XLIII. ON THE STABILITY OF TRYPTOPHAN IN BARYTA HYDROLYSIS.

By HERBERT ONSLOW.

From the Biochemical Laboratory, Cambridge.

Report to the Medical Research Committee.

(Received April 22nd, 1921.)

THE observations detailed in the following papers arose out of an attempt to estimate tryptophan, using baryta hydrolysis to set it free from the protein molecule. The method had to be abandoned, but certain observations were made on the stability of tryptophan, which seem worthy of record. The use of baryta hydrolysis for the estimation of tryptophan was first suggested by S. W. Cole, and I am greatly indebted to him for much valuable advice and assistance throughout the work, and in preparing the manuscripts.

It is well known that tryptophan is rapidly destroyed during the acid hydrolysis of proteins, so that if it is to be estimated in the free state, it must be liberated from the protein molecule, either by enzymic digestion or alkaline hydrolysis. Although digestion may prove satisfactory in the case of some proteins, von Fürth and Lieben [1920] have abundantly shown how difficult it is to set tryptophan completely free, even by prolonged digestion, about 60 % of it remaining in a peptide form. In any case, insoluble proteins like keratin could never be estimated in this way.

There is not much evidence available as to the stability of tryptophan in alkalies. Herzfeld [1913], estimating the indole produced by the action of various alkalies on proteins and tryptophan at blood heat, found that all alkalies could decompose tryptophan, sodium hydroxide being the most effective. Boiling with baryta, however, only produced a trace of indole (less than 0.5 mg.) from 1 g. of caseinogen. Miss Homer [1915], in an attempt to estimate tryptophan by bromine titration, showed that there is no apparent loss of tryptophan when caseinogen is hydrolysed with baryta for 40, 60, 80 and 120 hours respectively. Moreover, not more than 5 mg. of indole and no indole derivatives were produced during hydrolysis of 50 g. caseinogen. These conclusions are criticised by von Fürth and Lieben [1920], who argue that the figures for the tryptophan were so very low that considerable initial loss may have taken place. Further, they believe that there is an appreciable loss of tryptophan after six hours' heating with baryta on the water-bath. These statements depend on the accuracy of the formaldehyde colour reaction as a method of estimating tryptophan in unhydrolysed proteins. It may be men-

tioned, however, that the same authors are of the opinion that proteins may be heated in 30 % sodium hydroxide for 30 minutes without appreciable loss, whereas Herzfeld showed that with 6 % sodium hydroxide, 10 g. caseinogen, Witte's peptone and haemoglobin respectively produced 0.15, 1.2 and 3.8 mg. of indole. In spite of the evidence to be adduced in this paper, which serves to show that tryptophan is not continuously destroyed during baryta hydrolysis, it is quite possible that at some period there is an appreciable loss, as was suggested by von Fürth. The question must wait final decision until some accurate and reliable method has been devised for estimating tryptophan. The chief point of interest in the following experiments is the comparative stability of tryptophan in baryta, when in the presence of the other products of hydrolysis, compared with the ease with which it is decomposed when alone.

The effect of baryta hydrolysis on the nitrogen content of the mercury precipitate.

An attempt was first made to detect whether there was any loss of tryptophan during the baryta hydrolysis of a protein, by estimating the nitrogen of the mercuric sulphate precipitate. It was thought that, in addition to tryptophan, the mercuric sulphate reagent of Hopkins and Cole [1901] only precipitated cystine, tyrosine, and histidine. Since cystine is destroyed by alkaline hydrolysis, it seemed that, provided tyrosine and histidine were eliminated, the nitrogen content of the mercury precipitate would be a measure of the tryptophan. In the first place it was found that the precipitation of tyrosine can be almost prevented by raising the concentration of the sulphuric acid to 7 % by volume, before adding the mercury reagent, the last traces of tyrosine being easily washed away with 5-7 % sulphuric acid. No tryptophan appears to be removed during this process, especially if the acid for washing contains 2-3 % mercuric sulphate. Moreover, the increased concentration of the acid does not prevent the complete precipitation of tryptophan, even in very dilute solutions, provided the precipitate is allowed to stand from 3 to 4 days, as may be seen from Table I, in which the tryptophan recovered was calculated from the total nitrogen of the mercury precipitate.

Table I. *Influence of the percentage of tryptophan and of H_2SO_4 on the complete precipitation of the mercuric compound.*

Weight of tryptophan taken in mg.	Concentration of tryptophan in mg. %	Concentration of H_2SO_4 %	Weight of tryptophan recovered in mg.	Percentage of apparent loss or gain in mg.
40	20	6.0	39.9	- 0.25
40	20	7.0	39.9	- 0.25
50	20	7.0	49.1	- 1.80
40 (+ histidine HCl 0.064 g.)	20	7.0	37.7	- 5.75
40	20	8.0	30.6	- 23.50
50	4	7.0	50.1	+ 0.25
50	2	7.0	45.1	- 8.80
50 (+ amino-acids 2.5 g.)	2	7.0	50.5	+ 1.00

That the precipitation of tryptophan is facilitated by the presence of other amino-acids is seen by the last experiment, in which 2.5 g. of leucine, and

glutamic and aspartic acids were added to the tryptophan. When alone in 7 % acid, tryptophan is quantitatively precipitated in a concentration of 0.004 %.

Histidine appears to be fairly stable when boiled with baryta, as is shown by the following experiment:

0.5 g. histidine HCl was boiled in 10 % $\text{Ba}(\text{OH})_2$ on a sand-bath for 120 hours. The baryta was removed and the solution precipitated with HgCl_2 and Na_2CO_3 . The total nitrogen of the precipitate was equivalent to 0.345 g. histidine HCl, a loss of 31 %.

Some histidine is precipitated by the mercury reagent, especially in low concentrations of sulphuric acid, and in the presence of the other products of hydrolysis. Even in 7 % sulphuric acid, under the latter conditions, some histidine is precipitated, but this can be avoided by diluting the fluid until it contains only 0.02 % of the base (see Table IV). After having precipitated twice with these concentrations the final product fails to give a sulphur reaction, showing the absence of cystine.

After hydrolysis and the removal of the baryta¹ the caseinogen solutions were twice precipitated with mercuric sulphate, observing the concentrations specified, *i.e.* 7 % H_2SO_4 by volume, and a concentration of histidine not greater than 0.02 %. The total nitrogen of the mercury precipitate from a number of hydrolyses was then estimated, and the results are shown in Table II.

Table II. *Influence of baryta hydrolysis of caseinogen on the nitrogen content of the precipitate obtained with HgSO_4 in 7 % H_2SO_4 .*

No. of experiment		Weight of substance taken in g.	Period of hydrolysis in hours	Total nitrogen of HgSO_4 ppt. in g.	Apparent yield of tryptophan in g.
1	Tryptophan	0.3	60	0.0099	0.072
2	Tryptophan	0.3	120	Traces	—
3	Hammarsten's casein	20.0	40	0.081	0.530
4	"Pure casein"	20.0	40	0.089	0.649
5	"Pure casein"	20.0	70	0.090	0.656
6	"Pure casein"	20.0	80	0.096	0.700
7	"Pure casein"	20.0	80	0.104	0.758
8	"Pure casein"	20.0	100	0.100	0.729
9	"Laitproto No. 6†"	20.0	40	0.036	0.262
10	"Laitproto No. 6"	20.0	120	0.041	0.299
11	"Pure casein"	20.0	60	0.101	0.736
	glucose	0.2			
12	"Pure casein"	20.0	60	0.088	0.641
	glucose	1.0			
13	"Pure casein" (+ <i>N</i> NaOH 13 cc.)	20.0	80	0.051	0.372
14	"Pure casein" (in 10 % NaOH)	20.0	80	0.000	0.000

* "100 % pure casein" purchased from Casein Ltd., Culvert Works, Battersea.

† Commercial sample of caseinogen obtained from the above firm.

¹ A 10 % solution of caseinogen was hydrolysed in 10 % baryta, and some difficulty was encountered in washing the BaSO_4 quite free of tryptophan. It was found advantageous to use boiling water containing 10 % of alcohol and a little acetic acid for extracting the precipitate.

These estimations (Kjeldahl) were made in duplicate, and average figures are given throughout. Precautions were taken to add thiosulphate before distillation, and incineration, owing to the resistance of the indole ring [Dakin and Dudley 1914], was continued for an hour after the fluid was quite clear.

From experiments 1 and 2 in Table II it is clear that pure tryptophan is almost completely destroyed when it is boiled in the presence of baryta, the solution turning pink and giving a strong smell of indole as well as a positive vanillin reaction [Nelson 1916]. From experiments 3 to 8, it appears that the nitrogen of the mercuric sulphate precipitate reaches a maximum after about 70 hours, and remains constant after 100 hours' hydrolysis. Samples of commercial caseinogen were found to behave in a very different manner from the pure product, as is shown by experiments 9 and 10, the apparent yield of tryptophan being only 40 % of that of the "pure casein." That this was not due to contamination by traces of lactose is shown by experiments 11 and 12, in which a carbohydrate was added to pure caseinogen without there being any obvious change in the apparent yield of tryptophan. The small yield from commercial caseinogen is due to the large amount of sodium added to these preparations, as may be clearly seen from experiments 13 and 14. For when enough soda is added to a solution of 20 g. of pure caseinogen to make it neutral to phenolphthalein (13 cc. *N* NaOH), the apparent yield of tryptophan is about the same as from the commercial samples. In some preparations, so much alkali is introduced that much of the protein is racemised, and will not digest [Dakin and Dudley 1914]. If pure caseinogen is hydrolysed with 10 % sodium hydroxide, or barium hydroxide containing a sodium salt, no trace of mercury precipitate is obtainable and considerable quantities of indole are produced.

In order to test the effect of baryta hydrolysis on free tryptophan in the presence of the other products of hydrolysis, a number of experiments were carried out with gelatin (which contains no tryptophan) to which were added cystine and histidine to make the total amount of these substances about equal to that in caseinogen. The mercuric sulphate precipitate was prepared under the conditions described above, and the total nitrogen obtained is shown in Table III.

Experiments 1 to 4 show that the nitrogen contained by the mercury precipitate from gelatin is very low, even in the presence of the additional amino-acids, and experiment 5 shows that the same is true of zein. In experiments 6, 7 and 8, after allowing for the 7 mg. of nitrogen from the gelatin, nearly the whole of the tryptophan added is apparently recovered, it being assumed that the presence of the tryptophan does not cause the mercury reagent to precipitate anything extra from the hydrolysed gelatin. From the results recorded in the following communication, however, it is very necessary to put this to the test, as it has been found that the mercury precipitate obtained by the ordinary method for isolating tryptophan contains polypeptides,

Table III. *The nitrogen content of the mercury compound, obtained under the specified conditions, from gelatin and from gelatin + tryptophan, hydrolysed for 80 hours.*

No. of experiment		Weight of substance hydrolysed in g.	Period of hydrolysis in hours	Nitrogen of HgSO ₄ ppt. in g.	Equivalent weight of tryptophan
1	Gelatin	40.00	80	0.008	—
2	Gelatin	20.00	80	0.006	—
	Cystine	0.05			
3	Gelatin	20.00			
	Cystine	0.20	80	0.008	—
	Histidine	0.50			
4	Gelatin	20.00	80	0.006	—
	Tyrosine	1.00			
5	Zein	15.00	80	0.005	—
6	Gelatin	20.00			
	Cystine	0.20	80	0.041	0.301
	Histidine	0.50			
	Tryptophan	0.30			
7	Gelatin	20.00			
	Cystine	0.02	40	0.037	0.271
	Histidine	0.50			
	Tryptophan	0.30			
8	Gelatin	20.00			
	Cystine	0.02	60	0.044	0.322
	Histidine	0.50			
	Tryptophan	0.30			

the presence of which might raise the nitrogen content of the mercury precipitate, thus compensating for any tryptophan lost. Even allowing for this, it is fair to assume that the presence of other amino-acids in some way protects tryptophan from the destructive action of barium hydroxide. The effect of crystalline amino-acids on the stability of tryptophan was accordingly examined as follows:

The effect of baryta hydrolysis on pure tryptophan in the presence of other free amino-acids.

Tryptophan, with a mixture of tyrosine, glycine, leucine and aspartic acids, was boiled with 10 % baryta for 40 hours, but no smell of indole could be detected. Even a comparatively small amount of a single amino-acid exerts a considerable protective influence.

In flask *A* were placed 5 mg. of tryptophan and some baryta solution. In flask *B* were placed the same amount of tryptophan and 0.125 g. of aspartic acid with the same amount of baryta. After 15 minutes' boiling, neither flask smelt of indole, but after half an hour indole could be detected in flask *A*, and after 45 minutes the contents had turned pink. Flask *B* was boiled for 4½ hours without the appearance of any indole.

A further experiment was carried out to test the protective influence of the amino-acids more rigorously. It was devised so that any compensation for a loss of tryptophan by the precipitation of amino-acids might be detected, thereby serving to show the extent to which tryptophan is destroyed in baryta

hydrolysis. The monoamino-acids added were chosen so as to avoid those which are easily precipitated by mercuric sulphate, and the following were taken:

	g.
Tryptophan (from caseinogen) ...	0.2
Glycine (from gelatin)	1.5
Leucine (from caseinogen) ...	1.3
Alanine (from silk)	4.0
Aspartic acid (from asparagine)	2.0
Glutamic acid (from gluten flour)	1.0
	<hr/> 10.0

This mixture of amino-acids dissolved in 200 cc. of water contained 2 % of tryptophan. Half the solution was boiled for 40 hours with the addition of 5 g. of barium hydroxide. The other half was made up to a volume of 500 cc. containing 5 % sulphuric acid, to serve as a control, and precipitated directly with 150 cc. of the mercury reagent. Some 30–40 cc. of fluid were distilled from the boiled portion, extracted with xylene and tested with vanillin and *p*-dimethylaminobenzaldehyde, both reactions for indole were faint but positive. The distillate, however, gave no precipitate with mercuric sulphate. After the removal of the baryta, the total volume was made up to 500 cc. containing 5 % sulphuric acid, and precipitated with 150 cc. of the mercury reagent. The precipitates were now washed, decomposed with hydrogen sulphide, the mercury removed, and the volume concentrated to 200 cc. Estimations were then made of the total and of the amino-acid nitrogen, which were as follows:

Mixture of amino-acids boiled for 40 hours with 10 % Ba(OH)₂.

	Nitrogen in g.	Equivalent weight of tryptophan in g.
Nitrogen of tryptophan added ...	0.0137	0.100
Total nitrogen	0.0131	0.095
Amino nitrogen	0.0075	0.110
Non-amino nitrogen (by difference)	0.0056	0.081

Since in the boiled portion the tryptophan calculated from the amino nitrogen is more than the amount taken (0.1 g.), and that calculated from the total nitrogen rather less, it is clear that some other amino-acids have been precipitated with the tryptophan. The actual amount of tryptophan may be calculated from the non-amino nitrogen, since the contaminating amino-acids must be monoamino-acids, and amounts to 0.081 g. a loss of 19 mg. of tryptophan, which may easily be accounted for by the indole-like substance. The amount of monoamino-acids precipitated by the mercury is very small, being equivalent to not more than 2 mg. of nitrogen. In the control experiment 52.3 % of the total nitrogen was found to be amino nitrogen, and in a second control experiment carried out later, 50.2 % of the nitrogen was in the amino form,

showing that very little of the amino-acids was precipitated except tryptophan.

A somewhat similar experiment, but without a control, was carried out with tryptophan, to which were added the products of a 90-hour hydrolysis of gelatin in baryta. The fraction of the hydrolysed gelatin precipitated by HgSO_4 was carefully removed, and in addition the remaining histidine was precipitated with HgCl_2 and $\text{Ba}(\text{OH})_2$. All the reagents were removed, the fluid evaporated to a gum, and 0.2 g. of tryptophan added to 10 g. of these products of hydrolysis. The solution was then boiled for 40 hours in 100 cc. of 10 % $\text{Ba}(\text{OH})_2$. The boiled mixture was treated exactly as before, except that the tryptophan was precipitated in a litre of 7 % sulphuric, with 300 cc. of the mercury reagent. The figures obtained for the nitrogen were as follows:

	Nitrogen in g.	Equivalent weight of tryptophan in g.
Nitrogen of tryptophan added ...	0.027	0.200
Total nitrogen	0.031	0.227
Amino nitrogen	0.014	0.207
Non-amino nitrogen (by difference)	0.017	0.245

In this case the tryptophan calculated from the total nitrogen is rather more, and that calculated from the non-amino nitrogen considerably more than the amount added (0.20 g.). This is no doubt due to the large amount of proline and hydroxyproline present in gelatin (over 24 % [Dakin, 1920]), some of which appears in the mercury precipitate, thereby raising the amount of non-amino nitrogen, and consequently the apparent amount of tryptophan. In this case the amino nitrogen is probably a more accurate measure of the tryptophan than the non-amino nitrogen. Boiling produced a small amount of a volatile substance which gave the indole reactions and could moreover be precipitated by mercuric sulphate.

It is an interesting fact that this action of the products of protein hydrolysis in preventing the destruction of tryptophan by barium hydroxide, is the reverse of what is experienced in acid hydrolysis. Miss Homer [1915] found that only 50 % of pure tryptophan is destroyed when it is boiled with 25 % sulphuric acid for 42 hours, whereas when tryptophan is split from the protein molecule, it is destroyed so rapidly that it is only with difficulty that a portion can be recovered.

A 4-5 % solution of caseinogen was hydrolysed with H_2SO_4 . One portion was made to contain 5 % sulphuric acid, and another 7 %. After the addition of HgSO_4 to both, it was found that though a small precipitate giving the glyoxylic reaction was formed in the 5 % acid, none whatever separated in the 7 % acid, even after standing several days.

Thus the products of hydrolysis seem to protect tryptophan from the action of alkalis, but they assist the destructive action of acids. Further, tryptophan is rapidly destroyed by a univalent ion such as Na^+ , even when it is in the presence of the other products of hydrolysis. The fact that the bivalent Ba^{++} ion does not rapidly destroy tryptophan when in the presence of amino-acids, etc., is not easily explained; possibly Ba^{++} can form stable double salts with tryptophan and some other amino-acid, which are not easily decomposed.

The effect on the solubility of amino-acid compounds produced by the presence of other amino-acids in solution.

It has been mentioned that pure histidine monohydrochloride is not easily precipitated in acid solution, yet in the presence of the other products of hydrolysis it is precipitated with considerable ease. This is more clearly demonstrated by Table IV.

Some gelatin was hydrolysed with baryta. The solution was made 5 % acid with H_2SO_4 , HgSO_4 was added, and the small precipitate filtered off. The excess mercury was removed with hydrogen sulphide, the SH_2 boiled off, and the H_2SO_4 removed quantitatively with baryta. The solution was finally evaporated to dryness, and dissolved in 7 % H_2SO_4 to make a 0.8 % solution, to which histidine was added in varying amounts.

Table IV. *Influence of the products of protein hydrolysis on the solubility of the histidine mercury compound.*

Percentage of histidine in 7 % H_2SO_4	Precipitate after 2 days	Percentage of histidine in 7 % H_2SO_4 + 0.8 % hydrolysed gelatin	Precipitate after 2 days
0.200	—	0.200	++
0.100	—	0.100	+
0.050	—	0.050	±
0.025	—	0.025	—

This experiment forcibly illustrated a point familiar in the chemistry of the purines, and repeatedly observed during this work, namely that the ordinary laws of aqueous solubility for amino-acids and their salts, do not apply to amino-acids in solution with each other. The behaviour of the copper salt of tryptophan is a well-known example of this, for though quite insoluble in water, it is easily dissolved by even dilute solutions of other amino-acids. The failure to observe this fact frequently leads to error. Thus Miss Homer [1915] obtained a value for the tryptophan content of caseinogen lower than the amount actually crystallised by the discoverers. This was chiefly due to the use of phosphotungstic acid to remove polypeptides from the solution of tryptophan. Such precipitates always give the glyoxylic reaction.

Van Slyke [1911] observes that three-fourths of the tryptophan nitrogen is precipitated by phosphotungstic acid with the "base fraction." As a matter of fact pure tryptophan is not as a rule precipitated by most samples of phosphotungstic acid, but in the presence of polypeptides, etc., a certain amount is precipitated. Von Fürth and Lieben [1920] claim to separate free tryptophan from that bound as peptides with phosphotungstic acid, by keeping the concentration of tryptophan low and that of the acid high. No proof, however, is given to show that phosphotungstic acid will differentiate between tryptophan and di- or tri-peptides. For instance, the following experiment was tried in which 0.025 g. tryptophan and 0.018 g. of freshly dissolved alanyl-glycyl-glycine were taken in 25 cc. of 5 % HCl , but no matter how much phosphotungstic acid was added, the solution remained clear indefinitely.

CONCLUSIONS.

1. When caseinogen is hydrolysed in acid solution, tryptophan is rapidly destroyed. When pure tryptophan is boiled in acid solution, it is much more stable.

2. Tryptophan when alone is rapidly destroyed by all alkalies, but NaOH and Na_2CO_3 are more effective than $\text{Ba}(\text{OH})_2$.

3. When caseinogen is hydrolysed by NaOH, tryptophan is rapidly destroyed, but when $\text{Ba}(\text{OH})_2$ is used, there is evidence to show that tryptophan is much more stable.

4. The destruction of tryptophan by the univalent, but not by the bivalent ion, seems to depend on the presence of the other products of hydrolysis. The destruction of tryptophan may be largely prevented by the addition of crystalline amino-acids.

5. The precipitation of free tyrosine by the mercury reagent may be prevented to a very great extent by raising the concentration of the H_2SO_4 from 5 to 7 % by volume.

6. Histidine is fairly stable when boiled with baryta. In acid solutions it is not easily precipitated by HgSO_4 except in high concentrations, but in the presence of other amino-acids it is precipitated in 7 % H_2SO_4 , unless its concentration is reduced to 0.02 %.

7. The total nitrogen of the HgSO_4 precipitate, obtained under the specified conditions from hydrolysed caseinogen, reaches a maximum in 60–70 hours, and does not decrease after 100 hours.

8. Gelatin and zein, after baryta hydrolysis, and the specified treatment, yield only traces of nitrogen in the HgSO_4 precipitate. After the addition of pure tryptophan to gelatin the total nitrogen of the HgSO_4 precipitate appears equivalent to the tryptophan added.

REFERENCES.

- Dakin (1920). *J. Biol. Chem.* **44**, 499.
Dakin and Dudley (1914). *J. Biol. Chem.* **17**, 275.
von Fürth and Lieben (1920). *Biochem. Zeitsch.* **109**, 153.
Herzfeld (1913). *Biochem. Zeitsch.* **56**, 82.
Homer (1915). *J. Biol. Chem.* **22**, 369.
Hopkins and Cole (1901). *J. Physiol.* **27**, 418.
Nelson (1916). *J. Biol. Chem.* **24**, 528.
Van Slyke (1911). *J. Biol. Chem.* **10**, 40.