A New Method for the Direct Isolation of Glycine from Protein Hydrolysates

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A number of procedures have been proposed for the isolation of glycine from protein hydrolysates. The ester method originally described by Fischer (1901) is laborious and involves great losses, owing largely to incomplete precipitation of glycine ester hydrochloride and the volatility of the free ester. The precipitation of glycine as its barium carbamate (Kingston & Schryver, 1924) is time-consuming. Nitranilic acid, introduced by Town (1936) as a specific precipitant for glycine, has been shown by subsequent workers (Stein & Miller, 1938; Macara & Plimmer, 1940; Block, 1940) to precipitate histidine along with glycine. The difficulties inherent in the earlier methods may be partially overcome by the direct precipitation of glycine as a complex with potassium trioxalatochromate (Bergmann & Fox, 1935). This method, however, suffers from the disadvantage of introducing potassium ions, which are difficult to remove, into the solution.

The purpose of this paper is to describe a simple procedure for the direct isolation of glycine from protein hydrolysates. The present work originated from a study of the direct isolation of lysine picrate from gelatin hydrolysates (Robson & Selim, 1952). While isolating this picrate, it was found that when calcium hydroxide was used instead of sodium hydroxide to attain the appropriate pH a crystalline yellow compound was instantaneously formed. Preliminary examination of the product indicated that it was a complex compound of calcium picrate with one or more of the neutral amino acids. This property of calcium picrate has already been reported (Robson & Selim, 1953).

In order to throw further light on this problem, it was decided to investigate the behaviour of calcium picrate towards the α -amino acids which are usually present in a protein hydrolysate. It was found that this picrate forms a highly insoluble complex salt with glycine. A number of other monoamino acids were found to form similar complex derivatives, although the solubilities of the latter in water are not so low as that of the calcium picrate-glycine complex. While further study of the conditions under which calcium picrate can be employed for the analysis of certain neutral amino

acids is still in progress, a search has been made for other metal picrates capable of specifically precipitating glycine from protein hydrolysates.

Copper picrate, which is readily soluble in water, has been found to fulfil the above requirement. Under the conditions employed, glycine alone, of all the amino acids normally occurring in a protein hydrolysate, forms a sparingly soluble complex salt with copper picrate. Analysis of this complex proves it to be composed of 2 moles of glycine combined with 1 mole of copper picrate, its formula being $(C_2H_5O_2N)_2 \cdot Cu(C_6H_2O_7N_3)_2 \cdot 6H_2O$. It is readily obtained from aqueous solutions containing the two components in the ratio of 2 moles of glycine to 1 mole of copper picrate, but the same compound can also be isolated from solutions having the precipitant and glycine in different molecular proportions. Glycine-copper picrate complex crystallizes promptly from hot water in yellowish green plates. It is soluble in water to the extent of 0.9 g./100 ml. at 10°, but in the presence of a slight excess of copper picrate the solubility diminishes to 0.25 g./100 ml. Experiments with aqueous solutions containing 2 g. of glycine have indicated that under optimum conditions the amino acid can be recovered to the extent of 98.5% by treatment with copper picrate.

These facts suggest a new procedure for the direct precipitation of glycine from protein hydrolysates. In order to demonstrate the practical applicability of the method, two representative proteins were analysed, one (gelatin) rich in glycine and the other (egg albumin) relatively poor in this amino acid. In each case, glycine-copper picrate complex was obtained in good yield and purity.

With gelatin, the method consists mainly in treating a warm concentrated hydrolysate, previously freed from the sulphuric acid used for hydrolysis, with twice as much copper picrate as that required to combine with the glycine present. When all the precipitant has dissolved and the mixture has cooled to room temperature, yellowish green crystals of the glycine complex derivative begin to separate. After a few hours the heavy crystalline precipitate is collected and washed with ice-cold water. Recrystallization of the product from hot water gives pure glycine-copper picrate.

Assuming that the glycine content of moisture- and ash-free purified gelatin, calculated to 16.0 g. of nitrogen, is between 22.2 and 22.8 % (Block & Bolling, 1951), the yield of the final product corresponds to 92–94 % of the total amino acid present. Separation of the free amino acid is easily effected by decomposition of the complex salt with hot sulphuric acid, extraction of picric acid with hot benzene and removal of copper as sulphide. From the sulphuric acid-free solution pure glycine has been obtained in yields equivalent to 90 % of the theory.

With proteins having a smaller glycine content, such as egg albumin, the technique described above is varied slightly. When copper picrate is added to a concentrated egg-albumin hydrolysate in the proportion of one mole to each mole of glycine present no precipitation takes place. This is due to competition between glycine and the basic amino acids for the precipitant. To overcome this difficulty, several times as much copper picrate as that theoretically required for the formation of the glycine complex are dissolved in the hydrolysate. Furthermore, it has been found that the presence of large amounts of ammonia interferes with the precipitation of the complex salt through the formation of ammonium picrate. The isolation of the glycine complex derivative should, therefore, be preceded by the removal of ammonia. From egg-albumin hydrolysates, pure glycine-copper picrate complex has been isolated in amounts corresponding to approximately 90 % of the total amino acid present in the protein. This figure has been calculated on the assumption that the glycine content of purified egg albumin, corrected for moisture and ash, and calculated to 16 g. of nitrogen, is between 3.3 and 3.6 % (Block & Bolling, 1951).

The recrystallization of the glycine-copper picrate complex should be carried out from relatively large volumes of water at temperatures not exceeding 90°. When the complex salt is dissolved in a small volume of boiling water it undergoes partial decomposition, with the formation of a basic form of glycine-copper picrate. Treatment of the complex salt with boiling ethanol results in its complete conversion into a mixture of the basic form of glycine-copper picrate, glycine picrate and picric acid. It has been shown that the presence of a little water is an important factor in this decomposition, since the anhydrous complex is not affected by boiling with ethanol. The reaction may be represented by the equation

A study of the properties of a number of other heavy-metal picrates has shown that some of them

can be successfully employed as specific precipitants for certain monoamino acids. Further results will be reported later.

EXPERIMENTAL

Materials

Gelatin (E. Merck, Darmstadt) (N, 16.5 % of moisture-free protein; moisture, 14.6 %; ash, 2.08 %).

Egg albumin (British Drug Houses Ltd.) (N, 14.08 % of moisture-free protein; moisture, 10.9 %; ash, 4.95 %).

Copper picrate ($\text{Cu}(\text{C}_6\text{H}_3\text{O}_7\text{N}_3)_2 \cdot 5\text{H}_2\text{O}$) was prepared by the following procedure: to a suspension of commercial picric acid in hot water, an excess of pure copper carbonate was added in small portions with stirring. The mixture was then boiled for a few minutes and filtered while hot. After several hours in the refrigerator, the resulting green crystalline precipitate of copper picrate was filtered off, washed with a little ice-cold water and then air-dried.

Isolation of glycine-copper picrate complex from gelatin hydrolysates

Gelatin (200 g.) was added to 1200 ml. of 8N- H_2SO_4 and the protein brought completely into solution by heating on a steam bath. When the protein had dissolved the mixture was refluxed for 30 hr. The hydrolysate was diluted with 2 vol. of distilled water and the insoluble humin removed by filtration. The acid mixture was neutralized to litmus by the addition of an aqueous suspension of $\text{Ca}(\text{OH})_2$ and the precipitated CaSO_4 removed and extracted twice with boiling water. The SO_4^{2-} remaining in the combined filtrate and washings was removed with $\text{Ba}(\text{OH})_2$. Ca^{2+} , still in solution, was removed with oxalic acid, the small precipitate in each case being collected and washed on the filter with boiling water. The hydrolysate, freed from oxalate, SO_4^{2-} , Ca^{2+} and Ba^{2+} , was then concentrated *in vacuo* to about 0.5 l. and filtered to remove any insoluble material. To the filtrate (about 700 ml.) solid powdered copper picrate (395 g., 20 % in excess of double the amount calculated to combine with the glycine present in the ratio of 1 mole of picrate to 2 moles of amino acid) was added and brought into solution by gentle warming. On cooling to room temp., yellowish green crystals of the glycine-copper picrate complex salt began to separate, until after a few hours the mixture had turned into a heavy solid mass of crystals. After 2 days at 0° the crystalline ppt. was filtered off, sucked dry and then washed with 100 ml. of 5 % copper picrate solution in two portions. The product so obtained was recrystallized from a large volume of hot water. After keeping the soln. overnight at 0° the well-crystallized complex was collected at the pump, washed with a little ice-cold water and finally dried in a vacuum desiccator over H_2SO_4 . The combined mother liquor and washings from the recrystallized compound, on concentration *in vacuo* to a small volume, yielded a small crop of similar crystals. The total yield was 190 g.

Glycine-copper picrate complex contains 6 moles of water of crystallization, which are lost at 110°. (Found: water of crystallization, 13.95. Calc. for $(\text{C}_2\text{H}_5\text{O}_2\text{N})_2 \cdot \text{Cu}(\text{C}_6\text{H}_3\text{O}_7\text{N}_3)_2 \cdot 6\text{H}_2\text{O}$, 13.88 %. Found: C, 28.41; H, 2.16; N, 16.93; Cu, 9.37. Calc. for $(\text{C}_2\text{H}_5\text{O}_2\text{N})_2 \cdot \text{Cu}(\text{C}_6\text{H}_3\text{O}_7\text{N}_3)_2$: C, 28.67; H, 2.11; N, 16.73; Cu, 9.49 %).

Properties of the glycine-copper picrate complex. The new compound crystallizes rapidly from hot aqueous soln. in the form of shining yellowish green crystals which appear under the microscope as transparent flat plates. On heating the dry crystalline hydrate at 100–120°, a dark green compound is obtained which is the anhydrous form of the glycine-copper picrate complex. When the compound is allowed to crystallize from a small volume of boiling water two different crystalline substances are obtained. A dark-green picrate which crystallizes in clusters of needles separates faster than a light-green picrate which crystallizes as large individual plates. The dark green compound was found to be the basic form of the glycine-copper picrate complex, whereas the light-green substance was the original neutral complex. The filtrate from the two compounds, on concentration *in vacuo*, yields a mixture of glycine picrate and picric acid.

Decomposition of the glycine-copper picrate complex into the basic derivative was brought to completion by treating the finely powdered substance (15 g.) with refluxing ethanol for 15 min. On cooling to room temp., the resulting dark-green precipitate was collected, washed with a little ice-cold water and finally dried at 110° (7.2 g.). It was far less soluble in water than the original compound. Analysis showed that it was the basic form of glycine-copper picrate complex salt. The yield corresponds to 99.6% of the theoretical. (Found: N, 14.72; Cu, 16.95. Calc. for $(C_2H_3O_2N)_3 \cdot Cu(C_6H_3O_7N_3)_2 \cdot CuO$: N, 14.95; Cu, 16.97%.) The filtrate and washings from this operation were combined, concentrated *in vacuo* to dryness and the residue (5.73 g.) was repeatedly extracted with ether. The combined ether extracts, on evaporation to dryness, yielded 2.16 g. of pure picric acid, equivalent to 98% of the theoretical amount. The residue remaining after the removal of picric acid was taken up in the minimum volume of boiling water and the soln. filtered to remove any insoluble substance. After chilling the filtrate for 12 hr. the bright yellow crystalline picrate which had separated was collected, washed with a little ethanol and recrystallized from a small volume of boiling water. The resulting product had m.p. 198–199°, which is characteristic of pure glycine picrate (Cox & King, 1929).

Isolation of glycine. The glycine-copper picrate complex (150 g.) was heated with 700 ml. 10% (v/v) H_2SO_4 on a steam-bath for 1 hr. with frequent stirring and shaking. The liberated picric acid was removed from the mixture by extraction with successive portions of hot benzene and Cu^{2+} was removed from the acid aqueous solution by H_2S as CuS . The resulting solution was decolorized by boiling for several minutes with a little acid-washed charcoal and was then completely freed from H_2SO_4 with $Ba(OH)_2$. The filtrate and washings from $BaSO_4$ were concentrated *in vacuo* to a syrup and glycine was precipitated with ethanol. After standing overnight at 0° glycine was collected and washed with ethanol. It was recrystallized by dissolving it in the minimum amount of boiling water and precipitating it with ethanol. The recrystallized product weighed 26 g. and when heated slowly decomposed at 230–235°. A sample (0.5 g.) was converted into hippuric acid. The recrystallized hippuric acid had m.p. 192–193°, which was not depressed on admixture with an authentic specimen, m.p. 192–193°.

Isolation of glycine-copper picrate complex from egg-albumin hydrolysates

Egg albumin (100 g.) was hydrolysed with H_2SO_4 as described above. The hydrolysate was diluted with twice its volume of water and humin filtered off. The filtrate was decolorized by boiling for 5 min. with 20 g. of charcoal. To the combined filtrate and washings $Ca(OH)_2$ in slight excess of the H_2SO_4 present was added, and the $CaSO_4$ removed and washed. The free ammonia was removed by partial concentration of the filtrate *in vacuo*. SO_4^{2-} and Ca^{2+} were completely removed in the manner previously described and the solution was concentrated *in vacuo* to about 200 ml. The small amount of amino acids which had separated was removed and washed several times with ice-cold water. The combined filtrate and washings (about 300 ml.) were treated with 200 g. of copper picrate, gently warmed until all the solid had dissolved, and then cooled. After 3 days at 0° the mixture was filtered and the glycine-copper picrate complex washed with a little ice-cold water. The product was recrystallized as described above (12.5 g.). Analysis confirmed that it was pure glycine-copper picrate complex.

SUMMARY

1. A new method for the isolation of glycine from protein hydrolysates by its direct precipitation with copper picrate is described.
2. The method has the advantages that it is simple and economical in time and reagents. The glycine-copper picrate complex is readily decomposed by acids, and picric acid can easily be recovered.
3. The introduction of undesirable reagents is avoided, and after the removal of glycine the hydrolysate is left in a form suitable for the isolation of other amino acids.

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