32. THE SALTING-OUT OF AMINO-ACIDS FROM PROTEIN HYDROLYSATES. A METHOD FOR THE ISOLATION OF *L*-PHENYLALANINE

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L-PHENYLALANINE, despite its comparative insolubility in water, is difficult to isolate from protein hydrolysates. Indeed, it would appear from the literature that the only isolation of the fully optically active amino-acid, apart from that claimed by Brazier [1930] which will be discussed below, was that originally carried out by Schulze & Barbieri [1881] when they obtained it from the etiolated sprouts of lupin seedlings. Hitherto it has generally been separated by Fischer's method of ester distillation. The disadvantages of this procedure are too well known to require further comment here and in addition it is universally conceded that even under the best conditions obtainable with high vacuum distillation some 25% at least of the product is racemized in the process. On the other hand whilst synthetic *dl*-phenylalanine can readily be obtained in quantity the resolution of the product is tedious and the yields small.

For some time we have realized the probability that the mixture of amino-acids which separates from a partially or completely neutralized protein hydrolysate saturated with sodium chloride according to the technique of Barnett [1933] or of Hill & Robson [1934] contains phenylalanine in addition to leucine and methionine. Both Barnett's "leucine" fraction and Hill & Robson's "leucine-methionine" fraction, obtained at *pH* 2.2 and 6.0 respectively, give a strong positive Kapeller-Adler reaction for phenylalanine. Moreover, a study of the solubility curves of *L*-phenylalanine, obtained by resolving the synthetic compound, in aqueous and saturated sodium chloride solutions at varying *pH* (Fig. 1), shows that they are practically identical with the corresponding curves for leucine (Fig. 2) as determined by Sano [1926] and by Barnett, a fact which tended to confirm the correctness of our view. For such reasons efforts were directed in the first instance towards the elaboration of a method for the separation of phenylalanine from the leucine-methionine mixture salted-out at *pH* 6.0.

The only method for separating phenylalanine from leucine described in the literature which appeared to us to deserve serious consideration was that of Brazier referred to above. This worker made use of the insolubility of the copper salts of leucine, phenylalanine and aspartic acid to separate these amino-acids from a zein hydrolysate. After regeneration of these acids from their copper salts in the usual way, the aspartic acid was removed by alcohol precipitation of its barium salt in presence of excess baryta. Filtration at this point yielded a solution which, after removal of residual barium, deposited large quantities of leucine on concentration. The remaining solution, which still contained some leucine and most of the phenylalanine, was now boiled with freshly precipitated zinc hydroxide and allowed to cool. The solids which separated from the cold mixture were collected and dried at 100°. The filtrate was evaporated to dryness

and the solids remaining dried at 110°. The two solid fractions thus obtained were separately extracted with cold water, the extracts combined, boiled with more zinc hydroxide, and the previous treatment of the zinc salts repeated until

Fig. 1.

Fig. 2.

Fig. 1. $\bigcirc$ — $\bigcirc$ — $\bigcirc$ Solubility of phenylalanine in water. $\times$ — $\times$ — $\times$ Solubility of phenylalanine in water saturated with sodium chloride.

Fig. 2. $\bigcirc$ — $\bigcirc$ — $\bigcirc$ Solubility of leucine in water. $\times$ — $\times$ — $\times$ Solubility of leucine in water saturated with sodium chloride. $\bigcirc$ — $\bigcirc$ — $\bigcirc$ Solubility of methionine in water. $\times$ — $\times$ — $\times$ Solubility of methionine in water saturated with sodium chloride.

Fig. 3. $\bigcirc$ — $\bigcirc$ — $\bigcirc$ Solubility of phenylalanine in water saturated with sodium sulphate. $\times$ — $\times$ — $\times$ Solubility of leucine in water saturated with sodium sulphate.

the residue obtained was completely soluble in cold water. Brazier claimed that this solution, after removal of the zinc, yielded phenylalanine on evaporation.

Applying Brazier's technique to the "leucine" fraction salted-out from both casein and zein hydrolysates, we have obtained in each case only small quantities of a mixture which consisted chiefly of leucine with smaller proportions of

phenylalanine and methionine as shown by the Kapeller-Adler and nitroprusside reactions. In the light of these findings we have repeated Brazier's process on sulphuric acid hydrolysates of casein and zein, but in neither case have we been able to isolate a pure sample of phenylalanine. It is unfortunate that Brazier gives neither analytical figures, except for nitrogen (Kjeldahl), nor optical data for the material which she reported to be phenylalanine. Since the copper and zinc salts of methionine possess the same order of solubility as those of leucine and phenylalanine, and since, moreover, the methionine content of zein has been estimated by Baernstein [1936] to be 2.2 %, it would appear highly improbable that the phenylalanine isolated in this way is pure, while little reliance can be placed on the nitrogen value of 8.05 % which she quotes for her product (phenylalanine requires 8.48 % N). Her admission, too, that the specimen of phenylalanine which she isolated was difficult to crystallize and showed a tendency to hygroscopy is indicative, in our opinion, of its impurity. Finally, an instance that other workers have been unable to use the method for the purpose described is to be found in the communication of Jukes [1933] on the fractionation of the amino-acids of egg-livetin. On applying it to the amino-acids recovered from the insoluble copper salts obtained from a hydrolysate of this protein, Jukes was unable to separate phenylalanine from the mixture so obtained despite the fact that the Kapeller-Adler colour test showed its presence therein in appreciable quantity.

Another method we originally had in mind for use in attempting to solve this problem was that described by Boyd [1933] by which he separated amino-acids from protein hydrolysates by making use of the different solubilities of their hydantoin derivatives. Actual experiments, however, showed that this valuable method could not be utilized because, apart from the difficulty of avoiding partial racemization of the amino-acids when converting them into their hydantoins, the solubilities of these derivatives of leucine, phenylalanine and methionine are such that no definite separation by fractional crystallization could be achieved.

Attention was finally directed to the possible use of the compounds of phenylalanine with those aromatic substances, e.g. rufianic acid, *p*-benzeneazophenol, picrolonic acid etc. which have from time to time been reported as yielding insoluble crystalline precipitates with this amino-acid. Zimmermann [1930], for example, has reported that rufianic acid forms water-insoluble compounds with phenylalanine, tyrosine and tryptophan, those with alanine, valine, leucine etc. being soluble in water. Under the conditions described by him, we have been unable to obtain a definite crystalline product with either *l*- or *dl*-phenylalanine. The same observation applies to the molecular compound of phenylalanine and *p*-benzeneazo-phenol described by Pfeiffer & Angern [1926]. Of the three reagents mentioned picrolonic acid has found the widest application in this field of work. First introduced by Knorr [1897] as a precipitant for organic bases, the acid was used by Steudel [1903] for the separation of the hexone bases, whilst Mayeda [1907] later described the picrolonates of phenylalanine and tryptophan. Levene & Van Slyke [1912], describing the properties of the picrolonates of several amino-acids, drew attention to the much greater solubility in water of the picrolonate of *l*-phenylalanine over that of *dl*-phenylalanine and demonstrated the use of this reagent for the separation of the components of an artificial mixture of phenylalanine and aspartic acid. Much more recently Bergmann & Niemann [1937] used picrolonic acid to demonstrate the presence of phenylalanine in a digest obtained by the action of papain (proteinnase) on cattle-fibrin. Whilst they claimed that a considerable amount of the

amino-acid was liberated, they were able, however, to isolate only 0.3 g. of the 2.5 g. of phenylalanine reported to be present in 100 g. of this protein. In the light of these communications it appeared to us that the possibility of the use of this reagent for our purpose was worth investigating.

Of the three amino-acids, leucine, phenylalanine and methionine, which we now know to occur in the mixture salted-out at pH 6.0, the first-named is present in much greater amount than either of the others. From a study of the solubility curves (Figs. 1 and 2) it can be seen that if such a mixture is fractionally crystallized, the solid first recovered will consist wholly of leucine, but there comes a time when phenylalanine will commence to separate from solution. At this stage the mother-liquor will contain approximately equal amounts of leucine and phenylalanine. Accordingly we made up saturated aqueous mixtures of leucine and phenylalanine and of leucine, phenylalanine and methionine as described in the experimental section and to each was added a quantity of picrolonic acid slightly in excess of the amount required to combine with the phenylalanine known to be present. The mixture was boiled to dissolve the reagent, and the resulting solution allowed to cool in the air. When the solution was just warm, i.e. after 2–2.5 hr., the crystals which had separated were collected and twice recrystallized from water. The product thus obtained proved to be pure phenylalanine picrolonate. Moreover, when due allowance is made for the amounts of the picrolonate remaining in solution as determined by Levene & Van Slyke, the amount of the picrolonate actually isolated from the mixtures closely approximated to the theoretical figure.

With these findings as a basis, a method has been elaborated for the separation of *l*-phenylalanine from the "leucine" fraction of amino-acids salted-out at pH 6.0 and also from the monoamino-acid fraction obtained by the butyl alcohol extraction of both casein and zein hydrolysates. Briefly it is as follows. The solution of the amino-acids salted-out was concentrated until phenylalanine, as indicated by the Kapeller-Adler colour test, began to separate with the leucine. The mixture was then filtered and the solids, consisting mainly of leucine, washed with a little cold water. The mother-liquor and washings were united, boiled with copper hydroxide and the amino-acids from the insoluble copper salts which separated from the cold solution recovered in the usual way. A Kapeller-Adler estimation carried out on the solution of these amino-acids in water gave an approximate figure for the amount of phenylalanine present. The solution was then boiled down until the concentration of phenylalanine was roughly 2.5%, and picrolonic acid was added to the solution in an amount some 20% in excess of that required to combine with the phenylalanine present. After heating the mixture till all the reagent had dissolved, the resulting solution was allowed to cool in the air (2 hr.) and the long slender crystals of phenylalanine picrolonate which had separated were collected. These were recrystallized twice from minimum amounts of water. The mother-liquor on being left to cool overnight deposited long massive crystals of leucine picrolonate. To obtain the free amino-acid, the phenylalanine picrolonate was dissolved in boiling water, dilute sulphuric acid added until the reaction was definitely acid to thymol blue, and the mixture allowed to cool overnight. The picrolonic acid which separated was then removed by filtration, that remaining in solution being extracted with ether. The aqueous solution, after removal of sulphuric acid with baryta, was evaporated to dryness. Recrystallization of the solid so obtained yielded the characteristic four-sided plates of phenylalanine which after drying with alcohol and ether gave a negative nitroprusside (methionine) reaction, possessed full optical activity and was analytically pure.

From an examination of the solubility curve of phenylalanine in saturated aqueous sodium chloride it would appear that the greater part of this amino-acid present in the protein hydrolysate would be found in the mixture of amino-acids which is salted-out in the processes of Barnett and of Hill & Robson. Yet the amounts of the chemically pure amino-acid actually isolated from such a mixture were only 0.51 % in the case of zein and 0.29 % for casein. Other and unknown factors are apparently involved and as a source of phenylalanine such salted-out mixtures are of little value. On the other hand, application of the new technique to the monoamino-acid fraction obtained by extracting a protein hydrolysate by the butyl alcohol method of Dakin yielded 2.65 % in the case of zein and 1.7 % for casein. These figures are lower than those hitherto recorded. For zein, Osborne & Clapp [1907] recorded 4.8 %; Kapeller-Adler [1932] 5 %; Dakin [1923] quoted 7.2 % although too much reliance cannot be placed on this figure since it is not clear how the author isolated his amino-acid from the undistilled residue of amino-acid esters. For casein the figures are 2.72–3.2 % quoted by Fischer & Abderhalden [1903]; 2.64–3.40 % by Kollmann [1928] and 5 % by Kapeller-Adler, the last figure being the result of a colorimetric determination.

EXPERIMENTAL

Determination of the solubility of l-phenylalanine in aqueous NaCl

The *l*-phenylalanine required for these and other preliminary experiments was obtained from the synthetic amino-acid as follows. *dl*-Phenylalanine was synthesized by Erlenmeyer's method as modified by Lamb & Robson [1931] and the product converted into formyl *dl*-phenylalanine according to Steiger [1930]. The resolution of the formyl compound and the liberation of the *l*- and *d*-amino-acids from the resulting products were effected by the methods of Fischer & Schoeller [1907]. The acids obtained yielded the following data on analysis: *l*-phenylalanine $[\alpha]_D^{20} = -35.00^\circ \pm 0.20$. Schulze [1885] found -35.3° . N, 8.40 %. $C_9H_{11}O_2N$ requires 8.48 %. *d*-Phenylalanine $[\alpha]_D^{20} = +35.12^\circ \pm 0.20$. Fischer & Mouneyrat [1900] found $+35.08^\circ$. N, 8.46 %.

All solubility determinations were carried out in bottles of 50 ml. capacity. The mixture consisting of the solvent and an amount of amino-acid greater than that which would saturate it was shaken mechanically for 8 hr. and then left overnight after which time the solution was filtered directly into a micro-burette. Measured volumes of the solutions were taken for micro-Kjeldahl estimations. The *pH* of the solutions were determined colorimetrically and electrometrically, a double quinhydrone electrode being used for the latter purpose.

Preparation of the picrolonates of phenylalanine, leucine and methionine

dl-Phenylalanine picrolonate. *dl*-Phenylalanine (0.1 g.) and picrolonic acid (0.16 g.) were dissolved in boiling water (5 ml.). Rectangular prisms of the picrolonate separated immediately. Water (5 ml.) was added and the mixture again boiled and cooled. The crystals which had separated after 15 min. were collected, washed with much water and dried at 100° . They weighed 0.24 g. (almost theoretical) and melted at 255° (with decomposition) on slowly heating; 214° on rapidly heating. Levene & Van Slyke give 212° .

l-Phenylalanine picrolonate. *l*-Phenylalanine (0.083 g.) and picrolonic acid (0.132 g.) were dissolved in boiling water (9.3 ml.). On cooling the solution, the picrolonate immediately commenced to separate as long yellow needles. Water (5 ml.) was added, the mixture re-boiled and set aside to cool. The crystals

which separated were collected, dried first *in vacuo* and then at 100°. The yield of picrolonate was 0.19 g., an almost theoretical figure if allowance is made for the amount remaining in solution (its solubility in water as determined by Levene & Van Slyke is 0.34 g./100 ml.). The picrolonate does not melt when heated slowly; plunged into the bath at 200° it melts with decomposition.

l-Leucine picrolonate. *l*-Leucine (0.07 g.) and picrolonic acid (0.132 g.) were dissolved in boiling water (1.5 ml.). On cooling an oil separated. Water (6.5 ml.) was added and re-solution of the oil effected by heating. The picrolonate which separated as long slender rods when the solution was cold melted at 150°. On drying at 100°, the crystals broke down to a powder which on heating melted at 150°, then solidified to melt again at 184° (no decomp.). Levene & Van Slyke give 151°.

l-Methionine picrolonate. *l*-Methionine (0.075 g.) and picrolonic acid (0.132 g.) were dissolved in boiling water (3 ml.). On leaving the solution to evaporate slowly in a desiccator over H₂SO₄, the picrolonate was obtained as fine long needles, which melted at 165°. They were recrystallized from water, the product again only separating from solution on its being left in the desiccator for some days. The final product melted at 178–180° after softening at 174°.

The separation of l-phenylalanine (as picrolonate) from solutions containing a mixture of l-leucine and l-phenylalanine and of l-leucine, l-phenylalanine and l-methionine.

Amino-acids in mixture (A)	Vol. of water in which A was dissolved ml.	Wt. of picrolonic acid added g.	Wt. of phenylalanine picrolonate isolated g.	Theoretical yield less amount remaining in solution g.
0.1 g. P.	10	0.32	0.20	0.23
0.1 g. L.				
0.1 g. P.	15	0.20	0.18	0.21
0.1 g. M.				
0.1 g. P.	9	0.20	0.215	0.23
0.1 g. L.				
0.1 g. M.				
P. = <i>l</i> -phenylalanine. L. = <i>l</i> -leucine. M. = <i>l</i> -methionine.				

Isolation of phenylalanine from the mixture of amino-acids salted-out at pH 6.0 from protein hydrolysates

(a) Zein (500 g.; 90 % protein) was refluxed with 20 % HCl (1 l.) for 24 hr. The excess acid was distilled off under reduced pressure. To the syrupy mass of the amino-acid hydrochlorides remaining water was then added and the mixture again concentrated to a syrup, a process which was repeated several times. The final concentrate was dissolved in hot water (2 l.) and to the resulting solution 40 % aqueous NaOH was added until the pH of the mixture was 6.0. After being clarified with charcoal, the solution was left to cool overnight. The mixture of tyrosine and leucine which had separated from the cold solution was collected and recrystallized from boiling water. The tyrosine thus isolated weighed 14.5 g. The mother-liquor from this crystallization on evaporation to dryness yielded 56 g. of amino-acids which, as indicated by the Kapeller-Adler colour test, contained no phenylalanine. The original filtrate was further concentrated to approximately 1 l. when NaCl commenced to separate. The resulting mixture was placed in the ice chest overnight and the mass of amino-acids and salt which separated was collected and drained thoroughly by suction. It was then stirred with cold water (200 ml.) and finally sucked dry at the pump.

After being dried at 100° , this salted-out fraction weighed 106 g. and gave only a faintly positive test for chloride. The crude amino-acid mixture so obtained was dissolved in boiling water (1 l.) and the solution chilled overnight. The precipitate (23 g.) which separated was collected and the mother-liquor concentrated to 300 ml., cooled and the amino-acids which crystallized out, weighing 34 g., collected. Further concentration of the mother-liquor to 100 ml. yielded a solution from which was deposited a crop of amino-acids weighing 23 g. and reacting positively to the Kapeller-Adler test for phenylalanine. The filtrate obtained was boiled with an excess of $\text{Cu}(\text{OH})_2$, the mixture evaporated to dryness and the residual solids repeatedly extracted with cold water. The Cu salts remaining undissolved were suspended in dilute H_2SO_4 , decomposed with H_2S and the filtered solution was freed from SO_4^{2-} in the usual way. A Kapeller-Adler estimation carried out on the resulting filtrate showed that it contained 2.6 g. of phenylalanine. After addition of picrolonic acid (4 g.), the mixture was boiled to dissolve the reagent and then allowed to cool. Clusters of slender long needles separated almost immediately and were collected when the solution was just warm. Recrystallization of the product from boiling water yielded a solid which on drying weighed 5.5 g. and melted at 190° with decomposition. The melting point varied somewhat with the rate of heating but the decomposition was quite marked. After drying at 100° for some time, the crystals did not melt on heating strongly but only darkened at $192\text{--}194^{\circ}$; plunged into a bath at 192° , the crystals melted immediately with decomposition. Found: $[\alpha]_D^{20} = 24^{\circ}$ in alcoholic solution; N, 15.5%, $\text{C}_{19}\text{H}_{19}\text{N}_5\text{O}_7 \cdot \text{H}_2\text{O}$ requires N, 15.6%. The product was therefore considered to be pure phenylalanine picrolonate.

Recrystallization of the last fraction of the crude leucine collected (23 g.) from water provided a mother liquor which, concentrated to 100 ml. and treated with picrolonic acid in the manner described above, yielded a further quantity of phenylalanine picrolonate (1 g.). Hence the total yield of picrolonate obtained was 6.5 g.

To obtain the free amino-acid the picrolonate (2 g.) was boiled with *N* H_2SO_4 (20 ml.) and the solution left in the ice chest overnight. The picrolonic acid which had separated by that time was collected. The filtrate after being extracted twice with ether to remove the last traces of picrolonic acid was freed from sulphate with aqueous baryta in the usual way and the filtered solution concentrated to a small volume under reduced pressure. On cooling the concentrate, a mass of long needles of phenylalanine hydrate separated. Evaporation of an aqueous solution of the solid to a small volume on the boiling water bath led to the separation of the amino-acid in characteristic plates. The yield of the dried amino-acid was 0.72 g. Found: N, 8.40%. $\text{C}_9\text{H}_{11}\text{O}_2\text{N}$ requires 8.48%. $[\alpha]_D^{20} = -35.4^{\circ} \pm 0.7$. The total picrolonate (6.5 g.) would have yielded, on this basis, 2.3 g. of phenylalanine.

(b) Casein (500 g.; 84% protein) was hydrolysed with 20% HCl (1 l.). The amino-acid fraction salted-out at pH 6.0, when treated as described above, yielded phenylalanine picrolonate which after one recrystallization from water weighed 3.3 g., equivalent to 1.2 g. of the free amino-acid.

Isolation of phenylalanine from zein

Zein (100 g. = 90 g. protein) was hydrolysed with a mixture of H_2SO_4 (300 g.) and water (300 g.) for 24 hr. After quantitative removal of H_2SO_4 the solution was concentrated to 1 l. and set aside to cool. During the process tyrosine (3.75 g.) separated and was removed. The mother-liquor was further concentrated to about 250 ml. and the concentrate placed in the ice chest overnight.

The amino-acids (22.85 g.) which had separated in the interval were collected and washed with a little cold water. Phenylalanine was not found in this fraction. The filtrate and washings were combined and then extracted 15 times with warm butyl alcohol when all the phenylalanine, as indicated by the Kapeller-Adler test, had been removed from it. Removal of the butyl alcohol under reduced pressure resulted in the separation of solids weighing 18.8 g. These were dissolved in water, the solution obtained boiled with $\text{Cu}(\text{OH})_2$ and the mixture left in the air overnight. The insoluble Cu salts were then collected, washed with cold water and reconverted into the amino-acids in the usual way. A Kapeller-Adler estimation carried out on the resulting solution showed the presence therein of 3.5 g. of phenylalanine. The solution was then concentrated to approximately 100 ml. and treated with picrolonic acid (5 g.). The mixture was boiled to dissolve the reagent and allowed to cool in the air. The solid which had separated whilst the solution was still warm, i.e. after some 2 hr., was collected and recrystallized from water. The phenylalanine picrolonate so obtained, when dry, weighed 6.0 g. The total yield of picrolonate, if the quantities remaining in the various mother liquors (160 ml.) are taken into account, would therefore be 6.54 g.

The picrolonate (6.0 g.), decomposed in the manner already described, yielded 2.2 g. of dry, analytically pure, amino-acid.

SUMMARY

The solubility of *L*-phenylalanine in water and in water saturated with sodium chloride at differing pH has been determined.

A new method involving the use of picrolonic acid is detailed for the isolation of phenylalanine from protein hydrolysates.

L-Methionine picrolonate has been prepared and its melting-point determined.

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