XXX. IMPROVED METHODS FOR THE ISOLATION OF METHIONINE AND ERGOTHIONEINE.

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Methionine.

In a recent paper [Pirie 1932, 1] a method for the preparation of methionine was described. The principal difference between this method and that of Mueller lay in the use of mercuric acetate instead of mercuric sulphate, to avoid the inhibiting effect of the sulphate ion in an amino-acid mixture on the precipitation of methionine. The chloride ion has a similar interfering action and for the preparation of methionine it is best therefore to use an enzymic hydrolysate of caseinogen although a sulphuric acid hydrolysate from which the acid has been removed with baryta is fairly satisfactory.

Of the mercuric salts that are available commercially the acetate is the most suitable for the isolation of methionine; it seemed possible however that some other anion might improve the precipitation. After some search it was found that phosphotungstic acid, although it does not itself precipitate methionine either from pure solution or from an amino-acid mixture, increases the efficiency of precipitation by mercuric acetate. The mercury-phosphotungstic precipitate has the further merit of being more easily decomposed by baryta than the precipitate obtained with mercury alone; there is therefore no risk of racemisation during the extraction.

The amino-acid mixture recovered from this mercury-phosphotungstic precipitate is very similar to the mixture obtained by the original mercuric acetate precipitation. In each case the N/S ratio is about 3 and there is present no amino-acid, other than methionine, which is precipitated by mercuric chloride in slightly acid solution. The mixture has only been investigated in a superficial manner, but it seems to contain considerable quantities of phenylalanine- and proline-containing peptides. From this mixture the methionine may be precipitated directly by mercuric chloride, but if this is done, very much more mercuric chloride must be added than is necessary if the mixture is first fractionated with alcohol. By the latter means also a rather purer product is obtained.

Caseinogen and egg-albumin are the proteins which are most readily available for the preparation of methionine. The latter gives a very much larger yield (2·5 %) but it is more expensive. Furthermore, it is necessary to coagulate the commercial dried egg-white (by dissolving it in water and heating), partly to get rid of non-protein material and partly to facilitate the enzymic hydrolysis. For these reasons caseinogen is probably the most convenient starting-point.

Experimental.

1 kg. of caseinogen is suspended in 10 litres of water and the p_H adjusted to 8·4 with sodium hydroxide; a suitable amount of pancreatic extract is added and the mixture incubated at 37° in the presence of a little toluene, more sodium hydroxide being added from time to time to keep the digest at p_H 8·4. After 3 weeks it is brought to p_H 6 with acetic acid and allowed to stand in a cool place for a day; the tyrosine and undissolved materials are removed by filtration through a calico bag which is finally squeezed out in a press.

700 cc. of a 30 % solution of mercuric acetate in 2 % acetic acid are added to the clear filtrate; this will cause a slight precipitate to form most of which redissolves on vigorous stirring. 1000 cc. of 30 % phosphotungstic acid solution are now added in a thin stream with vigorous stirring, followed by 400 cc. more of the mercuric acetate solution. If the addition of a small amount of phosphotungstic acid to a filtered test portion causes further precipitation a further 100–200 cc. should be added to the main bulk of the preparation. If large amounts of either mercuric acetate or phosphotungstic acid are added a further precipitate will always be obtained but this is of no value in the preparation of methionine.

The precipitate is sucked as dry as possible on Büchner funnels and washed by suspending in 2–3 litres of water and filtering again. It is resuspended in water and heated to boiling while solid barium hydroxide is added, the addition being continued until the p_H remains at about 8·5 for 5 minutes without further addition of barium hydroxide.

The barium phosphotungstate is filtered off and extracted again with a litre of water and enough barium hydroxide to bring the p_H of the boiling suspension to about 9. The combined filtrates are brought to p_H 5 with sulphuric acid and filtered; a little kieselguhr on the filter-paper will ensure clear filtration. The filtrate will generally not distil easily *in vacuo* on account of frothing; it is therefore concentrated in a large evaporating basin over a flame until the volume is about 300 cc.; at this stage it is filtered from a trace of a mercury compound. The filtrate is evaporated on a boiling water-bath until a considerable amount of white solid separates (about 200 cc.). To this hot solution 400 cc. of alcohol are added and the mixture is left overnight at 0°. The solid is filtered off; if dried it should weigh about 50 g. and contain 6 % of sulphur. The filtrate will deposit more solid if it is distilled *in vacuo* and treated with more alcohol; care must be taken however that the concentration of alcohol does not exceed 80 % or a gum will be thrown out which has a very low sulphur content and interferes with the precipitation in the next stage. A little sodium hydroxide may with advantage be added to the alcoholic solution to bring its p_H to about 6. The precipitate obtained is mixed with the other lots. The alcoholic filtrate still contains some sulphur (estimated by the method recently described [Pirie, 1932, 2]); this is not easily recoverable because the N/S ratio may be as high as 15.

The solid thrown out by alcohol is dissolved in 300 cc. of hot water and the solution, filtered if necessary, is added to a boiling solution of 200 g. of mercuric chloride in 300 cc. of water. When cool a putty-like mass separates. The mixture is cooled in a freezing mixture and the solid separated as well as possible from the mother-liquor; the former is boiled up with 200 cc. of water and again chilled. Methionine is isolated from this mercury precipitate in the usual way [Pirie, 1932, 1].

The yield by this method is very constant; from 1 kg. of caseinogen 9–10 g. of pure methionine are obtained directly and a further 2–3 g. may be obtained by working up the mother-liquors in the manner outlined in the previous paper.

Ergothioneine.

It has long been known that thiolglyoxalines, like other mercaptans and like thiourea and its derivatives, form cuprous compounds which are insoluble in dilute acids [Wohl and Marckwald, 1889; 1892]. Although the cuprous compound of ergothioneine might have been expected to be a derivative well suited for the isolation and characterisation of that substance it has only very recently been prepared. Williamson and Meldrum [1932] have used the cuprous compound for the isolation of ergothioneine from blood; their method is simpler than any of the older ones and it gives a better yield. Blood, however, is a less convenient source of ergothioneine than ergot and the present paper describes a method, based on that of Williamson and Meldrum, for the isolation of the base from this material.

I have already described [Pirie, 1931] the cuprous derivatives of a number of mercaptans and have put forward the theory that only substances which contain a peptide linkage are soluble in the presence of an excess of cuprous oxide. Ergothioneine is another case in point for it is not soluble in dilute acid suspensions of an excess of cuprous oxide; the cuprous compound is however soluble in water. Cuprous ergothioneine therefore, like cuprous acetylcysteine, goes into solution if it is washed with distilled water; this fact is used to separate ergothioneine from a purine-like substance, similar to that already mentioned as a constituent of yeast [Pirie 1931, p. 626], which is precipitated by cuprous oxide from an aqueous extract of ergot.

The cuprous compound of ergothioneine is much less stable than that of glutathione and other similar mercaptans. It can be kept for some time in a vacuum but in air it rapidly goes grey and finally black. For this reason accurate analyses are impossible but the cuprous compound, on decomposition with hydrogen sulphide, gives rise to about half a molecule of sulphuric acid for each molecule of ergothioneine. This is in agreement with the structure that would have been expected *a priori* $(C_9H_{15}O_2N_3S)_2.SO_4Cu_2$.

Experimental.

900 g. of ground ergot are added to 4 litres of water and boiled for 5–10 minutes. The mixture is filtered through calico and the residue pressed. The solid is then re-extracted with a further 4 litres of water in the same way. Basic lead acetate solution is added, in considerable excess, to the combined milky extracts and the precipitate removed by filtration. This filtration is rather slow; it is advisable therefore to allow the precipitate to settle and to pour off as much as possible of the supernatant liquid. About 9 litres of clear filtrate will be obtained at this stage, and 200 cc. of concentrated sulphuric acid are added with thorough stirring. Lead sulphate is removed after about half an hour by filtering through kieselguhr. This filtrate is perfectly stable but the processes described in the next paragraph should be carried through without any avoidable delay.

Cuprous oxide suspension is added gradually until the initially formed white precipitate has become almost black; after about half an hour the supernatant fluid is discarded and the thick suspension centrifuged. The solid is stirred up thoroughly with 900 cc. of 0·3 *N* sulphuric acid and centrifuged again. It is now stirred up with distilled water and centrifuged; this extraction is repeated five times so that in all 4–5 litres of extract are obtained. Enough sulphuric acid is added to make it 0·5 *N*; this causes slight precipitation of cuprous ergothioneine, and the remainder of the ergothioneine is thrown out by the addition of cuprous oxide as before. This precipitate is allowed to settle somewhat and then filtered

off; it is suspended in 200 cc. of water to which a gram of barium chloride has been added and decomposed with H_2S.

The suspension of cuprous sulphide is heated nearly to boiling and filtered; the filtrate should contain a slight excess of sulphuric acid; if there is an excess of barium this must be removed with a little sulphuric acid. The filtrate is distilled *in vacuo* to small bulk and then allowed to crystallise in a vacuum desiccator after addition of a few drops of concentrated hydrochloric acid. The filtrate from the first crop is taken to dryness and the residue crystallised from a few cc. of hot water. The two lots of crystals are mixed and recrystallised from the minimum amount of boiling water. These crystals are dihydrated ergothioneine hydrochloride; when dried *in vacuo* 1·5–1·6 g. of the anhydrous hydrochloride are obtained. (Found: N 15·95; S 11·90; Cl 13·65 %. Calculated: N 15·82; S 12·05; Cl 13·37 %.) From the mother-liquor 0·2–0·4 g. of anhydrous ergothioneine may be recovered by evaporating to dryness, taking up in hot alcohol, filtering and adding a few cc. of pyridine. A crystalline precipitate separates on cooling; this is washed with alcohol and consists of pure anhydrous ergothioneine.

The total yield is therefore 0·18 % of the anhydrous base; the yields from ergot already given in the literature are: Tanret [1909] 0·1 % of the hydrochloride and Eagles [1928] 0·065 % of the free base. In each case the method of isolation is very much more laborious than that described in this paper.

Summary.

Methionine is isolated from an enzymic hydrolysate of caseinogen by precipitation with mercuric acetate and phosphotungstic acid (in one operation), followed by fractionation with alcohol and precipitation with mercuric chloride. The yield is 1·2 %. Ergothioneine is prepared by means of its cuprous derivative from an aqueous extract of ergot. The yield is 0·18 %.

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