XXIV. THE TITRATION CONSTANTS OF SOME AMIDES AND DIPEPTIDES IN RELATION TO ALCOHOL AND FORMALDEHYDE . TITRATIONS OF AMINO-N.

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MEASUREMENT of the titration constants of simple peptides is of biochemical interest for two reasons. Firstly, it provides information necessary for careful titrimetric estimation of amino-N in biological fluids or enzymic proteolysates [Richardson, 1934, 1, 2]. Secondly, it facilitates interpretation of protein titration curves by helping to define the change of titration constant which results from peptide formation [Cohn, 1931].

For gelatin hydrolysates and various synthetic dipeptides, suitable data are already available [Cannan and Muntwyler, 1930; Greenstein, 1933; and others]. There is therefore no difficulty in deducing the extent of error due to these substances in alkalimetric formaldehyde or alcohol titrations of amino-N [Richardson, 1934, 2]. Yet there exists an important group of substances containing amino-N about which there has been insufficient information hitherto. These, the amino-acid amides, may be quite plentiful in certain plant extracts and probably of enzymic proteolysates [*cf.* Melville, 1935]. Titration constants for certain typical derivatives have therefore been examined, together with data for some peptides of possibly distinctive behaviour. In addition, the usefulness of *iso*-glutamine as a readily synthesised buffer of value in peptidase experiments is described.

EXPERIMENTAL.

The glutamic acid derivatives were synthesised from *d*-glutamic acid by Bergmann's benzylchlorocarbonate method [Melville, 1935], while for other materials we are deeply indebted to Prof. Max Bergmann [Bergmann *et al.*, 1929; 1932]. In our experience, all materials required several recrystallisations to free them from traces of titratable contaminants, primarily affecting the alkaline curves. The glutamic acid derivatives, which showed this effect initially, yielded admirable data on recrystallisation [Melville, 1935, p. 182]. Other materials were not further recrystallised, and one of these, *d*-tyrosyl-*d*-arginine, has required suitable allowance for a titrated impurity (5 % of the group titre). Similar contaminants in *l*-isoasparagine and *d*-alanyl-*l*-proline had a sufficiently alkaline range (about 9.6, suggestive of free amino-acids) to give no serious interference in the constants.

Titration from a micro-burette was carried out with hydrogenated 0.1 *M* NaOH or HCl with the recognised precautions, the reagents being standardised against Na₂CO₃, potassium hydrogen phthalate and constant-boiling HCl as ultimate standards. Separate solutions were used for acid and alkaline curves

except in the cases of *d*-glutaminyl-*d*-glutamic acid and *d*-tyrosyl-*d*-arginine. Where a preparation was not originally isoelectric, a suitable correction (not exceeding 2 %) has been applied to both curves. Attempts to titrate the benzylcarbonato intermediaries at a hydrogen electrode were naturally abortive, owing to catalytic reduction with characteristic decay of electrode potential.

The apparatus has been described elsewhere [Melville and Richardson, 1934]; for titration at considerable acidity, however, a constricted vessel of 2 ml. minimum capacity was used. Junction with saturated KCl solution was made smoothly *in situ* about 10 min. before titration, and wandering potentials in acid solution were minimised by careful selection of bridges. Occasional renewal of the KCl surface was effected, in prolonged titration at acid extremes, by discharging small measured volumes of KCl from the bridge, the small salt increments being included in the estimated ionic strength. Electrodes were freshly palladised, gold-plated platinum wires, and working temperature was $25 \pm 0.02^\circ$. The reference potential assumed for a hydrogen electrode in 0.05 *M* potassium hydrogen phthalate was 0.2347 v. at this temperature [Clark, 1928]. Duplicate electrodes seldom disagreed by more than 0.0001 v.

As usual, observed titres were corrected for free acid and alkali at the p_H extremes, the simple Debye-Hückel relation modified by Simms [1926] being used to calculate hydrion concentrations from p_H ; thus, $p_H = \log \frac{1}{[H^+]} - \log \gamma$, where $-\log \gamma = 0.3 \sqrt{\mu}$ (Simms quotes $0.2 \sqrt{2\mu}$). μ , the ionic strength, was estimated without inclusion of the zwitterion. Hydroxyl ion concentrations were obtained correspondingly, the ionic (activity) product of water being accepted as $10^{-13.995}$ at 25° [Clark, 1928]. The calculated constants, G' etc., are titration constants [von Muralt, 1930] uncorrected for activity of the ions concerned. Thus since ionic strength was not constant during titration, a titre which provides the best carboxyl constant may differ slightly from that providing the best amino constant. Carboxyl and amino constants are both expressed as hydrion dissociation constants (Brønsted-Lowry) using the Sørensen logarithmic nomenclature, $p_{G'}$ etc.

Results.

In Table I, full data for *d*-glutamine and *d*-isoglutamine are quoted, data for other materials being suitably abbreviated. The possibility that the amides would suffer hydrolysis during titration has fortunately not been realised. Thus *d*-glutamine and its peptides are sufficiently stable even at 37° [Melville, 1935] to permit a reasonable titration; at 25° , steady titration produced the consistent data of Table I. Measurements on glycyl-*L*-proline and *d*-tyrosyl-*d*-arginine had been completed before Greenstein's paper [1933] appeared. Glycyl-*L*-proline gave good constants in accord with Greenstein's on an identical sample, so that further detail is not required (Table II). The samples of *d*-tyrosyl-*d*-arginine were apparently not identical, however, and results are more fully detailed as there is considerable divergence. The source of disparity is difficult to ascertain in the absence of more particulars than are given in his paper, but our constants seem consistent enough to be accepted (except that the fourth constant (guanidine) is impaired by the large free alkali correction and by a necessary assumption of 5 % of impurity of $p_{G'}$ 10.1).

In Table II, the mean constants of Table I are collected and compared with accepted constants for related compounds (expressed on a zwitterionic basis).

Table I. *Calculation of titration constants.*

Titrated solutions were all prepared by weight.

(a) <i>d</i> -Glutamine	(mol. wt. 146)	—COOH 3 ml. 0.25 <i>M</i> titrated with 0.197 <i>M</i> HCl. —NH ₂ 10 ml. 0.037 <i>M</i> „ 0.103 <i>M</i> NaOH.
(b) <i>d</i> -isoGlutamine	(146)	—COOH 10 ml. 0.030 <i>M</i> titrated with 0.103 <i>M</i> HCl. —NH ₂ 10 ml. 0.032 <i>M</i> „ 0.103 <i>M</i> NaOH.
(c) <i>l</i> -isoAsparagine monohydrate	(150)	—COOH 10 ml. 0.056 <i>M</i> titrated with 0.0995 <i>M</i> HCl. —NH ₂ 10 ml. 0.049 <i>M</i> „ 0.101 <i>M</i> NaOH.
(d) <i>d</i> -Alanyl- <i>l</i> -proline	(186)	—COOH 10 ml. 0.034 <i>M</i> titrated with 0.0995 <i>M</i> HCl. —NH ₂ 10 ml. 0.035 <i>M</i> „ 0.101 <i>M</i> NaOH.
(e) <i>d</i> -Glutaminylglycine	(203)	—COOH 2 ml. 0.21 <i>M</i> titrated with 0.103 <i>M</i> HCl. —NH ₂ 10 ml. 0.042 <i>M</i> „ 0.103 <i>M</i> NaOH.
(f) <i>d</i> -Glutaminy- <i>d</i> -glutamic acid	(275).	3.6 ml. 0.10 <i>M</i> solution in 0.2 <i>M</i> HCl titrated with 0.103 <i>M</i> NaOH (titres corrected for excess HCl).
(g) <i>d</i> -Tyrosyl- <i>d</i> -arginine	(337).	—COOH 10 ml. 0.021 <i>M</i> titrated with 0.0995 <i>M</i> HCl. Other groups. Back-titration with 0.101 <i>M</i> NaOH (corrected for excess HCl).

—COOH						—NH ₂					
Corrections						Corrections					
<i>p</i> _H	Titre ml.	μ	Acidity ml.	Over-lap ml.	<i>p</i> _G '	<i>p</i> _H	Titre ml.	μ	Alkal. ml.	Over-lap ml.	<i>p</i> _G '
(a)	3.301	0.26	0.018	-0.01	—	2.161	8.048	0.27	—	—	9.134
	3.035	0.47	0.029	0.02	—	2.176	8.393	0.55	—	—	9.130
	2.854	0.67	0.038	0.03	—	2.174	8.630	0.85	—	—	9.133
	2.708	0.89	0.047	0.05	—	2.176	8.804	1.14	—	—	9.131
	2.547	1.17	0.058	0.07	—	2.174	8.977	1.46	—	—	9.135
	2.387	1.52	0.068	0.11	—	2.177	9.126	1.76	—	—	9.136
	2.223	1.93	0.079	0.18	—	2.176	9.301	2.12	—	—	9.133
	2.044	2.42	0.090	0.31	—	2.167	9.498	2.48	—	—	9.137
	1.888	2.88	0.099	0.48	—	2.154	10.002	3.15	0.024	0.01	9.128
	(3.70) end-point						(3.56)				
(b)	4.865	0.23	—	—	3.803	7.270	0.61	—	—	—	7.886
	4.610	0.39	—	—	3.805	7.490	0.89	—	—	—	7.891
	4.344	0.65	—	—	3.808	7.649	1.15	—	—	—	7.884
	4.112	0.96	0.008	-0.01	—	3.804	7.811	1.43	—	—	7.886
	3.921	1.26	0.012	0.01	—	3.806	7.989	1.76	—	—	7.880
	3.751	1.55	0.013	0.02	—	3.806	8.170	2.07	—	—	7.880
	3.564	1.86	0.015	0.03	—	3.805	8.547	2.57	—	—	7.886
	3.388	2.14	0.017	0.06	—	3.803	8.782	2.78	—	—	7.882
	3.151	2.45	0.019	0.10	—	3.799	—	(3.13)	—	—	—
	(2.88) end-point										
(c)	4.295	0.22	—	—	2.976	6.716	0.21	—	—	—	8.026
	3.688	0.80	0.012	-0.02	—	2.976	6.998	0.39	—	—	8.022
	3.473	1.20	0.017	0.04	—	2.976	7.551	1.14	—	—	8.021
	3.256	1.71	0.023	0.07	—	2.971	7.840	1.79	—	—	8.020
	3.051	2.29	0.028	0.12	—	2.968	8.043	2.31	—	—	8.020
	2.832	3.00	0.034	0.22	—	2.971	8.289	2.92	—	—	8.022
	2.590	3.81	0.038	0.41	—	2.975	8.622	3.60	—	—	8.020
	(4.80) end-point						(4.50)				
(d)	4.105	0.25	0.006	-0.01	—	3.04	7.621	0.46	—	—	8.38
	3.706	0.55	0.013	0.02	—	3.03	7.841	0.70	—	—	8.38
	3.459	0.88	0.024	0.04	—	3.04	8.039	0.98	—	—	8.38
	3.002	1.69	0.045	0.13	—	3.03	8.345	1.50	—	—	8.38
	2.766	2.22	0.051	0.25	—	3.04	8.547	1.87	—	—	8.38
	2.475	2.88	0.057	0.51	—	3.03	8.910	2.44	—	—	8.37
	(3.03) end-point						(3.14)				

Table I (cont.).

TABLE I (Cont.)

—COOH						—NH ₂							
Corrections						Corrections							
	<i>p</i> _H	Titre ml.	<i>μ</i>	Acidity ml.	Over-lap ml.	<i>p</i> _G '		Titre ml.	<i>μ</i>	Alkal. ml.	Over-lap ml.	<i>p</i> _G '	
(e)	4.196	0.33	—	—	—	3.14		6.702	0.53	—	—	7.52	
	3.742	0.82	—	—	—	3.15		7.131	1.17	—	—	7.52	
	3.243	1.83	0.047	-0.03	—	3.14		7.500	1.97	—	—	7.52	
	2.897	2.67	0.057	0.07	—	3.15		7.700	2.43	—	—	7.52	
	2.700	3.11	0.061	0.12	—	3.15		8.124	3.23	—	—	7.52	
	2.280	3.93	0.066	0.35	—	3.15		8.713	3.79	—	—	7.51	
	(4.06)	end-point						(4.03)					
(f)	2.488	0.35	0.092	+0.30	-0.04	3.15		6.871	7.29	—	—	7.63	
	2.791	0.97	0.086	0.16	0.08	3.14		7.135	7.61	—	—	7.63	
	3.126	1.77	0.078	0.08	0.18	3.14		7.507	8.25	—	—	7.62	
	3.301	2.22	0.075	0.05	0.26	3.14		7.710	8.64	—	—	7.62	
	3.480	2.67	0.071	0.04	0.38	3.14		8.099	9.30	—	—	7.63	
	3.794	3.45	0.066	0.02	0.70	3.15		8.625	9.85	—	—	7.62	
	(3.395)	end-point						(10.15)					
	3.958	3.85	0.064	+0.02	+0.45	4.38		—	—	—	—	—	
	4.154	4.33	0.061	0.01	0.30	4.39		—	—	—	—	—	
	4.325	4.77	0.059	0.01	0.21	4.38		—	—	—	—	—	
	4.533	5.25	—	—	0.13	4.38		—	—	—	—	—	
	4.923	5.99	—	—	0.05	4.38		—	—	—	—	—	
	5.459	6.53	—	—	—	4.38		—	—	—	—	—	
	(6.79)												
—COOH						—OH							
(g)	(2.05)	—	—	—	—	—		9.192	2.38	—	—	+0.02	9.79
	3.461	2.37	0.030	-0.05	—	2.64		9.412	2.60	—	—	0.0	9.78
	3.163	2.61	0.031	0.10	—	2.63		9.656	2.88	0.040	-0.01	-0.04	9.80
	2.965	2.86	0.032	0.16	—	2.63		9.861	3.18	0.045	0.02	0.07	9.78
	2.710	3.27	0.036	0.29	—	2.63		10.093	3.46	0.046	0.03	0.12	9.81
	2.451	3.84	0.040	0.56	—	2.63			(4.00)				
	(4.10)	end-point											
—NH ₂						Guanidine							
	6.835	0.33	—	—	—	7.54		11.03	4.39	0.050	-0.27	0.0	12.23
	7.111	0.54	—	—	-0.01	7.55		11.23	4.63	0.051	0.43	-0.04	12.29
	7.374	0.81	—	—	0.01	7.55		11.44	5.02	0.053	0.71	0.06	12.29
	7.650	1.12	—	—	0.02	7.56		11.68	5.77	0.055	1.27	0.08	12.26
	8.298	1.76	—	—	0.07	7.56		—	(6.00)	—	—	—	—
	(2.00)	end-point											

DISCUSSION.

*Effect of structure on p_K .*¹ Many factors, not solely structural, take part in promoting hydron dissociation. It is, however, a commonplace that kationoid groups in a molecule ($-\text{C}_6\text{H}_5$, $-\text{I}$, $-\text{COOH}$, $-\text{COOC}_2\text{H}_5$, $-\text{CONH}_2$, $-\text{NH}_4^+$) will favour hydron dissociation (displace p_K to more acid regions), while anionoid groups ($-\text{CH}_3$, $-\text{COO}^-$, $-\text{NH}_2$) will retard it, the effect varying with the number, the potency and especially the proximity of the groups [see Allan *et al.*, 1926; Cohn, 1931]. Upon this basis, ample explanation of the major displacements of p_K in Table II is provided.

Thus, *glutamine* differs structurally from α -amino-*n*-butyric acid by a kationoid group, $-\text{CONH}_2$, in the γ -position, this distant group effecting a small decrease of both p_K (Table II); an enhanced effect with asparagine illustrates

¹ For purposes of discussion, $p_{G'}$ is accepted as equivalent to p_K . The approximation is small compared with the magnitudes discussed and the unspecified effects of salt concentration. Existence of optical isomerism is ignored as this is presumably without influence on p_K .

Table II. *Titration constants at 25°.*

	—COOH	—COOH	—NH ₂	—OH	Guan.	Reference
α -Amino- <i>n</i> -butyric acid	2.55	—	9.60	—	—	Czarnetzky and Schmidt [1932]
Glutamine (γ -amide)	2.17	—	9.13	—	—	This paper
Asparagine (β -amide)	2.02	—	8.80	—	—	Chibnall and Cannan [1930]
β -Hydroxyasparagine	2.12	—	8.26	—	—	Chibnall and Cannan [1930]
Glutamic acid	2.19	4.25	9.66	—	—	Kirk and Schmidt [1929]
<i>iso</i> Glutamine (α -amide)	3.81	—	7.88	—	—	This paper
<i>iso</i> Asparagine (α -amide)	2.97	—	8.02	—	—	This paper
α -Hydroxyasparagine	2.31	—	7.17	—	—	Chibnall and Cannan [1930]
Glutaminylglycine	3.15	—	7.52	—	—	This paper
Glutaminylglutamic acid	3.14	4.38	7.62	—	—	This paper
Glycylproline	2.84	—	8.53	—	—	This paper
Alanylproline	3.04	—	8.38	—	—	This paper
Alanylalanine	3.17	—	8.42	—	—	Simms [1928]
Phenylalanylgarginine	2.66	—	7.57	—	12.4	Greenstein [1933]
Tyrosylarginine	2.63	—	7.55	9.80	12.3	This paper
"	2.65	—	7.39	9.36	11.6	Greenstein [1933]

the result of structural proximity. The comparable behaviour of a kationoid —COOH group is shown in the first carboxyl constant of *glutamic acid*. The second carboxyl constant, however, is under the opposing influences of the anion, —COO[−], and a kation —NH₄⁺, and is not greatly removed from the constants of fatty acids ($p_{K'}$ 4.7), while the amino constant of glutamic acid is increased over that of glutamine owing to the replacement of the kationoid —CONH₂ group by the anion —COO[−]. In the case of *isoglutamine*, the carboxyl group is rather distant from the kationoid —CONH₂ group and the kation —NH₄⁺ and is consequently not so much stronger than a fatty acid; but in *isoasparagine* there is considerable enhancement. The amino-group of *isoglutamine* is in the α -position to a kationoid —CONH₂ group (as compared with the α -COO[−] anion of glutamine) and far removed from a —COO[−] anion; hence its $p_{K'}$ is displaced considerably in an acid direction. In *isoasparagine*, a corresponding displacement is retarded by the greater proximity of the —COO[−] anion. In the *hydroxyasparagines* similar considerations, including an effect of kationoid hydroxy-groups, are involved [Chibnall and Cannan, 1930].

There is no need for a detailed extension of the argument, but a few points require mention:

(a) A *dipeptide* may be regarded as a substituted amide R.CONH (CHR'. COO[−]), the substituent carrying an α -COO[−] anion (or kationoid —COOH) with perhaps further complicating groups. As a simple case, α -aspartylaspartic acid, $p_{K'_{NH_2}}$ 8.26 (from aspartylaspartic anhydride [Greenstein 1931]) is a substituted α -asparagine (8.02); whereas aspartylglycine, $p_{K'_{NH_2}}$ 9.07 [Simms, 1928]¹, and β -aspartyltyrosine, $p_{K'_{NH_2}}$ 8.92 [Greenstein, 1933; Bergmann *et al.*, 1934] are substituted β -asparagines (8.80). Compared with the amides, each peptide shows a slight increase of $p_{K'_{NH_2}}$ due to substituent —COO[−] anions. At the same time, however, it is the position of the —CONH— group which is most powerful in determining the magnitude of the peptide $p_{K'_{NH_2}}$.

(b) Therefore, a peptide carrying a —CONH₂ group in addition to its typical —CONHR group must be expected to show an abnormally low $p_{K'_{NH_2}}$. Indeed

¹ The exact structure of this peptide was not described, though Fischer's indeterminate synthesis from fumarylidyglycine was presumably employed. A paper from the same laboratory describes the preparation as stable to erepsin [Levene *et al.*, 1926], affording presumptive evidence of β -linkage [Bergmann *et al.*, 1934].

the displacement of $p_{K'_{NH_2}}$ in the glutamyl peptides is not exceeded in any amino-N compound of important biological distribution and has been equalled only when both residues of a dipeptide have contained kationoid groups (phenylalanylarginine, 7.57, tyrosyltyrosine 7.68 *etc.*, also cystine 7.5, 9.0). The possibilities of further depression by coupling such residues to glutamine, or particularly by preparing derivatives of α -hydroxyasparagine ($p_{K'_{NH_2}}$ 7.17) or β -hydroxyglutamine, should not be overlooked. In the meantime, it is likely that the mean $p_{K'_{NH_2}}$ for pepsin proteolysates at 7.9 [Cannan and Muntwyler, 1930] will be lower for proteolysates from other sources than gelatin which contains a minimum of kationoid groups. Of course, complexity of the polypeptides also operates to decrease the $p_{K'}$ (as in glycine hexapeptide, $p_{K'}$ 7.6 [Stiasny and Scotti, 1930]).

(c) It is noteworthy that introduction of a p -hydroxy-group into phenylalanine produces a negligible fall of $p_{K'_{NH_2}}$ in the resulting tyrosine (9.23 becomes 9.15). As it is unlikely that a different situation exists in their peptides (particularly after ionisation of the hydroxyl group), the analogy between our constants for tyrosylarginine and those for phenylalanylarginine (Table II) is satisfying. Again the hydroxyl constant of tyrosine (10.2) is not greatly decreased in the peptide, and resembles that of the similarly placed group in tyrosyltyrosine (9.80 [Greenstein, 1932]). Thus our new data show a uniformity previously lacking.

(d) There seems no essential difference between the acylproline and ordinary peptide linkages in their effect on $p_{K'}$.

Influence on the estimation of biological amino-N. In a detailed discussion of alcohol and formaldehyde titrations of biological fluids, it was shown [Richardson, 1934, 2] that a satisfactory and convenient indicator-point in the case of amino-acids (mean $p_{K'}$ 9.6) was the first visible colour of phenolphthalein (p_H 8.4). Now this discussion assumed a working temperature of 25°; at 15° the mean $p_{K'_{NH_2}}$ is more truly given at 9.9 with no accompanying change in the indicator range, so that the end-point could even be a little more alkaline than p_H 8.4. The more acid end-point favoured by Brown [1923] for his formaldehyde titration by a comparator method (phenol red, p_H 8.0) was due to the undue proportion of peptides or of amino-acids with kationoid groups which he investigated (*e.g.* asparagine, see below).

Now, the discussion quoted was concerned primarily with the single-indicator titration of simple amino-acids. It follows from the argument therein developed that where glutamine and asparagine may be present as bulk constituents, as in certain plant extracts, a lowered indicator-point is desirable to accommodate these lower values of $p_{K'}$ (9.13 and 8.80). Thus p_H 7.6 (phenol red) would be a likely point (Brown places it even lower for asparagine); yet at the same time the original indicator-points would not exclude any considerable portion of these amides from titration. A more important defect of the methods is that peptides (mean $p_{K'}$ 8.3) would largely escape estimation, as was noted in the discussion. A particularly serious example is presented by the peptides of glutamine and asparagine, or other peptides of abnormally low $p_{K'}$ (see previous section); in such cases the amino-groups would be 75 to 90 % titrated in the preliminary p_H adjustment and only the remaining 10 to 25 % would be included in the titration proper. The use of a lower indicator-point (p_H 6.5) to include peptide-N would automatically exclude the simpler amino-N. Conversely, a two-indicator method would include the desired groups but also various other unspecified substances.

Indeed, there is no titration method which can be safely applied without some foreknowledge of the chemical composition of the extract to be tested. Where

such foreknowledge is lacking, a two-indicator titration of lead-treated extracts by the Lüers formaldehyde method merits further development [Richardson, 1934, 2], or, as a temporary expedient, parallel titration of aliquots to different indicator end-points may be used to find the optimum conditions (as with peptones [Brown, 1923]). The other common method for amino-N (Van Slyke) is unfortunately liable to other error [Richardson, 1934; Melville, 1935] and its use requires similar foreknowledge of the composition of the extract.

Influence on the study of peptic scission. An unusually low $p_{K'}^{\text{NH}_2}$ in the dipeptides which result from protein scission will not adversely affect the customary Willstätter titrimetric estimation of the extent of hydrolysis [Richardson, 1934, 1]. On the other hand, the use of dipeptides with a low $p_{K'}^{\text{NH}_2}$ as substrates in dipeptidase studies raises a novel problem as to effective buffering capacity. Thus for a scission experiment at the customary p_{H} of 7.4 to 7.8, it is clear that such a dipeptide ($p_{K'}$ 7.6) must first be brought to the appropriate p_{H} by addition of 0.4 to 0.6 equivalent of NaOH. When this is followed by scission of the peptide, this one group ($p_{K'}$ 7.6) will be progressively replaced by two groups of $p_{K'}$ 9 to 9.6, these groups sharing the added NaOH. This leads, in the absence of buffering, directly to an increase of p_{H} to 8.5 to 9. Conversely, with buffers present, these must be sufficiently effective to engage without great p_{H} change almost the whole of the NaOH previously held by the peptide.

In such cases it is necessary to modify the current procedure of Grassmann [1927] to allow greater constancy of p_{H} during hydrolysis. Indeed, much of the present evidence of p_{H} constancy seems to depend on the use of dipeptides ($p_{K'}$ 8.3) requiring little p_{H} adjustment, the buffering capacity of the enzyme preparation itself being partly responsible for the preservation of p_{H} . Certainly, Grassmann's early use [1927] of only 0.8 equivalent of $\text{NH}_3\text{—NH}_4\text{Cl}$ buffer ($p_{K'}$ 9.3) at a p_{H} outside its effective buffering range (p_{H} 7.8) was grossly inadequate. At the same time, the subsequent general practice of using 0.03 *M* phosphate at p_{H} 7.4 to 7.8 to buffer peptides in 0.05 *M* to 0.1 *M* concentration [Bergmann *et al.*, 1934] is scarcely a sufficient improvement for critical p_{H} studies. Adequate buffering would need at least three equivalents of a buffer, whose $p_{K'}$ should be just alkaline to the experimental p_{H} in order to offer the maximum obstruction to the alkaline drift.

It is an added advantage if such a buffer is soluble in 90 % alcohol (or acetone), and is composed of some base or basic group and its salt (briefly because, in titration, the buffering range of a base is displaced by alcohol addition with respect to the phthalein indicator, so that encroachment of the buffer on the titration end-point is avoided [*cf.* Richardson, 1934, 1]; moreover, the temperature effects on the $p_{K'}$ of a basic buffer and of the titrated groups are comparable). In *isoglutamine* ($p_{K'}$ 7.9) these desirable properties are all combined and join with the convenient synthesis and stability of the material to mark it out for further study.

isoGlutamine as a buffer for study of peptide scission. Since the above criteria for buffering dipeptidase experiments are so admirably combined in *isoglutamine*, a few more details may be presented. *isoGlutamine* (α -glutamine) may be easily prepared from glutamic acid (for purity see Melville [1935, p. 182]) and has ample, though not great aqueous solubility. Used as a 0.03 *M* buffer for 0.01 *M* dipeptides, it does not interfere with alkalimetric acetone titration of amino-N, either by precipitating from solution or by encroaching on the end-point. In contrast the usual phosphate buffer is an acid substance with a $p_{K'}$ too low for adequate buffering against alkaline drifts and with insufficient solubility at the

end-point. Barbitone, as an alternative, has a suitable p_K' (8.2) and acetone solubility, but insufficient aqueous solubility (0.023 M) and being an acid is liable to interfere at the end-point.

Table III. *Stability of d-isoglutamine at 37°.*

0.124 g. *isoglutamine* adjusted to given p_H and made to 25 ml. (0.034 M) with phosphate or barbitone buffer. At intervals, 2 ml. were removed for NH_3 determination [cf. Melville, 1935, Tables II and III].

Time in hours	NH_3 liberated (as ml. $M/70$ HCl)		
	p_H 6.8	p_H 7.8	p_H 8.8
24	0.04	0.11	0.18
48	0.11	0.18	0.34
72	—	—	0.48
96	0.14	0.35	—
100 % scission	4.75	4.75	4.75

These features of *isoglutamine* would, of course, be valueless in presence of a latent tendency to hydrolysis of the α -amide group. Fortunately, Table III shows that this tendency is negligible under the conditions of ordinary experiment (it was interesting that simultaneous amino-N titrations showed no important increase in amino-N + NH_3 during liberation of NH_3 , virtual evidence of ring closure). Moreover, as *d-isoglutamine* showed no hydrolysis whatever during two hours with mixed yeast peptidases under conditions giving 60 % scission of *d*-glutaminylglycine, no fear of hydrolytic scission of the buffer need normally be entertained in experiments of the usual duration.

Finally, data for the preparation of 0.03 M *isoglutamine* buffers are interpolated from the data of Table I and presented in Table IV. Similar data for

Table IV. *Preparation of isoglutamine buffers (for immediate use).*

$M/30$ buffer prepared from 0.487 g. pure *isoglutamine* with given volume of 0.1 M NaOH, made to 100 ml. p_H at 25°.

p_H	7.0	7.2	7.4	7.6	7.8	8.0	8.2	8.4	8.6	8.8
ml. 0.1 M NaOH	3.85	5.7	8.2	11.4	15.1	18.9	22.5	25.5	28.0	29.7

isoasparagine (p_K' 8.0) may be readily prepared from Table I, should *isoglutamine* prove unsuitable in unusual circumstances.

SUMMARY.

Titration constants at 25° have been determined for *d*-glutamine, *d-isoglutamine*, *l-isoasparagine*, *d*-glutaminylglycine, *d*-glutaminy-*d*-glutamic acid and *d*-alanyl-*l*-proline, and re-determined for *d*-tyrosyl-*d*-arginine and glycyl-*l*-proline. Factors influencing the magnitude of the respective p_K' values are discussed. α -Amides and α -peptides possess constants characteristically different from their β - or γ -analogues.

In certain peptides the constants attributable to the amino-group are unusually low, and the influence of this fact on the titrimetric estimation of biological amino-N and on the study of peptidase action is discussed.

Advantages attending the use of *isoglutamine* as a buffer in peptidase studies are outlined, and the necessary data as to the composition and stability of the buffer mixtures are presented.

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REFERENCES.

- Allan, Oxford, Robinson and Smith (1926). *J. Chem. Soc.* 405.
Bergmann and Zervas (1932). *Ber. deutsch. chem. Ges.* **65**, 1192.
— — — — — Schleich and Leinert (1932). *Z. physiol. Chem.* **212**, 72.
— — — — — Salzmann and Schleich (1934). *Z. physiol. Chem.* **224**, 17.
— — — — — and du Vigneaud (1929). *Ber. deutsch. chem. Ges.* **62**, 1905.
Brown (1923). *J. Bact.* **8**, 245.
Cannan and Muntwyler (1930). *Biochem. J.* **24**, 1012.
Chibnall and Cannan (1930). *Biochem. J.* **24**, 945.
Clark (1928). The determination of hydrogen ions. (London.)
Cohn (1931). *Ergebn. Physiol.* **33**, 781.
Czarnetzky and Schmidt (1932). *Z. physiol. Chem.* **204**, 129.
Grassmann (1927). *Z. physiol. Chem.* **167**, 202.
Greenstein (1931). *J. Biol. Chem.* **93**, 479.
— (1932). *J. Biol. Chem.* **95**, 465.
— (1933). *J. Biol. Chem.* **101**, 603.
Kirk and Schmidt (1929). *J. Biol. Chem.* **81**, 237.
Levene, Simms and Pfaltz (1926). *J. Biol. Chem.* **70**, 253.
Melville (1935). *Biochem. J.* **29**, 179.
— and Richardson (1934). *Biochem. J.* **28**, 1565.
von Muralt (1930). *J. Amer. Chem. Soc.* **52**, 3518.
Richardson (1934, 1). *Proc. Roy. Soc. Lond.*, B **115**, 121.
— (1934, 2). *Proc. Roy. Soc. Lond.*, B **115**, 142.
Simms (1926). *J. Amer. Chem. Soc.* **48**, 1244.
— (1928). *J. Gen. Physiol.* **11**, 629.
Stiasny and Scotti (1930). *Ber. deutsch. Chem. Ges.* **63**, 2977.