C). To the azide was added the solvent (15 ml), previously equilibrated at the same temperature, and the mixture was swirled to effect dissolution. The stop-cock was then opened and the nitrogen evolved was collected. The volume of nitrogen was measured at intervals appropriate to the rate of the reaction. The reaction was followed to 80—90% completion. The bath was then removed and the flask was kept at room temperature overnight, then immersed again in the bath. The volume reading at this stage was taken as V_∞. In most cases, the observed values of V_∞ were 88—99% of the theoretical ones. The rate constant k_1 was calculated from equation (i), where V_t is the volume of nitrogen at time t. The half-life $t_{\frac{1}{2}}$ is expressed as (ln 2)/k_1.

$$k_1 t = \ln[V_\infty/(V_\infty - V_t)] \quad \text{(i)}$$

(b) *Peptide formation.* The rate was determined by measuring the consumption of an amine component by the ninhydrin method.

The flask containing Z-Gly-Phe-N_3, prepared from Z-Gly-Phe-NH·NH_2,HCl (0.407 g, 1 mmol) as described above, was immersed in the bath at a given temperature (±0.1 °C). A solution of an amino-acid t-butyl ester (amine component; 1 mmol) in ethyl acetate, which had been equilibrated at the same temperature, was then added. The concentration of the reactants was controlled by adjusting the volume of ethyl acetate used. At intervals samples were withdrawn from the mixture and immediately mixed with 0.5 ml of a ninhydrin solution [18] which had been kept in an ice-bath. The mixture was then heated on a boiling water bath for 15 min and cooled; after appropriate dilution with 50% ethanol, the absorbance at 570 nm was measured. A sample with no azide was also submitted to the ninhydrin reaction and the absorbance measured was taken as the value at time 0. The second-order rate constant k_2 was calculated from equation (ii), where a and x are the concentrations of amine component at times 0 and t, respectively; half life $1/(k_2 a)$.

$$k_2 t = x/[a(a - x)] \quad \text{(ii)}$$

Formation of Peptide and Urea.—(a) Z-Gly-Tyr-Gly-OBu^t and Z-Gly-NH·(CH)CH_2·C_6H_4·OH)·NH·CO-Gly-OBu^t (1). A solution of Z-Gly-Tyr-N_3, derived from the corresponding hydrazide (0.77 g, 2 mmol), in ethyl acetate (32 ml) was divided into four equal portions. The first portion was, after addition of H-Gly-OBu^t (0.10 g, 0.75 mmol), kept at 4 °C for 2 days (A). The second was evaporated *in vacuo* at a bath temperature of 0 °C and the residue was dissolved in DMF (10 ml). To this was added H-Gly-OBu^t (0.10 g) and the mixture was kept at 4 °C for 2 days (B). The third was kept at 4 °C for 21 h, after which H-Gly-OBu^t (0.10 g) was introduced. The mixture was then kept at 4 °C overnight (C). The last portion was treated in the same manner as the third, but the temperature was maintained at 25 °C throughout (D). T.l.c. in system B showed little or no urea formation in reaction mixtures (A) and (B). In (C), both peptide and urea were major products, but no peptide was detected in (D).

Reaction mixtures (C) and (D) were combined and chromatographed on a silica gel column (20 g) with 5% methanol in chloroform as solvent. The fractions containing peptide (I) and those containing urea (II) as main component were combined. Fraction (II) was evaporated and the residue was crystallised from methanol–ether to give the urea (0.19 g), m.p. 90—91°, $[\alpha]_D^{24}$ −6.7 ± 0.5° (*c* 1.0 in MeOH) [Found: C, 59.8; H, 6.2; N, 11.05. Calc. for compound (1) ($C_{25}H_{32}N_4O_7$): C, 60.0; H, 6.45; N, 11.2%]; amino-acid analysis (theoretical values in parentheses): Gly 1.31 (2), NH_3 1.17 (2), Tyr not detected (0).

Fraction (I) combined with reaction mixtures (A) and (B) was chromatographed on a silica gel column (20 g) with 2% methanol in chloroform as solvent. The fractions containing a major product as a single component were collected and evaporated *in vacuo* to give an amorphous

solid (0.51 g); $[\alpha]_D^{24.5}$ $-7.0 \pm 0.5°$ (*c* 1.0 in MeOH) [Found: C, 61.45; H, 6.55; N, 8.65. Calc. for Z-Gly-Tyr-Gly-OBu^t ($C_{25}H_{31}N_3O_7$): C, 61.85; H, 6.45; N, 8.65%].

(b) Z-Gly-Phe-Gly-OBu^t *and* Z-Gly-NH·CH(CH_2Ph)·NH·CO-Gly-OBu^t (2). A solution of the azide, prepared from Z-Gly-Phe-NH·NH_2,HCl (0.81 g, 2 mmol), in ethyl acetate (32 ml) was divided into four equal portions. To the first was added H-Gly-OBu^t (0.10 g, 0.75 mmol) and the mixture was kept at 4 °C for 2 days (A). The remaining portions were evaporated *in vacuo* at a bath temperature of 0 °C and the residues were dissolved in chloroform (8 ml each). To one of these was immediately added H-Gly-OBu^t (0.10 g) and the mixture was kept at 4 °C for 2 days (B). Another was kept at 4 °C for 21 h, after which H-Gly-OBu^t (0.10 g) was added. The mixture was then kept at 4 °C overnight (C). The last one was kept at 25 °C for 23 h before addition of H-Gly-OBu^t (0.10 g) and the mixture was kept at 25 °C overnight (D). The mixtures (A)—(D) were examined by t.l.c. (in system B; HBr–ninhydrin). (A) contained peptide and no detectable urea; (B) peptide and a smaller amount of urea; (C) urea and a smaller amount of peptide; (D) urea and no detectable peptide. These mixtures were then combined and evaporated *in vacuo*. The residue was chromatographed on a column of silica gel (50 g) with 3% methanol in chloroform as solvent. Fractions (7 ml) were collected and examined by t.l.c. in system B. The fractions corresponding to two major components were collected separately and evaporated *in vacuo*; tubes 25—38 and 39—60 afforded 0.33 and 0.40 g, respectively, of syrupy residue. The material derived from tubes 39—60 was crystallised from methanol–ether and recrystallised from ethyl acetate–ether; yield 0.30 g; m.p. 165—167°; $[\alpha]_D^{23.5}$ $-7.6 \pm 0.5°$ (*c* 1.0 in MeOH) [Found: C, 61.9; H, 6.9; N, 11.5. Calc. for compound (2) ($C_{25}H_{32}N_4O_6$): C, 61.95; H, 6.65; N, 11.55%]; amino-acid analysis: Gly 1.20 (2), NH_3 0.85 (2), Phe not detected (0).

The material derived from tubes 25—38 was treated with trifluoroacetic acid (TFA) at 25 °C for 60 min; the mixture was then evaporated *in vacuo* and the residue was crystallised from ether and recrystallised from ethyl acetate–ether; yield 0.20 g; m.p. 156—158°; $[\alpha]_D^{25}$ $-15.0 \pm 0.5°$ (*c* 1.0 in EtOH) [Found: C, 60.9; H, 5.75; N, 10.2. Calc. for Z-Gly-Phe-Gly-OH ($C_{21}H_{23}N_3O_6$): C, 61.0; H, 5.6; N, 10.15%]. This product was identical with authentic Z-Gly-Phe-Gly-OH.*

(c) [Z-Gly-NH·CH(CH_2Ph)·$NH]_2$CO (3). An ethyl acetate extract of Z-Gly-Phe-N_3, prepared from the corresponding hydrazide (0.81 g, 2 mmol) was kept, without drying, at 25 °C for 3 days. The crystalline precipitate (0.44 g) was recrystallised from ethanol; yield 0.17 g; m.p. 185—190° (decomp.); $[\alpha]_D^{23.5}$ $-3.1 \pm 0.5°$ (*c* 1.0 in DMF) (Found: C, 65.0; H, 5.95; N, 12.05. $C_{37}H_{40}N_6O_7$ requires C, 65.3; H, 5.9; N, 12.35%). This product was clearly distinguished from Z-Gly-Phe-NH_2 † on t.l.c. in system B.

Preparation of Ureas.—(a) Z-Lys(Boc)-Pro-Val-Gly-NH·CH($[CH_2]_4$·NH-Boc)·NH·CO-Lys(Boc)-Lys(Boc)-Lys(Boc)-NH_2 (4). Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-NH·NH_2 (0.24 g, 0.27 mmol) [19] was converted into the corresponding azide, which was dissolved in DMF–chloroform (1 : 6; 35 ml) and kept at 25 °C for 4 h, during which period the azide i.r. band at 4.75 μm had disappeared. To this was then added a solution of H-Lys(Boc)-Lys(Boc)-Lys(Boc)-NH_2 (free base; 0.16 g, 0.23 mmol)‡ in DMF (1 ml) and the mixture was, after concentration *in vacuo* to remove chloroform, allowed to stand at 25° C for 20 h. A gelatinous precipitate which had separated was filtered off (0.25 g) and crystallised from methanol (0.19 g, 54%); m.p. 223—225° (decomp.), $[\alpha]_D^{24}$ $-30.5 \pm 0.8°$ (*c* 1.0 in MeOH) (Found: C, 57.8; H, 8.25; N, 13.25. $C_{75}H_{129}N_{15}O_{20}$ requires C, 57.7; H, 8.35; N, 13.45%); amino-acid analysis: Lys 3.73 (4), NH_3 2.96 (3), Pro 0.97 (1), Gly 1.00 (1), Val 1.00 (1). The urea thus obtained could be distinguished from the corresponding peptide (Table 1)‡ by t.l.c. in system A.

(b) Z-Lys(Boc)-Pro-Val-Gly-NH·CH($[CH_2]_4$·NH-Boc)·NH·CO-Lys(Boc)-Arg-Arg-NH_2 (5). A solution of Z-Lys(Boc)-Pro-Val-Gly-Lys(Boc)-N_3 prepared from the corresponding hydrazide (0.44 g, 0.5 mmol) [19] in DMF–chloroform (1 : 6; 7 ml) was kept at 25 °C for 7 h. To this were then added H-Lys(Boc)-Arg-Arg-NH_2 acetate (0.32 g, 0.42 mmol) [15] and triethylamine (0.20 ml) with DMF (2 ml) as solvent. The mixture was kept at 25 °C overnight and evaporated *in vacuo*. To the residue were added ethyl acetate (30 ml) and M-acetic acid (30 ml), and the mixture was shaken vigorously. The organic phase was extracted with M-acetic acid (20 ml × 3). The aqueous solutions were combined, washed with ethyl acetate (20 ml × 3), concentrated *in vacuo* to *ca.* 20 ml, and extracted with water-saturated butan-1-ol (20 ml × 3). The extracts were combined, washed with M-acetic acid (20 ml × 3), and evaporated *in vacuo*, and the residue was precipitated from methanol–ether to give the desired *urea* in pure form (0.40 g, 66%); m.p. 148—150°; $[\alpha]_D^{23.5}$ $-31.1 \pm 0.8°$ (*c* 1.0 in MeOH) (Found: C, 53.0; H, 8.2; N, 17.8. $C_{65}H_{113}N_{19}O_{16}$,-$3H_2O$ requires C, 53.1; H, 8.15; N, 18.1%); amino-acid analysis: Lys 1.81 (2), NH_3 2.21 (3), Arg 1.89 (2), Pro 1.03 (1), Gly 0.98 (1), Val 1.00 (1). The product was homogeneous (HBr–ninhydrin and Sakaguchi reagent) on t.l.c. in system E, but could not be distinguished from the corresponding peptide (Table 1) [15] in the same solvent system.

(c) Boc-Gly-Tyr-Ser-NH·CH(CH_2·CH_2·SMe)·NH·CO-Gly-OBu^t. Boc-Gly-Tyr-Ser-Met-NH·NH_2 (0.58 g, 1 mmol) [16] was converted into the corresponding azide. The resulting solution in ethyl acetate was kept at 30 °C for 7.5 h (for kinetic measurements, see Table 4), then set aside at room temperature overnight. A small amount of precipitate was filtered off. To the filtrate was added H-Gly-OBu^t (0.13 g, 1 mmol) and the mixture was kept at room temperature overnight. The resulting precipitate (0.42 g) was chromatographed on a silica gel column (20 g)

* Z-Gly-Phe-Gly-OH was derived from the corresponding tripeptide t-butyl ester, which was synthesised stepwise from the *C*-terminal by the DCC procedure; m.p. 159—160°, $[\alpha]_D^{24}$ $-15.3 \pm 0.7°$ (*c* 0.9 in EtOH) {lit. m.p. 162.5—163°, $[\alpha]_D^{21}$ $-14.6 \pm 0.3°$ (*c* 1.3 in EtOH) (D. W. Clayton, J. A. Farrington, G. W. Kenner, and J. M. Turner, *J. Chem. Soc.*, 1957, 1398)}.

† Z-Gly-Phe-NH_2 was synthesised from Z-Gly-OH and H-Phe-NH_2 by the DCC method; m.p. 145—147°; $[\alpha]_D^{25}$ $0.0 \pm 1.0°$ (*c* 0.5 in DMF), $+6.0 \pm 1.0°$ (*c* 0.5 in MeOH).

‡ This compound was obtained as an intermediate in the synthesis of Z-Lys(Boc)-Pro-Val-Gly-[Lys(Boc)]$_4$-NH_2 (Table 1). Z-Lys(Boc)-NH_2, derived from Z-Lys(Boc)-OSu [15] by treatment with ammonia, was coupled with Z-Lys(Boc)-OSu repeatedly in combination with the removal of the Z group by catalytic hydrogenolysis to give H-[Lys(Boc)]$_4$-NH_2. This was then combined with Z-Lys(Boc)-Pro-Val-Gly-OH [19] by the DCC method to afford the octapeptide derivative; m.p. 221—223°, $[\alpha]_D^{24}$ $-36.6 \pm 0.8°$ (*c* 1.0 in MeOH) (K. Inouye, Y. Sumitomo, and M. Shin, unpublished data) {lit. m.p. 219—221°, $[\alpha]_D^{20}$ $-38°$ (*c* 1 in MeOH) (B. Riniker and W. Rittel, *Helv. Chim. Acta*, 1970, **53**, 513)}.

[19] H. Otsuka, K. Inouye, and Y. Jono, *Bull. Chem. Soc. Japan*, 1964, **37**, 1471.

with methanol–chloroform (2 : 98, 100 ml; 10 : 90, 200 ml) as solvent. The fractions containing the main product as a single component were collected and evaporated *in vacuo* and the *residue* was solidified by treatment with ethyl acetate (0.25 g, 36.5%); m.p. 156—158° (decomp.), $[\alpha]_D^{23}$ $-2.1 \pm 0.5°$ (*c* 1.0 in MeOH) (Found: C, 52.6; H, 7.2; N, 11.9; S, 4.75. $C_{30}H_{48}N_6O_{10}S$ requires: C, 52.6; H, 7.05; N, 12.25; S, 4.7%). The product was homogeneous on t.l.c. in system C.

(d) Boc-Gly-Tyr-Ser-NH·CH(CH_2·CH_2·SMe)·NH·CO-Glu(OBu^t)-His-Phe-Arg-Trp-Gly-OH (6). The azide prepared from Boc-Gly-Tyr-Ser-Met-NH·NH_2 (0.29 g, 0.5 mmol) [16] was allowed to undergo rearrangement as described above. To the resulting isocyanate were added H-Glu-(OBu^t)-His-Phe-Arg-Trp-Gly-OH (0.20 g, 0.2 mmol) [20] and triethylamine (0.07 ml, 0.5 mmol) with DMF (5 ml) as solvent, and the mixture was kept at room temperature. After several hours it was evaporated *in vacuo* and the gelatinous residue was solidified by trituration with ether (0.40 g). A portion (0.20 g) of this crude product was chromatographed on a column (3.4 × 150 cm) of Sephadex G-25 (medium) with butan-1-ol–acetic acid–water (4 : 1 : 2) as solvent. Fractions (10 ml) were collected and their absorption at 280 nm was measured. Tubes 41—50 corresponding to a main peak were combined and evaporated *in vacuo* and the residue was precipitated from methanol–ether to yield the desired urea (0.16 g); amino-acid analysis: Ser 0.99 (1), Glu 0.70 (1), Gly 2.00 (2), Met 0.02 (0), Tyr 1.01 (1), Phe 0.99 (1), His 1.00 (1), Arg 0.98 (1). The product was homogeneous (HBr–ninhydrin and Ehrlich reagent) on t.l.c. in systems D and E, but was inseparable from the corresponding peptide (Table 1) [16] in the same solvent systems. Amino-acid analysis revealed a small amount of methionine, indicating that the product was contaminated with some peptide.

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[20] K. Inouye, *Bull. Chem. Soc. Japan*, 1965, **38**, 1148.