LXVIII. THE BEHAVIOUR OF PEPTIDES IN AQUEOUS SOLUTIONS

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It has become clear of late that intermolecular forces are important in determining some of the characteristic features of the so-called "high-molecular substances" both in the solid and in the dissolved (colloidal) state. In the case of the gelatin sol, it has been shown by the present author [1927; 1928; 1931, 1] that if the temperature at which the gelatin solution is kept is varied from 10 to 37° the size of the physical unit (the gelatin micelle) is changed, being distinctly larger at lower temperature and smaller at higher temperature. These changes in the size of the physical unit are reversible, if the change in the temperature and the restitution of the original temperature occur within a short time interval, but become gradually irreversible if the solution is exposed to temperature changes for a longer period. Under these circumstances, apparently, the morphological arrangement of the molecules within the colloidal micelle is partly upset. The changes involved concern certain physico-chemical properties only; the chemical composition remains intact. Our findings, which have been confirmed by other authors [Gerngross *et al.* 1930; Liepatoff & Putilowa, 1934], were explained by the assumption that intermolecular forces play a part in the formation of the gelatin micelle. These forces are known to be influenced even by such slight changes in temperature as, in general, cause no chemical changes. The size of the physical unit of gelatin therefore varies according to the prevailing temperature and the history—in any case the thermal history—of the solution, whilst the chemical molecules remain intact. Such considerations are not limited to gelatin, but apply also e.g. to polysaccharides [*v.* Haworth & Hirst, 1936]. Here too a clear distinction must be drawn between the chemical molecule and the variable physical unit which is present in solutions.

In order to gain an insight into the nature of the intermolecular forces of proteins, and the action and dependence of these forces upon the size and the constitution of the chemical molecule, systematic studies of the various types of chemically known building stones of the proteins were undertaken.

It has already been shown [Frankel, 1930, 1] that intermolecular forces, recognizable by the appearance of association in aqueous solutions, come into play even in the case of simple α -amino-acids, such as glycine (confirmed by Cann [1932]; cf. also Hoskins *et al.* [1930]), arginine and others; though it was clear that association is not a common feature of all α -amino-acids. It therefore cannot depend exclusively upon the presence of the common structural group: $\cdot\text{CHNH}_2\cdot\text{COOH}$. As regards solutions containing two different amino-acids the existence of another type of intermolecular force, which leads to the formation in solution of salts between basic and acidic α -amino-acids, e.g. between arginine or histidine and glutamic or aspartic acid, was revealed. The necessity of taking into account such salt-like linkages in discussing the constitution of proteins,

both in the solid state and in the formation of protein micelles, was stressed [Frankel, 1931, 2]. Similar conclusions were reached for the keratin fibre by Speakman & Hirst [1931; 1933], through study of the action of acids on wool, and by Astbury [1933] on the basis of X-ray observations. These authors found that the salt linkages mentioned above constitute the lateral and longitudinal connexions between the long peptide chains of keratin which contains enough basic and acidic amino-acids to account for such linkages.

In continuation of previous work, the present paper deals with the behaviour of peptides in aqueous solution. On linking different amino-acids to form new molecules, several questions arise in regard to the intermolecular forces existing in solution.

(I) How does an amino-acid which exhibits association in pure solution behave in this respect:

(a) when it forms a peptide which contains two or more molecules of this amino-acid only;

(b) when in combination with another amino-acid which itself shows association in pure solution;

(c) when in combination with another amino-acid which itself does not associate?

(II) Does increase in length of the peptide chain alone cause an association; i.e. do peptides which contain two or more molecules of the same or of different amino-acids, neither of which is associated in pure solution, exhibit association?

The latter question was provoked by the following consideration. It has been suggested [Dunkel, 1928; Meyer & Mark, 1930] that, in homologous series, molecular cohesion as a measure of the intermolecular forces in the solid state is an additive property, which can be estimated by the summation of the increments contributed by the different structural groups of the molecule to the evaporation heat. Among the different structural groups contained in organic molecules, the group —CO.NH— , which is typical of the peptide linkage, is believed to contribute a specially high increment to the molecular cohesion. If these considerations can in principle be extended to the dissolved state, peptides might be expected to show an association in solution which would increase with increasing number of —CO.NH— groups in the molecule, independently of the specific properties of the amino-acids which constitute it.

Methods

Two methods were used in the experiments to be discussed: the cryoscopic and the refractometric. The former needs no special explanation, the well-known Beckmann arrangement, with electromagnetical stirring, being used: the usual correction was applied for supercooling. The refractometric method of investigating association is based on the rule which was experimentally demonstrated by previous investigations [Frankel, 1930, 2]. It was there shown that a relation exists between the values of the refractive indices of solutions of a pure substance in different concentrations and the state of the solute, and hence, also, changes in the state of the solute within the investigated concentration interval.

The refractive index of a given solution, n_a , at given temperature can be resolved into the constant refractive index of the pure solvent at the same temperature, n_s , and the increment of the refraction, A , caused by the dissolved substance. Expressing the concentration in g. solute per 100 g. solution, and assuming that no change in the molecular state of the solute occurs within the

concentration interval investigated, the increment, A , is proportional to the concentration, p , as defined above:¹

$$A = n_a - n_s = p \cdot \Delta_a \quad \dots (1);$$

Δ_a being the specific increment of the solute, and denoting the increment of the refractive index of the solvent per unit (1 g.) of dissolved substance in 100 g. solution. It will be shown elsewhere by the author & Katchalsky that this proportionality signifies the observance in this case of the law of mixture. On the other hand, if association occurs within the concentration range investigated, the observed values for the refractive indices will be lower than those calculated from the value of the lowest concentration investigated; in the case of dissociation, the observed refractive indices will be greater than those calculated. These generalizations were inferred from investigations of a considerable number of substances of known behaviour with regard to association and dissociation. All that is necessary in this method is the determination of the refractive indices of solutions of the substance in question in different concentrations and the comparison of the calculated values with those found.

When the solubility of the investigated peptides permitted, both methods were used. In all such cases, the dependence of the increase of the refractive indices upon the state of the solute was confirmed by the results of the cryoscopic measurements. In cases where, owing to lack of solubility or scarcity of material, no cryoscopic measurements could be carried out, the optical method alone was used. The latter can also be used in the investigation of comparatively weak solutions. The measurements were made with a Zeiss dipping refractometer, the temperature being kept constant to $\pm 0.05^\circ$ in a water-bath.

The investigated peptides

Twenty different peptides, prepared according to known methods, were studied. They may be divided into the following groups:

I. (a) Peptides built up of one species of amino-acid which is associated in aqueous solution: glycylglycine, diglycylglycine, triglycylglycine and tetraglycylglycine.

(b) Peptides built up of two (or more) different amino-acids each of which is associated: glycyl-*dl*-phenylalanine, glycyl-*l*-tyrosine and diglycylcystine. (The three other amino-acids besides glycine are probably associated as will be seen later.)

(c) Peptides containing both associating and normal (i.e. non-associating) amino-acids: glycyl-*dl*-leucine, *dl*-leucylglycine, *dl*-alanylglycine, diglycyl-*l*-leucine, *dl*-leucylglycylglycine, *dl*-leucyl-*l*-tyrosine and di-*dl*-leucylcystine. (The last two peptides probably, but not certainly, belong to this group.)

II. Peptides built up of amino-acids which do not associate singly in solution: the di-, tri- and tetra-peptides built of *dl*-alanine units; the di- and tri-peptides of *dl*-leucine and prolyl-*dl*-alanine.

Discussion and results

I. (a) *Peptides built up of an amino-acid unit which is associated in aqueous solution.* As mentioned above, glycine was chosen as an example of such an amino-acid and the series of its peptides, starting with the dipeptide and ending

¹ It should be noted that this regularity does not appear if the concentration is expressed in other terms than those stated above. As most authors have used other expressions for the concentration (e.g. g. or mol. per unit volume of the solvent or the solution), equation (1) has been overlooked.

with the pentapeptide, was investigated. The solubility of the first two compounds of this series permitted freezing-point determinations, as well as refractometric measurements. With the higher peptides, the optical method only could be used. The results are summarized in Table I (refractometric measurements) and Table II (cryoscopic measurements).

Table I. *Refractometric measurements of solutions of glycine peptides in different concentrations*

	g. solute in 100 g. solution	Refractive index		Difference $\times 10^5$ R.I. calculated - found
		Found 1.3.....	Calculated 1.3.....	
Glycyl- glycine $t = 17^\circ$	H ₂ O	33230	—	—
	0.4017	34054	—	—
	1.5989	36319	36504	18.5
	2.3901	37849	38152	30.3
	5.5020	43918	44520	60.2
Diglycyl- glycine $t = 19^\circ$	H ₂ O	33083	—	—
	0.3322	33736	—	—
	0.5029	34046	34070	2.6
	0.9997	34982	35048	6.6
	1.3932	35705	35821	11.6
	3.0498	38865	39077	21.2
Triglycyl- glycine $t = 20.4^\circ$	H ₂ O	32966	—	—
	0.2761	33523	—	—
	0.3509	33592	33674	8.2
	0.9094	34701	34801	10.0
	1.3599	35472	35710	23.8
	2.7040	37927	38422	49.5
Tetraglycyl- glycine $t = 22^\circ$	H ₂ O	32810	—	—
	0.06267	32939	—	—
	0.1264	33052	33070	1.8
	0.1613	33091	33142	5.1
	0.4184	33550	33670	12.0
	0.6268	33970	34100	13.0

Table II. *Cryoscopic measurements of glycylglycine and diglycylglycine in various concentrations*

	g. solute in 100 g. H ₂ O	Mol. weight found
Glycylglycine; mol. weight calculated: 132.08	9.077	146.8
	7.564	144.4
	6.483	143.9
	5.988	143.4
	3.010	134.0
	2.150	132.0
Diglycylglycine; mol. weight calculated: 189.11	5.387	205.9
	4.310	203.5
	3.078	197.4

All these peptides showed association in aqueous solution. The cryoscopic measurements carried out with glycine [Frankel, 1930, 1] and now with glycylglycine and diglycylglycine allow a comparison of the degrees of association (expressed as the association factor) of these three compounds which differ in the size of their molecules. Fig. 1 shows clearly that, with theoretically equimolar concentrations, the degree of association increases with increasing size of the molecule.

(b) *Peptides built up of different amino-acids each of which is associated in its pure solution.* No definite proof exists as to the association of tyrosine,

Fig. 1. Dependence of the association of glycine, glycylglycine and diglycylglycine on the molar concentration.

Table III. *Refractometric measurements of glycyl-L-tyrosine, diglycylcystine and glycyl-DL-phenylalanine in different concentrations*

	g. solute in 100 g. solution	Refractive index		Difference $\times 10^5$ R.I. calculated - found
		Found 1.3.....	Calculated 1.3.....	
Glycyl- L-tyrosine $t=26^\circ$	H ₂ O	32420	—	—
	0.2523	32966	—	—
	0.3197	33091	33113	2.2
	0.8301	34076	34217	14.1
	1.2415	34877	35107	23.0
	2.4712	37430	37769	33.9
Diglycyl- cystine $t=25^\circ$	H ₂ O	32518	—	—
	0.2918	33130	—	—
	0.3705	33246	33295	4.9
	0.9602	34350	34532	18.2
	1.4362	35244	35530	28.6
	2.8550	37849	38505	65.6
Glycyl-DL- phenyl- alanine $t=27.9^\circ$	H ₂ O	32192	—	—
	0.2568	32791	—	—
	0.3261	32889	32952	6.3
	0.8456	33989	34165	17.6
	1.2659	34896	35147	25.1
	2.5207	37695	38082	38.7

cystine and phenylalanine, as their very slight solubilities preclude measurements. One must therefore rely on inferences drawn from closely related compounds in classifying these acids: e.g. dihydroxyphenylalanine, which differs

from tyrosine by an additional hydroxyl group, is strongly associated [Frankel, 1930, 1]. For the three peptides grouped under this heading, the refractometric measurements (cf. Table III) indicate association.

(c) *Peptides built out of two different amino-acids, only one of which shows association.* In this group, seven different peptides (listed above) were investigated, four of them by both the cryoscopic and the refractometric methods, the remaining three (owing to insufficient amount at our disposal) by the latter method only. The associating amino-acids contained in these peptides are glycine, tyrosine¹ and cystine;¹ the non-associating amino-acids, alanine and leucine. As will be seen from Tables IV and V, which present the results of the cryoscopic and refractometric methods respectively, both methods gave consistent results: the molecular weights found for each peptide are practically identical with the calculated values, and remain constant within the investigated concentration

Table IV. *Refractometric measurements of glycyl-dl-leucine, diglycyl-l-leucine, dl-leucylglycine, dl-leucylglycylglycine, dl-alanylglycine, dl-leucyl-l-tyrosine and di-dl-leucylcystine*

	g. solute in 100 g. solution	Refractive index		Difference × 10 ⁵ R.I. calculated - found
		Found 1.3.	Calculated 1.3.	
Glycyl- dl-leucine <i>t</i> = 19.5°	H ₂ O	33052	—	—
	0.4123	33837	—	—
	1.5835	36033	36067	3.4
	3.1730	39074	39093	1.9
Diglycyl- l-leucine <i>t</i> = 29.5°	H ₂ O	32020	—	—
	0.2757	32502	—	—
	0.3501	32642	32628	- 1.4
	0.9075	33596	33603	0.7
	1.3582	34497	34391	- 10.6
	2.7032	36865	36746	- 11.9
dl-Leucyl- glycine <i>t</i> = 18.6°	H ₂ O	33122	—	—
	0.4242	33900	—	—
	1.5903	36014	36038	2.4
	3.2368	39055	39057	0.2
dl-Leucyl- glycyl- glycine <i>t</i> = 26.4°	H ₂ O	32374	—	—
	0.2641	32857	—	—
	0.3354	32978	32987	0.9
	0.8697	33970	33968	- 0.2
	1.3020	34740	34755	1.5
	2.5938	37115	37116	0.1
dl-Alanyl- glycine <i>t</i> = 25°	H ₂ O	32529	—	—
	0.2492	32986	—	—
	0.3164	33122	33107	- 1.5
	0.8202	34008	34028	2.0
	1.2277	34748	34773	2.5
	2.4434	36974	36995	2.1
dl-Leucyl- l-tyrosine <i>t</i> = 25°	H ₂ O	32517	—	—
	0.2341	32954	—	—
	0.2972	33044	33072	2.8
	0.7709	33970	33956	- 1.4
	1.1543	34720	34672	- 4.8
	2.3021	36917	36811	- 10.6
Di-dl-leucyl- cystine <i>t</i> = 30°	H ₂ O	31960	—	—
	0.2201	32370	—	—
	0.2795	32463	32480	1.7
	0.7246	33295	33310	1.5
	1.0847	33970	33980	1.0
	2.1598	35976	35983	0.7

¹ Cf. remark under heading I (b).

Table V. *Cryoscopic measurements of glycyl-dl-leucine, dl-leucylglycine, dl-leucylglycylglycine, and dl-alanyl-glycine*

	g. solute in 100 g. H ₂ O	Mol. weight found
Glycyl- <i>dl</i> -leucine; mol.	9.248	185.0
weight calculated: 188.4	7.707	185.7
	5.1377	189.4
<i>dl</i> -Leucylglycine; mol.	2.500	184.1
weight calculated: 188.4		
<i>dl</i> -Leucylglycylglycine;	8.0948	244.5
mol. weight calculated:	6.2971	245.0
245.17	5.6674	246.8
<i>dl</i> -Alanyl-glycine; mol.	8.0233	147.3
weight calculated: 146.1	6.2426	146.9
	5.1075	146.5
	3.7455	146.1

interval; the refractive indices for the different peptide solutions in different concentrations are practically the same as the calculated values, and thus indicate that no changes occur within the limits of these concentrations. In the cases in which refractometric measurements only were carried out, the optical method likewise revealed a practical identity between calculated and observed refractive index values. All these results show that peptides obtained by linking an associating with a non-associating amino-acid exhibit normal behaviour in solution, i.e. when an associating amino-acid enters into a peptide together with a non-associating amino-acid, the association of the former is entirely suppressed.

The fact, that unlike the corresponding compounds with glycine, leucyl-tyrosine and dileucylcystine do not show association may, in view of the experimental evidence that glycine is associated and leucine is not, be regarded as an additional indication that tyrosine and cystine themselves belong to the associating amino-acids.

II. *Peptides built up only of amino-acids which do not associate.* In previous work, it was found [Frankel, 1930, 1, 2] that alanine and leucine were "normal" amino-acids. These acids were mainly used to build up the peptides which were tested.

The *dl*-series, alanine, alanylalanine, dialanylalanine and trialanylalanine was investigated for the purpose of finding out whether repetition of the —CO.NH— group causes the phenomenon of association in a peptide which is entirely built up of non-associating amino-acids. With higher alanine peptides, the investigation becomes impossible as they are insoluble. Table VI gives the results of the refractometric method for the series of alanine peptides; the calculated and found values all agree closely. None of the peptides shows signs of association, although the number of —CO.NH— groups and the size of the molecule are both increased. It can therefore be safely stated that, in the case of the dissolved state, simple repetition of the —CO.NH— group does not itself cause association. Analogous results are furnished by the investigation of the series leucine, leucylleucine, and dileucylleucine (cf. Table VI and previous results [Frankel, 1930, 1]). Here, too, the investigation is limited by the insolubility of the higher peptides. The di- and tri-peptides built up by *dl*-leucine, like the amino-acid itself, are not associated. Prolylalanine is another example of a dipeptide containing two different amino-acids, neither of which is associated. This dipeptide, too, is normal in solution. Table VII gives the molecular weights

Table VI. *Refractometric measurements of solutions of alanine peptides, of leucine peptides and of prolylalanine in different concentrations*

	g. solute in 100 g. solution	Refractive index		Difference $\times 10^5$ R.I. calculated - found
		Found 1.3.....	Calculated 1.3.....	
<i>dl</i> -Alanyl- <i>dl</i> -alanine $t = 30^\circ$	H ₂ O	31962	—	—
	0.2696	32447	—	—
	0.3423	32580	32578	-0.2
	0.8874	33580	33559	-2.1
	1.3279	34358	34350	-0.8
	2.6430	36748	36716	-3.2
Di- <i>dl</i> -alanyl- <i>dl</i> -alanine $t = 25^\circ$	H ₂ O	32517	—	—
	0.2733	33001	—	—
	0.3471	33122	33126	0.4
	0.8995	34084	34097	1.3
	1.3465	34857	34901	4.4
	2.6788	37259	37262	0.3
Tri- <i>dl</i> - alanyl- <i>dl</i> - alanine $t = 27^\circ$	H ₂ O	32306	—	—
	0.2726	32783	—	—
	0.7071	33496	33543	4.7
	1.0589	34141	34159	1.8
	2.1107	35984	35999	1.5
<i>dl</i> -Leucyl- <i>dl</i> -leucine $t = 24^\circ$	H ₂ O	32615	—	—
	0.1105	32810	—	—
	0.1404	32849	32863	1.4
	0.3643	33200	33258	5.8
	0.5457	33580	33579	-0.1
	1.0890	34545	34536	-0.9
Di- <i>dl</i> -leucyl- <i>dl</i> -leucine $t = 25^\circ$	H ₂ O	32521	—	—
	0.2349	32927	—	—
	0.2984	33056	33037	-1.9
	0.7751	33861	33860	-0.1
	1.1620	34529	34530	0.1
	2.3240	36534	36537	0.3
Prolyl- <i>dl</i> - alanine $t = 28^\circ$	H ₂ O	32192	—	—
	0.2735	32697	—	—
	0.3473	32810	32833	2.3
	0.9005	33853	33854	0.1
	1.3478	34662	34680	1.8
	2.6835	37164	37144	-2.0

Table VII. *Cryoscopic measurements of dl-alanyl-dl-alanine and dl-leucyl-dl-leucine*

	g. solute in 100 g. H ₂ O	Mol. weight found
<i>dl</i> -Alanyl- <i>dl</i> -alanine; mol. weight calculated: 160.11	6.8883	153.2
	5.3575	156.2
	4.3834	154.1
	3.7091	156.8
<i>dl</i> -Leucyl- <i>dl</i> -leucine; mol. weight calculated: 244.2	0.7807	242

found for *dl*-alanyl-*dl*-alanine, and for *dl*-leucyl-*dl*-leucine; the results are consistent with those of the refractometric method and show normal behaviour. The experiments noted under I (a), on the other hand, prove that peptide molecules of the same or even smaller size will exhibit association if the amino-acids of which they are composed show association themselves.

SUMMARY

1. A number of peptides have been investigated, either by cryoscopic and refractometric measurements jointly, or by the latter method alone, as regards association in aqueous solution.

2. Peptides, which are built up of associating α -amino-acids only, show association in solution. Cryoscopic measurements of glycine and its di- and tri-peptides show an increasing association with increase in size of the peptide molecule.

3. Peptides built up both of associating and non-associating amino-acids are not associated in solution.

4. With a peptide series containing non-associating amino-acids only, no support was found for the assumption that simple enlargement of molecular size and repetition of the $-\text{CO.NH}-$ group, typical of the peptides, could induce a tendency of the molecule to associate in solution. Such a tendency in fact exists, however, if the α -amino-acids building up the peptides themselves possess this property.

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