IX. A METHOD OF ESTIMATING THE TRYPTOPHAN CONTENT OF CASEINOGEN, BASED ON DETERMINATIONS OF THE NITROGEN VALUES OF THE MERCURIC SULPHATE PRECIPITATE.

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IN spite of the several attempts that have been made to estimate tryptophan, a glance at the table given below will show that the results achieved hitherto have not been such as to call for much excuse for another attempt. A means of estimating tryptophan is sorely needed, not only in the analysis of proteins, where the figures for this amino acid are still represented by blanks, but also in the analysis of food stuffs connected with a multitude of problems, such as the etiology of pellagra, to mention only a single instance.

The Tryptophan Content of Caseinogen according to various Authors.

Percentage of tryptophan found	According to	Method of estimation
1.5	Hopkins and Cole [1901]	Isolated and weighed
0.5	Abderhalden and Kempe [1907]	Colorimetric (<i>p</i> -dimethylaminobenzaldehyde reaction)
0.6	Fasal [1912]	Colorimetric (glyoxylic acid reaction)
0.6	Moraczewski [1913]	From indole produced by bacteria
0.5	Herzfeld [1913]	Colorimetric (<i>p</i> -dimethylaminobenzaldehyde reaction)
1.75	Thomas [1914]	Colorimetric (ditto)
1.1	Homer [1915]	Titred with bromine
1.7	Dakin [1918]	Isolated and weighed
2.0	von Fürth and Nobel [1920]	Colorimetric (Voisenet's formaldehyde reaction)
1.7-1.8	Thomas [1920]	Colorimetric (<i>p</i> -dimethylaminobenzaldehyde reaction)
1.54	Folin and Looney [1922]	Colorimetric (phenol reagent)
1.1-1.39	Lüscher [1922]	Colorimetric (benzaldehyde and formaldehyde reaction)
1.50	May and Rose [1922]	Colorimetric (<i>p</i> -dimethylaminobenzaldehyde reaction)
2.24	Holm and Greenbank [1923]	Colorimetric (ditto)

It will be seen that the discoverers of tryptophan actually isolated as much of the crystalline amino acid as most workers claim to have estimated by their methods. Dakin, moreover, by extraction with butyl alcohol, obtained

1.7 % at the first attempt, and Onslow [1921] has shown that yields approaching this value may be obtained in routine work.

The colorimetric methods of Thomas, of von Fürth and Nobel and of Holm and Greenbank are the only ones that give values of 1.7 % or greater. As there must be a considerable loss of tryptophan in the mother liquors after crystallisation, it can hardly be doubted that the true value is higher than 2 %, and therefore nearer the figures given in this paper and those of Holm and Greenbank.

Though the colorimetric methods are difficult to carry out, and are liable, according to some of the authors, to an error of 20 %, they are certainly the best yet published. In addition, however, to the low values sometimes obtained, all the usual objections to colorimetric methods may be urged. These objections are especially weighty when the reaction depends not on a single coloured substance, but on two substances of different colours as is the case with tryptophan, *i.e.* "tryptophan blue" and "tryptophan red" [Fearon, 1920]. Any method, therefore, which is based on such familiar procedures as the estimation of nitrogen values, even though open to serious objections, is likely to be an advance in the direction of accuracy.

A description was given in a previous paper [Onslow, 1921] of the compound precipitated by mercuric sulphate from a solution of caseinogen which had been digested by enzymes. Among the amino acids found in addition to tryptophan, there were leucine, cystine, proline, glutaminic and aspartic acids and histidine, but neither lysine nor arginine. The absence of the last two diamino-acids is of considerable importance, as will be seen below.

The work was begun in the hope of obtaining an accurate method for estimating tryptophan. When, therefore, it was found that the total nitrogen of the mercuric sulphate precipitate was no criterion of the tryptophan content, owing to the other amino acids present, an attempt was made to obtain a value for the tryptophan from the difference between the amino nitrogen and the more resistant nitrogen of the pyrrole ring.

PRINCIPLE OF THE METHOD.

In the absence of any diamino-acid, or any heterocyclic acid other than histidine, the total nitrogen of the mercuric precipitate may be considered as belonging to the following fractions, many of which can be estimated:

$$\text{Total N} = \text{Tryptophan N} + \text{Histidine N} + \text{Monamino N} + \text{Peptide N}.$$

It will be seen that account is taken of the fact that a proportion of the amino acids of the mercury compound still remains combined as polypeptides, and, moreover, that proline is only present in an insignificant amount [Onslow, 1921].

In proteins, such as gelatin, which contain very large amounts of proline, special precautions would have to be taken, and, similarly, with proteins containing large amounts of cystine, an estimation of the total sulphur by Benedict's method [1909] would have to be made, so that the correction for

cystine could be applied when using Van Slyke's method for amino nitrogen. From the last equation the amino nitrogen may be expressed as follows:

$$\text{Amino N} = \text{Tryptophan N}/2 + \text{Histidine N}/3 + \text{Peptide amino N} + \text{Monamino N}$$

and hence, the non-amino nitrogen is given by:

$$\text{Non-amino N} = \text{Tryptophan N}/2 + \text{Histidine } 2\text{N}/3 + \text{Peptide non-amino N}.$$

This equation can, of course, be written:

$$\text{Tryptophan N} = 2 [\text{Non-amino N} - (\text{Histidine } 2\text{N}/3 + \text{Peptide non-amino N})].$$

Tryptophan, it will be seen, is the only unknown term in the equation, because the non-amino nitrogen may be calculated from the difference between the total nitrogen (obtained by Kjeldahl's method) and the amino-nitrogen (obtained by Van Slyke's method); the histidine nitrogen can best be estimated, under these conditions, by the methods of Koessler and Hanke [1919], while the peptide non-amino nitrogen is found from the difference between the amino nitrogen before and after complete hydrolysis. The only term in the last equation not determined is tryptophan, which may therefore be calculated.

The obvious method for completing the hydrolysis of any peptides remaining in the mercuric compound would be to submit them to acid hydrolysis as was done by Levene and Van Slyke [1912] in the case of urine. The great difficulty in doing this is the presence of so large an amount of tryptophan, both free and as polypeptides, which is rapidly destroyed by this treatment, being converted into humin. There is, however, no other method available, because, unfortunately, it has been shown to be impossible to effect complete hydrolysis by means of enzymes¹ [Onslow, 1921; von Fürth and Lieben, 1920; Frankel, 1916], and baryta hydrolysis, the only other possible method, is inapplicable, because the tryptophan, being unsufficiently protected by other amino acids, would break down to indole. Consequently, it is necessary to know the changes undergone by tryptophan during acid hydrolysis. Gortner and his co-workers [Gortner, 1916; Gortner and Blish, 1915; Görtner and Holm, 1917, 1920] have shown that tryptophan and tyrosine are the only two amino-acids² concerned in humin formation, and, since tyrosine can be removed from the mercury compound by thoroughly washing, the tryptophan alone is affected. Van Slyke [1912, 1] has shown that the acid hydrolysis of pure tryptophan in no way alters the ratio of the amino nitrogen to the total nitrogen. This important observation was confirmed, and it was also shown, by the following experiment, that when most of the tryptophan is converted to humin by the addition of a carbohydrate, the ratio of total to amino nitrogen remains 2 : 1.

0.25 g. tryptophan and 0.25 g. glucose were boiled in 25 % hydrochloric

¹ This conclusion has been denied by Abderhalden, but Henriques and Gjaldboek [1911] and Andersen [1915] have challenged this attitude. In any case the method would be useless on account of the large amounts of tryptophan that would be introduced in the erepsin and other enzymes needed.

² The data advanced by Roxas [1916] to prove that other amino acids are involved in humin formation, show such low values for these amino acids that they seem rather to support Gortner's contention that if traces of any other amino acids appear to have formed humin, it is by a physical process of adsorption and not by a chemical reaction.

acid for 24 hours on a sand-bath. Distillation with calcium hydroxide in the usual way produced no ammonia. The nitrogen of the humin formed, which amounted to about 79 % of the tryptophan N, was estimated by Kjeldahl, and the filtrate containing the residual tryptophan gave the following figures:

Total nitrogen	...	...	...	...	6.7 mg.
Amino nitrogen	...	...	...	...	3.5 mg.

In another experiment, 0.3 g. tryptophan and 0.3 g. glucose were boiled in 25 % hydrochloric acid with the following result:

Total nitrogen	...	...	...	...	27.7 mg.
Amino nitrogen	...	...	...	...	13.8 mg.

In this case 25 % of the total nitrogen became melanin. If the ratio of the nitrogen of the residual tryptophan remains undisturbed, the humin must contain equal proportions of nitrogen from the ring, and from the side chain. Thus, if half the melanin nitrogen, formed during complete acid hydrolysis, be added to the amino nitrogen, a correct figure for the total terminal NH_2 groups should be obtained. This, less the original amino nitrogen, should give the non-amino nitrogen bound in the peptides, which is the last term of the equation.

GENERAL SCOPE OF RESEARCH.

In all, 20 estimations¹ of the percentage of tryptophan in caseinogen were made. During the course of the work, several difficulties were encountered, but most of these were eventually overcome. Of the last eight estimations, six were finally considered to be satisfactory. Four of these, carried out on one sample of caseinogen (English "casein") gave concordant values. The remaining two, carried out on another sample (Merck's "casein") gave concordant, but rather higher, values. Many subsidiary experiments were performed in connexion with the difficulties arising at various points in the course of the estimations.

OUTLINE OF EXPERIMENTAL PROCEDURE IN ESTIMATION.

A known weight of caseinogen was digested with trypsin in a known volume of fluid. The digest was precipitated with a known volume of the Hopkins and Cole reagent. Free tryptophan, polypeptide of tryptophan, free tyrosine, histidine and probably small quantities of other amino acids were precipitated. The first precipitate was filtered off after four days. The filtrate was allowed to stand. A second precipitate was then deposited and filtered off after 14 days. The two precipitates were washed separately with 7 % sulphuric acid, containing 2-3 % mercuric sulphate, until the wash fluid gave no tyrosine reaction even after standing in contact with the precipitates for 24 hours. The two precipitates were united, suspended in water made slightly alkaline with baryta, and treated with hydrogen sulphide. The treatment was repeated, later in hot

¹ The majority of these were carried out with considerable skill and care by Mr John Lowndes, assistant to the late Mr Onslow. The research owes much to Mr Lowndes' accurate and careful work. (M. W. O.)

solution, until the filtrates from the mercuric sulphide gave no glyoxylic reaction. All the filtrates were added together. The residual mercuric sulphide was then boiled with 10 % baryta solution for an hour and filtered, and the filtrate added to the above combined filtrates. The solution now contained tryptophan, histidine, polypeptide and probably traces of other amino-acids. The barium was quantitatively removed from the solution, which was then reduced *in vacuo* and made up to a known volume. The total nitrogen and amino nitrogen were then determined. The histidine nitrogen was estimated by the method of Koessler and Hanke. Three portions of the original solution were hydrolysed with an equal volume of 6*N* hydrochloric acid in an autoclave for three different periods ranging from 1 to 2 hours at 140°, that is, under a pressure of 3½ atmospheres. In each case the hydrolysed solution was taken down to dryness *in vacuo*. The residue was distilled with calcium hydroxide, thus giving the ammonia nitrogen. The melanin was filtered off, and of this the total nitrogen was determined. The amino nitrogen and total nitrogen were determined in the filtrate. From the above values the tryptophan present can be calculated according to the theoretical equation previously given.

DETAILS OF EXPERIMENTAL PROCEDURE.

1. *Caseinogen preparations employed.*

For the first four estimations, English "casein¹" was used; for estimation V, a small sample of "casein according to Hammarsten" (Merck), and, for estimation VI, English "casein" again. The values of total nitrogen of the solutions given by the decomposition of the mercury precipitates were too low in the first six estimations to give, after allowance for impurities, a value for tryptophan of more than 1.7 %. It was therefore thought possible that racemisation had occurred to some extent in the preparation of the protein used, thus rendering it incapable of digestion and causing the low values.

Hence samples of caseinogen were prepared from milk by two methods. First, the method of Van Slyke and Bosworth [1913] was tried, employing 4 gallons of milk. The product, however, on digestion (estimation VII) did not give a higher value than hitherto obtained for the total nitrogen. Secondly, the method of Mellanby [1915] was tried, and again the product gave no higher value (estimation VIII).

At this stage it became obvious that the cause of low values was to be sought elsewhere. Consequently, for estimations IX to XVIII inclusive "casein according to Hammarsten" (purchased from Messrs Thomson, Skinner and Hamilton) was employed, and during these later estimations, other, hitherto unsuspected, sources of error, affecting the total nitrogen values and consequently the tryptophan values, were discovered. For estimations XIX and XX "casein according to Hammarsten" (Merck) was employed.

¹ 100 % pure casein obtained from Casein Co., Culvert Works, Battersea.

2. Determination of percentage of water and ash in caseinogen.

The percentage of water was determined by drying a known weight of caseinogen taken at the time when the digest was started. The drying was carried out in an electric oven heated to 105°. The ash was determined by incineration in a porcelain crucible.

The water content of Hammarsten's casein (T. S. and H.) varied from 8–11 %; the ash varied from 0.7–0.8 %. In the sample of "Hammarsten's casein" (Merck) used, the water content was 10 %, the ash 0.7 %. The actual values for each estimation are given in Table I.

3. Digestion of caseinogen.

The caseinogen was digested in a buffer solution, as described in the preparation of tryptophan [Onslow, 1921], in order to prevent the rise of hydrogen ion concentration during hydrolysis. This was found to remain very constant in a solution of *N*/10 sodium acetate, brought to p_H 8.1 by the addition of soda, but it was feared that the tryptophan might be slowly destroyed by oxidases present in the trypsin. When tested for oxidases in the usual way, the sample of trypsin appeared to be practically free. Nevertheless, the following experiment was carried out: 0.5 g. of pure tryptophan was dissolved in 200 cc. of buffer solution. 100 cc. were placed in a round flask *A*, so that a large surface of the fluid was exposed to the air. The remaining 100 cc. were placed in a measuring flask, *B*, so that the fluid just reached to the mark on the narrow neck. The measuring flask was tightly stoppered and fitted with a tube through which hydrogen could be passed. Flask *A* was plugged with wool. A brisk stream of pure hydrogen was passed through flask *B* for 30 minutes, and both flasks placed in the incubator at 37°. The treatment with hydrogen was repeated every two days for seven days, when the tryptophan in both flasks was precipitated as usual, and the nitrogen estimated in duplicate.

	Weight of tryptophan taken	Equivalent weight of tryptophan
A.	0.25 g. (aerobic)	0.248 g.
B.	0.25 g. (anaerobic)	0.244 g.
A.	0.25 g. (aerobic)	0.242 g.
B.	0.25 g. (anaerobic)	0.230 g.

From the above figures it appears that no considerable amount of tryptophan is destroyed by oxidation during the process of digestion.

In the next place an active enzyme is necessary, since von Fürth and Lieben [1920] have shown that when caseinogen is digested with trypsin, under the usual conditions, it does not yield more than one-third of the total tryptophan as the free acid. One of the purified dried products is best, since the various extracts prepared from fresh pancreas are too rich in tryptophan and purine bases. Such an enzyme should be strong enough to split off all the tryptophan in a 2 % solution of caseinogen in such a form that it is rapidly precipitated by the mercury reagent of Hopkins and Cole [1901]. Much time was spent in testing

various pancreatic extracts and preparations of trypsin; eventually a solution of Merck's trypsin was used as being most efficient and, at the same time, introducing the minimum quantity of extraneous nitrogen compounds.

The method of procedure was as follows. 50 g. of caseinogen were put into a dry Winchester quart bottle, of accurately known capacity (about 2650 cc.), and about one-third of the bottle was filled with the buffer solution of *N*/10 sodium acetate. The p_H was adjusted by carefully adding strong caustic soda solution until the mixture was alkaline to cresol-red but acid to phenolphthalein.

The enzyme extract was prepared by dissolving 2 g. of Merck's trypsin in 50 cc. of the buffer solution and allowing it to stand in the incubator at 38° for an hour, during which time a small deposit was formed which was filtered off. A solution containing 2 g. of sodium fluoride was also allowed to stand in the incubator and then filtered.

The enzyme extract and fluoride thus prepared were then added to the caseinogen and buffer solution. The reaction immediately became acid, and more soda solution was added until the mixture was faintly alkaline to phenolphthalein. The bottle was then nearly filled with the buffer solution. It is necessary to readjust the reaction continually as, for 24 hours or longer, it is liable to change. When it had ceased to change, the bottle was completely filled with buffer solution. From experience it has been found advisable to keep the reaction at first slightly alkaline to phenolphthalein. If this is done there is less likely to be any undigested deposit at the end of digestion.

The digest was kept in the incubator at a temperature of not less than 38° for 7-10 days. During the last two days, it is possibly advantageous to raise the temperature to 42°.

4. *Precipitation.*

The fluid after digestion was clear, with but a very slight deposit; nevertheless it was always filtered. In the early experiments the filter paper was washed free from tryptophan, and the washings added to the digest. This is undesirable as the washings alter in each case the concentration of the digest for precipitation. Hence, in later experiments, the following plan was adopted, which was quicker and avoided washing. An aliquot portion only of the filtered digest was used, representing 45 g. of caseinogen. Thus the concentration of amino acids and polypeptides in the digest was kept as constant as possible for each precipitation. The filtered digest was made 5 % acid with sulphuric acid, and 150 cc. of the Hopkins and Cole reagent was added for each 1000 cc. of digest. The mixture was allowed to stand for four days, during which time most of the tryptophan and tryptophan polypeptides were precipitated as mercury compounds. It was found, however, that after four days a precipitate continued to be formed which, though consisting mainly of the mercury compound of tyrosine (which deposits more slowly than that of tryptophan), always contained some tryptophan compound. Since the mercury compound

Table I. *Estimation of tryptophan in caseinogen.*

Number of estimation	Percent- age of water in caseinogen	Percent- age of ash in caseinogen	Time of digestion in days	Original solution			Hydrolysed portion of original solution					Total amino ni- trogen (by addition) mg.	Percentage of trypto- phan in casein- ogen mg.
				Total nitrogen mg.	Amino nitrogen mg.	Non- amino nitrogen mg.	Histidine non-amino nitrogen mg.	Time in auto- clave hours	Amino nitrogen mg.	Melanin nitrogen mg.	Ammonia nitrogen mg.	Total* nitrogen (by ad- dition) mg.	
XIII	10.9	0.674	7	160.756	53.580	107.176	8.438	1	56.044	30.068	16.130	163.263	1.92
XV	8.55	0.791	8	160.322	43.862	116.460	9.033	1	62.839	35.129	18.343	164.212	1.97
XVI	8.55	0.791	25	167.718	51.950	115.768	20.301	1	61.737	24.365	14.791	159.376	1.97
XVIII	8.0	0.673	27	177.742	55.628	122.114	28.251	1	60.863	30.220	16.639	158.450	1.97
XIX	10.0	0.667	14	187.954	56.581	131.373	25.415	1	59.424	22.516	21.570	168.004	1.97
XX	10.0	0.667	14	174.804	62.640	112.164	16.057	1	61.035	24.981	23.111	—	1.97
								1	59.466	27.755	24.035	—	1.87
								1	59.105	26.670	27.567	—	1.87
								1	62.243	32.489	28.486	—	1.87
								1	58.615	34.021	28.792	—	1.87
								1	60.667	28.202	15.186	187.462	2.15
								1	69.352	30.707	15.655	187.305	2.15
								1	65.949	36.656	15.968	187.931	2.14
								1	64.807	27.263	7.828	174.782	2.14
								1	66.088	32.585	12.524	174.780	2.14
								2	62.889	36.030	15.029	173.216	2.14

* All figures for the original solution are calculated on 50 g. of dry caseinogen.

† Includes the total nitrogen of the filtrate from the melanin together with that of half the melanin and of the calculated proportion of ammonia.

Table II. *Shows the distribution of nitrogen after hydrolysing mixtures of tryptophan and histidine in an autoclave.*

Exp.	Percentage of histidine of total nitrogen	Portion hydrolysed in autoclave							Deficiency of amino nitrogen	Percentage of total ammonia nitrogen derived from amino nitrogen
		Total nitrogen calculated mg.	Amino nitrogen calculated mg.	Amino nitrogen found mg.	Time in autoclave hours	Amino nitrogen mg.	Melanin nitrogen mg.	Ammonia nitrogen mg.		
I	13	125.800	73.950	73.971	1	—	3.315	2.536	—	—
II	10.5	153.240	78.625	79.460	4	—	11.177	10.145	—	—
III	18	167.275	—	—	4	70.146	12.445	10.398	—	—
(a)	—	—	—	—	6	—	4.837	3.551	3.092	29.7
(c)	—	—	—	—	18	65.021	19.039	16.485	—	—
IV	8	149.230	72.610	—	24	62.332	18.278	166.934	4.920	29.9
V	24	180.307	82.969	—	24	70.615	21.322	149.289	1.139	34.5
VI	0	137.200	68.600	68.556	24	61.278	12.952	179.616	1.693	30.3
"	"	"	"	"	"	"	"	"	0.846	47.7

of tyrosine has subsequently to be removed by washing, and this is a slow process, the second precipitate was filtered off separately, so as to avoid contaminating the first precipitate with more tyrosine compound than necessary. After 14 days a precipitate still continued to form, but this rarely contained tryptophan to any appreciable extent. If, however, the digestion is either not good or not sufficiently long in duration or both, the precipitate may contain tryptophan even after 16 days' deposit. It is possible that, in these circumstances, polypeptides of tryptophan were present to a greater extent, and were but slowly precipitated. But, for a good digest of 10 days' duration or more, 18 days should suffice for total precipitation of the tryptophan compounds.

5. *Washing to free precipitates from tyrosine compound.*

The two mercury precipitates were filtered on hardened folded papers on an ordinary funnel. They were then washed with a wash-fluid containing 2-3 % mercuric sulphate in 7 % sulphuric acid. In the majority of estimations, I-XII, the precipitates were washed with 7 % sulphuric acid alone, and, as the washings gave for some time no glyoxylic reaction, it was assumed that the 7 % acid only removed the mercury tyrosine compound. This is true at first; but, after prolonged washing, when the tyrosine compound is almost completely removed, it was found by accident, on testing the wash fluid again, that tryptophan was present and was being removed from the precipitate, probably owing to final removal of mercury by the 7 % acid. This loss vitiated the results of estimations I-XI. For the later estimations, the wash-fluid always contained mercuric sulphate, and the washings were repeatedly tested with glyoxylic reagent at every stage. There was thus no loss of tryptophan during the process.

The washings were tested for tyrosine with Millon's reagent, and washing was continued until this test was negative. It was found, towards the end of the washing, that if the wash fluid was allowed to run through the precipitates, it would give a negative tyrosine reaction, but if the fluid was allowed to stand on the precipitate by blocking the neck of the funnel, after 24 hours a strong tyrosine reaction might be given. Hence, towards the end of the washing operations, the fluid was always allowed to remain in contact with the precipitates for 24 hours or longer until it gave a negative Millon reaction.

The first precipitate was free from tyrosine in about three weeks, but the second precipitate, though containing but little tryptophan, needed six weeks' washing or more. If, however, the digests were set up in advance, the later stages of estimation could be carried out while the preparation of further precipitates was in progress. The amount of tryptophan in the second precipitate was so small, that it was thought inadvisable to attempt to reprecipitate it with mercury reagent with a view to removing the tyrosine compound in this way.

6. *Decomposition of mercury precipitate with hydrogen sulphide.*

After tyrosine was completely removed, the precipitates were washed once or twice with water. They were then washed off the papers (these being reserved) and suspended in water which was made slightly alkaline with baryta. Hydrogen sulphide was then passed in for a considerable time, and the liquid filtered. This process was repeated several times. The filter-papers also were suspended and treated separately. Finally, the papers and mercuric sulphide were suspended in water, heated on a water-bath, and again hydrogen sulphide passed in. This was repeated until the filtrate and washings from the sulphide gave no glyoxylic reaction. The various filtrates and washings were then all united. For estimations I–XII, the treatment of the sulphide precipitate ceased at this stage. Even, however, after the precautions mentioned in sections 4 and 5 above as regards precipitation and washing had been taken, the value obtained for tryptophan content, on completing the estimation, was only about 1.7 % as a rule. As mentioned in the Introduction, 1.7 % of crystalline tryptophan has been obtained by Dakin from a caseinogen digest¹; therefore the total content should be higher. It was difficult to imagine where any loss of tryptophan had taken place, but the fact that the mercuric sulphide was found to contain nitrogen, even after well washing with hot water and with alcohol, led to the suggestion that some tryptophan might be retained in the sulphide, either by adsorption or from some other cause, such as incomplete decomposition. The mercury sulphide residue from one estimation, therefore, after the final washing with hot water, was boiled with 10 % baryta for one hour as a method of extracting any residual tryptophan. The barium was quantitatively removed from the filtrate from the sulphide, and the solution tested for tryptophan with glyoxylic reagent. Quite a good reaction was given. Hence in all subsequent estimations, the mercuric sulphide was always extracted with 10 % baryta, and the extract, after removal of barium, was added to the original filtrate from the hydrogen sulphide decomposition.

7. *Reduction in vacuo.*

The solution obtained from 6 was quantitatively freed from barium with dilute sulphuric acid; this left the solution slightly acid owing to the hydrogen sulphide. The barium sulphate was filtered off, and the filtrate concentrated *in vacuo* on a water-bath. As the hydrogen sulphide passed off, the solution became slightly alkaline; hence there should be no danger of the tryptophan being altered by heating on the water-bath. After concentrating the main bulk, the last washings of the barium sulphate precipitate may be concentrated separately and added later, in order to avoid heating the main bulk of tryptophan solution longer than is absolutely necessary. The reduction *in vacuo* was carried sufficiently far to enable the residue and washings to be made up to a volume of 100 cc. Henceforth, this solution will be referred to as the original solution.

¹ Due, no doubt, to the fact that fresh extract of pancreas was used, which is impossible, as already explained, in the present procedure.

8. *Determination of total and amino nitrogen.*

The total nitrogen of 5 cc. (in duplicate) of the original solution was then estimated (Kjeldahl), incineration being carried on for three-quarters of an hour after the mixture was clear. In the majority of estimations I–XII, this figure was too low, as mentioned above; for, when the value of the histidine present was subtracted, the remaining figure, even if it represented tryptophan entirely (which was unlikely owing to presence of traces of other amino acids), would only give a value at most of 1.7 % or, more frequently, of 1.3–1.5 %. After the method of extraction of the mercuric sulphide with 10 % baryta solution was adopted, the total nitrogen value was raised; it still varied considerably, however, as was to be expected, since the concentration of polypeptides and histidine in each digest varied and, consequently, different amounts of these substances were precipitated.

The total amino nitrogen was estimated on 1 cc. of the original solution (Van Slyke). This value also varied in each estimation for the same reasons as caused variation of the total nitrogen.

9. *Determination of histidine.*

Several methods have been described for this purpose, and much time was spent in testing them. Lautenschlager's [1918] titanium method was given an exhaustive trial, but theoretical results could not be obtained, even when the titanium procedure was replaced by Limpricht's [1878] method of reduction with stannous chloride (technical method still employed in Germany). There can be no doubt that under the given condition, diazobenzenesulphonic acid does not react quantitatively. According to Lautenschlager, one molecule of the diazo reagent reacts with one molecule of titanium and of histidine; according to Meyer [1909], four molecules of titanium react with each azo-group, and, according to Pauly [1915], the histidine compound is a bi-azo compound, which decomposes, yielding 20 % of its nitrogen when heated in either acid or alkaline media. The work of Weisz and Ssoblew [1914] shows that the concentration of histidine has a powerful influence on the production of the bi-body, and the observations of Hanke and Koessler [1920] show that the temperature at which, and the order in which, the reagents are added have a further influence.

The copper method of Kober and Sugiura [1913] was also tried. It was found to be very accurate for some of the amino-acids, but the observation of these authors that amino-caproic acid (*nor*-leucine) quantitatively precipitates all the copper except that of histidine, could not be verified, at least in mixtures containing the products of hydrolysis.

Finally, the colorimetric method of Koessler and Hanke was adopted as preferable to that of Weisz and Ssoblew. It is convenient for very small amounts of histidine, and even if not very accurate, the error introduced cannot be very great.

The procedure was as follows. To 5 cc. of the original solution in a small glass basin, 2.25 cc. of concentrated hydrochloric acid were added and the mixture made up to 12.5 cc. This was heated in the basin on a water-bath, and 2.5 cc. of 15 % phosphotungstic acid solution added, which precipitates the histidine both free and as polypeptide. The solution was immediately taken off the bath, stirred well and allowed to stand on ice for four days. The histidine precipitate was then filtered off, washed with fluid consisting of 15 g. of phosphotungstic acid, 18 cc. of concentrated hydrochloric acid and 200 cc. of water saturated with histidine phosphotungstate. The histidine precipitate was dissolved in the smallest quantity of 10 % caustic soda and made up to 250 cc. This solution was then used for estimation.

The method of Koessler and Hanke is based on the colour reaction given by histidine with *p*-diazobenzenesulphonic acid. This was compared colorimetrically with a standard indicator solution.

The reagent was prepared as follows: 4.5 g. of sulphanilic acid were dissolved in 45 cc. of 37 % hydrochloric acid (sp. gr. 1.19) and made up to 500 cc. 25 g. of 90 % sodium nitrite were dissolved in water and diluted to 500 cc. Then 1.5 cc. each of the sulphanilic acid and sodium nitrite solutions were measured into a 50 cc. volumetric flask. The flask was then immersed in an ice-bath for five minutes. Then 6 cc. more of the sodium nitrite solution were added, and the well-mixed solution again allowed to lie in the ice-bath for five minutes. The mixture was made up with distilled water to 50 cc., and the flask returned to the ice-bath where it was kept. This reagent must not be used for at least 15 minutes after diluting with water. It is best to prepare a fresh reagent every day.

The standard solutions for the indicator were prepared as follows: 0.5 g. of Grüber's methyl orange was dissolved in water and diluted to 500 cc.: 2.5 g. of Grüber's Congo red were mixed with 50 cc. of absolute alcohol and made up to 500 cc. with water.

For the estimation of histidine, 1 cc. of the Congo red and 1.1 cc. of the methyl orange were allowed to flow into 250 cc. of water contained in a 500 cc. volumetric flask. It was then made up with water. The indicator solutions must not be mixed in concentrated form.

The estimation now proceeded as follows: ($l-x$) cc. of water and 5 cc. of a 1.1 % sodium carbonate (Merck) solution were measured into the left-hand cylinder of a Kober colorimeter. Then 2 cc. of the reagent were measured into a 5 seconds delivery 2 cc. pipette, the time noted to the second, and the reagent allowed to flow into the alkali. The contents of the cylinder were then thoroughly mixed. Finally, x cc. of the histidine solution were allowed to flow into the cylinder exactly 1 minute after the reagent began to mix with the alkali. The contents of the cylinder were again thoroughly mixed, the cylinder transferred to the colorimeter and the instrument set at 20 mm. 8 cc. of the indicator solution were put in the right hand cylinder which was then adjusted to match.

Before estimations can be made, a curve must be plotted using a solution of known strength of histidine hydrochloride. The number of mm. on the scale of the colorimeter can then be read off as values of histidine.

It was found advisable to take readings about every minute for half an hour in order to record the maximum development of colour. Also the tube of the colorimeter should be repeatedly screwed up and down in the cup in order to secure thorough mixing. The values read on the right-hand scale should not exceed 20 mm.

10. *Hydrolysis in the autoclave and determination of amino, melanin and ammonia nitrogen.*

The next question to be considered was the length of time of hydrolysis necessary to decompose the peptides completely. Van Slyke [1912, 2] in his study of this subject, has shown that one and a half hours in an autoclave at 150° will complete the hydrolysis of caseinogen and most other proteins. Owing to the very different composition of the present mixture, it seemed necessary to repeat this work, and as the figures show (Table I), complete hydrolysis, as defined by the maximum figure for the amino nitrogen, was certainly obtained at the end of one and a half hours at 140°, if not earlier.

The method of procedure was as follows. Amounts, each of 20 cc., of the original solution were heated in a flask with an equal volume of 6*N* hydrochloric acid, for three periods ranging between one and two hours, in an autoclave at 140°. The hydrolysed mixture was then distilled *in vacuo* on a water-bath to dryness, whereby the hydrochloric acid was removed. The residue in the flask was washed out into a Claisen flask, made alkaline with 15 cc. of a 10 % suspension of calcium hydroxide, 100 cc. of alcohol were added and the mixture distilled at a temperature below 45° for half an hour into a known volume of standard alkali. (It is important to wash out into the Claisen flask, otherwise fumes of hydrochloric acid may not be completely removed.) In this way any ammonia present was determined. The melanin was filtered off from the residue in the flask and carefully washed until free from chlorides. The total nitrogen of the melanin on the filter paper was then estimated (Kjeldahl). The filtrate was concentrated *in vacuo*, and the volume made up to 50 cc.; of this the total nitrogen and the amino nitrogen were then determined (Kjeldahl and Van Slyke).

VALUES FOR TRYPTOPHAN CONTENT OBTAINED.

The figures for six of the last eight estimations are given in Table I. The first twelve estimations, though affording experience in the method, were vitiated by the errors mentioned in sub-sections 4, 5 and 6. Two of the last eight estimations were discarded on account of a suspected loss due to incomplete precipitation. A point to be noted is that the amount of histidine precipitated appears to increase with the time of digestion. This is probably due to the fact

that more free histidine is present, and hence the concentration is greater and more is precipitated.

The greatest difficulty in the interpretation of the results is offered by the production of ammonia during hydrolysis in the autoclave. The important point at issue is whether any, and, if so, how much of the ammonia arises from the amino groups. Apart from general interest, its origin is irrelevant, except in so far as it affects the total amino nitrogen value (made up of the amino value of the hydrolysed solution together with half the total nitrogen of the melanin). The whole question is considered in detail in the next section, where the results of a separate research on the origin of ammonia will be given, and it will be shown how the proportion of ammonia arising from the amino groups was determined for each estimation.

The next point to be considered is the total amino nitrogen after hydrolysis in the autoclave, for it is this figure that is essential in solving the equation (p. 65).

Fig. 1, constructed from the values obtained in estimation XX, represents the distribution of nitrogen during hydrolysis: this is also confirmed, in essential, by some preliminary experiments on hydrolysis for different periods and also by graphs constructed from the values obtained in ten, at least, of the other estimations—in fact in all where such values were determined.

From Fig. 1 it will be seen that during hydrolysis the amino value of the filtrate from the melanin, at first increases, but is eventually falling at the end of two hours. On the other hand, both the melanin and ammonia continue to increase. Since the ratio of total to amino nitrogen in the melanin remains unaltered, it will be seen that half the melanin value at any point should be added to the amino values. Further, it will be shown on p. 81, as we have said, that a certain proportion of the ammonia is also derived from the amino nitrogen. Hence the true amino value at any point is the sum of the amino nitrogen estimated, together with half the melanin value and a certain proportion of the ammonia value. Moreover, the total amino value (as calculated above) should remain constant, once complete hydrolysis has taken place; that is, the falling amino value (estimated) should always be compensated by the due proportions of melanin and ammonia, provided, as there is reason to believe, there is no other source of loss of nitrogen. This final value, therefore, is a measure of the total amino nitrogen of the original solution after hydrolysis, and has been used in solving the equation in every estimation.

Since the amount of polypeptide in the original solution may always vary, it will be seen that separate hydrolyses are essential in the case of each estimation. For the amount of caseinogen employed in these estimations, it is not possible to conduct more than three hydrolyses in the autoclave in each estimation. Several preliminary trials, however, were made, before the estimations were commenced, of hydrolysis in the autoclave for periods between $\frac{1}{2}$ –2 $\frac{1}{2}$ hours, and the values all indicated the same course of events as is recorded in the accompanying figure.

In estimations XIII and XV, unfortunately, the period of one and a half hours was not tried. Hence the value at one and a quarter hours has been taken, but there is little doubt that this is the maximum when the remaining estimations are considered, for in these the amino values at one and a quarter and one and a half hours are practically identical. In estimation XVIII, it

Fig. 1.

is true, the amino value at one and a third hours is too high, owing to the melanin value being too great as is seen on plotting a graph. For estimations XVI-XX inclusive, therefore, the mean of the amino values at one and a quarter and one and a half hours has been taken as the final value.

As the result of employing the values in Table I for solving the theoretical equation, the percentage of tryptophan in 100 g. of dry caseinogen (corrected for ash) is as follows:

English "casein"	Merck's "casein"
%	%
1.92	2.15
1.97	2.14
1.97	
1.87	

SOURCES OF ERROR IN ESTIMATIONS.

The possible sources of error in the values given above must now be considered. The chief possibility of error lies, no doubt, in the origin of ammonia formed in hydrolysis. Other, very slight, errors, as it will be shown, may be introduced by the enzyme extract employed and by the presence of proline. All these will be dealt with under the following sub-sections.

1. *Production of ammonia during hydrolysis in the autoclave.*

To the problem as to the origin of ammonia much time and experiment were given. The suggestions made and experiments performed are given under the following headings:

(a) That the ammonia is derived from amide nitrogen present in the original solution. Such nitrogen is said to be driven off by heating with 20 % hydrochloric acid for one hour on a water-bath, and then estimating as ammonia in the usual way. No amide nitrogen was obtained when the original solution from pure caseinogen was used.

(b) That the ammonia is derived from tryptophan. 100 mg. of tryptophan were heated with an equal quantity of 6N hydrochloric acid in an autoclave at 140° for two hours. 1.13 mg. of melanin nitrogen were found, but no ammonia. A mixture of tryptophan and glucose was treated similarly; 10.1 mg. of melanin were found but only a very small amount of ammonia nitrogen (0.5 mg.). After hydrolysis, the amino nitrogen was found to be 50 % of the total, as should be expected on the hypothesis that the ratio in the melanin remains unchanged.

(c) That the ammonia is derived from tryptophan in the presence of small quantities of other amino acids such as might occur in the original solution. Hence tryptophan (200 mg.), alanine (10 mg.), aspartic acid (15 mg.), cystine (5 mg.), glutaminic acid (15 mg.), glycine (10 mg.), leucine (30 mg.), tyrosine (5 mg.) and phenylalanine (10 mg.), together with lactose (10 mg.) were hydrolysed as usual in an autoclave for two hours; 0.7 mg. of ammonia nitrogen was found.

(d) That the ammonia is derived from purine introduced by trypsin. 100 mg. of uric acid, heated in the autoclave as usual for two hours, gave 1.41 mg. of ammonia nitrogen. It will be seen, however, on p. 83 that the amount of purine nitrogen introduced with the trypsin solution could only provide a very small amount of ammonia.

(e) That some substance precipitated with the mercury precipitate catalyses the production of ammonia. 100 mg. of tryptophan together with 3 cc. of the original solution were hydrolysed as usual for one and a half hours. 0.01 mg. of ammonia nitrogen was found.

(f) That the presence of polypeptide leads to production of ammonia. A solution of peptone, polypeptides and amino acids (alcoholic extract of Witte's peptone) was hydrolysed in the autoclave as usual for one and a half hours. 6.64 mg. of ammonia nitrogen were found.

(g) That tryptophan, in the presence of other amino acids, is catalysed by some substance in the original solution. A mixture of tryptophan and amino acids similar to those employed in (c), together with 5 cc. of the original solution, was heated in the autoclave as usual for one and a quarter hours. 0.25 mg. of ammonia nitrogen was found.

(h) That the presence of polypeptide affects the production of ammonia from tryptophan. A mixture of tryptophan (100 mg.), alanyl-glycyl-glycine (300 mg.) and lactose (10 mg.) was heated in the autoclave as usual for two hours. 0.28 mg. of ammonia nitrogen was found.

Except in the case of the polypeptide mixture in (f), which contained substances other than are present in the original solution, none of the experiments (a) to (h) offers a solution as to the source of ammonia.

Hence this remained uncertain, until a number of additional estimations had been made: from these it appeared that the amount of ammonia produced bears a relation to the amount of histidine present. This is illustrated by the following figures taken from graphs constructed from values given in Table I.

No. of estimation	Percentage of histidine N of total N	Ammonia mg. formed during hydrolysis in autoclave	
		During 1 hour	During 1½ hours
XIII	8	15	23
XV	8	15	22
XVI	18	19	28
XVIII	24	22	32
XX	17	7	11
XIX	20	12	18

In the early stages of the work, the amount of histidine present had been thought too small to account for the ammonia produced. However, the presence of sufficient histidine in the majority of the estimations, coupled with the above relationships, led to the suggestion that much of the ammonia might be produced from the histidine.

Hence solutions of pure tryptophan and pure histidine monohydrochloride were made up, in which the total nitrogen was of a value comparable to that found in the original solution from the digest, and in which the histidine nitrogen was present to the extent of 8, 10, 13, 18 and 24 %; in one solution tryptophan alone was employed. Aliquot portions of these solutions were heated with an equal volume of 6N hydrochloric acid in the autoclave for various periods at the usual temperature and pressure. The ammonia, melanin,

amino and total nitrogen values were determined in the usual way. The results are seen in Table II.

Certain deductions can be made from the above data. First, that ammonia is, without doubt, formed from both tryptophan and histidine, but in greater quantity from the latter under the above conditions. Secondly, that the rate of formation of melanin (and probably ammonia) is catalysed by some substance present in the original solution from the digest, which is absent from the pure amino acids, tryptophan and histidine. In all the above experiments, much less ammonia and melanin were formed than from the original solution, although the length of time of hydrolysis was much greater. It could also be seen that melanisation took place very slowly, for in Exps. III (c), IV and V, the hydrolysed solution was taken out of the autoclave and examined at intervals of about four hours. Examination showed that, unlike what occurs in the original solution in which 20–30 mg. of melanin are formed in the first hour, as a rule very little melanin was formed during the first four hours, and the amount gradually increased with the time of hydrolysis.

That a very small influence may greatly affect the rate of melanisation is also illustrated in the above experiments; for in Exps. I, II and III (a), one sample of hydrochloric acid was used, for Exps. III (b), (c), IV and V another sample. Thus, in spite of the fact that the tryptophan solution used in Exps. III (a), (b) and (c) was identical, the two different samples of acid (which were initially of the same strength and apparently equally pure) brought about melanisation at different rates. A similar phenomenon is illustrated in Exps. IV and V, as contrasted with III (c), for in IV and V, with the same acid, a sample of tryptophan was used which was more pure (as determined by the total and amino nitrogen, see Table II) than that employed in Exps. I, II and III. Consequently in IV and V, melanisation was slower.

The above results make all the preliminary hydrolyses of various mixtures of amino acids (a) to (f) unsatisfactory, for it is obvious that the pure amino acids should be subjected to many hours' hydrolysis in the autoclave before any deductions can be drawn as to formation of ammonia.

The conclusions finally to be drawn are that, in the solution from the digest, some substance catalyses melanisation and, very probably, the formation of ammonia; and that histidine contributes largely to the ammonia.

The main question at issue with regard to the ammonia in the estimation of tryptophan is, however, the determination of what proportion of it arises from deamination. For we are concerned with the ammonia nitrogen, only in so far as it affects the total amino value after hydrolysis.

The difficulty experienced in obtaining a correct estimation of the proportion of ammonia derived through deamination in the original solution from the digest is brought about by the possible presence of traces of aliphatic amino acids. Since, however, the latter can only be present in relatively very small amounts, the greater part of the ammonia no doubt arises from the histidine and to some extent from the tryptophan. The only course, therefore,

to adopt is to take a percentage of the ammonia, formed on hydrolyses of the original mixture, based on that formed by deamination in the mixtures used above in Exps. III, IV and V. This has been obtained as follows.

By reference to Table II, it will be seen that even when half the value of the melanin nitrogen in each case is added to the amino value determined after hydrolysis, there is a deficit in the amino value as compared with that originally determined before hydrolysis. The deficit of nitrogen, therefore, represents the proportion of ammonia nitrogen arising from deamination. Expressed as a percentage of the total ammonia it is for four experiments as follows:

	Percentage of histidine N of total N	Percentage of total ammonia N derived from amino N
Exp. III (a)	18	29.7
" III (c)	18	29.9
" IV	8	34.5
" V	24	30.3
" VI	0	47.7

From the above values Fig. 2 has been plotted, thus enabling one to obtain the proportion, approximately, of ammonia derived from the amino groups for any percentage of histidine within the range of the actual estimations made.

Fig. 2.

2. *Tryptophan and purine bases present in the trypsin employed.*

It was thought essential to make an estimation of these substances. For this purpose, 13.25 g. of Merck's trypsin was dissolved in *N*/10 sodium acetate, and the clear solution transferred to a Winchester quart bottle of 2650 cc. capacity. 2 g. of sodium fluoride (previously dissolved and filtered) were added, and the whole made up to about 2600 cc. with *N*/10 sodium acetate. The p_H was adjusted to 8.1 with caustic soda solution, and the enzyme digest placed in an incubator at 38°. After 20 hours, the digest had become acid to cresol-red and the p_H was again adjusted. This procedure was continued until the digest ceased to change, and, after four days, it was then filtered. In order to maintain the concentration of enzyme as near as possible to that employed in the original solution, 2 litres of the filtrate were added to 8 litres of 7 % sulphuric acid, thereby obtaining 10 litres of fluid of about 5 % acid. 1200 cc. of the Hopkins and Cole reagent were added, and the precipitate allowed to stand for four days. The remainder of the filtrate (about 600 cc.) was treated separately but, otherwise, in all particulars like the main bulk.

The latter, smaller, precipitate was also filtered off separately and used for a qualitative test for tryptophan with the glyoxylic reagent. A fairly strong reaction was given. The larger precipitate from the bulk of the filtrate was filtered through hardened filter paper, and then washed with the usual wash-fluid (3 % mercuric sulphate in 7 % sulphuric acid). No tyrosine could be detected in the washings. The filtrate from the larger precipitate was allowed to stand for a further 14 days; a slight precipitate was deposited, but gave no test for tryptophan.

The mercury precipitate from the 2 litres of digest (which was equivalent to 10 g. of trypsin) was washed with water, suspended and treated as usual with hydrogen sulphide, etc. The filtrate was reduced *in vacuo* to 100 cc. The total and amino nitrogen were then determined; the results are given on p. 83.

The purine bases were then estimated as follows: 50 cc. of the above 100 cc. obtained from the decomposition of the mercury precipitate were diluted to 100 cc. The purines were first precipitated with copper by the method of Krüger and Schmid [1901], that is, 6 g. of sodium acetate, 10 cc. of 40 % sodium bisulphite and 15 cc. of 10 % copper sulphate were added. The resulting precipitate was allowed to stand for two days. It was then filtered off, washed free from sulphate, suspended in water made slightly acid with hydrochloric acid, boiled and treated with hydrogen sulphide. The sulphide precipitate was filtered off and washed free from chloride. The combined filtrates were boiled to remove hydrogen sulphide and made up to 100 cc. In this the purines were then precipitated with silver by the method of Arnstein [1897], that is, 10 cc. of magnesia mixture, 10 cc. of 20 % ammonia and 10 cc. of 1 % ammoniacal silver nitrate were added. The resulting precipitate was allowed to stand in the dark for 24 hours. It was then filtered off, washed free from ammonia, and boiled with a little magnesium oxide to remove the last traces of ammonia;

the total nitrogen of the whole precipitate was then determined (Kjeldahl). The results, both for tryptophan and purine content, were as follows:

	Total nitrogen mg.	Total amino nitrogen mg.	Total purine nitrogen mg.	Total nitrogen other than purine mg.	Total non- amino nitrogen mg.
10 g. trypsin	11.231	4.252	8.021	3.210	6.979

From the above it will be seen that the total non-amino nitrogen in the 2 g. of trypsin used is only 1.396 mg. As it is impossible with so small an amount to apportion the nitrogen between purines, tryptophan and other possible substances present, this correction has been abandoned.

3. *Proline precipitated by the Hopkins and Cole reagent.*

This has already been shown [Onslow, 1921] to amount only to 10 mg. in 200 g. of commercial caseinogen, *i.e.* 2.5 mg. in 50 g. From pure caseinogen it is probably less.

It has therefore been decided to omit the corrections for nitrogen introduced by proline and by the trypsin extract. They probably only partially compensate for the inevitable loss of some tryptophan during the estimation.

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