CL. THE PREPARATION OF METHIONINE FROM CASEINOGEN.

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IN the course of some work on the unidentified sulphur compounds in proteins I have found that the methods that have been used for the isolation of methionine from a protein hydrolysate are very unsatisfactory. All the methods that have been described, except that of Otake [1925] who isolated 0.0001 % of methionine from yeast, are simply modifications of Mueller's [1923] original method and involve the precipitation of the mercury compound of methionine mixed with a large amount of basic mercuric sulphate. The following yields have been obtained from caseinogen. Mueller [1923] 0.2–0.4 %, Barger and Coyne [1928] 0.1–0.3 %, du Vigneaud and Meyer [1931] 0.1–0.2 %. These low yields, and the laborious nature of the isolation, justified Barger and Weichselbaum [1931] in concluding that it was easier to make this amino-acid synthetically.

In this paper a method of preparation is described which is very much simpler than those that have already been described and which gives better yields; 1.0 % of methionine is readily obtained from caseinogen and, by working up the final mother-liquor the yield may be raised to 1.4 %.

It became obvious during the preliminary work that the precipitation of methionine as its mercury compound was unsatisfactory in the presence of large amounts of other amino-acids. An initial partial separation of the hydrolysate is therefore imperative and is most easily carried out by means of butyl alcohol. In strongly acid solution methionine is appreciably soluble in the alcohol, it may therefore be concentrated by the ordinary Dakin [1918] procedure. I have found it more convenient, however, to extract the methionine from the butyl alcohol with sodium hydroxide instead of distilling off the alcohol from the amino-acid mixture. I have carried out this double extraction in a separating funnel but it would be easier on a large scale to do it continuously. The hydrolysate should be purified somewhat, before extracting, by precipitation with lead acetate; if this precipitation is omitted a semi-solid foam is obtained when the hydrolysate is shaken with butyl alcohol. Picric acid is equally efficacious if for any reason the presence of lead in the extracted fluid is to be avoided. It does, however, precipitate a tar from the hydrolysate and for this reason I prefer lead.

EXPERIMENTAL.

200 g. caseinogen are hydrolysed by boiling for 14 hours with 200 cc. of water and 400 cc. of concentrated hydrochloric acid. The hydrolysate is distilled *in vacuo* till it becomes very viscous; the residue is then dissolved in 500 cc. of water. It dissolves slowly and it is generally necessary to heat the mixture. When it has cooled, 200 cc. of saturated normal lead acetate are added and the precipitate of lead chloride and tarry material is removed by filtration.

A sample of the filtrate is titrated with sodium hydroxide using bromophenol blue as an external indicator. It should be equivalent to 1-1.5 times its volume of *N* NaOH. This strongly acid solution is now extracted with 2 litres of technical butyl alcohol in a large separating funnel and the aqueous layer is run off. Sometimes it is difficult to see the interface since each layer is dark brown in colour; this difficulty can be overcome by having the apparatus in a poorly lit room and holding a powerful electric lamp close to it. The butyl alcohol in the funnel is now shaken with sufficient 10 % sodium hydroxide solution (about 250 cc.) to make it neutral. A large excess of alkali should be avoided, but it makes little difference whether the mixture is left neutral or is made definitely alkaline. The aqueous layer is separated after being left for an hour or so to settle thoroughly. It is important that the sediment which collects at the interface during the first (acid) extraction should be removed with the aqueous layer whereas that which collects during the second (alkaline) extraction should be left in the separating funnel. The acid aqueous solution from the first extraction is now returned to the funnel and extracted again with the same butyl alcohol and the butyl alcohol re-extracted with 5 % sodium hydroxide (about 300 cc. will probably be required). This alkaline aqueous extract is mixed with the first lot.

After two more double extractions as described above the greater part of the methionine, along with other amino-acids, is obtained in the alkaline solution. The same amount of butyl alcohol has been used successfully to extract as much as 800 g. of hydrolysed caseinogen, in this case the double extraction was repeated 12 times. After every second extraction 30 cc. of conc. hydrochloric acid should be added to the hydrolysate. After the last alkaline extraction a further extraction with 200 cc. of water will bring out a little more methionine.

The combined alkaline extracts are now distilled *in vacuo* till all the dissolved butyl alcohol has been removed and the volume is about 500 cc. When cold the solution is filtered and the solid is boiled with about 200 cc. of water, cooled and filtered again. The combined filtrates are brought to p_H 5 with HCl, and 200 g. of mercuric acetate dissolved in about 500 cc. of 3 % acetic acid are added. The volume should now be 1000-1500 cc.; if it is smaller than this the precipitation will be incomplete. Precipitation is complete in a minute or two and the mixture may be filtered immediately. The solid is sucked dry on the

filter, immediately ground up with 400–500 cc. of water and again filtered. The mercury precipitate should never be allowed to become parched or the subsequent extraction with baryta will be unsatisfactory. The precipitate is suspended in water and added to 600 cc. of boiling $N/3$ barium hydroxide. The mixture is heated to boiling, solid barium hydroxide being added if necessary to make the solution strongly alkaline to thymolphthalein. The mixture is filtered hot and the solid is extracted again in the same way with 700 cc. of $N/7$ barium hydroxide.

Barium is removed fairly exactly from the combined filtrates with sulphuric acid. The amount of sulphuric acid finally present should not be greater than 1 cc. of $2N$. The mixture is filtered and the filtrate distilled *in vacuo* to dryness to remove as much as possible of the acetic and hydrochloric acids. The residue is dissolved in 30 cc. of water and poured into a boiling solution of 60 g. HgCl_2 in 120 cc. of water. The mixture is cooled in a freezing mixture till crystals of ice separate. It is then left for an hour or two at 0° and filtered. The putty-like mass of methionine-mercury compound is kneaded with ice-cold water; this is added to the filtrate. A further quantity of less pure methionine can be obtained by adding 6–10 g. of mercuric acetate, in the form of a 30 % solution, to this filtrate and working up the precipitate as if it were a mercuric chloride precipitate.

The mercury compound is suspended in 250 cc. of water and decomposed with H_2S ; the decomposition is rather slow. The filtrate from mercuric sulphide is distilled *in vacuo* nearly to dryness. Care is required at this point, for if the distillation is not carried far enough a large amount of HCl will remain with the methionine. This will necessitate the addition of a large amount of pyridine in the next stage and the yield of methionine will be small because it is appreciably soluble in alcohol in the presence of pyridine hydrochloride. If on the other hand the distillation is too violent solid methionine hydrochloride¹ will blow down the condenser as a fine dust. I have found it safest to connect the distillation flask to the condenser by means of a piece of pressure tubing, this can be clamped off at the critical moment. 30 cc. of alcohol are used to dissolve the solid methionine hydrochloride; if necessary the alcoholic solution is filtered and the flask washed out with a further 15 cc. of alcohol.

Pyridine is now added until a drop of the solution on a tile is alkaline to bromophenyl blue; 2 cc. more of pyridine are added and the mixture cooled for some hours in a freezing mixture. It is filtered and the solid washed with a little alcohol.

Yield, 1.8 g. of methionine of 95 % purity.

When measured in 3 % aqueous solution $[\alpha]_{5461}^{21} = -7.9^\circ$.

From the mercuric acetate precipitate a further 0.8 g. is obtained. This is only 75 % pure but may be purified by reprecipitation with mercuric chloride.

¹ Methionine, like ethyleysteine, forms a hydrochloride, which crystallises from strong hydrochloric acid in clusters of long needles and does not lose hydrochloric acid when dried *in vacuo*. It melts sharply at 174° (uncorr.) but solidifies again, and on further heating melts at 210 – 223° . Found: N, 7.2; S, 17.22; Cl, 19.4. Calculated: N, 7.54; S, 17.25; Cl, 19.12 %.

In each case the alcoholic mother-liquor contains sulphur to the extent of about 1.5 mg./cc. In this case there is therefore about 150 mg. of sulphur still present in the mother-liquor. The greater part of this is in the form of methionine and I have isolated it in about 70 % yield (reckoned on the sulphur content) by making the combined mother-liquors from several preparations alkaline (with barium hydroxide) and distilling *in vacuo* to get rid of pyridine, followed by acidification (with sulphuric acid) and precipitation with mercuric chloride.

The yield of methionine isolated in this way is therefore 1.4 %. This is considerably less than the actual methionine content of caseinogen, for more may be obtained by more prolonged extraction with butyl alcohol and also by precipitating with a larger amount of mercuric acetate. Both of these procedures, however, involve more labour than the extra yield is worth.

Methionine may also be isolated by following the method already described but with the omission of the butyl alcohol extractions. In this case the yield is much smaller and is very variable; the method then differs from those that have already been described by other workers only in the shortening of some of the processes and gives a very similar yield, *i.e.* 0.25–0.7 %.

DISCUSSION.

The commercial caseinogen which has been used in this work contains 0.6 % of sulphur (uncorrected for moisture or ash) and from it 1.4 % of methionine has been isolated; this, since methionine contains 21.5 % of sulphur, corresponds to 0.3 % of sulphur in the protein. It is clear therefore that methionine is the principal sulphur-containing constituent of caseinogen.

The yield of methionine is not quite high enough definitely to rule out the possibility of a third sulphur-containing amino-acid. Most workers have put the cystine content of caseinogen at 0.3 % [*e.g.* Sullivan, 1929]; on this basis there is a considerable amount of sulphur in caseinogen still unaccounted for but it is probable that part of this is methionine. Baernstein [1930], by oxidation with iodine, finds 0.94 % of cystine in caseinogen; on this basis there can be no other sulphur-containing constituent. Sherman and Woods [1925], by feeding experiments, found a still higher value, 1.2–2.5 %. This value is clearly impossible and it seems from the recent work of Jackson and Block [1931] and of Weichselbaum, Weichselbaum and Stewart [1932] that their method of estimation does not distinguish between methionine and cystine. Steudel and Schumann [1929] found, by the Folin-Looney [1922] technique, 2.6 % of "cystine" in caseinogen which had been treated with nitrous acid. This value, like that of Sherman and Woods, appears to be the sum of the cystine and the methionine contents of the protein, but the nature of the reaction involved is obscure; free methionine, on treatment with nitrous acid, does not give a product that reacts with the Folin-Looney reagent.

SUMMARY.

The preparation of methionine from the butyl alcohol extract of an acid hydrolysate of caseinogen is described.

The yield is 1.4 %.

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