XXX. THE ESTIMATION OF TYROSINE IN PROTEINS BY BROMINATION.

By ROBERT HENRY ADERS PLIMMER and ELIZABETH COWPER EAVES.

From the Institute of Physiology, University College, London, and the Physiological Laboratory, University of Sheffield.

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The tyrosine content of a protein is usually ascertained by hydrolysing the protein with sulphuric acid, removing the acid as barium sulphate, concentrating the solution and washings and then weighing the tyrosine which separates out. The data so obtained by various workers are in some cases very concordant, but it is generally considered that the real tyrosine content of a protein is higher than these figures represent since it is frequently impossible to isolate the whole of the tyrosine. Osborne and Clapp [1906] and Osborne and Guest [1911] have particularly emphasised this point and they consider that the tyrosine content of a protein so obtained is only a minimal one, the true content being from one half to one per cent. higher. The presence of cystine in the tyrosine isolated has also never been taken into consideration. The two substances are so alike in their solubility in water, in acids and in alkalies that their separation is a matter of considerable difficulty [Plimmer, 1913]. Losses occurring in the isolation of the tyrosine may be compensated for by admixture with cystine.

It was shown by J. H. Millar [1903] that tyrosine was readily brominated and converted into dibromotyrosine, and that the amount of tyrosine in a simple mixture of amino-acids could be accurately estimated by means of this reaction.

A. J. Brown and E. T. Millar [1906] using this reaction showed that the tyrosine was completely liberated at a very early stage in the hydrolysis of edestin by trypsin. Their data gave the tyrosine content of edestin as 4·06 per cent., a figure which is considerably higher than that obtained by direct isolation (2·1 per cent.). They made no estimations of the tyrosine

content of other proteins. The higher value agrees with Osborne's supposition that the tyrosine content of proteins as obtained by isolation is only a minimal one and it seemed very desirable that further estimations should be made by this method.

It was found that it was better to alter Millar's method as it was not sufficiently delicate for estimating small amounts of tyrosine such as are obtained by the hydrolysis of proteins. The procedure of Brown and Millar also involved a large deduction for the amount of bromine absorbed by the control (protein or other decomposition products, possibly histidine) which prevented an accurate estimation. The absorption of bromine by the unchanged protein and its product of hydrolysis, histidine, which according to Knoop [1908] reacts with bromine, has been eliminated by the use of phosphotungstic acid. Another disturbing factor is the presence of tryptophane with the tyrosine amongst the products of hydrolysis. Though tryptophane may be destroyed by boiling with acids, its decomposition products still absorb bromine. The estimation of tyrosine amongst the products of the acid hydrolysis of proteins was therefore impossible. It remained to take advantage of the rapid and complete liberation of tyrosine during the early stages of tryptic digestion and the slower liberation of tryptophane, which is only complete after several days when a moderately active trypsin solution is employed [Hopkins and Cole, 1901]. By estimating the bromine absorption after short intervals of digestion in the filtrate from the phosphotungstic acid precipitate, values for the tyrosine content of proteins have been obtained which agree closely with those found by direct isolation.

Our work was withheld since we felt considerable diffidence as to the exactness of our data, but since the publication of much higher figures for the tyrosine content of proteins by Folin and Denis [1912] by a new colorimetric method and since these figures have been criticised by Abderhalden and Fuchs [1913] our data are of some value in support of the results of Abderhalden and Fuchs. Folin and Denis do not seem to have taken sufficient account of the presence of tryptophane and oxytryptophane, which react with their reagent. If their figures express the total tryptophane and tyrosine content, and our values the tyrosine content alone, then the difference will give the tryptophane content of proteins which is at present unknown and of considerable importance.

Experimental.

(1) *The Estimation of Small Amounts of Pure Tyrosine by Bromination.*

J. H. Millar's method for the estimation of tyrosine depends upon the formation of dibromotyrosine when tyrosine is treated with nascent bromine; this is liberated by adding sodium bromate to an acid solution of potassium bromide. The equations representing the reaction are:

$$NaBrO_3 + 5KBr + 6HCl = NaCl + 5KCl + 3Br_2 + 3H_2O,$$

$$C_6H_4(OH).CH_2.CH(NH_2).COOH + 2Br_2 = C_6H_2Br_2(OH).CH_2.CH(NH_2).COOH + 2HBr,$$

from which we find that 1 g. of tyrosine absorbs 1·765 g. of bromine and corresponds to 0·5558 g. of sodium bromate.

His procedure was to add 10–15 cc. of a 20 per cent. solution of potassium bromide to a solution of tyrosine in hydrochloric acid and to titrate with M/5 sodium bromate solution until it assumed a persistent deep yellow colour. If the solution were coloured, starch and potassium iodide were used as an indicator.

Millar found that 1·808 g. of bromine were absorbed by 1 g. of tyrosine, a figure which is slightly higher than the theoretical value but of sufficient accuracy to show that tyrosine can be estimated in solution by bromination; in one of the experiments 0·2 g. tyrosine required 3·8 cc. of the bromate solution.

The estimation of smaller amounts of tyrosine than 0·2 g. was not investigated by J. H. Millar. The amount of tyrosine in a protein does not usually exceed 3 per cent. except in the case of caseinogen which contains from 4·5–5 per cent. and silk-fibroin which contains about 10·5 per cent. One gram of protein would therefore usually give a solution containing 0·01–0·04 g. tyrosine. To estimate 0·01 g. tyrosine, 0·19 cc. of M/5 bromate solution would be required and an error of 0·05 cc. in the titration would make an error of 25 per cent. in the estimation. It was therefore necessary to ascertain if tyrosine could be brominated by a more dilute solution of sodium bromate. Estimations were therefore made with an N/5 (= M/30) solution of bromate in the same way as described by J. H. Millar, thus:

0·1911 g. tyrosine in 25 cc. hydrochloric acid solution required 21·05 cc. M/30 bromate solution;

i.e. 1 g. tyrosine absorbs 1·75 g. bromine (theoretical 1·765).

The bromination of tyrosine by the M/30 solution of sodium bromate is slower than by the M/5 solution and it appeared that the method might be more convenient if the bromination were effected by adding excess of the sodium bromate solution to the tyrosine solution, allowing the reaction to proceed for 10–15 minutes in a closed flask, adding potassium iodide and titrating the excess of halogen with thiosulphate solution using starch as indicator. 2 g. of tyrosine were dissolved in 500 cc. hydrochloric acid;

50 cc. + 30 cc. M/30 $NaBrO_3$ solution; 7·1 cc. thiosulphate solution; 22·9 cc. bromate required.

50 cc. + 30 cc. M/30 $NaBrO_3$ solution; 7·2 cc. thiosulphate solution; 22·8 cc. bromate required.

i.e. 50 cc. contain 0·207 g. tyrosine.

The absorption of bromine for 1 g. of tyrosine is 1·825 g., a value slightly higher than the theoretical (1·765), but comparable with that found by J. H. Millar (1·808). In subsequent experiments this high figure was not obtained. It seems to have been due to the presence of a rather large excess of bromate solution; the vapour in the flask appeared yellow and loss occurred during the titration.

Small quantities of tyrosine can thus be accurately estimated by making this alteration in the procedure.

(2) *Estimation of Tyrosine in the Presence of Protein and its Products of Hydrolysis.*

It has been shown by A. J. Brown and E. T. Millar that proteins absorb bromine under the conditions employed for the estimation of tyrosine by J. H. Millar's method, but this did not preclude the estimation of tyrosine in the presence of unaltered protein if the amount of bromine absorbed by the protein was deducted from the amount absorbed by the mixture of protein and tyrosine. They showed further that there was no increase in the absorption of bromine by gelatin during its digestion by trypsin. Gelatin does not contain tyrosine or tryptophane, and since it was found that tryptophane did not absorb bromine under the same conditions it was concluded that the increase in the absorption of bromine when edestin was digested by trypsin was entirely due to the liberation of tyrosine. The absorption of bromine due to the separation of tyrosine increased very rapidly and reached a maximum in about one hour. The result gave the tyrosine content of

edestin as 4·06 per cent. which differs very greatly from the value (2·1 per cent.) obtained by the isolation and weighing of tyrosine.

An examination was made for possible sources of error occurring during the estimation of tyrosine by bromination.

Brown and Millar used M/5 sodium bromate solution and 50 cc. of a 1 per cent. solution of edestin.

0·80 cc. of the bromate solution was required for the bromination and from this 0·42 cc. was deducted for the control. The difference of 0·38 cc. gave the tyrosine content.

As shown above an error of 0·05 cc. in the titration, if M/5 sodium bromate solution be used, corresponds to a difference of 25 per cent. in the amount of tyrosine when estimating 0·01 g. of tyrosine, which is equivalent to a variation of 1 per cent. in the tyrosine content of edestin. A proportionate error must be considered when the deduction for the control is made. The error in titration can be reduced by employing M/30 bromate solution. An error of 0·23 cc. in the titration will now correspond to a difference of 10 per cent. in the amount of tyrosine, which is equivalent to a variation of 0·4 per cent. for the tyrosine content of edestin. Dilution of the reagent will thus reduce the error but it is not in itself sufficient to make the method an accurate one.

The amount which has to be deducted for the control is greater than the amount used in the actual estimation; greater accuracy can therefore only be obtained by reducing or eliminating this deduction.

The constituents of a protein which are known to absorb bromine are tyrosine, tryptophane and histidine [Knoop, 1908]. According to Brown and Millar tryptophane does not absorb bromine under the conditions existing during the estimation of tyrosine so that it is most probably the histidine which absorbs the bromine and necessitates the large deduction for the control. Histidine can be removed by precipitation with phosphotungstic acid which leaves the tyrosine (and tryptophane) in solution in the filtrate. Hence if the deduction for the control be due to the presence of histidine, the absorption of bromine by the filtrate should be due solely to the tyrosine contained in the enzyme preparation. A preliminary experiment with gelatin showed that this was the only deduction necessary.

A 2 per cent. solution of trypsin was allowed to digest in the presence of chloroform; at intervals 20 cc. were removed and placed in 50 cc. of 5 per cent. sulphuric acid + 20 cc. of 10 per cent. phosphotungstic acid. To 50 cc. filtrate (= 0·333 g. trypsin) 10 cc. of sodium bromide (2 per cent.) and 10 cc. of the sodium bromate solution (1 cc. = 0·012 g. Br) were added;

after 10–15 minutes 10 cc. sodium iodide (4 per cent. solution) were added and the mixture titrated with sodium thiosulphate solution (1 cc. = 0·00663 g. Br) using starch as indicator[1].

Time	Thiosulphate required	Bromine absorbed
0	15·1 cc.	0·02 g.
4 hours	11·1	0·046
6 ,,	11·8	0·042
24 ,,	12·1	0·040

Taking the absorption after 6 hours, 1 g. of trypsin absorbs 0·126 g. bromine.

A 2 per cent. solution of gelatin in 0·4 per cent. sodium carbonate solution was digested in the presence of chloroform with 0·2 per cent. trypsin. 50 cc. samples were removed at intervals and placed in 50 cc. of 5 per cent. sulphuric acid + 20 cc. of 10 per cent. phosphotungstic acid. 100 cc. of the filtrate (= 0·833 g. gelatin) were treated with 10 cc. sodium bromide solution + 10 cc. sodium bromate solution (1 cc. = 0·012 g. bromine) and after half an hour the mixture was titrated with sodium thiosulphate (1 cc. = 0·00663 g. bromine) after adding sodium iodide and starch as indicator. Each sample contains 0·833 g. trypsin.

Time	Thiosulphate required	Bromine absorbed	Bromine absorbed by 0·0833 g. trypsin	Bromine absorbed by gelatin
0	17·4 cc.	0·0046	0·0025	0·0021
4 hours	16·2	0·0126	0·0104	0·0022
6 ,,	16·25	0·0126	0·0104	0·0022
24 ,,	15·6	—	—	—

The slight absorption by the gelatin (= 0·24 per cent.) is most probably due to the presence of tyrosine; the sample (gold label) gave a distinct reaction with Millon's reagent.

This procedure was then applied to the estimation of the tyrosine in caseinogen.

100 cc. samples of a 1 per cent. caseinogen solution were digested with 5 cc. of a trypsin solution for periods of 1–5 hours and were then precipitated with 25 cc. phosphotungstic acid solution in hydrochloric acid; 50 cc. of the filtrate were used for the titration:

[1] In the presence of phosphotungstic acid it is better to use sodium bromide instead of potassium bromide and sodium iodide instead of potassium iodide as potassium phosphotungstate is precipitated when potassium salts are present and the precipitate interferes with the titration with thiosulphate when starch is used as indicator. Auld and Mosscrop [1913] have maintained that starch and potassium iodide cannot be used when estimating tyrosine in digests of protein by the Millar method. Colourless filtrates are obtained after precipitation with phosphotungstic acid and with the alteration in the procedure the disappearance of the blue colour when the solution is titrated with thiosulphate is quite sharp. No difficulty has been experienced in determining the end point under these conditions.

Time (hours)	M/30 bromate absorbed	Difference
0	2·4 cc.	—
1	3·8	1·4
2	4·1	1·7
3	4·6	2·2
4	4·6	2·2
5	4·55	2·15

The maximum absorption of bromine occurred after 3 hours and then remained constant. The percentage of tyrosine in caseinogen calculated from the above difference is 5·08, a figure which agrees well with those usually given (4·5–5).

The following duplicate experiments with another solution of caseinogen show the reliability of the procedure[1].

	(1)			(2)		
Time (hrs.)	Bromate added	Thiosulphate required	Bromate absorbed	Bromate added	Thiosulphate required	Bromate absorbed
0	5 cc.	9·65 cc.	0·2 cc.	5 cc.	9·55 cc.	0·25 cc.
0·5	,,	7·45	1·3	,,	7·3	1·35
1	,,	5·95	2·05	,,	5·9	2·05
2	,,	4·8	2·6	,,	4·85	2·6
3	,,	4·35	2·85	,,	4·35	2·85

The estimation of tyrosine in the phosphotungstic acid filtrate is thus possible if no other products which absorb bromine are present. Gelatin does not contain tryptophane and cystine and it only contains a small amount of phenylalanine. Cystine and phenylalanine have been found not to absorb bromine, but the behaviour of tryptophane, which according to Brown and Millar does not react with bromine under the conditions adopted by J. H. Millar, probably because of the presence of hydrochloric acid, required further investigation as the method had been modified and as Dr Hopkins had informed us that tryptophane did react with bromine when treated in this way. A quantity of tryptophane was kindly supplied to us by Dr Hopkins for this purpose and the following experiments were carried out:

0·0631 g. tryptophane was dissolved in hydrochloric acid and titrated directly with sodium bromate by Millar's method.

7·3 cc. bromate were required. Bromine absorbed = 0·112 g.

0·0238 g. tryptophane was dissolved in hydrochloric acid: 10 cc. sodium bromide and 10 cc. sodium bromate solution (= 1536 g. Br) were added: after

[1] These experiments were carried out by one of us in conjunction with Mr S. H. Wood and the results were communicated to a meeting of the Physiological Society in March 1907. Continuation of the work was not then possible and no further experiments were made until 1909. The results of these later experiments were communicated to the Biochemical Club in March 1912.

15 minutes titrated with sodium thiosulphate solution (1 cc. = 0·00663 g. Br) after adding sodium iodide and starch: 3·2 cc. were required. Bromine absorbed = 0·1321 g.

0·0200 tryptophane was treated as in the previous experiment: 6·4 cc. thiosulphate were required. Bromine absorbed = 0·1105 g.

0·57 g. tryptophane was dissolved in 1000 cc. water; 100 cc. of this solution were used in each of the following experiments:

(1 cc. $NaBrO_3$ = 0·012 g. Br and 1 cc. thiosulphate = 0·00663 g. Br.)

	HCl	NaBr	$NaBrO_3$	Titrated after	Thiosulphate required	Bromine absorbed
100 cc.	5 cc.	10 cc.	10 cc.	15 mins.	2·05 cc.	0·1064 g.
	10	10	10	,,	1·40	0·1107
	1·5	10	10	,,	2·30	0·1048
	5	10	20	,,	17·95	0·1210
	10	10	20	30 mins.	17·0	0·1270
	5	10	20	,,	16·5	0·1306
	10	10	20	1·5 hrs.	15·05	0·1400

Another series of experiments gave similar results.

Tryptophane thus absorbs bromine under the conditions adopted by Millar; it absorbs a greater amount of bromine under the modified conditions, the absorption increasing with a larger amount of bromate and with the time allowed for the reaction. The absorption corresponds to about 6 atoms of bromine by 1 molecule of tryptophane.

The presence of tryptophane in solution with tyrosine will thus interfere with the estimation of tyrosine.

According to Hopkins and Cole tryptophane is destroyed by prolonged boiling with acids. If its products of decomposition do not absorb bromine the estimation of tyrosine should be possible after acid hydrolysis. Some experiments were therefore made to see if tryptophane still absorbed bromine after boiling with acids:

100 cc. of the above solution of tryptophane were heated for 10 hours with excess of concentrated hydrochloric acid (50 cc.). 10 cc. NaBr + 20 cc. $NaBrO_3$ were added and after 15 minutes titrated with thiosulphate (16·5 cc. required). Bromine absorbed = 0·110 g.

0·058 g. tryptophane was dissolved in 100 cc. water: 10 cc. were titrated directly: 10 cc. after boiling with concentrated hydrochloric acid and 10 cc. after boiling with 25 per cent. sulphuric acid. The bromine absorbed was respectively 0·0361 g., 0·0057 g. and 0·0106 g.

0·0998 g. tryptophane was dissolved in 50 cc. water: 10 cc. were titrated directly and 10 cc. after boiling for 5 hours with hydrochloric acid. The bromine absorptions were respectively 0·1017 g. and 0·074 g.

Absorption of bromine still occurs, but to a less extent, after boiling tryptophane with acid, so that the estimation of tyrosine is not possible after the hydrolysis of protein by acids.

Brown and Millar have shown that the whole of the tyrosine is very rapidly liberated by the action of trypsin and our preliminary experiments with trypsin and caseinogen have confirmed their results. It has been shown by Hopkins and Cole that the liberation of tryptophane does not occur rapidly with moderately active trypsin solutions and that its amount in solution only reaches a maximum after several days. This difference in the rate of liberation of the two substances may therefore permit of the estimation of the tyrosine content of a protein when the bromine absorption is measured at intervals during the digestion and when only those amounts absorbed in the early stages, from 6–24 hours, are taken as a measure of the tyrosine content.

(3) *Absorption of Bromine during the Tryptic Digestion of Proteins.*

Specimens of several animal and vegetable proteins have been procured and examined by the method described above. The vegetable proteins were most kindly sent to us by Prof. Osborne, the specimens in most cases being the same as those in which he and his co-workers had determined the tyrosine content by direct isolation and weighing.

In general, a 1 per cent. solution of the protein in 0·25 per cent. sodium carbonate was digested in the presence of chloroform or carbon tetrachloride with a 0·1 per cent. solution of trypsin. 50 cc. samples were removed immediately and after various intervals of time and then precipitated with 100 cc. of 10 per cent. phosphotungstic acid in 5 per cent. sulphuric acid. 100 cc. of the filtrate were then taken for the estimation. 10 cc. sodium bromide (2 per cent.) and 10 cc. sodium bromate solution were added; after 15 minutes the excess of bromine was displaced by adding 10 cc. of sodium iodide solution (4 per cent.) and the liberated iodine titrated with sodium thiosulphate solution using starch as indicator. The estimations were generally made after periods of 6 hours and 24 hours, since the amount of material at our disposal did not allow of observations at more frequent intervals. These times were chosen as the preliminary experiments with caseinogen showed that the absorption began after about 1 hour and reached a maximum in from 3–5 hours and that another rise sometimes occurred in about 24 hours. The presence of tryptophane in solution was tested for by the bromine reaction in some experiments; it was generally absent in the 6-hour period but was faintly visible in the 24-hour period.

The estimation with silk-fibroin was performed after hydrolysis with 20 per cent. sulphuric acid for 18 hours as trypsin has only a very slight action upon this protein. Silk-fibroin does not contain tryptophane.

Peptone Roche, which is prepared from silk-fibroin by acid hydrolysis, gave only a slight precipitate with phosphotungstic acid; it seems to contain tyrosine or a polypeptide containing tyrosine which reacts with bromine. Like silk-fibroin this protein contains no tryptophane.

It was impossible to estimate the tyrosine content of the alcohol soluble proteins—the gliadins—since they are only digested with extreme slowness by trypsin.

The following are the data:

Trypsin. (Used in experiments with caseinogen, "peptone Roche.")

1 per cent. in 0·25 per cent. Na_2CO_3 solution digested in presence of carbon tetrachloride. 50 cc. samples in 100 cc. phosphotungstic acid solution. 100 cc. filtrate (=0·33 g. trypsin) for estimation. 5 cc. sodium bromate solution (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br).

Time (hours)	Thiosulphate required	Br absorbed
0	8·3 cc.	0·0210 g.
6	6·3	0·0345
7	6·65	0·0321
24	6·7	0·0318

Caseinogen. (Hammarsten.)

12·233 g. in 487·27 cc. of 0·25 Na_2CO_3 solution + 122·33 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid solution. 100 cc. filtrate (=0·667 g. caseinogen +0·0667 g. trypsin). 10 cc. sodium bromate solution (=0·1536 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·00642 for trypsin	Tyrosine content per cent.
0	22·9	—	—	—
5 hours	12·5	0·0696	0·0632	5·35
6 ,,	12·65	0·0695	0·0631	5·34
23 ,,	10·75	0·0814	0·0749	6·35
30 ,,	7·4	0·1036	0·0972	8·23
48 ,,	7·9	0·1005	0·0941	7·98
4 days	7·55	0·1036	0·0972	8·23

"*Peptone Roche.*"

5·735 g. in 514·2 cc. of 0·25 Na_2CO_3 solution + 57·3 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid solution. 100 cc. filtrate (=0·333 g. peptone + 0·0333 g. trypsin) for estimation. 10 cc. sodium bromate (=0·1536 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br).

Time	Thiosulphate required, cc.		Total Br absorbed, g.	Br absorbed after deducting 0·0021 at 0 hrs. & 0·0032 afterwards for trypsin	Tyrosine content per cent.
0	14·85		0·0998	0·0538	8·79
2·5 hours	14·2		0·0954	0·0582	9·32
3·75 ,,	13·5				
7 ,,	13·1	13·4	0·090	0·0636	10·23
9 ,,	13·3				
24 ,,	13·6				

Silk-fibroin.

4·39 g. were hydrolysed with 20 per cent. H_2SO_4, and the solution made up to 500 cc. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·293 g. fibroin) for estimation. 5 cc. bromate solution (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br). Thiosulphate required=4·1 cc. Br absorbed=0·0493 g. Tyrosine content=9·53 per cent.

Conglutin (Merck) containing 5·2 per cent. moisture.

5·6158 g. in 222·7 cc. Na_2CO_3 solution + 56·1 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·632 g. dry conglutin) for estimation. 5 cc. bromate (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br)

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br. absorbed after deducting 0·00642 for trypsin	Tyrosine content per cent.
0	11·7	—	—	—
4 hours	8·55	0·0193	0·0129	1·15
6 ,,	8·3	0·0210	0·0146	1·31
24 ,,	5·35	0·0415	0·0351	3·15

Excelsin. (Own preparation.)

2·697 g. in 306 cc. of 0·25 per cent. NaOH + 2 cc. $CHCl_3$ + 2 "holadin" capsules. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·2937 g. excelsin) for estimation.

Time	Total Br absorbed, g.	Br absorbed after deducting 0·002 for holadin	Tyrosine content per cent.
0	0·00287	0·0008	—
5·5 hours	0·0149	0·0129	2·5
24 ,,	0·0258	0·0238	4·6
28 ,,	0·026	0·024	4·61
48 ,,	0·0282	0·026	5·0

Legumin (Osborne) containing 4·07 per cent. moisture.

2·9866 g. in 266·6 cc. Na_2CO_3 solution + 30 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·03198 g. dry legumin + 0·0033 g. trypsin) for estimation. 5 cc. bromate (=0·08375 g. Br). Titrated with thiosulphate (1 cc.=0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·45	0·0012	—	—
6·75 hours	8·15	0·0185	0·0152	2·69
27·5 ,,	7·0	0·0272	0·0239	4·23
3 days	6·0	0·0351	0·0318	5·63

Edestin (Osborne) containing 13·07 per cent. moisture.

4·6052 g. in 182·3 cc. Na_2CO_3 solution + 46 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (=0·58 g. dry edestin + 0·0667 g. trypsin) for estimation. 5 cc. bromate (=0·0768 g. Br). Titrated with thiosulphate (1 cc.=0·00672 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·00642 g. for trypsin	Tyrosine content per cent.
0	11·4	—	—	—
4 hours	8·35	0·0207	0·0143	1·4
6 ,,	7·85	0·0241	0·0177	1·73
24 ,,	5·0	0·0432	0·0368	3·6
48 ,,	2·6	0·0593	0·0529	5·17

Vignin (Osborne) containing 7·03 per cent. moisture.

1·8982 g. in 169 cc. Na_2CO_3 solution + 18·9 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (= 0·310 g. dry vignin + 0·033 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Titrated with thiosulphate (1 cc. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·9	—	—	—
6·75 hours	7·95	0·0223	0·0190	3·4
27·5 ,,	6·35	0·0360	0·0327	5·97

Squash Seed Globulin (Osborne) containing 10·02 per cent. moisture.

1·912 g. in 170·1 cc. Na_2CO_3 solution + 19·1 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (= 0·3 g. dry protein + 0·0333 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Thiosulphate (1 c.c. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·8	—	—	—
5·75 hours	8·2	0·0205	0·0172	3·24
27 ,,	6·5	0·0339	0·0306	5·8

Amandin (Osborne) containing 10·46 per cent. moisture.

3·3404 g. in 298·6 cc. Na_2CO_3 solution + 33·4 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (0·3 g. dry amandin + 0·0333 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Thiosulphate (1 cc. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·6	—	—	—
6 hours	8·2	0·01896	0·0157	2·9
24 ,,	6·75	0·0304	0·0271	5·1

Glycinin (Osborne) containing 9·06 per cent. moisture.

1·9166 g. in 170·5 cc. Na_2CO_3 solution + 19·2 cc. trypsin solution + 2 cc. CCl_4. 50 cc. samples in 100 cc. phosphotungstic acid. 100 cc. filtrate (= 0·302 g. dry glycinin + 0·0333 g. trypsin) for estimation. 5 cc. bromate (= 0·08375 g. Br). Thiosulphate (1 cc. = 0·0079 g. Br).

Time	Thiosulphate required, cc.	Total Br absorbed, g.	Br absorbed after deducting 0·0033 g. for trypsin	Tyrosine content per cent.
0	10·4	—	—	—
6 hours	9·3	0·0091	0·00058	1·1
28 ,,	7·7	0·0218	0·0185	3·47

In most cases there was an increase in the bromine absorption between the periods of 6 hours and 24 hours. The exact time at which the increase occurred could not be ascertained since it was impossible to make more frequent determinations owing to the scarcity of material. An increase after a constant period would show the point when all the tyrosine was liberated and that at which the tryptophane became set free. There is a very close

agreement between the figures for the tyrosine content after the 6-hour interval and the figures obtained by isolation and weighing, as is shown by the following table:

Protein	Percentage of tyrosine after 6 hours' digestion	Percentage by weighing
Caseinogen	5·34	4·5
"Peptone Roche"	10·23	—
Silk-fibroin	9·53	9–10·5
Conglutin	1·31	2·1
Legumin	2·69	2·4
Edestin	1·73	2·1
Vignin	3·4	2·3
Squash seed globulin	3·24	3·1
Amandin	2·9	1·1
Glycinin	1·1	1·9
Excelsin	2·5	3·1

The correspondence in the figures in the cases of edestin, glycinin, squash seed globulin, excelsin, legumin and silk-fibroin is very close. The value is 1 per cent. higher for caseinogen, and the value is also higher for amandin and vignin. The result should be slightly higher for excelsin as the material was not dried. The amounts of amandin and glycinin available were very small, so that much stress cannot be placed on these figures.

The method of bromination therefore appears to be of use for the estimation of the tyrosine content of proteins if measurements of the absorption are made at frequent intervals during a tryptic digest of the protein, but it must be used with precautions and the figures carefully criticised.

Summary.

The estimation of small quantities of tyrosine—0·01–0·04 g.—can be effected by J. H. Millar's method of bromination, when a more dilute solution of sodium bromate is used, but it is preferable to modify his procedure by adding excess of the reagent and titrating the non-absorbed halogen with thiosulphate solution, using potassium iodide and starch as indicator. Tyrosine cannot be directly estimated by bromination in the presence of protein and its decomposition products, since histidine and tryptophane also absorb bromine. Histidine can be removed by precipitation with phosphotungstic acid. The absorption of bromine by tryptophane is not completely eliminated after boiling with acids so that tyrosine cannot be estimated by this method in solutions containing the products of acid hydrolysis of proteins which contain tryptophane. Values for the tyrosine

content of proteins, agreeing with those obtained by isolation and weighing, are obtained when the bromine absorption of a tryptic digest is measured after an interval of about 6 hours.

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