bilirubin/100 ml. of final solution, absorption density increases relatively little with concentration.

4. Prompt, biphasic and delayed direct reactions encountered in obstructive jaundice or acute hepatitis depend solely upon the quantity of bilirubin present and are explained by this failure of Beer's law.

5. In the Rappaport & Eichhorn method total bilirubin in sera from cases of haemolytic jaundice requires 5-6 hr. for complete coupling, instead of less than 30 min. required for bilirubin in sera from cases of obstructive jaundice and acute hepatitis. In haemolytic jaundice two types of direct reaction are encountered: (1) a very greatly delayed and exceedingly weak development of colour, and (2) a biphasic response in which the less slow phase occurs during the first hour of the reaction.

6. Reasons are given for disregarding most measurements of direct and indirect bilirubin hitherto reported in the literature.

7. The significance of these findings is discussed.

Our thanks are due to all those clinicians who have given us such free access to their cases. Grants from the Central Research Fund of the University of London, from the Research Fund, Diabetic Department, King's College Hospital, and from the Medical Research Council enabled special necessary apparatus and chemicals to be purchased. Dr O. Rosenheim of the National Institute for Medical Research kindly supplied a specimen of bilirubin originally prepared by Thudichum. Finally, particular thanks are due to Mr A. H. Dawson and Miss M. Sandiford for their able technical help.

ADDENDUM

Since this work was completed, cells of 2 cm. thickness and of only 15 ml. capacity have become available for use with the Spekker instrument and low concentrations of bilirubin may be measured more accurately with them than with the 1 cm. cells.

REFERENCES

- Acil, D. & Goldgruber, G. (1932). *Z. ges. exp. Med.* **84**, 787.
 Barron, E. S. G. (1931). *Medicine*, **10**, 77. Baltimore.
 Bollman, J. L., Sheard, C. & Mann, F. C. (1927). *Amer. J. Physiol.* **80**, 461.
 Cabello-Ruiz, J. (1943). *Rev. Soc. argent. Biol.* **19**, 247.
 Cantarow, A., Wirts, C. W. (Jr.) & Hollander, G. (1942). *Arch. intern. Med.* **69**, 986.
 Delgado, E. (1943). *La Bilirrubinemia*, quoted by Ducci & Watson (1945).
 Ducci, H. & San Martin, L. (1943). *Rev. méd. Chile*, **71**, 1094.
 Ducci, H. & Watson, C. J. (1945). *J. Lab. clin. Med.* **30**, 293.
 Evelyn, K. A. (1936). *J. biol. Chem.* **115**, 63.
 Feigl, J. & Querner, E. (1919). *Z. ges. exp. Med.* **109**, 153.
 Fischer, H. & Orth, H. (1937). *Die Chemie des Pyrrols*. Leipzig: Akad. Verlagsgesellschaft.
 Fowweather, F. S. (1932). *Biochem. J.* **26**, 165.
 Grunenberg, K. (1923a). *Z. ges. exp. Med.* **31**, 119.
 Grunenberg, K. (1923b). *Z. ges. exp. Med.* **35**, 128.
 Harrison, G. A. (1937). *Chemical Methods in Clinical Medicine*. London: Churchill.
 Haslewood, G. E. D. & King, E. J. (1937). *Biochem. J.* **31**, 920.
 Lepehne, G. (1920). *Dtsch. Arch. klin. Med.* **132**, 96.
 Lepehne, G. (1942). *J. Lab. clin. Med.* **27**, 1447.
 Lichtman, S. S. (1942). *Diseases of the Liver, Gallbladder and Bile Ducts*. Philadelphia: Lee and Febiger.
 MacInnes, D. A. & Belcher, D. (1933). *Industr. Engng Chem. (Anal. ed.)*, **5**, 199.
 Malloy, H. T. & Evelyn, K. A. (1937). *J. biol. Chem.* **119**, 481.
 McNee, J. W. (1922). *Brit. med. J.* **1**, 716.
 McNee, J. W. & Keefer, C. S. (1925). *Brit. med. J.* **2**, 52.
 Newman, C. E. (1928). *Brit. J. exp. Path.* **9**, 112.
 Rappaport, F. & Eichhorn, F. (1943). *Lancet*, **1**, 62.
 Soejima, R. (1927). *Arch. klin. Chir.* **149**, 206.
 Stein, H. B. (1941). *S. Afr. J. Med. Sci.* **6**, 104.
 Van den Bergh, A. A. H. (1918). *Der Gallenfarbstoffe im Blute*. Leiden: Van Doesburg. Quoted by Watson, C. J., in *Handbook of Haematology*, edited by H. Downey (1938). London: Hamish Hamilton.
 Van den Bergh, A. A. H. & Muller, P. (1916). *Biochem. Z.* **77**, 90.
 Watson, C. J. (1942). *Proc. Soc. Exp. Biol., N.Y.*, **49**, 641.

Problems Associated with the Acid Hydrolysis of an Impure Protein Preparation

By J. W. H. LUGG, *Division of Biochemistry and General Nutrition, Commonwealth Council for Scientific and Industrial Research, University of Adelaide, South Australia*

(Received 11 October 1945)

Products of the hydrolysis of proteins, the amino-acids, were being listed in qualitative and quantitative studies of protein composition long before the introduction of the peptide theory (Fischer, 1902; Hofmeister, 1902) of protein structure pro-

vided a satisfactory theoretical basis for the practice. In general, acid hydrolysis has been more widely used than alkali hydrolysis and, since its introduction by Ritthausen (1868), hydrochloric acid has tended to replace sulphuric acid as hydrolyzing

agent. Some of its advantages have recently been discussed by Chibnall, Rees & Williams (1943). The difficulties attending subsequent removal of the hydrolyzing agent preclude, for many purposes, exploitation of the high rates of hydrolysis by acids like cetyl sulphonic acid (Steinhardt & Fugitt, 1942).

It has been customary to conduct acid hydrolyses at the boiling-point (under atmospheric pressure) of the mixtures concerned, and for greatly varying periods. Sometimes the aim has been to secure conditions believed to be most satisfactory for the estimation of one amino-acid; sometimes it has been intended to estimate many amino-acids in the hydrolysate and the aim has been to achieve the maximum content of free amino-acids (estimated as amino and/or carboxyl groups).

When impurities of certain types are present in large quantity in the protein preparations, much 'humin' is produced during acid hydrolysis, and in association with this there may be serious vitiation of the estimation of certain amino-acids (e.g. see Lugg, 1939). Humin formation also introduces mechanical problems. When much insoluble humin is present it is extremely difficult to maintain smooth boiling of the mixture, even with the aid of several hollow-tipped boiling-sticks, and there is in consequence, the risk of loss by bumping, overheating, or actual charring. These mechanical hazards can be avoided by hydrolyzing at a temperature below the boiling-point. The literature affords little guidance, however, in regard to the period required to achieve the maximum content of free amino-acids, except that it might be expected to lie between 24 and 48 hr. at 100° when 6N-HCl is used (e.g. see Van Slyke, 1912) and to be diminished by approximately a half for every 10° increase in temperature (e.g. see Van Slyke, 1912; Nasset & Greenberg, 1929).

The purpose of the present investigation was to establish conditions suitable for the acid (HCl) hydrolysis of a particular impure protein preparation (one of a group of preparations apparently similar not only in regard to their amino-acid composition but also in regard to the nature and quantity of contaminating impurity) as a step in the estimation principally of the monoamino-dicarboxylic acids and many of the monoamino-monocarboxylic acids. This type of contaminant has been observed to yield substances of an acid nature (other than the 'humin') during the acid hydrolysis (unpublished observations), and as these may interfere with the estimation of some of the amino-acids a means was sought of removing them whilst leaving the amino-acids in the hydrolysate. The interpretation of the findings of the main experiments, however, has considerable bearing also upon problems associated with the hydrolysis of relatively pure protein preparations.

EXPERIMENTAL

Hydrolysis of the preparation

The material used was the extracted protein preparation from leaves of *Phalaris tuberosa*, described by Lugg & Weller (1944). It contained, when dry, 14.90% N and 1.7% ash, and about 10% of impurity (Lugg, 1939). It was treated with constant boiling HCl (6.08N at 760 mm. Hg pressure) in the proportion of 100.0 ml. of acid / 10.02 g. of air-dry preparation (9.44 g. dry). The mixture was contained in a flask fitted with a loosely closed reflux condenser and maintained at a temperature of 99° by immersion in boiling water. The flask was shaken at intervals, more frequently during the early stages of hydrolysis, since the preparation very rapidly formed a gel with the acid. This had largely disintegrated within 15 min., most of it appearing to go into solution but with the formation of particles of insoluble humin. Tests showed the volume of the mixture (when cooled to 20°) to be 107.35 ml. from shortly after the commencement of hydrolysis. Samples were withdrawn from time to time for examination.

Examination of samples of hydrolysate

In earlier studies the liberation of amino groups during hydrolysis of proteins has usually been followed by one or other of two methods or modifications of them: Van Slyke's (1911) gasometric method, and Sorensen's (1907) 'formol' titration. Richardson (1934a), stressing the fact that the 'formol' titration and Linderström-Lang's (1928) 'acetone' titration actually estimate, respectively, amino and carboxyl groups, and not carboxyl and amino groups as had frequently been assumed, expressed the opinion that a comprehensive titration method could be made extremely informative. Accordingly, he outlined a general titrimetric procedure for the estimation of mineral acid, carboxyl groups and amino groups in mixtures containing them. Subsequent studies (e.g. Wadsworth & Pangborn, 1936; Neuberger, 1944) have emphasized the complexity of the reactions between formaldehyde and various amino-acids, but the broad principles of the comprehensive titrimetric procedure remain valid and have been followed in the present work. New methods based upon the 'ninhydrin' reaction (e.g. Schott, Rockland & Dunn, 1944) may prove valuable in studies of this type.

Samples of 0.5000 ml. of the withdrawn, cooled and stirred hydrolysate, were diluted to 10.00 ml. after partial neutralization with about 2.2 ml. of N-NaOH solution. 1.000 ml. portions of the dilute solution were taken for titration at about 20° with N/70 NaOH solution in 'acetone', 'plain' and 'formaldehyde' solution to give estimates respectively of mineral acid, mineral acid plus 'carboxyl', and mineral acid plus 'carboxyl' plus 'amino' groups. The so-called 'carboxyl' groups, as estimated here, include non-carboxylic acid groups and partly exclude true carboxyl groups; and so-called

'amino' groups as estimated here, may include ammonia, proline-N, hydroxyproline-N and some other non-amino-N groups, together with certain weak acid groups, and exclude the NH_2 group in the guanidinium ion of arginine.

'Acetone' titrations. The sample was treated with 10 ml. of acetone containing 0.05 mg. of naphthylamine orange (as a few drops of its solution in 75% ethanol-water mixture), and titration was commenced. As titration proceeded, the acetone solution of the dye was added to maintain the volume ratio of acetone to water at 10 : 1 and the dye concentration constant. Titrations were made to two end-points, the first corresponding to 0.31 ml. of $\text{N}/70$ HCl in 1 ml. of aqueous solution plus 10 ml. of acetone (referred to as 'pH' 3.4), and the second corresponding to 0.077 ml. of $\text{N}/70$ HCl under similar conditions (referred to as 'pH' 4.0). Blanks, by which the titrations were corrected, were estimated by titrating NaCl solutions of approximately the same ionic strength as the partially neutralized hydrolysates. These blanks varied with the final volume and also with the final concentration of NaCl, but were about three times as large as the corresponding blanks in absence of the NaCl.

'Plain' titrations. The sample together with the volume of $\text{N}/70$ NaOH required for titration did not exceed 6 ml., and if less than this, the volume was made up with water to 6 ml. Titrations were made to pH's 6.0, 6.5, 7.0 and 7.5, these being established with appropriate phosphate buffers containing bromothymol blue. The mixtures contained 0.04 mg. of the dye (as a few drops of its solution in ethanol).

'Formaldehyde' titrations. The sample was diluted with water and then with 1 or 2 ml. of 15M-formaldehyde solution (40% (w/v) formaldehyde solution) and titrated within a few minutes, the volume being adjusted with water so as to be 11 or 12 ml., respectively, at the end of the titration. The mixtures sometimes contained 0.10 mg. of phenolphthalein, and sometimes twice this amount. The end-points were established against three borate buffer-formaldehyde mixtures, made by adding 1 or 2 ml. of the formaldehyde solution to 10 ml. of the buffer, the mixtures containing phenolphthalein at concentrations corresponding with those used in the titrations. The buffers contained 20 g. of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) per litre and some contained HCl in addition, to reduce the pH from 9.2 to about 8.7. 10 ml.

of the pH 8.7 buffer plus 1 ml. of the formaldehyde solution gave a solution of pH 8.5, and 10 ml. of the pH 9.2 buffer plus 1 or 2 ml. of the formaldehyde solution gave a solution of pH 9.0 or 8.9, respectively. Values of pH were estimated by means of the glass electrode neglecting liquid junction p.d. corrections. Blanks, by which the titrations were corrected, were estimated by titrating water containing 1 or 2 ml. of the formaldehyde solution to the appropriate end-points.

The test solutions always contained a brown coloured component (usually attributed to 'soluble humin'), and in most instances it was necessary to conduct the titrations with compensation for this coloration, in an apparatus like that described by Lüers (1920). All the 'acetone', many of the 'formaldehyde' and several of the 'plain' titrations, were conducted in this manner.

Richardson (1934 *b, c*) customarily used higher amino-acid concentrations than was practicable in the present study. If the diluted hydrolysates were permitted to be even 1.2 times as concentrated as those actually used in this work, substances were precipitated from them on adding the acetone for the 'acetone' titrations.

The procedure was checked briefly with solutions of glycine, ammonium chloride, arginine hydrochloride and histidine hydrochloride containing HCl. These solutions, of which 1 ml. samples were taken for titration, contained per 100 ml., 0.4000 g. of glycine plus 0.2372 g. of HCl, 0.2367 g. of NH_4Cl plus 0.0292 g. of HCl, 0.3976 g. of arginine monohydrochloride plus 0.1031 g. of HCl, and 0.3000 g. of histidine monohydrochloride plus 0.0984 g. of HCl, respectively.

The titration results, corrected for the appropriate blanks, are shown in Table 1, and are there compared with values calculated on the simple assumptions, that in the 'acetone' and 'plain' titrations one molecule of HCl remains bound by every NH_3 molecule or NH_2 group, and that in the 'acetone', 'plain' and 'formaldehyde' titrations, the free HCl, free HCl plus carboxyl groups, and free HCl plus carboxyl groups plus NH_3 and NH_2 groups, respectively, are titrated. The guanidinium ion of arginine is so strongly basic that its titration could only just have begun at pH 9; and histidine,

Table 1. *Titrations with N/70 NaOH of samples of solutions containing known amounts of HCl and of glycine, ammonium chloride, arginine monohydrochloride and histidine monohydrochloride*

Substances	Type of titration								
	'Acetone'		'Plain'				'Formaldehyde'		
	'pH' 3.4	'pH' 4.0	pH 6.0	pH 6.5	pH 7.0	pH 7.5	1.36M. pH 8.5	1.36M. pH 9.0	2.5M. pH 8.9
	(ml.)	(ml.)	(ml.)	(ml.)	(ml.)	(ml.)	(ml.)	(ml.)	(ml.)
Glycine + HCl (actual)	0.98	0.95	4.54	4.55	4.56	4.57	8.30	8.32	8.32
" (calculated)	0.82	0.82	4.55	4.55	4.55	4.55	8.28	8.28	8.28
NH_4Cl + HCl (actual)	0.61	0.56	0.56	0.58	0.61	0.64	—	3.68	3.67
" (calculated)	0.56	0.56	0.56	0.56	0.56	0.56	3.66	3.66	3.66
Arginine + HCl (actual)	0.69	0.76	1.98	1.99	2.00	2.02	—	3.34	3.37
" (calculated)	0.65	0.65	1.97	1.97	1.97	1.97	4.61	4.61	4.61
Histidine + HCl (actual)	—	1.29	2.62	2.78	2.90	2.94	—	3.89	3.87
" (calculated)	1.89	1.89	2.89	2.89	2.89	2.89	3.89	3.89	3.89

(Actual and calculated titration values are shown.)

which has a weakly basic imidazole group, did not titrate satisfactorily. The correspondence otherwise was considered to be good (see also the subsequent discussion), and it was felt that reasonable precision could be attained in spite of the low concentrations and the magnitudes of the blanks involved.

In Table 2 are shown the results of titrating the samples of partially neutralized protein hydrolysate.

in the same way as that used in the volatile base estimation and comparing with a Nesslerized ammonia solution. The tints appeared to be entirely similar to those produced with the ammonia. The results are shown in Table 3, the volatile base being assumed to comprise ammonia and substituted ammonia, and the values are represented as percentages of the protein-N appearing as volatile base-N and ammonia-N.

Tests made with methylamine and cyclohexylamine showed these amines to be quantitatively estimated in the

Table 2. *Titrations with N/70 NaOH of 0.05 ml. portions of hydrolysate (9.44 g. of protein preparation plus 0.58 g. moisture plus 100.0 ml. 6.08N-HCl; total vol. 107.35 ml.) after various periods of hydrolysis*

(The titrations have been corrected for the amount of alkali required to adjust the pH of the hydrolysate to 7.0.)

Period of hydrolysis (hr.)	Type of titration								
	'Acetone'		'Plain'				'Formaldehyde'		
	pH' 3.4 (ml.)	pH' 4.0 (ml.)	pH 6.0 (ml.)	pH 6.5 (ml.)	pH 7.0 (ml.)	pH 7.5 (ml.)	1.36M. pH 8.5 (ml.)	1.36M. pH 9.0 (ml.)	2.5M. pH 8.9 (ml.)
0.5	-1.56	-1.44	-0.19	-0.12	±0.00	0.08	0.87	0.90	0.90
1	-1.92	-1.81	-0.20	-0.12	±0.00	0.15	1.10	1.18	1.19
2	-2.51	-2.29	-0.24	-0.15	±0.00	0.13	1.38	1.47	1.46
4	-2.70	-2.50	-0.17	-0.13	±0.00	0.08	—	1.91	1.90
8	-2.94	-2.80	-0.15	-0.09	±0.00	0.09	—	2.20	2.21
12	—	-2.86	—	-0.07	±0.00	—	—	—	2.35
24	-3.06	-2.95	-0.10	-0.05	±0.00	0.04	2.08	2.42	2.45
48	-3.21	-3.01	-0.08	-0.03	±0.00	0.05	2.22	2.65	2.67
72	-3.13	-3.04	-0.08	-0.05	±0.00	0.03	—	2.70	2.74
101	—	-3.05	—	-0.06	±0.00	0.04	—	—	2.75
147	-3.08	-3.04	—	-0.05	±0.00	0.05	—	2.72	2.69
192	-3.20	-3.08	—	-0.05	±0.00	0.05	—	2.76	2.78
266	-3.12	-2.93	-0.10	-0.06	±0.00	0.05	2.31	2.75	2.80
337	-3.02	-2.99	-0.10	-0.05	±0.00	0.04	—	2.65	2.66
385	-3.05	-2.98	-0.09	-0.05	±0.00	0.03	—	2.70	2.72

The most precise titrations were the 'plain' titrations to the end-point at pH 7.0. These have been assigned a zero value in the table, inasmuch as the degree of partial neutralization was fairly arbitrary, and a common reference point is needed for each series of titrations. Consequently, although in fact all the titrations were positive with respect to the N/70 NaOH solution employed, the table shows some titrations to be positive and some negative. Although the hydrolysis was performed in duplicate the removal of samples was not always duplicated. However, many of the values shown in Table 2 are the means of duplicates which agreed fairly closely.

Volatile base and ammonia estimations. Volatile base estimations were made by exhaustively distilling samples of hydrolysate at pH 10.0 under low pressure (temp. 45°) into standard acid and back-titrating the excess acid. Ammonia estimations were made by Nesslerizing a distillate obtained

procedure for volatile base. In the procedure for the estimation of ammonia, the cyclohexylamine gave a milky 'cloud' with the Nessler reagent, and the methylamine gave a coloration of somewhat different tint and only about 1.5% as intense to a normal eye as that given by an equivalent amount of ammonia. In larger quantities, methylamine was found to give a yellow cloud. The methylamine coloration may have been due, in part, to the presence of a trace of ammonia as impurity, but certain amines (of which methylamine is one) are reputed to give with Nessler's reagent coloured substances soluble in KI solutions and of different tint from that given by ammonia (Charitshkov, 1906). Thus the presence of more than a trace of a substituted ammonia would probably be revealed by notable differences between the estimations of volatile base and ammonia, or by departure from normal tint of the Nessler coloration.

'Insoluble' humin-N estimations. Samples of well-stirred hydrolysate were brought to pH 2.0 by adding NaOH solution, and the humin was filtered off after a few hours

Table 3. *Amounts of volatile base-N, ammonia-N and 'insoluble' humin-N (expressed as percentages of the protein-N) present in the hydrolysate after various periods of hydrolysis*

	Period of hydrolysis (hr.)									
	0.5	1	2	4	8	24	48	96	192	384
% volatile base-N	4.03	4.46	4.76	4.87	5.07	5.48	5.92	6.68	8.16	10.3
% ammonia-N	—	—	4.67	—	4.94	5.43	—	6.62	8.16	10.2
% insoluble humin-N	—	—	—	—	—	—	2.8	—	—	3.1

and washed. The N contents of humin and filtrate were estimated by the Kjeldahl method and served to check the calculated total N content of the system. Humin-N is shown in Table 3 as a percentage of total N.

Removal of organic acids. One of the hydrolysates, after hydrolysis had proceeded for 385 hr. was evaporated down, under reduced pressure at the boiling point (below 50°), to a viscous syrup. The syrup was taken up in about 50 ml. of water and the evaporation was repeated in all seven times. The syrup was then dissolved in slightly diluted constant-boiling HCl and the volume was adjusted to about that of the hydrolysate before evaporation. Samples were taken for the usual titrations, which indicated that 3% of the 'carboxyl' and none of the 'amino' groups had been lost. Only 0.05% of the total N was found in the distillate and this probably represented traces of material transferred with spray during the evaporations.

The hydrolysate was then submitted for 88 hr. to continuous extraction at about 20° with ether freed from peroxide and ethanol, the ether percolating through the hydrolysate in drops at the rate of 1 l./hr. Portions of the ether extract were taken and submitted to the usual titration procedures after removal of ether. It was found that 4% of the 'carboxyl' groups were present in the ether after 41 hr. and 6.5% after 88 hr. Of the total N, 0.8% was found in the ether and in several drops of syrup which had separated from the ether and probably represented coalesced droplets of hydrolysate which had been carried over mechanically by the ether. The extraction was then stopped and the hydrolysate was freed from ether by evaporation under reduced pressure and made up to about its original volume with slightly diluted acid as before. Samples were taken for the usual titrations, which revealed the loss of 7% of 'carboxyl' groups and less than 1% of 'amino' groups from the hydrolysate. Actually, the extracted 'carboxyl' groups were underestimated by about 10%, judging from the buffer capacity in 'plain' titrations in the range of pH 6.25-7.5.

The correspondence between the quantities of 'carboxyl' and 'amino' groups lost from the hydrolysate in these treatments, and those found in the distillate or extract, shows that anhydride formation (see Wilson & Cannan, 1937) must have been inappreciable.

DISCUSSION

In Fig. 1 smooth curves have been drawn through points representing the data in Table 2. It will be seen that the scatter of points from the curves (attributed here to lack of precision) was most pronounced in the 'acetone' titrations, especially those at 'pH' 3.4.

It appears that the chief contaminant of the extracted plant-leaf protein preparations is of a polyuronide or polysaccharide nature, such as pectin or mucilage (Lugg, 1939). On digestion with mineral acid these substances may yield sugars and uronic acids as primary products, and CO₂, formaldehyde, organic acids (not necessarily carboxylic) and unidentified substances (see, for example, Link & Niemann, 1930; Hirst & Jones, 1937; Aso, 1939) by secondary reactions. The carboxyl groups of most carboxylic acids are too strongly dissociating

for them to exert more than a trace of buffer action over the pH range 6.0-7.5.

It will be seen that during the very early stages of hydrolysis there was considerable change in the buffer capacity of the hydrolysate in 'plain' titrations over the pH range 6.0-7.5. Even after 0.5 hr. the buffer capacity was rather high. Then, presumably after most of the long polypeptide chains had been broken down to small fragments, the buffer capacity was still higher. Data presented by Simms (1928) and Greenstein (1931) have shown that the ionization constants of the carboxyl and amino groups of peptides are generally much closer together than are those of the individual amino-acids, and it is principally to this fact that the rather high buffer capacity of the hydrolysate at this stage must be attributed, as the contribution of the acids arising from the contaminant could not have been great. Finally, when almost all the peptide (including acyl proline and acyl hydroxyproline) linkages had been broken (after 24 hr.), the buffer capacity had become small and it remained there after very steady.

'Plain' titration values appropriate to pH 6.25 (cf. Richardson, 1934*a*) have been selected as probably most nearly corresponding with the point at which the sum of the 'carboxyl' groups still un-titrated and of the 'amino' groups beginning to be titrated, was a minimum. Judging from the work of Batchelder & Schmidt (1940) the influence of NaCl in the concentrations here encountered, upon the ionization constants of the amino-acids, must have been very small.

It will be noticed that the 'acetone' titrations of the hydrolysate to 'pH' 4.0, were invariably a little smaller (shown as more negative in Table 2) than those carried to 'pH' 3.4. Ordinarily this would suggest the presence of rather strong organic acid groups, and the values at 'pH' 3.4 would be selected as more likely to differentiate the mineral acid from the rest. But the salt concentration was high and blanks were correspondingly large (particularly in titrations at 'pH' 3.4), and uncertain, both because of their magnitude and because specific ion effects may become more important at the higher salt concentrations. Even in the 'acetone' titrations of the glycine plus HCl and the NH₄Cl plus HCl solutions, the uncertain blanks must be held responsible for the slightly anomalous titration values reported (cf. Richardson, 1934*c*). For the reason that the direction as well as the magnitude of the blank errors is uncertain in the 'acetone' titrations of the hydrolysate, it has been deemed advisable to accept means of the titration values at 'pH' 3.4 and 4.0 as most satisfactorily differentiating the mineral acid from the 'carboxyl' groups.

'Formaldehyde' titrations of the hydrolysate at pH 8.5 (unlike that of the glycine plus HCl) were

Fig. 1. Variation, with period of hydrolysis, of titration fractions in the hydrolysate of Table 2. a_1 , a_2 : 'Acetone' titrations to 'pH' 3.4 and 4.0, respectively. p_1 , p_2 , p_3 , p_4 : 'Plain' titrations to pH 6.0, 6.5, 7.0 and 7.5, respectively. f_1 , f_2 , f_3 : 'Formaldehyde' titrations to pH 8.5 (1.36M-formaldehyde), pH 9.0 (1.36M-formaldehyde) and pH 8.9 (2.5M-formaldehyde), respectively.

Fig. 2. Variation, with period of hydrolysis, of 'carboxyl' and 'amino' groups and volatile base in the hydrolysate of Table 2. A, 'carboxyl' groups; B, 'amino' groups; C, volatile base; D, 'amino' groups minus volatile base plus amide-NH₂; E, 'amino' groups minus volatile base.

notably smaller than those at pH 9.0 or 8.9, except during the early stages of hydrolysis. Discrepancies of this magnitude were anticipated from Levy's (1933) study of the influence of various concentrations of formaldehyde upon the apparent ionization constants of the amino-acids. It was considered, in the light of aspects discussed later, that the titrations in presence of 2.5M-formaldehyde (most of the values were slightly greater than those obtained in presence of 1.36M-formaldehyde) would afford the most satisfactory estimates of 'amino' groups.

Points representing arithmetic mean positions between curves a_1 and a_2 , and curve f_3 in Fig. 1, have been used together with points representing arithmetic mean positions between curves p_1 and p_2 , in constructing the smooth curves A and B in Fig. 2, showing the production of 'carboxyl' and 'amino' groups, respectively. Curve C has been drawn through points calculated from the data in Table 3 for the production of volatile bases. As was found by Shore, Wilson & Stueck (1936) for ovalbumin, the early, short-lived, rapid production of volatile base (essentially NH_3) was followed by a long slow production, and these phases may almost certainly be attributed substantially to deamidation and deamination (including, perhaps, decarboxylation) reactions, respectively. Whereas Shore *et al.* found the deamination with ovalbumin to be almost linear with time of hydrolysis, the rate of deamination with the *P. tuberosa* leaf-proteins had begun to fall perceptibly towards the end of the period of hydrolysis. The amide content of this preparation has already been reported as 4.74% of the protein-N (Lugg & Weller, 1944), this value being calculated from the volatile base produced during a 3 hr. hydrolysis at 100° with 2N-HCl. Back extrapolation of the more nearly linear portion of curve C , Fig. 2 (preferably drawn to larger scale), subsequent to the virtual completion of the presumed deamidation (10–20 hr.), suggests approx. 4.8% for the amide-N, a value agreeing closely with that found at 2 hr. and with that reported by Lugg & Weller (1944). Curve E has been constructed by subtracting values on curve C from corresponding values on curve B , and curve D by adding quantities representing the amide NH_3 produced (after about 2 hr. this was 0.155) to all points on curve E . Thus curve E shows the net production (i.e. liberation minus destruction) of non-volatile 'amino' groups, and curve D purports to show the net production of amide- NH_3 plus amino-acid 'amino' groups.

The general impression gained from a study of Fig. 2 is that both 'carboxyl' and 'amino' groups were liberated by rapid reactions in the early stages of hydrolysis and by slow reactions thereafter. During the early stages, 'carboxyl' groups were liberated more rapidly than were 'amino' groups, and the difference is probably to be sought in the

liberation of acid groups from the impurity. By about 15 hr. the rate of net production (i.e. rate of liberation minus rate of destruction) of 'carboxyl' groups had fallen below the rate of net production of 'amino' groups (i.e. below the rate of liberation of 'amino' groups, as their rate of destruction seems to have been zero).

The decarboxylation of a dicarboxylic amino-acid and the deamination of a diamino-acid should yield amino-acids in the first step, but the decarboxylation of a monocarboxylic amino-acid and the deamination of a monoamino amino-acid should yield an amine and a carboxylic acid, respectively. In the event of a simple amino-acid undergoing decarboxylation and deamination, the only titratable residue in the procedure adopted here would be the NH_3 . Volatile basic groups may thus be produced from amino-acids by deamidation and deamination reactions (giving NH_3) and by decarboxylation reactions (giving amines). This last-mentioned potential source of volatile base seems generally to have been ignored. However, Table 3 suggests that even after 385 hr. hydrolysis, by which time more than half of the volatile base must have arisen in deamination and decarboxylation reactions, only a trace of 'amine' (if any) could have been included. If decarboxylation of amino-acids is appreciable under these conditions, then the tentative conclusion must be drawn that the amino groups too are usually involved or that any amines formed are rapidly deaminated.

Whereas a decarboxylated amino- or carboxylic-acid ceases to provide an acid group for the titrations (the CO_2 , being lost in the procedure adopted here, fails to affect even the 'formaldehyde' titrations), a deaminated amino-acid, yielding NH_4Cl , provides a basic group as before. But because of the presence of acid groups derived from the impurity, the 'carboxyl' group net production curve (showing a maximum at about 60 hr.) cannot be given much consideration when judging optimum conditions of hydrolysis. Attention must be concentrated rather upon curve D , which shows a maximum at about 75 hr. If any considerable fraction of the 'amino' groups were to remain unliberated at this point it would be preferable to hydrolyze beyond it, and to apply correspondingly greater corrections for the losses of amino-acids by the degradation reactions, than to have considerable quantities of unknown peptides in the hydrolysate. In accepting this as the most satisfactory stage of hydrolysis for the object in view, I have taken as a criterion of 'completeness' of hydrolysis a condition differing formally from that of Henriques & Gjaldbak (1910), who chose the stage at which the 'amino'-N minus ammonia-N reaches a maximum.

An important question arises in regard to the deamination. If ammonia were to come from the

guanidinium group of arginine and the imidazole group of histidine, the non-volatile 'amino' groups would not be decreased correspondingly and might even be increased. Deaminative destruction of amino-acids could thus be obscured, and the accepted criterion of 'completeness' of hydrolysis would be unsatisfactory. In tests under conditions similar to those obtaining in the hydrolysis of the protein, arginine (which represents such a large fraction of the protein-N) yielded only 0.02 % of its N as volatile base in 24 hr. and only 0.08 % in 96 hr. This extremely small production of volatile base is reassuring.

By 75 hr. about 0.3 % of the total N still remained to be liberated as 'amino'-N, but (in addition to the amide-N) 1.4 % had been produced as NH_3 by degradation of amino-acids. And, although together with the apparently large destruction of acids coming from the impurity some decarboxylation of amino-acids must have occurred, it seems from the above discussion that this degradation of amino-acids (if appreciable) is already represented, at least in large part, by a portion of the 1.4 % deamination. The considerations are complicated by the possibility of partial decarboxylation of the dicarboxylic amino-acids, but in a general way it seems that the over-all corrections for loss due to decarboxylation and deamination would be substantially the same as those calculated from the deamination alone.

Although repeated evaporation at low temperature removed some of the free organic acids in the final hydrolysate (3 % of the 'carboxyl' groups), protracted extraction with ether removed much more (an additional 7 %). In an experiment with a more impure protein preparation from the leaves of *Trifolium subterraneum*, containing only 13.1 % N and therefore (Lugg, 1939) probably 20 % of impurity, the hydrolysis period was about 75 hr., and of the free organic acids produced it is probable that a much larger fraction had originated in the impurity. Extraction with ether as in the other experiment removed 4 % of the total 'carboxyl' groups in 12 hr., 7 % in 62 hr., 8 % in 86 hr. and 9 % in 180 hr., and, as in the earlier experiment, these quantities were judged to be underestimated by about 10 %. It seems that protracted extraction with ether would generally be more effective than repeated evaporations at low temperature in removing organic acids from the hydrolysates. It is possible, too, that end-products of amino-acid deamination and decarboxylation (presumably, mainly alcohols, if the deamination is hydrolytic) would be removed almost quantitatively by the ether.

From actual and presumptive knowledge of the composition of this protein (Lugg & Weller, 1944; Lugg, 1943), knowledge of the general effects of the impurities upon the estimations of the amino-acids

and knowledge of the behaviour of the various amino-acids in the titration procedure, it is possible to calculate the total 'amino'-N approximately. Thus, of the (say) 15 % of arginine-N not more than about 26 %, of the 2.08 % of tryptophan-N not more than about 50 %, and of the (say) 2 % of histidine-N not more than about 39 %, could appear as 'amino'-N, even if these amino-acids were wholly free in the solution. Of the 3 % insoluble humin-N, probably two-thirds would be represented by the tryptophan and the remaining third probably by fractions of the tyrosine, cyst(e)ine and methionine, for the most part (Lugg, 1939). It is unlikely that more than a very small amount of N could have been associated with the soluble humin (Lugg, 1939), but it is not known whether it would or would not be estimated as 'amino'-N. Thus, not more than about 84.6 % of the total N could be expected to appear as 'amino'-N. This value would be still further decreased, by the fact that the titrations of the 'amino' groups of the other amino-acids must have been short of completion (e.g. see Levy, 1933) by from about 0.1 % in the case of glycine (say 0.4 % of the protein-N) to about 7 % in the case of proline (say 2 % of the protein-N), the over-all discrepancy being of the order of about 1 %. On the other hand, the value would be increased in consequence of the phenolic OH group in tyrosine being about 10 % titrated in the 'formaldehyde' titration and by the fact that the 'plain' titration at pH 6.25 of the acids formed from the contaminant was found to be about 10 % short of completion. The actually estimated 'amino'-N, calculated from curve B and the known volume of hydrolysate, was 85.4 % of the total N.

SUMMARY

1. A study has been made of the liberation and destruction of 'carboxyl' and 'amino' groups, during the acid hydrolysis for 385 hr. at 99°, of an impure protein preparation obtained from plant leaves.

2. The maximum net production of amide- NH_3 plus amino-acid 'amino' groups (essentially NH_2 groups except that in the guanidinium ion of arginine, proline-N and hydroxyproline-N), has been formulated as a criterion of 'completeness' of the protein hydrolysis. The net production of 'carboxyl' groups could not be used in the formulation, because some of them came from the impurity.

3. Under the conditions employed (using for hydrolysis 10 ml. of 6.08N-HCl/g. protein) the period needed to meet the requirements of this criterion was about 75 hr. This is notably in excess of the time which might have been presumed necessary from the work of earlier investigators with other proteins. The net production of 'carboxyl' groups was at a maximum at about 60 hr. At 75 hr. the

'amino'-N represented 85.4 % of the total N, and although about 0.3 % of the total N yet remained to be liberated as 'amino'-N, some 1.4 % had already appeared as NH_3 (in addition to amide-N) from degradation of amino-acids.

4. Tests indicated that volatile amines (substituted ammonia) could have been present only in traces in the hydrolysate, and the conclusion has been drawn that when an amino-acid is decarboxylated under the above conditions, the amino group also must usually be involved or the amine first formed must be deaminated rapidly.

5. Organic acids arising from decomposition of the impurity (believed to be a polysaccharide or polyuronide, like mucilage) and by deamination of amino-acids, were found to be more readily removed by prolonged extraction of the hydrolysate with ether, than by repeated evaporation with water at the boiling-point under low pressure.

I am indebted to Mr R. J. Cowan of the Institute of Medical and Veterinary Science for certain preliminary pH measurements, and to Mr D. W. Dewey for assistance with later measurements of the pH values.

REFERENCES

- Aso, K. (1939). *J. agric. chem. Soc. Japan*, **15**, 161.
 Batchelder, A. C. & Schmidt, C. L. A. (1940). *J. phys. Chem.* **44**, 893.
 Charitshkov, K. V. (1906). *J. Soc. phys.-chim. russe*, **7**, 1067.
 Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 372.
 Fischer, E. (1902). *Ber. dtsch. chem. Ges.* **35**, 1095.
 Greenstein, J. P. (1931). *J. biol. Chem.* **93**, 479.
 Henriques, V. & Gjaldhæk, J. K. (1910). *Hoppe-Seyl. Z.* **67**, 8.
 Hirst, E. L. & Jones, J. K. N. (1937). *J. Soc. chem. Ind.* **56** (*Chem. & Ind.* **15**), 724.
 Hofmeister, F. (1902). *Ergebn. Physiol.* **1**, 759.
 Levy, M. (1933). *J. biol. Chem.* **99**, 767.
 Linderström-Lang, K. (1928). *Hoppe-Seyl. Z.* **173**, 32.
 Link, K. P. & Niemann, C. (1930). *J. Amer. chem. Soc.* **52**, 2474.
 Lüters, H. (1920). *Biochem. Z.* **104**, 30.
 Lugg, J. W. H. (1939). *Protein Metabolism in the Plant*, Appendix I (A. C. Chibnall). New Haven: Yale Univ. Press.
 Lugg, J. W. H. (1943). *Biochem. J.* **37**, 132.
 Lugg, J. W. H. & Weller, R. A. (1944). *Biochem. J.* **38**, 408.
 Nassett, E. S. & Greenberg, D. M. (1929). *J. Amer. chem. Soc.* **51**, 836.
 Neuberger, A. (1944). *Biochem. J.* **38**, 309.
 Richardson, G. M. (1934 a, b, c). *Proc. roy. Soc. B*, **115**, 121, 142, 170.
 Ritthausen, H. (1868). *J. prakt. Chem.* **103**, 193.
 Schott, H. F., Rockland, L. B. & Dunn, M. S. (1944). *J. biol. Chem.* **154**, 397.
 Shore, A., Wilson, H. & Stueck, G. (1936). *J. biol. Chem.* **112**, 407.
 Simms, H. S. (1928). *J. gen. Physiol.* **11**, 629.
 Sorensen, S. P. L. (1907). *Biochem. Z.* **7**, 45.
 Steinhardt, J. & Fugitt, C. H. (1942). *Bur. Stand. J. Res., Wash.*, **29**, 315.
 Van Slyke, D. D. (1911). *J. biol. Chem.* **9**, 185.
 Van Slyke, D. D. (1912). *J. biol. Chem.* **12**, 295.
 Wadsworth, A. & Pangborn, M. C. (1936). *J. biol. Chem.* **116**, 423.
 Wilson, H. & Cannan, R. K. (1937). *J. biol. Chem.* **119**, 309.

Ascorbic Acid Content of Cornea

By ANTOINETTE PIRIE, *Nuffield Laboratory of Ophthalmology, Oxford*

(Received 13 October 1945)

In several animal species the aqueous humour contains 20–30 times as much ascorbic acid as the blood plasma. This was first shown by Müller (1933) and the average values collected by Bellows (1944) are: cattle 20, rat 15, rabbit 25, and guinea-pig 13 mg./100 ml. This high concentration in a body fluid is exceptional for, in general, ascorbic acid is more concentrated in the body cells than it is in the fluids. For example, Ralli & Sherry (1941) found that human white blood cells contain 25–38 mg. ascorbic acid/100 g., while average values for human plasma are below 1.0 mg./100 ml.

Other parts of the eye also contain high concentrations of ascorbic acid. The retina (Sullmann & Schmidt, 1942), the vitreous humour (Johnson, 1936) and the ciliary processes (Friedenwald, Buschke & Michel, 1943) in cattle, rabbits and guinea-pigs have about 15–20 mg./100 g.: the concentration in the lens may be higher or lower than it is in the aqueous humour according to the species of animal studied (Bellows, 1944). Friedenwald *et al.* (1943) consider that ascorbic acid is secreted into the aqueous humour by the ciliary body and that this is the source of the ascorbic acid in the lens. The aqueous