absolute values of tyrosine and tryptophan in 'pure' proteins. The proteins quoted in Table 6 are normal commercial products and no attempt has been made to assess their purity.

SUMMARY

1. The ultra-violet absorption spectra of tyrosine and tryptophan have been determined in 0·1N-NaOH and the curves found to intersect at 257·15 and 294·4 mμ (ε, 2748 and 2375, respectively).

2. By using intensities of absorption at 294·4 and 280 mμ, determined by means of a photoelectric spectrophotometer, mixtures of tyrosine and tryptophan may be analyzed with considerable accuracy provided the molar ratios are not greater than 20 : 1 either way.

3. Using about 25 mg. of material, proteins may be analyzed with comparable accuracy if due correction is made for irrelevant continuous absorption.

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The Estimation of Threonine and Serine in Proteins

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Although serine was discovered by Cramer as long ago as 1865, its isolation by the older gravimetric methods was so laborious and intractable, except perhaps from the proteins of silk, that the values recorded in the literature for the amounts present in other proteins were no more than minimal estimates. Threonine, discovered as late as 1935 by McCoy, Meyer & Rose, was likewise in the same category; feeding experiments showed that its presence in proteins must be widespread, but quantitative data were entirely lacking. As a consequence, the new and indirect method introduced by Nicolet & Shinn (1939) for the estimation of these two hydroxy-amino-acids has attracted attention.

Nicolet & Shinn showed that β-hydroxy-α-amino-acids react with periodates. Serine and threonine give ammonia, glyoxylic acid and a free aldehyde, and under suitably chosen conditions the reaction is quantitative and practically instantaneous. The formaldehyde and acetaldehyde thus formed were made the basis for the determination of serine and threonine in protein hydrolysates.

Shinn & Nicolet (1941) found that the acetaldehyde produced from threonine is carried over by aeration at a pH close to 7, and preferably pH 7·0–7·2. In the case of protein hydrolysates, the formaldehyde produced from the serine does not cause contamination as it is held back by the large excess of primary amino groups present; with relatively pure solutions of serine and threonine the tendency for inconvenient amounts of formaldehyde to volatilize may be sufficiently restrained by the addition of an adequate excess (10 mol. or more) of alanine. In their procedure for the estimation of threonine, the periodate reaction is carried out in the presence of a bicarbonate buffer and the pH maintained within the suggested range by aeration with CO_2. The acetaldehyde is absorbed in sodium bisulphite solution and a subsequent titration gives a measure of threonine. As a routine procedure in protein analysis they admit that such evidence is not specific for acetaldehyde but, according to their experience, treatment of the bisulphite solution to yield again the aldehyde for precipitation with dimedone (Vörlander, 1929) always gave an approximately pure dimedone derivative. Martin & Synge (1941), exploring the possible use of the periodate reaction for the same purpose, could find no evidence for the presence of higher homologues of acetaldehyde, and hence of higher homologues of threonine, on treatment of a number of protein hydrolysates.

Continuing their studies, Nicolet & Shinn (1941*a*) found that the formaldehyde produced from serine could be determined as the dimedone derivative by adding the reagent to the residual acetaldehyde-free solution. From known mixtures, the recovery of serine ranged from 95 to 98%. Meanwhile Van Slyke, Hiller, MacFadyen, Hastings & Klemperer (1940) had made use of the ammonia produced in the periodate reaction in their investigations on the structure of hydroxylysine, and had incidentally reported

that synthetic β-hydroxyglutamic acid gave the expected quantitative yield of ammonia under such treatment. Nicolet & Shinn (1942) subsequently pointed out that the ammonia produced on periodate oxidation of a protein hydrolysate gave a measure of the 'total hydroxyamino-acids present' and that in the absence of β-hydroxyglutamic acid (which yields no volatile aldehyde), the amount of ammonia formed should be quantitatively equivalent to the total volatile aldehyde given by serine, threonine and, if present, hydroxylysine. Their balance sheets for casein, lactalbumin and lactoglobulin showed that no more than a relatively insignificant amount of β-hydroxyglutamic acid could be present in any of these proteins after hydrolysis.

Modifications of Nicolet & Shinn's procedures have been introduced by subsequent workers. Winnick (1942) has adapted the micro-diffusion technique of Conway to the estimation of threonine as acetaldehyde, while Boyd & Logan (1942) prefer to estimate serine as formaldehyde by a colorimetric procedure after removal from the reaction mixture by distillation. Apart from their applicability in the estimation of smaller amounts of the hydroxyamino-acids concerned, neither of these modifications is necessarily superior to the original. Desnuelle, Antonin & Naudet (1944*a*, *b*) have also made use of colorimetric procedures for estimating the aldehydes without previous removal from the periodate oxidation mixture.

The present work has been of somewhat slow growth. Initiated as nothing more ambitious than the simple application of the methods of Nicolet & Shinn to various proteins of interest in this laboratory, apparent improvements have suggested themselves from time to time as experience has been gained. After adequate trial many of these have been subsequently discarded, yet some have stood the test and the modified procedures now presented, especially in the estimation of serine, incorporate certain features which experience suggests as advantageous. Much time has been devoted to the estimation of formaldehyde and to the question of whether the full amount of this aldehyde given on oxidation of protein hydrolysates can legitimately be ascribed to serine. This latter problem has been simplified by the development of the new procedure described later for the quantitative estimation of hydroxylysine, so that due allowance for the formaldehyde given by this base can be made. The data given later in Table 2, showing good equivalence between the NH_3 and the total volatile aldehyde obtained on periodate oxidation of various protein hydrolysates, serve to reinforce the conclusion to be drawn from the researches of earlier workers (Dakin, 1941; Nicolet & Shinn, 1942; Bailey, Chibnall, Rees & Williams, 1943) that β-hydroxy-glutamic acid, which would give ammonia but no volatile aldehyde, is not a constituent of the proteins concerned.

The present estimation of threonine as acet-aldehyde follows closely that of Shinn & Nicolet (1941). The bicarbonate buffer is replaced by a phosphate buffer so that acidification of the residual acetaldehyde-free solution can be made without copious evolution of carbon dioxide, and excess arsenite is added (Boyd & Logan, 1942) to prevent the distillation of iodine during the subsequent removal of formaldehyde in steam. Under the conditions employed no significant oxidation of acetaldehyde occurs when this is carried over by aeration with air. To estimate serine, the formal-dehyde is removed by distillation in steam under appropriate conditions; this is trapped in sodium bisulphite solution, which is subsequently titrated with iodine in the usual way. Such a procedure, though non-specific, is speedy and makes use of the same reagents as are required for the estimation of threonine. As a precaution when dealing with protein hydrolysates, the steam distillate from one estimation is received direct in dimedone solution so that both the yield and the melting-point of the dimedone derivative can be checked. The procedure of Van Slyke, Hiller & MacFadyen (1941) with only one minor modification, has been used for the estimation of periodate ammonia-N.

During the course of this work it has been necessary in all cases of protein or control mixture analysis to obtain a value for ammonia-N given after hydrolysis for 24 hr. with 20% (w/w) HCl. Such a value is always in excess of the true amide-N, and as both serine and threonine undergo a slow decomposition to give ammonia during hydrolysis, opportunity has been taken to compare the ammonia-N thus produced with the excess just mentioned.

EXPERIMENTAL

Procedures for the estimation of threonine, serine and periodate ammonia

Reagents required. Potassium arsenite solution: 25 g. $KAsO_2$ are dissolved in water, diluted to 100 ml., and the alkalinity adjusted with N-H_2SO_4 so that on addition of 1 ml. 0·5M-periodic acid to 1·5 ml. of the solution in 20 ml. water the pH is brought to 7·0 (bromthymol blue). In practice, it is more convenient to determine the amount of H_2SO_4 required to bring 1·5 ml. of the arsenite solution in 20 ml. of water to the correct pH and add each reagent separately to the reaction tubes, as the adjusted reagent crystallizes from solution after 3–4 days.

Commercial sodium bisulphite solution, sp.gr. 1·34. 5 ml. are diluted to 100 ml. as required.

Phosphate buffer, pH 7·16. Made up as required by mixing 30 ml. of KH_2PO_4 solution (18·156 g./l.) and 70 ml. Na_2HPO_4 solution (19·536 g./l.).

Periodic acid, 0·5M (114 g./l.).

0·01N-*iodine*. Prepared as required from stock 0·1N-solution.

Starch solution. 0·5% in 20% (w/v) NaCl (filtered).

Apparatus required. That of Shinn & Nicolet (1941) is used for the estimation by aeration of acetaldehyde and of periodate-ammonia. Four sets of tubes are usually run in series. For the separation of formaldehyde by steam distillation the same apparatus as that used for micro-

Kjeldahl ammonia is employed. The distillation flask, through which the stream is passed, is of 100 ml. capacity and is not surrounded by the usual vacuum jacket. This distillation flask stands on an asbestos plate provided with a window covered with wire gauze, under which is placed a gas burner so that the contents of the flask can be concentrated if required. The apparatus is set up in quadruplicate.

Estimation of threonine as acetaldehyde. The procedure is essentially that of Shinn & Nicolet (1941). One of the four sets of tubes contains a control mixture (approximately 5 mg. serine, 5 mg. threonine and 50 mg. alanine) so that the percentage recovery of acetaldehyde in the other three can be estimated. Aeration is obtained by suction and a flow-meter is introduced between the suction pump and the complete apparatus. The incoming air is drawn through two wash bottles containing approximately 80 ml. dilute $NaHSO_3$ solution and then through another containing 5% (w/v) NaOH. The four sets of tubes are connected in series through wash bottles containing 50 ml. 5% NaOH (changed after three estimations).

To carry out the estimation approximately 3·5 and 2·5 ml. of bisulphite solution are added to the first and second receiver tubes respectively and diluted to 25 ml. A sample of the protein or test solution, containing between 1·5–7 mg. threonine and 2–7 mg. serine respectively is pipetted into the reaction tube and adjusted to pH 7·0 with N-NaOH. Arsenite solution (1·5 ml.) is then added, followed by the requisite amount of N-H_2SO_4 and 6 ml. phosphate buffer, and the contents of the tube thoroughly mixed after finally adjusting the vol. to approximately 18 ml. When each of the four sets of tubes have been prepared in this way the stop-cocks of the separating funnels are closed and into each funnel is introduced 1 ml. 0·5M-periodic acid and 3 ml. water. The whole apparatus is then connected up, air drawn through slowly and the stop-cocks opened. After 3–4 min. the rate of aeration is increased to 1 l./min. and continued for 1 hr. The suction is then stopped and the contents of each pair of receiver tubes washed into a titration flask. These flasks are stoppered and set aside until the formaldehyde distillations described below are completed.

To estimate the acetaldehyde, the excess bisulphite is oxidized with approximately 0·1N-iodine and the final end-point obtained with 0·01N-iodine, 2 ml. of starch solution being used as indicator (cf. Clausen, 1922). Approximately 7 ml. M-$NaHCO_3$ are now added and the liberated bisulphite titrated with 0·01N-iodine until the fading rate of the blue colour slows down. Another 2 ml. of the $NaHCO_3$ solution are now added and the titration continued; after adding a further 2 ml. the titration is taken to completion. When the final end-point is reached the blue colour is fairly stable and persists for 1 min. or longer. If need be, the end-point can be confirmed by adding an additional 1 ml. $NaHCO_3$ solution.

Estimation of serine as formaldehyde. After removal of the acetaldehyde by aeration the contents of each reaction tube are quantitatively transferred without undue delay to a micro-Kjeldahl distillation flask, into which 0·6 ml. N-H_2SO_4 has already been run. By varying the size of the micro-burner flame and the rate of entry of steam, the distillation proceeds so that the contents of the flask are concentrated to a final vol. of 5 ml. in 30 min. The receiver flask, containing approximately 250 ml. of distillate, is stoppered and set aside until the acetaldehyde titrations have been completed. The procedure for the titration of the formaldehyde is similar to that for acetaldehyde, except that 4 ml. of starch solution are used and 5% (w/v) Na_2CO_3 replaces M-$NaHCO_3$. These threonine-serine determinations are usually carried out in triplicate, one of the formaldehyde estimations being made with dimedone. In this latter case, the formaldehyde is distilled into 25 ml. dimedone solution (saturated at room temperature). Crystallization of the dimedone derivatives is allowed to proceed for 24 hr. or longer after adjusting the solution to pH 4·6 (cf. Yoe & Reid, 1941).

Estimation of periodate-ammonia. The preformed NH_3 present in the protein hydrolysate or in the control mixture was determined in the micro-Kjeldahl distillation apparatus, using N/70-HCl and N/70-NaOH. The measured sample, containing 1·2–1·7 mg. NH_3 was brought to pH 4 with N-NaOH and then treated with 5 ml. borate-NaOH buffer, pH 11·0. The determination of periodate-NH_3 follows closely that of Van Slyke *et al.* (1941), with the exception that the NH_3 (periodate + preformed) was first aerated (for 45 min.) into excess H_2SO_4 and then estimated by distillation as described above.

Analysis of control mixtures

The recoveries obtained with threonine and serine were 99 ± 1% and 100 ± 2% respectively as aldehyde and 99 ± 1% and 99 ± 1% respectively as periodate ammonia-N. During acid hydrolysis, however, both of these hydroxyamino-acids undergo a progressive decomposition linear with time, as represented in Figs. 1 and 2 (cf. Lowndes, Macara & Plimmer, 1941). The aldehyde and periodate ammonia-N estimations on the unchanged material are in good agreement in each case, and the N representing the decomposition products is present quantitatively as free NH_3. After hydrolysis with 20% (w/w) HCl for 24 hr. (the standard procedure for proteins) the aldehyde recovery of threonine was 94·7% and of serine 89·5%. To ascertain whether corrections based on these recoveries are permissive in the case of protein hydrolysates, appropriate analyses have been made on amino-acid control mixtures (cf. Tristram, 1946) representing hydrolysates of insulin, edestin, horse globin and β-lactoglobulin respectively. Interference might be expected if one or more of five amino-acids are present. Formaldehyde condenses readily with tryptophan. In keeping with this observation the recovery of serine as formaldehyde on analysis before hydrolysis of the control mixtures containing tryptophan (edestin, horse globin, β-lactoglobulin) was irregular, and duplicate determinations differed by several per cent. After hydrolysis, which destroys the tryptophan, the recoveries were normal and reproducible. Under certain conditions formaldehyde may condense with histidine (Neuberger, 1944) and thus reduce the recovery of serine as this aldehyde. The results for the globin control mixture, which contains a very high proportion of histidine show that under the experimental conditions employed, no appreciable loss

occurs. Glycine may also interfere with the formaldehyde estimation of serine, but only if present in amount representing 50 % or more of the

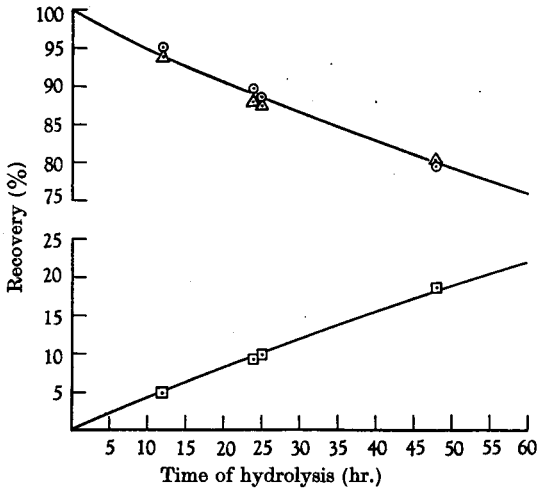

Fig. 1. Decomposition of serine on HCl hydrolysis. ⊡ Ammonia obtained on hydrolysis. ⊙ Formaldehyde obtained on periodate oxidation. △ Ammonia obtained on periodate oxidation.

total N of the amino-acid mixture, e.g. when used to replace alanine in the preceding operation to estimate threonine as acetaldehyde. Even under these rather extreme conditions, which are outside those likely to be met with when dealing with protein hydrolysates, the recovery of formaldehyde is lowered by no more than 4 %. Nicolet (1943) has suggested that the presence of cystine might give rise to high values for periodate ammonia-N. The analysis of the insulin control mixture, which

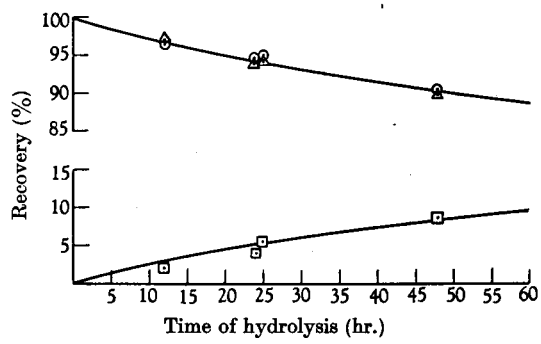

Fig. 2. Decomposition of threonine on HCl hydrolysis. ⊡ Ammonia obtained on hydrolysis. ⊙ Acetaldehyde obtained on periodate oxidation. △ Ammonia obtained on periodate oxidation.

contained 11·1 % cystine-N (as % of total N), and experiments with cystine itself, show that under the conditions now employed no appreciable error is involved. There is a suggestion nevertheless that the presence of a relatively large amount of cystine or perhaps of tyrosine during hydrolysis has reduced by 2–4 % the subsequent recovery of serine as formaldehyde.

Table 1. *Estimation of serine and threonine in various control mixtures before and after hydrolysis*

			Before hydrolysis		After hydrolysis			
Control mixture	Type of N	Amount of N	Found (N as % total N)	Recovery (%)	Found (N as % total N)	Recovery (%)	Corrected for decomposition (N as % total N)	Recovery (%)
Serine alone	Serine-N	100	100	100	89·5	89·5	100	100
Threonine alone	Threonine-N	100	99·0	99·0	94·7	94·7	100	100
(Edestin)	Serine-N	4·51	*	*	4·03	89·2	4·50	99·8
	Threonine-N	2·43	2·41	99·2	2·26	93·3	2·39	98·4
	Total aldehyde-N	6·94	*	*	6·29	90·6	6·89	99·3
	Periodate-N	6·94	6·79	97·8	6·14	88·5	6·74	97·1
(Horse globin)	Serine-N	4·60	*	*	4·03	87·6	4·50	97·9
	Threonine-N	3·05	3·04	99·5	2·92	95·9	3·08	101·0
	Total aldehyde-N	7·65	*	*	6·95	90·8	7·58	99·1
	Periodate-N	7·65	7·76	101·4	7·01	91·6	7·65	100·0
(Insulin)	Serine-N	4·48	4·41	98·5	3·85	86·0	4·30	96·0
	Threonine-N	1·57	1·56	99·3	1·47	93·8	1·55	98·7
	Total aldehyde-N	6·05	5·97	98·9	5·32	87·9	5·85	96·7
	Periodate-N	6·05	5·97	98·8	5·40	89·4	5·95	98·3
(β-Lactoglobulin)	Serine-N	3·34	*	*	2·98	89·4	3·33	99·7
	Threonine-N	3·85	3·84	99·7	3·62	94·0	3·82	99·3
	Total aldehyde-N	7·19	*	*	6·60	91·8	7·15	99·4
	Periodate-N	7·19	7·08	98·5	6·52	90·6	7·06	98·2

* Recoveries were irregular, due to the presence of tryptophan (see text).

In all other respects the control analyses given in Table 1 show normal recoveries, and the data given in columns 8 and 9 show that corrections of 100/94·7 and 100/89·5 to allow for the decomposition during hydrolysis of threonine and serine respectively are permissive. These corrections have accordingly been applied to the protein analyses given later in Tables 3 and 4, yet it should be emphasized that in these cases the application may not be strictly valid, since during the early stages of hydrolysis the hydroxyamino-acid concerned will be still in peptide combination, and hence perhaps more prone to decomposition than when in the free state. It is known that when serine is present in the protein in the form of an ester with phosphoric acid—as it is, for instance, to a small extent in casein—it undergoes considerable destruction on acid hydrolysis (Posternak, 1927; Damodaran & Ramachandran, 1941).

Analysis of various proteins

The protein preparations used in the present work have already been described (Chibnall, Rees & Williams, 1943; Macpherson, 1946). In all cases hydrolysis was effected by boiling with 20% (w/w) HCl for 24 hr. The data given in Table 2 show an excellent agreement between the total aldehyde recovery, expressed as N, and the periodate ammonia-N, in all cases except that of ovalbumin, which is referred to later.

Table 2. *Aldehyde-ammonia balance on periodate oxidation of various protein hydrolysates*

	N as % protein-N		
Protein	N equivalent of volatile aldehydes	Periodate ammonia	Difference
Amandin	4·65	4·61	+0·04
Casein	7·69	7·80	−0·11
Collagen*	4·70	4·57	+0·13
Edestin	6·34	6·28	+0·06
Gelatin*	4·59	4·72	−0·15
Gliadin	4·64	4·58	+0·06
Haemoglobin (horse)	7·01	7·08	−0·07
Insulin	5·49	5·55	−0·06
β-Lactoglobulin	6·78	6·74	+0·04
Myosin (rabbit)	6·52	6·43	+0·09
Porcupine quill tip	7·23	7·33	−0·10
Ovalbumin	9·76	9·02	+0·74
Salmine	3·07	3·00	+0·07
Silk fibroin	11·34	10·98	+0·36
Wool keratin	11·71	11·82	−0·11

* Contains hydroxylysine.

The analyses for threonine and serine are given in Tables 3 and 4, the data in columns 4 and 6 being based on recoveries of 94·7 and 89·5% respectively. Values (uncorrected for decomposition on hydrolysis) obtained by other workers who have used the periodate method in one or other of its forms, or the method of microbiological assay, are given for comparison. Comment is perhaps premature as all the various procedures are new and have not yet been widely employed: nevertheless the data given in Table 2 make it difficult to believe that the values now reported can be much in error. As Chibnall (1946) has recently pointed out, the method of micro-biological assay has so many factors in its favour that it is likely to replace in the near future all others demanding a more laborious technique, provided that it passes the necessary stringent tests. The data for serine and threonine now presented may perhaps contribute towards this ultimate goal.

The estimation of serine as formaldehyde in the presence of carbohydrate

Formaldehyde is produced on periodate oxidation of certain carbohydrates, so that the estimation of serine as this aldehyde in hydrolysates of proteins that contain a carbohydrate moiety (e.g. ovalbumin) or a carbohydrate adulterant (e.g. crude plant products) may well give fictitiously high values. Moreover, the fate of the carbohydrate material during the acid hydrolysis cannot be predicted with certainty, so that it is impossible to suggest any simple and direct correction for the error thus introduced. The point can be illustrated by reference to ovalbumin. In Table 2 the value for total aldehyde-N exceeds that of periodate ammonia-N by 0·74%. According to Neuberger (1938), the carbohydrate moiety in 100 g. ovalbumin contains 1·8 g. mannose and 0·9 g. glucosamine. A mixture of mannose and glucosamine in these proportions, and in amount equivalent to that present in an actual analysis of ovalbumin itself, was passed through the usual hydrolysis and periodate procedures. The serine-N equivalent to the formaldehyde produced was equal to 0·38% of the total N of the ovalbumin assumed to be present. It should be pointed out here that in this, as in all such estimations, any volatile aldehyde (e.g. furfuraldehyde) produced during the hydrolysis with acid will have been removed during the treatment preceding periodate oxidation. With the knowledge at present available it is thus clearly impossible to account for the full excess of formaldehyde given by the ovalbumin itself. With proteins of this type therefore the serine-N, as a first approximation, must be estimated as the difference between the periodate NH_3-N and the threonine-N. Such a procedure is reminiscent of the pioneer work of Martin & Synge (1941). In the case of ovalbumin the value for serine-N is thereby reduced to 6·17% of the protein-N (Table 4).

Table 3. *Threonine contents of various proteins*

Protein	Total N (%)	Threonine-N as % protein-N: Found	Threonine-N as % protein-N: Corrected	Wt. threonine/100 g. protein: Found	Wt. threonine/100 g. protein: Corrected	Literature Wt. threonine/100 g. protein: Periodate oxidation method	Literature Wt. threonine/100 g. protein: Microbiological assay method
Amandin	18·75	1·64	1·73	2·62	2·77	—	—
Casein	15·65	3·22	3·40	4·31	4·55	3·5[11], 4·6[9], 3·2[2], 4·32[3], 3·95[4]	4·1[8], 4·2[1] 3·8[12]
Collagen	18·20	1·39	1·47	2·16	2·28	—	—
Edestin	18·65	2·30	2·43	3·64	3·85	2·2[11]	—
Gelatin	18·00	1·36	1·44	2·08	2·20	—	2·0[1]
Gliadin	17·66	1·33	1·40	1·98	2·09	2·92[3]	—
Haemoglobin (horse)	16·80	2·89	3·05	4·14	4·37	4·0[11]	—
Insulin	15·54	1·48	1·57	1·97	2·07	2·66[7], 3·2[10]	—
β-Lactoglobulin	15·58	3·66	3·87	4·84	5·11	4·72[4], 5·85[6] 5·35[3]	4·6[1]
Myosin	16·80	3·42	3·61	4·89	5·17	—	—
Porcupine quill tip	16·1	2·69	2·84	3·68	3·89	—	—
Ovalbumin	15·76	2·85	3·02	3·83	4·05	4·8[11]	3·6[1]
Silk fibroin	18·71	0·94	0·99	1·49	1·57	1·54[5]	1·36[1]
Wool keratin	16·3	4·38	4·63	6·08	6·42	—	—

(1) Stokes, Gunness, Dwyer & Caswell (1945).
(2) Borchers, Totter & Berg (1942).
(3) Winnick (1942).
(4) Nicolet & Shinn (1942).
(5) Nicolet & Saidel (1941).
(6) Brand & Kassell (1942).
(7) Nicolet & Shinn (1941*b*).
(8) Hier, Graham, Friedes & Klein (1945).
(9) Williamson (1944).
(10) Brand (private communication).
(11) Desnuelle, Antonin & Naudet (1944*b*).
(12) Greenhut, Schweigert & Elvehjem (1946).

Table 4. *Serine contents of various proteins*

Protein	Total N (%)	Serine-N as % protein-N: Found	Serine-N as % protein-N: Corrected	Wt. serine/100 g. protein: Found	Wt. serine/100 g. protein: Corrected	Literature: Serine-N as % protein-N (periodate oxidation method)	Literature Wt. serine/100 g. protein: Periodate oxidation method	Literature Wt. serine/100 g. protein: Microbiological assay method
Amandin	18·75	3·01	3·36	4·24	4·74	—	—	—
Casein	15·65	4·47	5·00	5·28	5·90	4·75[1]	5·82[2], 5·0[5], 5·3[6]	6·4[6]
Collagen*	18·20	2·21	2·46	3·01	3·37	3·22[1]	—	—
Edestin	18·65	4·04	4·51	5·66	6·32	—	2·0[8]	—
Gelatin*	18·00	2·11	2·36	2·85	3·18	—	3·32[9]	3·8[6]
Gliadin	17·66	3·31	3·70	4·38	4·90	—	—	—
Haemoglobin (horse)	16·80	4·12	4·60	5·18	5·79	4·43[1]	4·15[8]	—
Insulin	15·54	4·01	4·48	4·67	5·22	—	3·57[4], 5·8[7]	—
β-Lactoglobulin	15·58	3·12	3·49	3·64	4·07	—	3·12[2], 5·0[7]	3·2[6]
Myosin (rabbit)	16·80	3·10	3·46	3·91	4·37	—	—	—
Porcupine quill tip	16·10	4·54	5·07	5·47	6·11	—	—	—
Ovalbumin†	15·76	6·17	6·90	7·29	8·14	6·28[1]	4·85[8]	—
Silk fibroin	18·71	10·40	11·60	14·53	16·24	—	13·57[3]	14·5[6]
Wool keratin	16·30	7·33	8·19	8·96	10·01	—	—	—
Salmine	24·70	3·07	3·43	5·57	6·35	3·24[1]	—	—

(1) Boyd & Logan (1942).
(2) Nicolet & Shinn (1942).
(3) Nicolet & Saidel (1941).
(4) Nicolet & Shinn (1941*b*).
(5) Williamson (1944).
(6) Stokes & Gunness (1945).
(7) Brand (private communication); values carry a 10% correction for decomposition during hydrolysis.
(8) Desnuelle *et al.* (1944*a*)
(9) Nicolet & Shinn (1941*a*).

* Allowance made for hydroxylysine.

† See text.

The ammonia produced on the complete acid hydrolysis of proteins

Many protein chemists have long considered that the ammonia produced on hydrolysis of proteins (20% (w/w) HCl for 24 hr.) must be in excess of that arising from acid-amide (asparagine and glutamine) residues on account of the decomposition undergone by certain of the amino-acid residues while still in peptide combination or after their appearance as free amino-acids. The progressive decomposition of serine and threonine illustrated in Figs. 1 and 2

shows that these two hydroxyamino-acids must contribute to this excess ammonia and data are now available to investigate the matter in detail.

Values for the true amide-N of a series of proteins were obtained some years ago by the method of Gordon, Martin & Synge (1941) and have been quoted by Chibnall (1942, 1946), but the protocols have not yet been published. The protein (*c.* 0·25 g.) and 100 ml. conc. HCl were maintained in an incubator at 37°. The protein dissolved fairly rapidly in all cases to give a clear solution. Samples were withdrawn at intervals, excess HCl removed *in vacuo* at 37°, and the free ammonia was determined in the micro-Kjeldahl apparatus after neutralization with NaOH and the addition of pH 11·0 buffer. The results are given in Table 5, which also includes data showing that 99 % of the amide-N of asparagine is set free in 220 hr., and that threonine, serine and citrulline give no more than 0·1 % of their total N as ammonia-N under these conditions. The ammonia-N set free on hydrolysis of various control mixtures and proteins with 20 % HCl for 24 hr. was estimated as part of the procedure for the determination of periodate-N, and the values obtained are given in Table 6.

Inspection of the data collected in Table 6 shows, in the first place, that the ammonia-N in excess of the true amide-N of the globin and edestin control mixtures is quantitatively accounted for in terms of threonine and serine decomposition. The first of these mixtures is rich in histidine and lysine, the second in arginine, so that all three bases are apparently stable to hydrolysis, confirming Macpherson (1946). With the insulin control there is

Table 5. *Amide-N of various proteins*

Protein	Amide-N as % total protein-N (Time of hydrolysis, conc. HCl, 37°) (20 hr.)	(32 hr.)	(46 hr.)	(70 hr.)	(130 hr.)	(180 hr.)	(220 hr.)	(300 hr.)	(1000 hr.
Amandin	10·8	—	13·87	—	15·64	—	15·70	15·84	15·80
Casein	5·82	7·27	7·91	8·59	8·79	—	8·99	9·05	9·07
Edestin	3·50	5·10	6·27	8·20	9·25	9·39	9·47	9·49	—
Excelsin	—	—	—	—	—	—	—	8·01	7·96
Gliadin	16·0	19·2	—	24·80	25·35	25·37	—	25·40	25·60
Haemoglobin (horse)	—	3·59	—	4·15	4·37	4·60	4·56	4·58	4·58
Insulin	—	—	—	—	—	—	8·92	—	9·15
β-Lactoglobulin	4·33	5·67	6·18	6·68	6·87	6·86	6·94	7·00	—
Ovalbumin	3·00	4·15	—	5·95	6·35	6·52	6·54	6·58	—
Salmine	—	—	—	—	—	—	0·0	—	—
Asparagine	—	—	48·4	—	—	49·40	49·80	49·80	—
Serine	—	—	—	—	—	—	0·08	—	—
Threonine	—	—	—	—	—	—	0·11	—	—
Citrulline	—	—	—	—	—	—	—	0·25	—

Table 6. *Details of the ammonia-N produced on hydrolysis of various control mixtures and proteins with* 20 % (*w*/*w*) HCl *for* 24 *hr.*

Protein	(Results as % total N.) Ammonia-N given on hydrolysis with 20 % HCl	Amide-N	Difference	Ammonia-N due to decomposition of serine and threonine	Unaccounted excess ammonia-N
Edestin (Amino-acid control mixture)	10·14	9·50	0·64	0·60	+0·04
Globin (horse) (Amino-acid control mixture)	5·23	4·56	0·67	0·63	+0·04
Insulin (Amino-acid control mixture)	9·94	8·99	0·95	0·45	+0·50
β-Lactoglobulin (Amino-acid control mixture)	7·62	6·85	0·77	0·55	+0·22
Amandin	16·74	15·84	0·90	0·44	+0·50
Casein	10·10	9·05	1·05	0·71	+0·34
Edestin	10·36	9·49	0·87	0·60	+0·27
Gelatin	1·45	0·50(1)	0·95	0·33	+0·62
Gliadin	26·10	25·4	0·70	0·46	+0·24
Haemoglobin (horse)	5·54	4·56	0·98	0·64	+0·34
Insulin	10·03	8·95	1·08	0·56	+0·52
β-Lactoglobulin	7·86	6·85	1·01	0·58	+0·43
Myosin (rabbit)	7·97	7·20(2)	0·77	0·55	+0·22
Ovalbumin	7·80	6·58	1·22	0·90	+0·32
Porcupine quill tip	8·17	7·15	1·02	0·68	+0·34
Salmine	0·39	0·0	0·39	0·36	+0·03
Wool keratin	8·60	7·20(1)	1·40	1·11	+0·29

(1) Gordon *et al.* (1941). (2) Bailey (1944).

an unaccounted excess of 0·5% ammonia-N and this probably reflects the high content of tyrosine which is known to be slowly destroyed on acid hydrolysis. Compared with their control mixtures, edestin, haemoglobin and β-lactoglobulin show an unaccounted excess of about 0·3% ammonia-N, a value of the same order as that exhibited by most of the other proteins concerned, suggesting that certain amino-acid residues—and those of serine especially and of threonine must be suspect—are more prone to decomposition when still in peptide combination or during peptide splitting. It would be wise therefore to treat the values for threonine and serine in columns 4 and 6 of Tables 3 and 4 with reserve, as the corrections applied may not adequately represent the decomposition suffered during the acid hydrolysis. The possible error nevertheless will not be very large, even if weighted strongly in favour of one or other of the two hydroxyamino-acids concerned. It is interesting to note that the ammonia given by salmine on 20% HCl hydrolysis is quantitatively accounted for in terms of serine decomposition.

Hydroxylysine content of various proteins

Van Slyke *et al.* (1941) showed that hydroxylysine gave one equivalent of NH_3 on periodate oxidation, and made use of the observation to estimate the amount present in various proteins by applying the principle to a phosphotungstate precipitate of the bases. The only proteins in which they could find any significant amount of the hydroxy-base were gelatin and collagen. The electrodialysis procedure for the separation of the basic amino-acids (Macpherson, 1946) forms an ideal starting-point for the estimation of this particular hydroxyamino-acid, as the cathode solution thus obtained will contain no other (known) substance reacting with periodic acid. The periodate oxidation procedure has been accordingly applied to the base (catholyte) solutions from all the proteins analyzed by Macpherson. In no case was any acetaldehyde, formaldehyde or periodate-NH_3 found except with gelatin and collagen, where the values for hydroxylysine-N were 1·2 and 1·1% respectively in terms of total protein-N. In both cases the value is higher than that recorded by Van Slyke and colleagues, showing the superiority of the electrodialysis procedure over that of phosphotungstic acid precipitation, which must be susceptible to solubility factors. The new values nevertheless are uncorrected for losses on decomposition during hydrolysis, as samples of pure hydroxylysine have not been available for test purposes.

SUMMARY

1. A modification of the Nicolet and Shinn periodate oxidation method for the estimation of serine and threonine is described.

2. Details of the destruction of threonine and serine during hydrolysis with 20% (w/w) HCl are given.

3. Serine and threonine values are presented for various amino-acid control mixtures and protein hydrolysates, obtained by estimating the aldehydes given on periodate oxidation.

4. The total amount of aldehyde produced on periodate oxidation has been checked against the ammonia simultaneously produced. Agreement in all cases is good.

5. The true amide-N and also the ammonia given on hydrolysis of the proteins with 20% HCl have been determined, and the difference between the two values is discussed.

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Tetryl Dermatitis

2. THE INTERACTION OF AROMATIC NITRO-COMPOUNDS WITH AMINO-ACIDS AND PROTEINS

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It is generally assumed that simple chemical substances, which cause sensitization, do so by virtue of their power to couple with proteins, thus producing antigens after introduction into the body. Harington (1944) has established that substances related to tetryl (2:4:6-trinitrophenylmethylnitramine) but lacking the full complement of aromatic nitro groups, fail to give a reaction when skin tests are made on guinea-pigs. Gell (1944) examined a number of compounds in an attempt to find those able to elicit skin reactions in tetryl sensitized guinea-pigs. He also described methods of making picryl-antigens from picryl chloride and rabbit serum, or guinea-pig globulin, but found that guinea-pigs were only weakly or not at all sensitized by intradermal injection of these picryl proteins.

Such results indicated that a more direct chemical investigation of the interaction of tetryl and related compounds with skin protein was desirable. Astbury (1937) has pointed out that fibrous proteins such as keratin, and globular proteins such as egg albumin possess fundamentally the same structure and arrangement of amino-acids. It was decided, therefore, to use crystallized egg albumin in preliminary experiments, where indication of a general reaction was sought, and then by using pure amino-acids, to decide which, if any of these, reacted with the nitro compound used. The amino-acid constitution of crystallized egg albumin is given by Calvery (1932) and Vickery & Shore (1932), and that of human skin by Eckstein (1935) and Block (1935).

METHOD

The following materials have been employed:

Nitro-compounds: tetryl (2:4:6-trinitrophenylmethylnitramine), picryl chloride, picramide (2:4:6-trinitroaniline), *N*-methyl picramide, 2:4:6-trinitrophenetole, 2:4:6-, 2:3:4-, 2:4:5-trinitrotoluene, and picric acid.

Proteins and amino-acids: crystallized egg albumin, *l*(−)-cystine, *l*(−)-tyrosine, *l*(−)-histidine, *l*(+)-arginine, *l*(−)-tryptophan, *l*(+)-lysine, *l*(−)-methionine and *l*(+)-glutamic acid. Experiments were set up to determine the reactivity of each aromatic nitro-compound towards egg albumin and each of the amino-acids listed above.

Solutions were prepared in 100 ml. conical flasks previously cleaned with chromic acid solution. For each experiment 50 ml. amino-acid solution (0·0005 M) and 50 ml. nitro-compound solution (0·0002 M) were mixed, but for the experiments with crystallized egg albumin the protein was dissolved in 50 ml. nitro-compound solution (0·0002 M), and the solution diluted to 100 ml. with distilled water. A complete system of control experiments was also set up. The solutions were kept in stoppered flasks at 37·5° in an electric oven with thermostatic control for periods from 2 to 14 days. The pH of each solution was determined at the conclusion of the interaction period by means of a Cambridge pH recorder and a Morton glass electrode assembly, and the ultra-violet absorption spectrum determined using a Hilger E498 medium quartz spectrograph and Spekker photometer, the light source being a 15,000 V. tungsten-steel spark, and the slit width 0·02 mm. ϵ is the molecular extinction coefficient defined by $\epsilon=(\log_{10} I_0/I)/cl$, where I_0 and I = intensity of incident and of transmitted light,