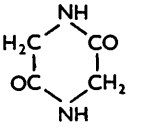
293. *Glycine Peptides. Part II.* The Heat and Entropy of Formation of the Peptide Bond in Polyglycine.*

By A. B. MEGGY.

Piperazine-2 : 5-dione polymerises to polyglycine in the presence of a limited amount of water, at 60—180°. The reaction at 140° has been studied in detail, and from a comparison with the results at 180° it is concluded that both the heat and the entropy of the reaction are small. From the degree of polymerisation of polymers obtained over the range 120—180°, the heat and the entropy of formation of the peptide bond in solid polyglycine have been calculated as 3·3 kcal. mole^{-1} and 17 cal. mole^{-1} degree^{-1}, respectively. Glycine polymerises to polyglycine at temperatures above 140°, in the presence of water, and of solid glycine. From the partial pressure of water in the system, it is calculated that for this reaction ΔH = 3·3 kcal. mole^{-1}, and ΔS = 5·9 cal. mole.$^{-1}$ degree^{-1}. On the assumption that the values are similar for the formation of peptide bonds from other amino-acids, synthesis of proteins under biological conditions is possible thermodynamically if the formation of the peptide bond is coupled with the hydrolysis of adenosine triphosphate, pyrophosphate, or polyphosphate.

IT has been shown [1] that heating piperazine-2 : 5-dione (I) with water at 180° yields a polymer of glycine. The reaction has now been studied at temperatures down to 60°. At 140° the reaction is complete in about two days, and at 120° in about three weeks; at 100° the reaction was incomplete after 14 weeks, and at 60° some polymer had been formed after three months. At 40° there was no sign of polymerisation after two years. The small amount of polymer formed at 60° was partially soluble in boiling water; the insoluble portion was identified as polyglycine by its *X*-ray diffraction pattern.



(I)

The reaction was studied in detail at 140°, the irreversible decomposition to ammonium carbonate and other products, which takes place at 180°, being then inappreciable. The results are summarised in Tables 1 and 2. The analytical methods are described in Part I. Table 1*a* shows the course of the reaction when the ratio of water to dione is 1 : 1 by weight. Column 2 gives the percentage of dione converted into insoluble

TABLE 1.

Time (hr.)	Percentage of total N as: polymer	$-NH_2$	dione	peptide	NH_3 or $(NH_4)_2CO_3$	Mean D.P. of sol. peptides
1	2	3	4	5	6	7
			a. Dione : water = 1 : 1 (*w*/*w*).			
7	2·5	3·4	83	5·5	—	1·62
19	60	21·5	20·9	20·3	2·0	0·95
24	65	18·8	17·9	17·9	1·6	0·95
42	60·5	23·8	10·9	27·6	1·8	1·18
114	50	33·8	7·3	35·9	5·9	1·06
			b. 10% *of dione replaced by glycylglycine.*			
7	39·5	9·5	40·2	17·5	—	1·84
17	55·5	13·2	22·0	25	—	1·90
30	56·5	16·9	15·8	27·3	0·9	1·62
51	60·5	22·7	10·8	26·8	1·3	1·18

polymer, which for all practical purposes is equal to the percentage of dione-nitrogen present as polymer-nitrogen. Columns 3, 4, and 5 refer to the aqueous phase. The degrees of polymerisation recorded in column 7 are obtained by dividing the figures in column 5 by those in column 3, and so refer to the soluble peptides. It is apparent from column 7 that from 19 hours onwards the aqueous phase contains only piperazine-2 : 5-dione, glycine, and a small

* Part I, *J.*, 1953, 851.

[1] Meggy, *J.*, 1953, 851.

amount of ammonia (or its carbonate), higher peptides being almost completely absent. (The minimum real value for column 7 is 1; the value of 0·95 arises from accumulating errors in the analytical methods.)

Table 1*b* shows the result of replacing 10% of the dione by glycylglycine. The initial stages of the reaction are greatly accelerated; after 7 hours there is about 40% polymer formation, compared with 2·5% without glycylglycine. However, the degree of polymerisation of the soluble peptides is between 1·5 and 2 for the first 30 hours, showing that glycylglycine is being formed by the hydrolysis of dione as quickly as it is being consumed in the formation of polymer. A similar catalytic effect is shown by small amounts of acids and bases. The results suggest that the first stage in the reaction is the hydrolysis of dione to glycylglycine, this being followed by the addition of dione molecules to the peptide. The later stages of the catalysed and the uncatalysed reaction are the same. Glycine and alanine also catalyse the reaction, though less effectively; however, when a polymer prepared with alanine as catalyst was hydrolysed with acid, and the hydrolysate was tested on a paper chromatogram, no spot for alanine could be detected; so it seems that, if alanine is present in the polymer in the early stages of the reaction as a terminal residue, it is removed later by hydrolysis.

Table 2 shows the effect of varying the ratio of water to dione. Polymer formation ceases when this ratio is between 3 : 1 and 4 : 1. With the water–dione ratio 3 : 1, the concentration of dione after 24 hours (Table 2*a*) is 43·4/3 = 14·3 g. per 100 g. of water; after 41 hours (Table 2*b*) there is a small increase in polymer formation. With a water–dione ratio of 4 : 1 there is no polymer formation after 24 hours, and the concentration of dione is 46·0/4 = 11·5 g. per 100 g. of water. The lower limit of dione concentration for polymer formation to take place must therefore lie between 11·5 and 14·3 g. per 100 g. of water. The estimated solubility of the dione at 140° is 16·6 g. per 100 g. of water, so that ΔG for the reaction: dione (solid) ⟶ polymer (solid), lies between −0·11 and −0·31 kcal. mole^{-1} at 140°. The value estimated[1] for 180° was −0·26 kcal. mole^{-1}, so that ΔG appears to change little with temperature. It seems probable that for this reaction

TABLE 2. *Effect of varying the dione : water ratio.*

Water/ Dione	Percentage of total N as polymer	$-NH_2$	dione	peptide	NH_3 or $(NH_4)_2CO_3$	Mean D.P. of sol. peptides
			Time: 24 *hr.*			
0·5	33	6·0	51·5	14·4	0·6	2·4
1	65	18·8	17·9	17·9	1·6	0·95
2	31·5	21·6	45·5	20·7	0·4	0·96
3	5·0	41·8	43·4	47·7	1·2	1·15
4	0	26·2	46·0	53·0	0·9	1·79
			Time: 41 *hr.*			
1	60·5	23·3	10·9	27·6	1·8	1·18
2	40·0	24·5	26·3	32·2	1·0	1·32
3	11·0	42·0	29·8	55·5	2·2	1·32

both ΔH and ΔS are small, in accordance with the calculations by Dainton, Devlin, and Small[2] for polymerisation of six-membered rings.

The equivalent weight of the polymers was determined by titrating them in saturated ealcium chloride solution with alkali after the addition of formaldehyde. A blank titration was necessary, in order to allow for acid in the formaldehyde and for ammonium salts in the calcium chloride. This method, which is a modification of that described by Sluyterman and Labruyere,[3] gave very reproducible results. The equivalent weight was also estimated by means of the absorption of Orange II (sulphanilic acid→β-napthol),[4] but this method showed certain anomalies; when these were eliminated, there was equivalence between the alkali titrations and the Orange II absorption, showing that the polymers contained equal numbers of carboxyl and amino-groups. A full account of this investigation will be published later.

[2] Dainton, Devlin, and Small, *Trans. Faraday Soc.*, 1955, **51**, 1710.
[3] Sluyterman and Labruyere, *Rec. Trav. chim.*, 1954, **73**, 347.
[4] Fraenkel-Conrat and Cooper, *J. Biol. Chem.*, 1944, **154**, 239.

In a polyamide melt the degree of polymerisation is determined by the homogeneous reaction:

$$x\text{-mer} + y\text{-mer} = (x + y)\text{-mer} + H_2O \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

which may be expressed as:

$$-CO_2^- + -NH_3^+ = -CO{\cdot}NH- + H_2O \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

Then
$$K_1 = \{-CO{\cdot}NH-\}\{H_2O\}/\{-CO_2^-\}\{-NH_3^+\} \quad . \quad . \quad . \quad (3)$$

K_1 is dimensionless and is the same for all values of x and y, including the occasion when x or $y = 1$.[5] The symbols in braces refer to activities.

It will be assumed that eqns. (1)—(3) can be applied also to the solid polyglycine phase in the present case, but with the exception that K has a special value when x or $y = 1$. In glycine the carboxyl and amino-groups are sufficiently near each other to influence each other; this is not so for the higher peptides, or for the systems for which eqns. (1)—(3) have been tested experimentally.

In solid polyglycine the different polymer species are all present in a single phase, as they are in a melt, and from *X*-ray diffraction data on the lower peptides of glycine it is very probable that the carboxyl and amino-groups are closely associated, so that reaction is possible without disruption of the crystal lattice. Application of eqns. (1)—(3) to the solid polymer phase is therefore plausible, as a working hypothesis, and since it leads to a heat of reaction which is in agreement with the value deduced from heats of combustion it appears justified.

In order to apply eqn. (3), it is necessary to find expressions for the activities of water, $-CO{\cdot}NH-$, $-CO_2^-$, and $-NH_3^+$ in terms of experimentally measurable quantities.

Consider a weight of the polymer, having degree of polymerisation n, which contains 1 g.-atom of nitrogen. The molecular weight of the repeating unit in the polymer is 57, so that the required weight will be $(57 + 18/n)$ g. This quantity of polymer will contain $(1/n)$ equiv. of $-CO_2^-$ groups, $(1/n)$ equiv. of $-NH_3^+$ groups, and $(1 - 1/n)$ equiv. of $-CO{\cdot}NH-$ groups.

The following are taken as standard states for the reactants: for water, liquid water at the temperature of the reaction; for $-CO{\cdot}NH-$, the $-CO{\cdot}NH-$ group in the polymer of infinite chain length; for $-CO_2^-$ and $-NH_3^+$, these groups in the polymer of infinite chain length.

Let the number of equivs. of any of the groups per g.-atom of nitrogen be (x). In the polymer of infinite chain length the activity of the carboxyl and amino-groups will be (x), and we may put $a = (x)$, or $a \rightarrow (x)$ as $n \rightarrow$ infinity. Since charged groups are involved, the ratio $a/(x)$ will diverge from unity as the concentration of charged groups in the polymer increases, *i.e.*, as n decreases. But over a narrow range of values of n the ratio $a/(x)$ may be taken as constant (denoted by γ), in analogy with an activity coefficient in an aqueous solution of an electrolyte. Then, $\gamma \rightarrow 1$ as $n \rightarrow$ infinity.

In the calculations in the present paper, n varies from 9 to 13, and it will be assumed that over this range the variation in γ may be neglected. Further, it is assumed that γ does not vary appreciably with temperature, again in analogy with aqueous solutions.

The value of (x) for the $-CO{\cdot}NH-$ group varies only from 0·89 to 0·92; moreover, this group carries no charge. It should be possible to put $a = (x)$ without appreciable error.

Inserting the appropriate quantities in eqn. (3) gives:

$$K_1 = a_{H_2O}.n(n - 1)/\gamma^2 \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

The equation is dimensionless. As γ cannot be evaluated from the data at present available, it will be assumed that it is unity.

The degree of polymerisation of the polymers from 100° to 180°, and the corresponding values of K_1 calculated by means of eqn. (4) are given in Table 3. Since in the reaction some of the piperazine-2 : 5-dione is hydrolysed to glycine, the liquid phase is a solution of

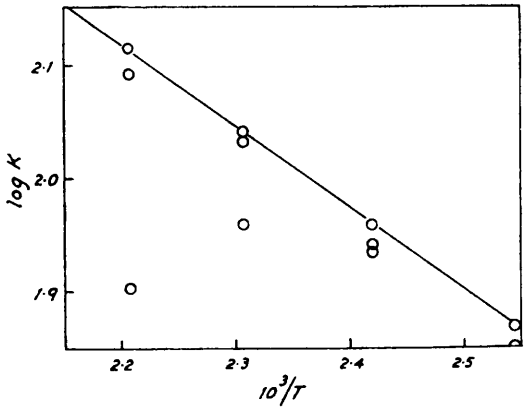
[5] Hermans, *J. Appl. Chem.*, 1955, **5**, 493.

glycine, in which the activity of water is less than unity. It is assumed that the activity of water in the polymer phase is the same as in the aqueous phase. From the yield of polymer the amount of glycine in the aqueous phase was calculated. The boiling points of

TABLE 3.

Temp.	Time (hr.)	Ratio, H_2O : Dione	Yield (%)	n	a_{H_2O}	K_1
180°	4	1	52	12·5	0·86	124
	7½	1	62	12·6	0·89	130
	3	2	13	10·1	0·87	80
160	8	1	60	10·7	0·88	91
	18	1	62	11·7	0·88	110
	48	1	60	11·6	0·88	108
140	41	1	60	10·7	0·88	91
	66	1	62	10·4	0·89	87
	120	1	58	10·7	0·88	91
	44	2	41	10·2	0·92	86
120	480	1	58	9·7	0·88	74
	650	1	60	9·5	0·88	71
100	720	1	68	7·7	0·91	47
	2500	1	68	7·9	0·91	49

a series of glycine solutions were determined in a Landsberg apparatus. If a particular solution boils at a temperature $T°$ and the vapour pressure of pure water at the same temperature is p atm., the activity of water in the glycine solution is $1/p$, approximately. It was assumed that in a solution of given concentration the activity of water relative to liquid water at the same temperature was constant from 100° to 180°. A graph was



constructed, which was linear, of a_{H_2O} against g. of glycine per 100 g. of water, and the activity of water in the aqueous phase was read off from it.

The values for K_1 obtained at 3 hours at 180°, at 8 hours at 160°, and at 30 and 104 days at 100° were not equilibrium values. The others may be. An attempt was made to approach the equilibrium from the other side. Polymer prepared at 180° was heated with water at 160° and 140°. After 18 hours at 160°, or 44 hours at 140°, there was no change in n. Heating for longer periods caused some decomposition, and both increases and decreases in n were observed. These results were without significance for determination of the equilibrium.

The values for K_1 given in Table 3 may be regarded as minimum values. The true equilibrium values may be greater; they can hardly be less.

In the Figure $\log K_1$ is plotted against $1/T$. The line is drawn with a bias towards the higher values of K_1, in view of the possibility that they may be on the low side. The line is given by the expression:

$$\log K_1 = 3{\cdot}72 - 725/T \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (5)$$

From this, for the reaction, x-mer + y-mer = $(x + y)$-mer + H_2O (liquid); $\Delta H = 3{\cdot}3$ kcal. mole^{-1} and $\Delta S = 17$ cal. mole^{-1} degree^{-1}.

Since glycine can be dehydrated to piperazine-2 : 5-dione at 140—180° in glycerol [6] or ethylene glycol,[7] and since it followed from the present work that the dione is thermodynamically unstable with respect to polyglycine at all temperatures from 60° to 180°, direct polymerisation of glycine to polyglycine appeared possible, and this was confirmed by experiment.

When glycine is heated with a small amount of hydrochloric acid in a sealed tube at a temperature above 140°, it is converted into polyglycine. The yield of polymer depends on the ratio of glycine to hydrochloric acid, and the results obtained by using 10N-hydrochloric are given in Table 4. No polyglycine is formed at 130°, and if tubes which have been heated to 140° are kept at 130° for 24 hours, the yield of polymer is reduced.

The essential conditions for the reaction appear to be a temperature above 140° and the presence of glycine as solid phase. Hydrochloric acid is not essential to the reaction; phosphoric acid, ammonium chloride, and sodium hydroxide are effective catalysts, or the reaction may be carried out with water only. However, hydrochloric acid gives the best yield and quality of polymer.

It is possible to carry out the reaction at atmospheric pressure in the following way. Sufficient water was added to the mixture of glycine and hydrochloric acid to dissolve all the solid at the boiling point. If this solution was evaporated immediately, glycine began to separate when the boiling point of the solution had risen to 115—117°. If the solution was first refluxed for 2 hours, and then evaporated, no solid separated. The boiling point of the solution rose steadily as the water was removed, and the residue became increasingly viscous. When the temperature had risen to 150—160° it was held there for a few hours, and the product was then extracted with water. The yield of polyglycine by this method varied greatly. It seems that during the preliminary refluxing some lower peptides of glycine are formed, and their presence interferes with the crystallisation of glycine, so that a supersaturated solution is obtained which can be heated to a temperature at which glycine can polymerise.

Polymerisation of glycine to polyglycine may be considered to take place by a heterogeneous reaction between glycine in solution and solid polyglycine, in which successive glycine molecules are added on to a polymer chain, thus :

$$\text{glycine} + x\text{-mer} = (x + 1)\text{-mer} + H_2O \quad . \quad . \quad . \quad . \quad . \quad . \quad (6)$$

This process by itself would lead to a continuous increase in the length of the polymer chains, and in the number average (n) of the degree of polymerisation. At the same time, however, hydrolysis of internal peptide bonds takes place. For every n molecules of glycine converted into polyglycine by reaction (6), one peptide bond is hydrolysed by the reverse of reaction (1), so that n remains constant. The degree of polymerisation is determined by K_1, according to eqn. (3). Glycine in the aqueous phase is also in equilibrium with soluble peptides and with piperazine-2 : 5-dione, but these are not here concerned. The equilibrium constant for eqn. (6) is given by :

$$K_2 = \{(x + 1)\text{-mer}\}\{H_2O\}/\{x\text{-mer}\}\{\text{glycine}\} \quad . \quad . \quad . \quad . \quad . \quad (7)$$

The standard state for glycine is solid glycine at the temperature of the reaction. The standard state for water is liquid water at the temperature of the reaction, as for eqn. (3). The standard state for the polymer is the solid polymer at the temperature of the reaction. In a polymer of number average degree of polymerisation n, the mole ratio [8,9] $(x + 1)$-mer to (x-mer) is $(n - 1)/n$, and the activity ratio will be the same. Eqn. (7) then becomes :

$$K_2 = (n - 1)\{H_2O\}/n\{\text{glycine}\} \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (8)$$

Eqn. (8) defines the activity of water in a system containing solid glycine and solid polyglycine. If the system also contains a liquid phase, that is, if glycine is in equilibrium with its saturated solution, the system will contain four phases ; glycine solution, solid

[6] Balbiano, *Ber.*, 1901, **34**, 1501; Maillard, *Ann. Chim.*, 1914, **1**, 519; **2**, 210.
[7] Sannie, *Bull. Soc. chim.* (*France*), 1942, **9**, 487.
[8] Flory, *Chem. Rev.*, 1946, **39**, 174.
[9] Meggy, *J.*, 1953, 796.

glycine, solid polyglycine, and water vapour. The system contains only two components, since all glycine peptides and polymers can be regarded as (x Glycine $-$ H_2O). Therefore in the presence of a liquid phase the system is invariant. There can be only one temperature at which four phases can co-exist, and this is a transition temperature. At all other temperatures, if a liquid phase is present, the solid phase may be either glycine or polyglycine. If both are present, no liquid phase can exist at equilibrium.

These conclusions are in agreement with the experimental results. The transition temperature lies between 130° and 140°. Below this temperature glycine is the stable solid phase in the presence of glycine solution; above it, polyglycine is the stable solid phase. Since polyglycine is stable at the higher temperature, the reaction n Glycine→ Polyglycine (n-mer) + $(n-1)H_2O$ must be endothermic.

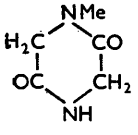
Experimental determination of K_2 is difficult. It is not possible to measure the activity of water for the system (solid glycine—solid polyglycine) over a range of temperatures, since equilibrium is not established in the absence of an aqueous phase. Polymer is not present in the presence of an aqueous phase below the transition temperature, at any rate in measurable amounts, since its solubility is very low. Consequently it is not possible to determine n. Above the transition temperature polymer is present in quantity, and n can be determined. The activity of water can also be determined from its partial pressure. But it is difficult to devise a simple method for determining the activity of glycine in the aqueous phase; even the determination of its concentration is rendered difficult by the presence of appreciable amounts of soluble peptides.

A single determination of K_2 at the transition temperature is possible, since at this temperature the activity of glycine is unity. It is only necessary to determine n, and the partial pressure of water.

Glycine was polymerised at 140°, in the presence of hydrochloric acid in a tube attached to a mercury manometer. After 24 hours at 140° the tubes were placed in a Silicone bath at 137°, and the manometer was read at intervals; a value was reached after 1 hour which remained constant for 6 hours. From the manometer reading the partial pressure of water was 1·2 atm. Since the vapour pressure of water [10] at 137° is 3·27 atm., $a_{H_2O} = 0{\cdot}36$. The degree of polymerisation was 12; from eqns. (4) and (5) the expected value is 16. If the transition temperature is assumed to be 137°, $K_2 = 0{\cdot}33$. If the transition temperature differs by a few degrees from 137°, this will not affect the value of K_2 at the transition temperature appreciably, since the variation of $(n-1)/n$ with temperature will be negligible, and the variation of a_{H_2O} will be small.

The transition temperature for the reaction, glycine(solid)→polyglycine(solid) + H_2O (liquid), being taken as 137°, $\Delta G = 0{\cdot}89$ kcal. mole^{-1} (glycine) at 137°.

Reactions of Other Piperazine-2 : 5-diones and Amino-acids.—Attempts to polymerise substituted piperazine-2 : 5-diones and amino-acids other than glycine were not successful. Heating sarcosylglycine with an equal weight of water at 140° for 20 hours converted it quantitatively into 1-methylpiperazine-2 : 5-dione (II), and no polymer was formed.



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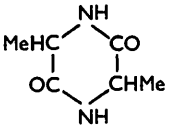

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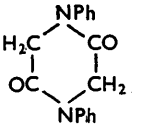

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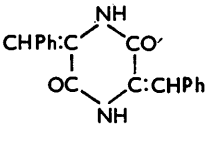


(V)

Under the same conditions glycylglycine gave polymer. 3 : 6-Dimethylpiperazine-2 : 5-dione (DL-alanine anhydride) (III) was hydrolysed to alanine and alanylalanine without polymer formation. 1 : 4-Diphenylpiperazine-2 : 5-dione (IV) (from N-phenylglycine [11]) and 3 : 6-dibenzylidenepiperazine-2 : 5-dione (V) (from benzaldehyde and piperazine-2 : 5-dione [12]) remained unchanged.

[10] International Critical Tables, Vol. III, p. 233.
[11] Bischoff and Hausdorfer, *Ber.*, 1892, **25**, 2271.
[12] Sasaki, *Ber.*, 1921, **54**, 163.

Tazawa [13] states that silk is depolymerised when heated in anhydrous ethylene glycol or glycerol to a mixture of diketopiperazines. This appears to be the reverse of the polymerisation of piperazine-2 : 5-dione; however, attempts to convert polyglycine into piperazine-2 : 5-dione by heating it in anhydrous glycerol have not been successful.

Attempts to polymerise amino-acids other than glycine were not successful. α-Amino-*iso*butyric acid was unchanged when heated with a little hydrochloric acid at 140° for 24 hours. At 220—240° a trace of a sublimate was formed, which may have been the anhydride, 3 : 3 : 6 : 6-tetramethylpiperazine-2 : 5-dione. On prolonged heating at this temperature, considerable pressure was developed, and the tubes burst. From the odour it seemed that decomposition had taken place to *iso*propylamine and carbon dioxide.

DL-Phenylalanine was partly decomposed under the reaction conditions, with the formation of tar. L- and DL-Alanine gave dark viscous products, completely soluble in water.

The higher amino-acids are less soluble than glycine; consequently the activity of water in their saturated solutions at 140° is greater than it is in the saturated solution of glycine at this temperature, and this would tend to prevent polymerisation. Two experiments were carried out in which glycine, DL-alanine, and 10N-hydrochloric acid were heated at 140°. The proportions of glycine to acid were 2 : 1 and 2·5 : 1, which, from Table 4, is

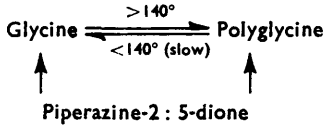
TABLE 4. *Direct polymerisation of glycine at* 140°.

Time of heating, 18 hr.; 1 ml. of 10N-HCl.

Glycine (g.)	2·0	2·2	2·6	2·8	3·5	5·0
Polymer (%)	0	0	?	5	20	53
Mean D.P. of sol. fraction	—	1·33	1·52	1·58	1·60	1·65

about the marginal ratio for polymer formation. Consequently, the activity of water in these experiments should be about the same as in the polymerisation of glycine. The product was almost completely soluble in water in both cases. However, the biuret reaction was pink, and the ratio of total nitrogen to amine-nitrogen was about 2·5. It seems that some peptide formation takes place under these conditions.

Discussion.—The relation between solid glycine, solid piperazine-2 : 5-dione, and polyglycine, in the presence of water, is :



In the presence of an aqueous phase, the dione is unstable with respect to glycine and polyglycine at all temperatures.

In the polymerisation of piperazine-2 : 5-dione, equilibrium was not reached between the polymer phase and glycine in solution. The solubility of glycine in water is 112 g./100 g. of water at 140°, by extrapolation from the data of Dunn, Ross, and Read,[14] and at or near this temperature polyglycine should be in equilibrium with the saturated solution of glycine. From Table 1, the concentration of glycine in the aqueous phase at 140° increases from 50 g./100 g. of water at 24 hours, to 80 g./100 g. of water at 114 hours, if the whole of the soluble nitrogen is regarded as glycine-nitrogen. This is still below the saturation value, and it seems that hydrolysis of the polymer is extremely slow, presumably because it is controlled by the rate of diffusion of the glycine formed by hydrolysis.

Although equilibrium was not established between the polymer phase and glycine in solution, it is believed that equilibrium was established between the various polymer species in the polymer, and water in the aqueous phase. Replicate experiments at the same temperature gave the same value of K_1, which did not change with time. The plot of log K_1 against $1/T$ was linear, and the heat of reaction calculated from the slope was

[13] Tazawa, *Acta Phytochim.* (*Japan*), 1942, **13**, 57.
[14] Dunn, Ross, and Read, *J. Biol. Chem.*, 1933, **103**, 579.

consistent with the heat of formation for the peptide bond in glycine-peptides calculated from combustion data.

The heats of combustion of glycine-peptides have been determined by Wrede.[15] The heat of formation of glycylglycine has been calculated by Borsook and Dubroff[16] from Huffman's data.[17] The calculated values for the heat of formation of the peptide bond in a number of glycine peptides (Table 5) show that the heat of formation of the peptide

TABLE 5. *Heat of formation of the peptide bond.*

Reaction	H (kcal.)	H per –CO·NH– (kcal.)	Ref.
$G + G = G_2 + H_2O$	5·50	5·50	16
$3G = G_3 + 2H_2O$	9·68	4·84	15
$4G = G_4 + 3H_2O$	13·10	4·36	,,
$G + G_2 = G_3 + H_2O$	4·18	4·18	,,
$G + G_3 = G_4 + H_2O$	3·42	3·42	,,
$2G$ = piperazinedione + $2H_2O$	7·90	3·95	,,

bond for successive glycyl residues tends to a value between 3 and 4 kcal. From the heat evolved in the enzymic hydrolysis of lactoglobulin, Linderstrøm-Lang[18] arrived at a similar estimate for the peptide bond in proteins. The value of 3·3 kcal. calculated from eqn. (5) is in good agreement with the thermochemical results. From the heat of formation of piperazine-2 : 5-dione, the heat of the reaction, piperazine-2 : 5-dione→polyglycine, is −0·65 kcal. per –CO·NH–. This is probably within the limits of error of the combustion data, but it indicates that ΔH for this reaction is small, in agreement with theory[2] and with the polymerisation experiments.

Since only a single determination of K_2 could be made it was not possible to determine ΔH for reaction (6) from the experimental results. However, it is probably not greatly different from the value 3·3 kcal. mole^{-1} for the reaction between two peptides, since from Table 5, for $G + G_3 = G_4 + H_2O$, $\Delta H = 3{\cdot}42$ kcals. If it be assumed that ΔH for reaction (6) is 3·3 kcal., then since $\Delta G = 0{\cdot}89$ at 137°, $\Delta S = 5{\cdot}9$ cal. mole^{-1} degree^{-1}.

Thus, assuming that $\Delta H = 3{\cdot}3$ kcal. mole^{-1} for reactions (1) and (6), $\Delta S = 17$ cal. mole^{-1} degree^{-1} for reaction (1) and 5·9 cal. mole^{-1} degree^{-1} for reaction (6). The difference of 11·1 cal. in the entropy for the two reactions is due mainly to the difference in the entropy of ionisation of glycine and the higher peptides.[19] For glycine, for the reaction,[20]

$$NH_3^+{\cdot}CH_2{\cdot}CO_2^- = NH_2{\cdot}CH_2{\cdot}CO_2H$$

$\Delta S = -1{\cdot}6$ cal. mole^{-1} degree^{-1}. For the higher glycine peptides[21] at 25°, $pK_a = 3{\cdot}00$, $pK_b = 7{\cdot}60$. For the conversion of the ionised into the non-ionised form, $\Delta G = 6{\cdot}24$ kcal. mole^{-1}. The heat of ionisation of glycine[20] is 9·65 and for glycyl-glycine 10·21 kcal. mole^{-1} at 25°. It has not been determined for the higher peptides, but if it is assumed to be the same as for glycylglycine, then for the higher peptides $T\Delta S = 3{\cdot}97$ kcals. at 25°, and $\Delta S = 13{\cdot}3$ cal. mole^{-1} degree^{-1}. The difference in the ionisation entropy of glycine and the higher peptides is therefore about 14·9 units at 25°, compared with a difference of 11·1 for the entropies of reactions (1) and (6) at 140°.

The difference in the ionisation entropy of glycine and the higher peptides is due to the increasing separation of the charged centres in the peptides.[19] For the tripeptide the separation is already so great that the carboxyl and amino-groups no longer influence each other. The same is observed when the groups are separated by a hydrocarbon chain. For 6-aminohexanoic acid,[21] $pK_a = 4{\cdot}43$, $pK_b = 10{\cdot}75$; these are practically the same as

[15] Wrede, *Z. phys. Chem.*, 1910, **75**, 92.
[16] Borsook and Dubroff, *J. Biol. Chem.*, 1940, **132**, 308.
[17] Huffman, *J. Phys. Chem.*, 1942, **46**, 885.
[18] Linderstrøm-Lang, Proc. 9th Solvay Congr., Brussels, 1953.
[19] *Idem*, Proc. 6th Int. Congress Exp. Cytology, Stockholm, 1950, p. 1; Meggy, *J. Appl. Chem.*, 1954, **4**, 154.
[20] Smith, *J. Biol. Chem.*, 1942, **146**, 193.
[21] Glasstone and Hammel, *J. Amer. Chem. Soc.*, 1941, **63**, 243; Cohn and Edsall, " Proteins, Amino-acids, and Peptides," Reinhold Publ. Corp., New York, 1943.

for an aliphatic acid and an aliphatic amine respectively. Consequently the values for the equilibrium constants K_1 and K_2 should be the same; this has been confirmed [5] for the polymerisation at 220°.

The dissociation constants of other α-amino-acids are approximately the same as for glycine,[21] and the dissociation constants for the α-amino- and the carboxyl groups in higher peptides are about the same as for the glycine-peptides.[21] It is probable that the heat and entropy for the formation of a peptide bond from an uncharged amino- and an uncharged carboxyl group are about the same for all α-amino-acids and peptides. It follows that the heat and entropy for the formation of a peptide bond between any two amino-acids will not differ greatly, on the average, from the values for glycine. This is supported by the estimate for the heat of hydrolysis of the peptide bonds in β-lactoglobulin.

At 37° the value of K_1 is 23·8, from eqn. (5), and $n = 5{\cdot}4$. It follows that if polyglycine of high molecular weight is treated with an endopeptidase capable of hydrolysing internal, but not terminal, peptide bonds, the polymer will be degraded to a number average degree of polymerisation of 5·4, corresponding to the hydrolysis of 15·7% of all the peptide bonds. The value of K_2 at 37° is 0·09; if the partially hydrolysed polymer is treated with a carboxy- or amino-peptidase, it will be hydrolysed completely to glycine. On the other hand, the original polymer of high molecular weight will be only slowly attacked by a carboxy- or amino-peptidase, as the number of terminal groups present will be small.

These conclusions resemble qualitatively and quantitatively the action of digestive enzymes on proteins. Pepsin hydrolyses only 15—20% of the peptide bonds; complete hydrolysis of the remainder takes place in the presence of trypsin and erepsin. The action of these enzymes on intact protein is slow, but it is rapid on protein which has been treated with pepsin. However, the action of enzymes on proteins is greatly influenced by specific action on particular types of peptide bond.

Since polyglycine is thermodynamically unstable with respect to glycine at 37°, direct formation of polymers from glycine at this temperature is not possible. Yet in biological systems the synthesis of proteins takes place from very low concentrations of amino-acids. If the heat and entropy of formation of the peptide bond in proteins are not greatly different from those for polyglycine, this synthesis must require a supply of energy. The most probable source is the so-called "high-energy" phosphate bond, usually a P–O–P bond, such as exists in adenosine triphosphate. It is known that this can bring about the synthesis of glutamine from glutamic acid and ammonia,[22] and of hippuric acid from glycine and benzoic acid,[23] and that the reactions are reversible. For the reaction,[24]

$$\text{ATP} + \text{H}_2\text{O} = \text{ADP} + \text{phosphate} \quad . \quad . \quad . \quad . \quad . \quad . \quad (9)$$

$\Delta G = -8{\cdot}00 \pm 0{\cdot}80$ kcal. at 25° and pH 7·0. For reaction (6), $\Delta G = 1{\cdot}83$ kcal. at 37°. Assuming −8·00 kcal. for reaction (9) at 37°, and combining reactions (6) and (9), we have:

$$\text{Glycine} + (x\text{-mer}) + \text{ATP} = (x+1)\text{-mer} + \text{ADP} + \text{phosphate} \quad . \quad . \quad (10)$$

for which $\Delta G = -6{\cdot}17$ kcal. at 37° and $K_3 = 2{\cdot}3 \times 10^4$, where:

$$K_3 = \{(x+1)\text{-mer}\}\{\text{ADP}\}\{\text{Phosphate}\}/\{x\text{-mer}\}\{\text{ATP}\}\{\text{Glycine}\} \quad . \quad (11)$$

The concentration of amino-acids in blood plasma is approx. 4×10^{-3} mole/l.; the concentration of phosphate [25] is approx. 10^{-3} mole/l. For eqn. (6) the standard state for glycine is the solid; at 37° the saturated solution is about 4M, so that the activity of glycine (solid) in a 4×10^{-3}M-solution is about 10^{-3}. For phosphate in eqn. (9) the standard state is the 1M-solution at pH 7·0. By inserting these values in eqn. (11), and putting $\{(x+1)\text{-mer}\}/\{x\text{-mer}\} = 1$, since the degree of polymerisation will be large, we have $\text{ADP}/\text{ATP} = 2{\cdot}3 \times 10^4$.

This signifies that in a solution 4×10^{-3}M with respect to glycine and 10^{-3}M with respect

[22] Levintow and Meister, *J. Biol. Chem.*, 1954, **209**, 265.
[23] Lipmann, *Fed. Proc.*, 1949, **8**, 597.
[24] Gutfreund, *Ann. Reports*, 1954, **51**, 301.
[25] Cameron, "Textbook of Biochemistry," Churchill, London, 1933.

to phosphate, and containing adenosine di- and tri-phosphate, the formation of polyglycine is possible thermodynamically if the molar ratio ADP/ATP is less than $2{\cdot}3 \times 10^4$. It should be noted that the ratio of the adenosine phosphates, not their total concentration, is the determining factor. The total concentration may be quite small. Provided that glycine is supplied at the right level, and the ADP/ATP ratio is maintained at the right level by some other reaction, *e.g.*, one involving the oxidation of carbohydrate, the synthesis of polyglycine could continue indefinitely.

The degree of polymerisation of the polymer will be determined by the reaction :

$$-CO_2^- + -NH_3^+ + ATP = -CO{\cdot}NH- + ADP + Phosphate \quad . \quad . \quad (12)$$

which is the combination of eqns. (2) and (9). Here we have :

$$K_4 = n(n-1)\{phosphate\}\{ADP\}/\{ATP\} \quad . \quad . \quad . \quad . \quad . \quad (13)$$

Since the solutions are 10^{-2}—10^{-3}M, a_{H_2O} may be taken as 1. For reaction (2) at 37°, $\Delta G = -2{\cdot}00$ kcal.; for reaction (9), $\Delta G = -8{\cdot}00$ kcal.; so that for reaction (12), $\Delta G = -10{\cdot}00$ kcal., and $K_4 = 10^7$. Putting $\{phosphate\} = 10^{-3}$, and $(ADP)/ATP = 2{\cdot}3 \times 10^4$, gives $n = 660$.

If the ratio $\{ADP\}/\{ATP\}$ is less than $2{\cdot}3 \times 10^4$, synthesis is possible from concentrations of glycine less than 4×10^{-3}, and the degree of polymerisation of the polymers will be greater than 660. It seems that, if the heat and entropy of formation of the peptide bond between any two naturally occurring amino-acids are on the average not greatly different from the values for glycine, the energy necessary for protein synthesis from amino-acids at the concentrations at which they are present in biological systems, to degrees of polymerisation such as are found in proteins, can be supplied by adenosine triphosphate or by any other compound at the same energy level, such as phosphocreatine. The energy available from the hydrolysis of a P–O–(C) bond, such as is found in adenosine monophosphate, glucose phosphates, or nucleic acids, is probably insufficient for protein synthesis. The energy available from the hydrolysis of an inorganic P–O–P bond, as in pyrophosphate or polyphosphate, is sufficient in principle to polymerise glycine in aqueous solutions at low temperatures. But it is necessary to provide a mechanism for coupling the hydrolysis of the P–O–P bond with the synthesis of the peptide bond. If solutions of glycine and sodium pyrophosphate or sodium polyphosphate (Calgon) are heated, there is a slow hydrolysis of the P–O–P bond, but no synthesis of polyglycine.

Experimental.

Determination of the Degree of Polymerisation.—A weighed amount of the polymer, dried at 100° for 1 hr., was dissolved in 42% calcium chloride solution, with warming if necessary. Dissolution of the polymer required ½—1 hr. Formaldehyde solution (40%; 10 ml.) was added, and the solution titrated with 0·1N-potassium hydroxide (to phenolphthalein). A control titration was carried out. The degree of polymerisation of the polymer was given by (equiv. wt.—18)/57.

Polymerisation of Glycine.—(a) *With hydrochloric acid.* Glycine (10 g.) was heated with concentrated hydrochloric acid at 140° (sealed tube) for 24 hr. and the product then extracted with boiling water. The solid material was separated at the centrifuge, washed twice with water, then twice with methanol, and dried at 100° or in a vacuum-desiccator ($CaCl_2$). The yield (4 g., 53%) was the same with 2N-acid and was not increased by heating for 65 hr.

(b) *With sodium hydroxide.* Glycine (5 g.) and 2N-sodium hydroxide (1 ml.) at 150° gave, in 19 hr., 1·3 g. (33%) of polymer.

Glycine (5 g.) with ammonium chloride (1·5 g.) in water (1 ml.) at 150° for 19 hr., with 85% phosphoric acid (2 ml.) at 140—150° for 60 hr., or with water at 140—150° for 68 hr. gave 20, 26, or 8%, respectively of polymer.

Polymerisation under Atmospheric Pressure.—Glycine (75 g.), concentrated hydrochloric acid (25 ml.) and water (60 ml.) were heated under reflux for 2 hr., then the water was distilled off during 1—2 hr., until the temperature was 140°. The mixture was kept at 140—150° for 18 hr. The product was boiled with water (150 ml.) until it disintegrated, then dissolved in a solution of calcium chloride (anhyd.; 800 g.) in water (1·2 l.), stirred with charcoal (10 g.) for 30 min. and filtered. The pale yellow solution was diluted to 5 l. and the polymer collected

at the centrifuge, washed with water and methanol, and dried at 100°. It formed a white powder (24 g., 42%).

Co-polymerisation of Glycine and DL-*Alanine.*—Glycine (2·5 g.), DL-alanine (2·5 g.), and concentrated hydrochloric acid (1 ml.) were heated for 19 hr. at 140°. The product was liquid when hot, except for a small residue, but solidified on cooling. It was dissolved in hot water (50 ml.) in which 0·1 g. was insoluble. The solution was decolorised with charcoal and filtered. The biuret reaction was magenta-red. Total N was determined by Kjeldahl's method, terminal N by neutralisation to methyl-red, addition of formaldehyde, and titration with alkali (to phenolphthalein). The ratio, total N : terminal N, was 2·6. In a second experiment, with 2 g. of glycine and 3 g. of alanine, the ratio was 2·5. No crystalline material was obtained from the solutions.

α-Amino*iso*butyric acid (5 g.) and 2N-hydrochloric acid (2 ml.) were unchanged during 19 hr. at 140—150°. Heating for 18 hr. at 220—240° gave only a trace of sublimate.

DL-Phenylalanine (1 g.) and 2N-hydrochloric acid (1 ml.) in 16 hr. at 140—150° gave only a little tar and an odour of phenylacetaldehyde.

p-Aminobenzoic acid (3 g.) was decomposed by water (3 g.) in 66 hr. at 140—160° completely to aniline and carbon dioxide.

Sarcosylglycine (1 g.) and water (1 g.) in 20 hr. at 140° gave a clear yellow solution; addition of alcohol (8 ml.) gave a trace of crystalline material (glycine ?). Evaporation of the filtrate and crystallisation of the residue (0·9 g.) from ethyl acetate gave material of m. p. 139—142° alone or mixed with sarcosylglycine anhydride.

1 : 4-Diphenylpiperazine-2 : 5-dione (3 g.) was unchanged by water (3 g.) in 65 hr. at 140—160°.

3 : 6-Dibenzylidenepiperazine-2 : 5-dione (3 g.) was unchanged by water (3 g.) for 65 hr. at 140—160°. After 24 hr. at 220—240° it was decomposed (odour of phenethyl alcohol): crystallisation of the product from water (charcoal) gave a small amount of crystals, m. p. 69—72°, probably impure phenylacetic acid.

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