CCLVI. THE MICRO-DETERMINATION OF GLYCINE IN PROTEIN HYDROLYSATES.

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THE separation of the amino-acids obtained on protein hydrolysis into three fractions by means of the differing solubilities of their copper salts as indicated in previous publications [Town, 1928; 1936] is generally satisfactory in that the amino-acids are largely found in those fractions to be expected from the known properties of their copper salts. Overlapping due to the formation of mixed copper salts and to changes in the solubility of the copper salts due to the presence of other soluble salts occurs only to a very small extent.

Although however this separation into large fractions is workable, separation of the individual amino-acids by fractional crystallization is very difficult. The author has therefore been carrying out concurrently a search for specific precipitants for some of the amino-acids. In particular, substances analogous to flavianic acid have been studied, all being aromatic compounds containing the *o*-nitrophenolic grouping. The only success was obtained in the case of nitranilic acid which is 2:5-dihydroxy-3:6-dinitro-*p*-benzoquinone of the formula:

Nitranilic acid acts as a strong dibasic acid and forms a salt with glycine of the formula $(C_2H_5O_2N)_2 \cdot C_6(OH)_2(NO_2)_2O_2$. This salt is soluble in water to the extent of 0.8 %, but is practically insoluble in 80–100 % alcohol. The acid gives no precipitate with alanine, valine, leucine, phenylalanine, glutamic acid or tryptophan, although with the latter the solution becomes very dark. Glycine nitranilate crystallizes in large yellow octahedral crystals which darken rapidly on heating to 120°; it is therefore dried *in vacuo* over H_2SO_4 . Nitranilic acid solutions decompose readily on standing, so that in forming the derivative the requisite amount of acid is weighed out, dissolved in a few ml. of alcohol and added to the solution to be precipitated.

As indicated below the precipitant has been used to separate an artificial mixture of glycine and alanine, and also to estimate the glycine in gelatin and in caseinogen, these two proteins being chosen as respectively rich and poor in glycine. A solution containing 100 mg. glycine and 162 mg. alanine was separated to the extent of 98 % by treatment with this precipitant. The presence of

mineral acids has no harmful effect and the amino-acids are conveniently dissolved in alcohol in the form of their hydrochlorides.

In applying the process to the estimation of glycine in protein hydrolysates, it is important to ensure the absence of inorganic cations, among which Na, K, NH_4 , Ca and Ba all form sparingly soluble nitranilates. Once these interfering substances are removed it is possible to estimate the glycine content of a protein using only 200–300 mg. of material.

Determinations carried out on gelatin and caseinogen gave the following results:

	% of glycine in protein	
	As nitranilate	Accepted figures
Gelatin	25.9	25.5
	25.3	—
Caseinogen	3.73	0.45
	3.40	—

In the case of gelatin an attempt was made to isolate glycine from the nitranilate as its 3:5-dinitrobenzoyl derivative according to Saunders [1934] and a crude yield of 82% was obtained, the yield of pure substance being 27%. This confirms that it was actually glycine that was precipitated from the gelatin by the nitranilic acid. The figures quoted for gelatin are those of Dakin [1920] and for caseinogen those of Foreman [1919]. In view of the utility of nitranilic acid as a specific precipitant for glycine, the preparation and investigation of substances of analogous constitution is being undertaken.

EXPERIMENTAL.

Preparation of nitranilic acid. 1:4-Diacetoxybenzene (20 g.; by acetylation of quinol) was treated very slowly with fuming HNO_3 (about 10 ml.) at a temperature not exceeding 5° and the resulting solution added very slowly to 70 ml. fuming HNO_3 at 2° with continuous mechanical stirring and maintenance of the temperature below 5° . This process occupied about an hour. Conc. H_2SO_4 (65 ml.) was then added dropwise with stirring, below 5° and during about 3 hours. On continued stirring for a further hour the nitranilic acid crystallized out. The mixture was poured into 10 vol. of ice in a beaker, cooled in ice-salt, and the crystals were collected rapidly on a sintered glass funnel. The crude substance was dried *in vacuo* over H_2SO_4 and KOH and recrystallized from boiling ethyl acetate; yellow prisms; yield 10.6 g.

Separation of a mixture of alanine and glycine. 162.2 mg. alanine and 100.8 mg. glycine were treated with 1 ml. conc. HCl and 12 ml. water, and the solution was evaporated to 4 ml.; 25 ml. absolute alcohol were added and then 300 mg. nitranilic acid in 5 ml. absolute alcohol. A fine yellow precipitate formed almost immediately, but was kept in the ice-chest for several hours. The precipitate was filtered off, washed with alcohol and dried over H_2SO_4 *in vacuo*; yield 247.8 mg., corresponding to 98% recovery.

Estimation of glycine in gelatin. 7.079 g. Coignet's gold label gelatin were kept overnight with 100 ml. conc. HCl and then refluxed for 24 hours. The solution was evaporated to dryness on a water-bath to remove excess HCl, the residue dissolved in water and made up to 250 ml. 10 ml. of this solution (equivalent to 283.2 mg. gelatin) were treated with 15 ml. baryta (0.328 *N*) to make it distinctly alkaline to phenolphthalein and air was then drawn through the solution for 1 hour in a water-bath at 70° to remove ammonia. The liquid was just acidified

to Congo red by addition of 2 ml. of 4*N* H₂SO₄, the BaSO₄ filtered off and washed and the filtrate and washings evaporated to 4 ml. Absolute alcohol (30 ml.) was now added and a further slight precipitate of BaSO₄ filtered off. To the clear alcoholic solution 300 mg. nitranilic acid in 5 ml. of alcohol were added and the whole kept overnight. A well crystallized precipitate formed almost immediately, which after due time was filtered off, washed with absolute alcohol, dried *in vacuo* over H₂SO₄ for 24 hours and weighed. Yield 181.6 mg. A second experiment gave 185.7 mg. These figures correspond to yields of glycine of 25.35 and 25.9 % respectively.

Estimation of nitranilic acid in the glycine nitranilate. 122.4 mg. of the glycine nitranilate obtained above were dissolved in 35 ml. cold water (nitranilic acid decomposes readily in hot solution), and 2 ml. *N* BaCl₂ added; a yellow crystalline precipitate of barium nitranilate immediately separated in shining plates. After some hours the precipitate was filtered off, washed with alcohol, dried at 100° for an hour, cooled and weighed. The weight of barium nitranilate was 116.8 mg. corresponding to a nitranilic acid content of 60.0 % (calc. 60.5 %). 160.1 mg. glycine nitranilate obtained in the second experiment gave 153.2 mg. barium nitranilate corresponding to 60.2 % of nitranilic acid. These figures indicate that the material precipitated by the nitranilic acid from the hydrolysed gelatin is essentially glycine.

Isolation of 3:5-dinitrobenzoylglycine from the glycine nitranilate. The filtrate from the barium nitranilate obtained from the 122.4 mg. glycine nitranilate used above was treated with 0.8 ml. 4*N* H₂SO₄ to remove excess Ba, and the filtrate from the BaSO₄ was evaporated to 5 ml. NaOH was added till the solution was just alkaline to phenolphthalein followed by 1.4 ml. *N* NaOH and 160 mg. 3:5-dinitrobenzoyl chloride and the whole shaken for 5 min. The mixture was now acidified with dil. HCl and the precipitated 3:5-dinitrobenzoyl compound filtered off. Yield 122 mg., corresponding to a crude yield of 82.3 %. The crude material (M.P. 160°) was boiled once with benzene to extract any free 3:5-dinitrobenzoic acid and then recrystallized from hot water, after which it had M.P. 175°; a second crystallization gave 34.7 mg. pure 3:5-dinitrobenzoylglycine, M.P. and mixed M.P. 180°, corresponding to 25 % of the glycine present and again confirming the identity of the material precipitated by the nitranilic acid.

Estimation of glycine in caseinogen. 0.7855 g. caseinogen was hydrolysed by refluxing with 25 ml. conc. HCl for 24 hours; the solution was evaporated to dryness on a water-bath and the residue dissolved in water, filtered from humin material and made up to 25 ml. 10 ml. of this solution treated exactly as described under the hydrolysis of gelatin, yielded 29.7 mg. glycine nitranilate corresponding to 3.73 % glycine in the caseinogen. A second experiment gave 27.0 mg. nitranilate, equivalent to 3.40 % of glycine. These two precipitates contained 61.0 and 62.5 % of nitranilic acid respectively.

SUMMARY.

A method has been evolved for the micro-determination of glycine in protein hydrolysates. The method has been applied to two representative proteins: one, gelatin, rich in glycine, and the other, caseinogen, poor in this amino-acid. The results in the case of gelatin agree fairly closely with those previously recorded, whilst in the case of caseinogen they are much higher than the accepted values. This may be due to the volatility of glycine ester which caused losses in the isolation by the ester method.

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REFERENCES.

- Dakin (1920). *J. biol. Chem.* **44**, 499.
Foreman (1919). *Biochem. J.* **13**, 378.
Saunders (1934). *Biochem. J.* **28**, 580.
Town (1928). *Biochem. J.* **22**, 1083.
—— (1936). *Biochem. J.* **30**, 1837.