XLIV. ON THE NATURE OF THE SUBSTANCES PRECIPITATED BY MERCURIC SULPHATE FROM HYDROLYSED CASEINOGEN, WITH REFERENCE TO THE ESTIMATION AND ISOLATION OF TRYPTOPHAN.

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In the preceding communication [Onslow, 1921] a procedure was described, by which the mercuric sulphate precipitate from hydrolysed caseinogen could be prepared, containing no free cystine, tyrosine or histidine. It was hoped that this precipitate would consist entirely of the tryptophan compound, which might be used as the basis of a method for estimating tryptophan. The nitrogen content, however (calculated for ash- and water-free caseinogen), corresponded to 4 % of tryptophan[1], which suggested that other amino-acids were precipitated, possibly in the form of polypeptides.

Redigestion of the mercury precipitate owing to the difficulty of effecting complete hydrolysis of the peptides.

To investigate the nature of the precipitate an attempt was first made to use Dakin's method [1918] for extracting tryptophan with butyl alcohol, using caseinogen which had been hydrolysed by baryta as previously described. Although Dakin obtained 1·7 % of tryptophan, very little was found on this occasion. It was impossible by extraction to free the fluid from the glyoxylic reaction. Moreover, when the butyl alcohol was evaporated under reduced pressure, a pigmented substance remained as a gum, while little or no tryptophan crystallised[2]. Further experiments were made, in which caseinogen was hydrolysed by a pancreatic extract instead of by baryta, and the resulting mercury precipitate after decomposition was extracted with butyl alcohol. Only a little

[1] The molecular weight of caseinogen was calculated by Van Slyke and Bosworth [1913] as 8888. This would give 2·3 % of tryptophan, supposing that each molecule of the protein contained one molecule of tryptophan. The most recent colorimetrical methods for estimating tryptophan, though very inadequate, give values for caseinogen of about 1·7–2 %.

[2] An attempt was also made to crystallise the racemic sodium salt of β-naphthalenesulphotryptophan from the products extracted by butyl alcohol, but even allowing for the usual yield of 84 %, the tryptophan obtained only amounted to 1 % of the caseinogen taken.

more tryptophan was obtained than before, and it was concluded that most of it must be present as polypeptides, which would dissolve in the butyl alcohol and leave a gum after evaporation. This appeared all the more probable, because Dakin obtained over 4 % of peptide anhydrides, after 14 hours acid hydrolysis, and von Fürth and Lieben [1920] have since shown that even after prolonged pancreatic and ereptic digestion, about two-thirds of the tryptophan remains in the peptide form.

In order to obtain the maximum amount of hydrolysis, the decomposed mercury precipitate was subjected to a second tryptic digestion, in the absence of the other products of hydrolysis, and again extracted with butyl alcohol. The tryptophan *now* separated in an amount hardly inferior to that obtained by Dakin. It was, however, necessary to extract the fluid for a long time before the glyoxylic reaction became negative, but the amount of nitrogen still remaining in the extracted fluid indicated that a quantity of dicarboxylic, or diamino-acids had been thrown down in the mercury precipitate.

Since all the tryptophan was not obtained in a free state by this treatment, some caseinogen was first digested with pepsin, then with trypsin at a reaction of $P_H = 8{\cdot}1$[1], and finally, with erepsin at the same reaction.

The mercury compound obtained was washed and decomposed as usual, and the solution again digested with trypsin and erepsin. In one or two experiments, in which digestion had been complete, the fluid obtained from the decomposed mercury compound would not give the reaction for polypeptides described by Kober [1911]. This is carried out by making the copper salts of the mixture. The copper of all the amino-acids is precipitated by boiling in weak alkaline solution, whereas the copper of the peptides remains unchanged, appearing as a blue filtrate. As a rule, however, it was impossible to obtain complete hydrolysis, and this agrees with the observations of Frankel [1917], who used Van Slyke's method to estimate the amino nitrogen. He found that after digesting proteins 100 hours with pepsin, they gave 20 % of amino-acid nitrogen, with pepsin and trypsin 70 %, with trypsin alone 50 %, and with pepsin, trypsin and erepsin 80–90 %. The difference between these results and those of von Fürth depends apparently on the fact that the latter removed the polypeptides with phosphotungstic acid, so that their amino nitrogen could not react during the formaldehyde titrations.

The amino-acids precipitated by the mercury reagent.

It was found necessary to identify as many as possible of the amino-acids in the mercury precipitate, and for this purpose the usual methods for separating them were adopted. The exact procedure can be followed most easily by reference to the scheme given below, in which the successive operations are shown in tabular form. The amino-acids could not be subjected to Fischer's

[1] According to various authors the optimum reaction for digestion of caseinogen by trypsin is about $P_H = 7{\cdot}2$, but according to a recent investigation by S. W. Cole [1920] maximum digestion occurs at $P_H = 8{\cdot}1$.

ester method owing to the small quantities available, consequently it was impossible to isolate some of the units.

Experiment I. A sufficient amount of the mercury compound was prepared by digesting 1 kilo. of caseinogen with trypsin and precipitating with mercuric sulphate. This precipitate, after being washed free from tyrosine, was decomposed and again well digested with trypsin, in order to carry the hydrolysis of the peptides as far as possible.

Table showing the procedure followed in separating the amino-acids.

(1) 10 % solution of caseinogen digested with trypsin, precipitated in 5% H_2SO_4 with $HgSO_4$, and filtered.

(2) Precipitate* decomposed with SH_2, redigested with trypsin, and again precipitated with $HgSO_4$ in 7 % H_2SO_4, and filtered.

<table>
<tr><td rowspan="2">Precipitate A.
(Contains tryptophan, etc.)

Discarded</td><td colspan="4">Filtrate B.
(Contains amino-acids liberated by second digestion)

Excess mercury removed with SH_2. Fluid precipitated with phosphotungstic acid in 5 % H_2SO_4, and filtered</td></tr>
<tr><td colspan="2">Precipitate C.
(Contains diamino-acids)

Precipitate decomposed with hot baryta, excess barium removed and histidine precipitated with $HgCl_2$ and Na_2CO_3; filtered</td><td colspan="2">Filtrate D.
(Contains monoamino-acids)

Excess phosphotungstic acid and H_2SO_4 removed. Dicarboxylic acids precipitated by $Ca(OH)_2$ and alcohol (Foreman [1914]); filtered</td></tr>
<tr><td></td><td>Precipitate E.

Precipitate of histidine decomposed by SH_2 and picrolonic acid added to isolate the HISTIDINE, which was identified by melting-point and N-content</td><td>Filtrate F.

Excess Hg removed. Phosphotungstic acid added in 5 % H_2SO_4. No precipitate of LYSINE or ARGININE</td><td>Precipitate G.

Precipitate of Ca salts dissolved in H_2O. Ca removed with oxalic acid and $Cu(OH)_2$ added to isolate ASPARTIC and GLUTAMIC acids</td><td>Filtrate H.
(Contains mono-amino-acids)

Alcohol evaporated and residue allowed to crystallise. Deposited LEUCINE and traces of TYROSINE. Other amino-acids remained which would not crystallise</td></tr>
</table>

* This precipitate after decomposition gave a faint reaction for sulphur showing the presence of cystine.

From precipitate *G* crystals of what appeared to be a mixture of the copper salts of aspartic and glutamic acids were obtained, but no attempt was made to isolate hydroxyglutamic acid.

The other monoamino-acids contained in filtrate (*H*) were allowed to crystallise slowly, typical striated nodules of impure leucine being deposited. These were dissolved and the fluid recrystallised from traces of tyrosine. The resulting product sublimed on heating, but the amino nitrogen value was lower than that of pure leucine. The filtrate still contained other monoamino-acids which would not crystallise, and as the material was very scarce, an

unsuccessful attempt was made, at the suggestion of Mr Raistrick, to separate the remaining acids as the picrates of the methyl ester hydrochlorides.

Experiment II. In order to make quite certain that arginine and lysine are not precipitated in the mercury compound, the diamino-acids were treated according to the method of Kossel and Kutscher. For this purpose 500 g. of caseinogen were digested and treated as in Experiment I, until the filtrate (*B*) had been obtained. The arginine and histidine were then precipitated as silver salts in a solution made alkaline with baryta and filtered off. The filtrate was tested for lysine with phosphotungstic acid which failed to give any precipitate. The arginine-histidine fraction was next decomposed, the histidine separated as a silver salt in neutral solution, and the filtrate tested for arginine by saturating it with baryta. No precipitate could be obtained, thus confirming the absence of arginine and lysine.

Experiment III. In order to find out whether proline was present among the monoamino-acids, a further 200 g. of caseinogen were digested and precipitated with the mercury reagent. Special pains were taken to ensure that this precipitate was washed free from all tyrosine. This was done by grinding the precipitate with 5 % sulphuric acid until the filtrate gave a negative Millon's reaction. The washing was then continued until 200 cc. of the filtrate, after being freed from mercury and sulphuric acid, failed to give the reaction for tyrosine with diazobenzenesulphonic acid [Totani 1915]. It was observed that after the tyrosine had been completely removed, a strong histidine reaction could still be obtained.

The washed mercury precipitate was now decomposed and boiled for 14 hours in 25 % sulphuric acid, to hydrolyse the peptides. The resulting black fluid was diluted until it contained 6 % of acid. Enough baryta was added to reduce the concentration to 5 % and the barium sulphate, together with most of the humin, filtered off. The filtrate was precipitated with mercuric sulphate, to separate the tryptophan, and the filtrate, after removing the reagents, was treated according to Dakin's method [1918] for preparing proline, by thoroughly extracting with butyl alcohol. The alcohol was concentrated to remove the monoamino-acids which separate, and the filtrate after taking to dryness under reduced pressure was dissolved in absolute alcohol and set aside to crystallise. The traces of peptide anhydrides deposited were filtered off, and the residue after taking to dryness was boiled in water with a little Merck's charcoal. As no more peptide anhydrides separated, the fluid was made up to a known volume, and the total and amino nitrogen estimated in duplicate, with the following result: amino nitrogen by Van Slyke, 3·1 mg.; total nitrogen by Kjeldahl, 13·1 mg.; non-amino nitrogen by difference, 10 mg.; equivalent weight of proline, 82 mg. It thus appears that there is a very small but definite amount of proline precipitated in the mercury compound which amounts to about 82 mg. from 200 g. of caseinogen.

It was impossible to overlook the fact that arginine or lysine might have been absent from filtrate (*B*) in experiments I and II, because they were

precipitated with the tryptophan in precipitate (*A*). In order to decide this, advantage was taken of the fact that nearly all the polypeptides must have been hydrolysed by boiling with strong acid, thus insuring the most complete separation possible between the tryptophan and the other amino-acids. In addition proline and the other monoamino-acids had been removed as described above, so that the aqueous layer after extraction with the butyl alcohol must contain all the dicarboxylic acids and the bases. This fraction was accordingly treated with mercuric chloride and sodium carbonate to precipitate the histidine. After removing the reagents, phosphotungstic acid was added to precipitate any lysine or arginine. No precipitate resulted, except after long standing, and this gave a strong histidine reaction, showing that this amino-acid had not been completely removed by treatment with mercuric chloride.

As there was some doubt whether the trace of tyrosine observed in filtrate (*H*) of experiment I, was due to the precipitation of a tyrosine polypeptide, or to the incomplete washing of the mercury precipitate from free tyrosine, great pains were taken to wash the precipitate thoroughly, as already described. Advantage was now taken of this precaution, to test for tyrosine in the fraction of the monoamino-acids extracted by butyl alcohol[1]. Tyrosine was present only in the peptide anhydrides separated from the ethyl alcohol, showing that a minute quantity of tyrosine peptide is precipitated with the mercury compound. This cannot be washed away with acid and appears to be hydrolysed with great difficulty.

As the result of the foregoing experiments, it may be concluded that the mercuric sulphate compound contains the following amino-acids in addition to tryptophan: cystine, tyrosine (traces), leucine and other monoamino-acids, glutamic and aspartic acids, histidine and a little proline, but neither lysine nor arginine. It is probable that most of these amino-acids are precipitated or adsorbed in the peptide form, possibly as tryptophan polypeptides. The amount of amino-acids precipitated by the mercury depends on the degree of hydrolysis. It was frequently noticed, especially when digestion was incomplete, that the mercury compound was precipitated in two layers, the first yellow, the other white. The latter possibly consisted mainly of polypeptides.

Is tyrosine split from the protein molecule early in digestion?

The fact that tyrosine was recognised in the peptide form after hydrolysis of a small fraction of the total protein in 25 % sulphuric, made it doubtful whether this amino-acid is split from the protein molecule as early as is sometimes claimed. Plimmer and Eaves [1913] have based a method of estimating

[1] It is impossible to test the decomposed mercury precipitate directly for tyrosine because the tryptophan present interferes with Millon's reagent, and the histidine masks the colour of the diazo reaction. The phenol reagent of Folin and Denis [1912] is said to give a blue colour with tyrosine alone of the protein decomposition products. Although this reagent was tried, it was found useless, since it gave a blue colour with the purest samples of tryptophan procurable. Abderhalden and Fuchs [1913] and Gortner and Holm [1920] have since shown that tryptophan and other amino-acids also give this blue colour.

tyrosine on the assumption that in pancreatic digestion this amino-acid is yielded quantitatively within six hours. Totani [1916] isolated 0·174 g. of tyrosine by acid hydrolysis after the removal of all the tyrosine formed during digestion, that is to say, nearly 0·3 % of the caseinogen taken. The tenacity with which tyrosine remains bound in the peptide linkage was therefore tested in the following way:

10 g. of caseinogen were digested for 6 hours, as directed by Plimmer and Eaves. The polypeptides, etc., were precipitated with phosphotungstic acid and after decomposition with baryta and the removal of the excess Ba, the filtrate was submitted to a thorough redigestion. After concentrating, 0·137 g. of a mixture of tyrosine and leucine was obtained, which on recrystallisation yielded 0·02 g. of pure tyrosine, or 0·2 % of the caseinogen taken. The filtrate still gave a strong Millon's reaction.

The preparation of tryptophan by the use of butyl alcohol.

It will be recollected that extraction of the decomposed mercury precipitate with butyl alcohol, as described by Dakin, only gave very small yields of tryptophan, not more than 0·2–0·5 %, which is the average yield of the Hopkins and Cole method. If, however, the decomposed mercury precipitate was subjected to a second digestion in the absence of the other products of hydrolysis, much larger yields of an almost pure product were secured. Owing to the great difficulty usually experienced in securing a good yield of tryptophan, a method by which 1·3–1·7 % may consistently be obtained by any laboratory assistant of ordinary skill, appears worthy of description.

Digestion of the caseinogen. To prevent the rise of the hydrogen ion concentration due to the liberation of the amino-acids, it is advisable to digest the caseinogen in a buffer solution. This solution is best prepared as follows: to 10 litres of tap water in a large vessel add 136 g. of crystalline sodium acetate, and gradually stir in 1 kilo. of caseinogen[1]. To this suspension add strong sodium hydroxide, until a drop of the mixture reacts alkaline to cresol red (*o*-cresol-sulphon-phthalein) and acid to phenolphthalein. The P_H will then be somewhere close to 8·1. Next add 15–20 g. trypsin[2], 20 g. sodium fluoride and 50 cc. of toluene. Fill into Winchester quart bottles and place in an incubator at a temperature of not less than 38°. If crystals of tyrosine do not form on the sides of the bottles after three or four days, add more enzyme and return to the incubator.

Separation of the mercuric sulphate compound. When digestion is complete, filter the fluid with the aid of kieselguhr on several large Buchner funnels acidify the filtrate with 13 cc. of ice-cold 50 % sulphuric acid to every 87 cc. of the fluid, and add 120–150 cc. of the mercuric sulphate reagent to

[1] "Laitproto No. 6," sold by Casein Ltd., Culvert Works, Battersea, is a fairly good commercial product, but it is important to remember that, owing to treatment with alkali, many commercial samples are racemised to such an extent that they will only partially digest.

[2] An active ferment is sold by the British Drug Houses as "trypsin," prepared by precipitation with ammonium sulphate. A less active preparation precipitated with alcohol is sold as "pancreatin." Excellent results may also be obtained with an acid extract of the pancreas [Cole 1920], or even by adding the minced gland itself.

every litre. Allow the precipitate to stand overnight, filter off, wash with 7 % H_2SO_4 until the filtrate no longer gives a Millon's reaction, and then with water. Suspend in a litre of water, add a hot saturated solution of baryta (about 20 g. in 50 cc. of water) until distinctly alkaline, and decompose with SH_2. Filter off the mercury sulphide and remove the SH_2 under reduced pressure, bring the fluid to a reaction of about $P_H = 8{\cdot}1$ by the addition of baryta or sulphuric acid, and digest with trypsin for the second time (5 to 7 days). Neutralise to litmus and concentrate under reduced pressure to 150–200 cc.

Extraction with butyl alcohol. It is preferable to carry out the extraction with butyl alcohol[1] in a stout flask rather than in a continuous extraction apparatus, so that the alcohol may be concentrated under reduced pressure. The flask should be fitted with two corks, one of which is supplied with a "pipette" similar to that used for blowing off the ether in Meig's method of fat extraction [see Cole, 1920]. Shake the flask intermittently and keep it at a temperature of about 70° on the water-bath. Blow off the hot, supernatant layer of alcohol into a Claisen flask prepared for distillation under reduced pressure, and repeat the extraction with a second volume of butyl alcohol. In the meanwhile, the distillate from the Claisen flask has separated into two layers, since the alcohol is more soluble in hot than in cold water. For the third extraction the alcoholic layer only should be used, thus obtaining a gradual reduction in the volume of the fluid extracted. Most of the tryptophan will have separated after 10–15 extractions carried out in this way. Reduce the alcohol in the Claisen flask to 40–50 cc. and filter off the tryptophan which at first usually crystallises in slightly discoloured shining plates. A second and third crop may be obtained by concentrating still further, but when the tryptophan begins to separate in a gummy condition, it is advisable to drive off the rest of the alcohol, to dissolve the residue in water, and to reserve it together with the extracted fluid for the next preparation, when it is added to the main portion just before precipitation with the mercury reagent. The amount of tryptophan obtained should be 10–15 g. which may be rendered pure by boiling in 60 % ethyl alcohol with a little Merck's charcoal, and recrystallising. Estimation of the amino nitrogen in Van Slyke's micro apparatus gave: amino N found, 6·93 %; calculated, 6·86 %.

Conclusions.

1. The mercuric sulphate compound precipitated from hydrolysed caseinogen was found to contain a number of amino-acids, probably combined with tryptophan as polypeptides. After removal of the free tyrosine, the following amino-acids were identified: leucine, cystine, tyrosine (traces) and other monoamino-acids; glutamic and aspartic acids, histidine and a little

[1] Owing to the ease with which acetone and aldehydes destroy tryptophan, the commercial butyl alcohol should be shaken with a saturated solution of sodium bisulphite, and fractionated, the procedure being repeated three times.

proline but neither lysine nor arginine. The degree of hydrolysis no doubt regulates the quantity of peptides precipitated, and possibly, to some extent, even the nature of the individual amino-acids.

2. The presence of combined tyrosine after severe acid hydrolysis indicates that this amino-acid does not separate quantitatively as early in digestion as is sometimes assumed.

3. It is difficult to secure digestion of caseinogen sufficiently complete to yield 1·7 % of free tryptophan, except by redigestion of the decomposed mercury precipitate. After this procedure, however, extraction with butyl alcohol results in a yield of tryptophan which compares favourably with that obtained by Dakin. A detailed account of the procedure for preparing tryptophan by this method is given.

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