CXLI. THE SALTING-OUT OF AMINO-ACIDS FROM PROTEIN HYDROLYSATES.

I. THE ISOLATION OF TYROSINE, LEUCINE AND METHIONINE.

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Requiring a supply of *l*-methionine for experiments designed for an attempt to throw light on the intermediate metabolism of this amino-acid, we were beset at the outset of our work with the problem of obtaining it in quantity. The useful and interesting paper by Pirie [1932] on its isolation from caseinogen hydrolysates had not then been published, and the only method which was likely to give us the amount deemed necessary for our purpose was that of synthesis. A quantity of the racemic acid was actually synthesised on the lines described by Barger and Weichselbaum [1931] with satisfactory results. It was soon realised, however, that resolution would involve so much material and time that we were obliged to turn to the reconsideration of Mueller's [1923] original method of isolating the active amino-acid and attempt to improve his yields.

At this stage of our investigation Pirie's paper appeared and we were able by his method to isolate a quantity of the active amino-acid. The serious difficulty in the procedure, as the author himself indicates, lies in the butyl alcohol extraction of the acid hydrolysate, both layers being so darkly coloured that at times it is impossible to distinguish the interface. It appears to us, moreover, that the method adopted for removing mercury from the mercury-methionine precipitate by heating the latter with a solution of barium hydroxide is liable to lead to loss of amino-acid. Mercuric oxide in hot alkaline solution is an excellent reagent for the rapid oxidation of amino-acids; we have avoided its use by decomposing the precipitate with hydrogen sulphide. It must be added, however, that Pirie [1933] has since described a second method which avoids the extraction by butyl alcohol. Of this we have had no experience.

In repeating Pirie's [1932] procedure a second time, we sought some means of purifying the acid hydrolysate before the butyl alcohol extraction. Pirie himself used lead acetate with only partial success. After several unsuccessful attempts with various reagents, we finally obtained a transparent and only slightly coloured solution by boiling the hydrolysate with a good grade of charcoal, the p_H of the solution having previously been raised to 4·5 by the addition of sodium hydroxide. On the occasion when this was done, the solution had to remain overnight before the extraction by butyl alcohol could be carried out. Next morning there had separated a copious crystalline precipitate which, as might be expected, consisted mainly of tyrosine. Leucine was also present, but to our great interest, the mixture, after alkaline fusion, gave an intense nitroprusside reaction. That this reaction was not due to cysteine was proved by boiling a portion of the solid with a solution of sodium plumbate, when no precipitate of lead sulphide was obtained. The probability that cystine is not present in the precipitate receives support from other directions. Pfeiffer and

Angern [1924], for example, showed that whilst ammonium sulphate can precipitate cystine from aqueous solution, sodium chloride will not do so; again, Okabe [1928] has found that the solubility of cystine in sodium chloride solution is considerably greater than in distilled water. Ruling cystine out in this way, it was apparent that the nitroprusside reaction was due to methionine, and it became clear that, if the above tyrosine complex contained methionine in any quantity, the problem of the isolation of the latter amino-acid would be considerably simplified.

The investigation was proceeding on these lines when our attention was drawn to a paper by Barnett [1933] on the salting-out of leucine from protein hydrolysates. It will be seen from Barnett's curve (Fig. 1) that the minimum solubility of leucine in saturated sodium chloride solution lies at p_H 2·4, at which point, as the author shows, the amino-acid is precipitated as dileucine hydrochloride. In studying Barnett's paper it appeared to us that methionine might reasonably be expected to be precipitated with leucine from saturated salt solution at p_H 2·4. Subjecting a caseinogen hydrolysate to Barnett's procedure, we found that methionine was actually present in the precipitate obtained at p_H 2·4, and that Barnett had apparently overlooked its presence therein. Unfortunately from our point of view, a sulphur determination carried out on the precipitate showed us that methionine was present in only very small amount.

Finding that Barnett's leucine fraction would not yield us methionine in promising yield, we turned to another aspect of this salting-out phenomenon. We have included in Fig. 1 a curve showing the results obtained by Sano [1926] in an investigation of the solubility of leucine in aqueous solution at different values of p_H. Comparison of the curves of Barnett and Sano shows that the effect of sodium chloride in reducing the solubility of leucine is at a maximum at p_H 2·4 and almost as great in the neighbourhood of p_H 6, *i.e.* at the isoelectric point of the amino-acid. From a knowledge of their chemical constitutions it can safely be assumed that the isoelectric points of leucine and methionine cannot be far removed from one another. The question remained whether the yield of methionine would be increased if the protein hydrolysate, after adjustment to p_H 6, were saturated with sodium chloride. An experiment based on this reasoning appeared worth undertaking.

The tyrosine-leucine-methionine mixture which had been precipitated after decolorising the protein hydrolysate at p_H 4·5 was dissolved in the minimum quantity of water and kept overnight. Next morning the tyrosine had separated in exceptionally good quality and yield. After filtration, the mother-liquor containing some leucine and methionine was added to the original bulk of the hydrolysate. The combined solutions, the p_H of which had been adjusted to 6 by the addition of sodium hydroxide, were then concentrated *in vacuo*. Ultimately crystals of sodium chloride began to separate; the evaporation was then stopped and the mixture placed in the ice-chest overnight. During this time the separated salt had settled to the bottom, whilst floating near the surface was a crystalline mass consisting, as shown by examination, chiefly of leucine and methionine. By decanting the solution from the salt, the separated amino-acids were collected, redissolved in water, and the methionine isolated according to Pirie's procedure. After a second precipitation, the yield of methionine, which, from sulphur determinations, was found to be of a high degree of purity, only amounted to some 0·6 % of the caseinogen used. Examination of the leucine-methionine solution from which the methionine had been precipitated showed that there still remained dissolved some of this amino-acid which had escaped precipitation by mercuric acetate. It was clearly evident that the conditions

for precipitating most of the methionine from the solution were not ideal for the purpose.

A series of semi-quantitative experiments on the following lines proved that this was not only true, but that it would probably be a matter of some difficulty, owing to the presence of other amino-acids, to regulate the conditions so that

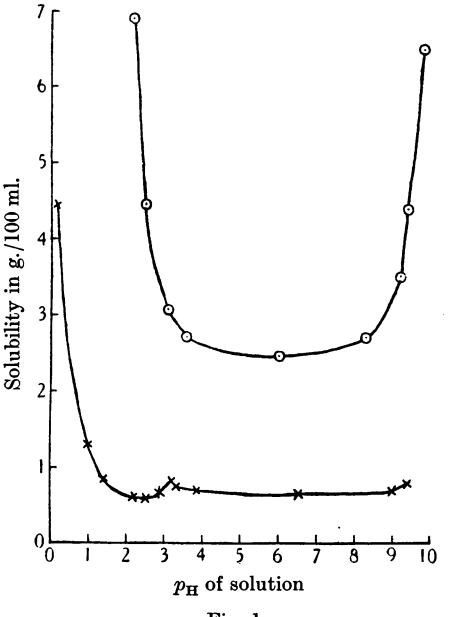

Fig. 1.

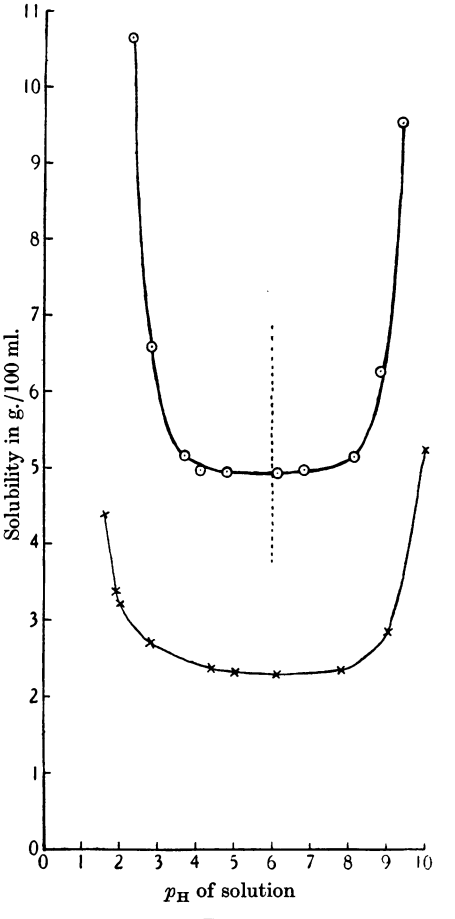

Fig. 2.

Fig. 1. ⊙—⊙ Solubility of leucine in water (Sano); ×—× Solubility of leucine in saturated sodium chloride solution (Barnett).

Fig. 2. ⊙—⊙ Solubility of methionine in water; ×—× Solubility of methionine in saturated NaCl solution.

most of the methionine would be precipitated. If, for example, to a solution of leucine, a solution of mercuric acetate in acetic acid be added drop by drop, a definite precipitation occurs. This precipitate however dissolves on the addition of (1) further mercuric acetate solution, or of (2) sodium chloride solution. Similar experiments with a solution of methionine showed that the precipitate produced by the addition of the mercuric acetate solution was soluble only in a

very much greater excess of the latter reagent, and that, while the presence of sodium chloride certainly favours the complete precipitation of the methionine, if the concentration of the sodium chloride exceeds a certain value, re-dissolution of the mercury-methionine precipitate occurs. Experimentation on these lines is being continued and will be reported in an early communication. At the present time it will suffice to add that if, after the precipitate obtained by treating the above leucine-methionine solution with mercuric acetate reagent has settled, sodium chloride solution be carefully added, a further precipitation takes place with the result that the ultimate yield of methionine obtained is raised from the above figure of 0·6 % to one of 1·5–1·6 %. Such yields have been obtained from caseinogen on several occasions. Egg-albumin, subjected to the same procedure, has given yields averaging 2·5 %.

The leucine was finally obtained by treating the solution, from which the mercury-methionine compound had been filtered, with hydrogen sulphide, filtering off the precipitated mercuric sulphide, concentrating the filtrate *in vacuo* to a syrup, dissolving the latter in the minimum quantity of water and raising the p_H to 6, when the leucine crystallises as the solution cools. Recrystallisation of the amino-acid follows in the usual way.

It was considered of general interest to include in this communication curves showing the solubility of methionine in aqueous and saturated sodium chloride solutions and these are shown in Fig. 2. The upper curve (solubility of methionine in aqueous solutions) appears to be symmetrical about an axis which cuts the p_H abscissa in the close neighbourhood of 6, a figure which may be taken as a close approximation to the isoelectric point of methionine. It is noteworthy moreover that the curve showing the solubility of methionine in saturated sodium chloride solution does not exhibit that dissymmetry which is seen in the corresponding curve for leucine (Fig. 1).

The average value of several actual yields of recrystallised amino-acids obtained from caseinogen (ash content 1·1 %, moisture 9 %, N 15 %) and egg-albumin (ash content 4·9 %, moisture 7·4 %, N 15·2 %) are as follows:

	Caseinogen %	Egg-albumin %
Tyrosine	3·0	1·35
Leucine	4·7	6·1
Methionine	1·55	2·5

Experimental.

1 kg. of caseinogen was hydrolysed with 2 litres of 20 % hydrochloric acid for 18–20 hours. The excess hydrochloric acid was removed in the usual way by concentration *in vacuo*, the residue being redissolved in hot water and evaporated until viscous.

Separation of tyrosine. The final residue was dissolved in 4 litres of boiling water, the solution treated with sufficient 40 % sodium hydroxide solution (approx. 460 ml.) to bring the p_H to 2·4, 40 g. of charcoal added, and the mixture, after being boiled for 20–30 minutes, was filtered hot. The charcoal-humin residue was extracted with about 1 litre of boiling water, and the filtered extract added to the main solution. The combined solution, which was quite clear and only faintly coloured, was allowed to cool overnight when the tyrosine crystallised out. This was filtered off and the filtrate used for the isolation of the methionine and leucine as described below. To increase the purity of the tyrosine thus obtained, it was recrystallised from boiling water in the usual way.

Separation of methionine. To the mother-liquor from the tyrosine, sodium hydroxide solution was cautiously added to p_H 6·0, and the resulting solution was evaporated under reduced pressure until sodium chloride commenced to separate (at approximately 1800 ml.). After cooling, the sodium chloride had settled to the bottom and the separated amino-acids were mainly floating near the surface of the liquid. The mother-liquor and the amino-acids were decanted to a Büchner funnel, and the acids sucked as dry as possible. The sodium chloride remaining in the container was separately dissolved in the minimum amount of water, and the solution thus obtained was used to wash the amino-acids on the Büchner free from mother-liquor.

The crude mixture of solid leucine and methionine obtained in this way was dissolved in 2 litres of water, and a cold saturated solution of mercuric acetate in 3 % acetic acid was added until no further precipitation occurred (about 250 g. mercuric acetate). After keeping for some time, a saturated solution containing approximately 60 g. of sodium chloride was added, when a further heavy precipitate formed. The mixture was kept overnight, and the mercury precipitate was then filtered off, suspended in about 2 litres of warm water and decomposed with hydrogen sulphide. The mercuric sulphide was boiled out with water, the mixture filtered and the filtrate added to the main methionine solution.

The solution containing the crude methionine was now evaporated almost to dryness, the residue dissolved in some 200 ml. of alcohol, the p_H of the alcoholic solution adjusted to 6·0 by the addition of pyridine and the mixture left in the ice-chest overnight. Next morning, the precipitate of crude methionine was filtered off, washed with alcohol to remove any pyridine and finally dried with ether.

For purification the product was redissolved in boiling water (1 litre for every 10 g. of crude methionine), a boiling saturated solution of mercuric chloride (10 g. of mercuric chloride per g. of the amino-acid) added, and the mixture, after being boiled for some minutes, was set aside until cold. The mercury precipitate then formed a solid mass, from which the mother-liquor was easily decanted. The solid was transferred to a mortar, ground up with water, the mixture filtered and the recovered solid suspended in warm water and decomposed with hydrogen sulphide. The further stages of the separation of the methionine followed the lines described above.

A further recrystallisation may be carried out from 75 % alcohol; it is inadvisable to use water since the solubility of methionine in this medium is approximately 5 % (Fig. 2). If, as sometimes happens, it is found difficult to remove the last traces of pyridine from the methionine, recrystallisation from 75 % alcohol, to which a few drops of acetic acid have been added, will enable this to be done.

Separation of leucine. The solution, from which the methionine was first precipitated by mercuric acetate, was freed from mercury by treatment with hydrogen sulphide. The mercuric sulphide was filtered off, the sulphide washed with boiling water, and the combined filtrates were concentrated *in vacuo* to a small bulk. The concentrate was dissolved in a small quantity of water and the p_H adjusted to 6·0, when the leucine at once began to crystallise. After some hours, the separated leucine was filtered off and recrystallised from dilute alcohol. This completed the procedure.

Summary.

A simple procedure is described for the isolation from caseinogen hydrolysates of tyrosine, methionine and leucine. The yields, especially those of tyrosine and methionine, obtained compare very favourably with figures already in the literature for these amino-acids.

The work forming the basis of this paper was done during the tenure by one of us (E. M. H.) of a Berridge Studentship awarded by the Delegacy of the University of London King's College.

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