CCXXXVIII. EXPERIMENTS ON AMINO-ACIDS

III. A METHOD FOR THE ISOLATION OF HYDROXY-AMINO-ACIDS FROM PROTEIN HYDROLYSATES

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(Received 5 October 1939)

FOUR hydroxyamino-acids may now, by the criteria of Vickery & Schmidt [1931], be regarded as authentic protein constituents. These are hydroxyproline, serine, threonine and tyrosine. The status of β -hydroxyglutamic acid must still be regarded as dubious. A number of other compounds belonging to this group have been reported as occurring in protein hydrolysates, but have not been authenticated [Dunn, 1938].

No general method for the isolation of this group of compounds has yet been described, and it is probably for this reason that so much confusion exists. Isolation in this group has hitherto depended on the chance insolubility of the amino-acid or a derivative, or on a laborious process of fractional crystallization. The isolation of threonine by Sharp [1939] from a Fischer ester distillate suggests the possibility of extending the use of this technique. A possible approach to the problem not involving isolation has been indicated by Nicolet & Shinn [1939], who have studied the reaction of serine and threonine with periodic acid. It would clearly be desirable if some method of isolation could be found which made use of the chemical function of the hydroxyl group of these compounds.

In Papers I and II of this series reference has already been made to the fact that *N*-acetyl-hydroxyamino-acids are practically unextractable from aqueous solution into chloroform. The possibility of reversing this state of affairs by benzoylating the hydroxyl group of these compounds was therefore considered. The use of benzoyl chloride in pyridine, as for the benzoylation of hydroxyl groups in carbohydrates, gave rise to undesirable coloured products. The use of pyridine was also to be avoided if it was wished to study the distribution of N in the fractions from a protein hydrolysate.

Sørensen & Andersen [1908] studied the benzoylation of some hydroxyamino-acids by benzoyl chloride and NaOH in aqueous solution. They found that:

(1) Benzoylation in the presence of excess alkali results in the formation of the *N*-benzoyl derivative.

(2) Benzoylation of the free amino-acid or its *N*-benzoyl derivative at 0° in the absence of excess alkali results largely in the formation of the *ON*-dibenzoyl derivative.

(3) Saponification in dilute aqueous alkali at room temperature converts the *ON*-dibenzoyl derivative into the *N*-benzoyl derivative.

In view of this it was decided to investigate the possibility of benzoylating the hydroxyl groups of *N*-acetyl-hydroxyamino-acids by the action of benzoyl chloride and NaOH at neutral reaction. Serine and hydroxyproline were investigated. The amino-acids were acetylated by the method of du Vigneaud & Meyer [1932] and the resulting alkaline mixture was kept for a time to ensure saponification of any *O*-acetyl groups that might have resulted. No attempt was

made to work up the resulting *N*-acetyl compound. The mixture was neutralized, and benzoylated directly, under the conditions described by Sørensen & Andersen for *O*-benzoylation. In each case a good yield of the *N*-acetyl-*O*-benzoyl derivative was obtained. These compounds are highly crystalline, and are readily extractable from aqueous solution into chloroform. Dilute aqueous alkali at room temperature rapidly converts them to the *N*-acetyl derivatives, liberating benzoic acid. Bergmann & Miekeley [1924] have shown that when *O*-benzoyl-*dl*-serine is treated with dilute aqueous alkali at room temperature acyl migration occurs, resulting in the formation of *N*-benzoyl-*dl*-serine. The present experiments show that when the amino-group is already acetylated this migration does not occur, and saponification proceeds in a normal manner. This fact is essential for the success of the method of isolation outlined below.

The behaviour on acid hydrolysis of *N*-acetyl-*O*-benzoyl-*l*-hydroxyproline was also investigated. On refluxing this compound with acid until the mixture showed constant optical rotation, there resulted a mixture of *l*-hydroxyproline and a compound which was thought to be *O*-benzoyl-*l*-hydroxyproline. Confirmation of the suggested structure of this compound was obtained by analogy: acid hydrolysis of *N*-acetyl-*O*-benzoyl-*dl*-serine yielded the known compound *O*-benzoyl-*dl*-serine, which had been described by Bergmann & Miekeley [1924].

This preferential removal of *N*-acyl groups in acid hydrolysis is probably to be explained as follows: removal by acid hydrolysis of the *N*-acetyl group sets free a positively charged NH_3^+ ($:\text{NH}_2^+$) group, which lowers the H^+ concentration in its immediate neighbourhood and thereby inhibits hydrolytic removal of the *O*-benzoyl group. A closely analogous effect was observed by Moggridge & Neuberger [1938] (cf. also Neuberger & Pitt-Rivers [1939]) in the case of the hydrolysis by acid of *N*-acetyl-methylglucosaminide.

The acid hydrolysis of *ON*-dibenzoylserine and *ON*-dibenzoyltyrosine was studied by Goldschmidt & Fünér [1930], who observed incomplete liberation of benzoic acid in each case. But as the hydrolytic agent employed was 70 % H_2SO_4 at 100° , it seems likely that the phenomenon is not the same as that described here; Goldschmidt & Fünér suggested the possibility of intramolecular anhydride formation.

The procedure described here seems likely to afford a general method (at least in cases where the hydroxyl and amino groups are close to one another) for preparing *O*-benzoyl-hydroxyamino-acids, a class of compound of which the only member hitherto described is *O*-benzoyl-*dl*-serine, which was prepared by Bergmann & Miekeley [1924], by acid hydrolysis of 2-phenyl-oxazoline-4-carboxylic acid.

In attempting to make use of these results for the isolation of hydroxyamino-acids from a protein hydrolysate, the following procedure was used.

After removal of the bases by precipitation with phosphotungstic acid, the hydrolysate is acetylated by the method of du Vigneaud & Meyer [1932], and the resulting mixture, after being kept alkaline overnight, is benzoylated at neutral reaction under the conditions described by Sørensen & Andersen [1908]. The resulting mixture might be expected to contain acetamino-acids (A), *N*-acetyl-*O*-benzoyl-hydroxyamino-acids (B), and benzamino-acids resulting from benzoylation of amino-groups that have escaped acetylation (C). Benzoic acid is also present. The mixture is acidified, and extracted continuously in the Neuberger [1938] apparatus, with sufficient chloroform to ensure fairly complete extraction of *N*-acetyl-*O*-benzoylserine. The chloroform extract would now be expected to contain some of the components of group A, most of group B, most of group C and benzoic acid. The chloroform is removed by evaporation, and

the residue is dissolved in dilute alkali at room temperature. This saponifies the *O*-benzoyl group of compounds in group B, leaving the other compounds unaffected. Subsequent acidification of the mixture and prolonged extraction by chloroform removes the components of group A, group C and the benzoic acid, leaving a solution consisting largely of *N*-acetyl-hydroxyamino-acids. The free amino-acids are prepared from this by acid hydrolysis.

The results of applying this procedure (of which full details are given in the experimental section of the paper) to hydrolysates of fibrin (pig), wool (Merino 64s, dry-combed top) and gelatin (Coignet "Gold Label"), were as follows: the final fraction contained in the case of fibrin 6% of the total N of the protein, of which 89% was in the form of amino-N; in the case of wool, 5.3% (96% amino-N); and in the case of gelatin 10% (33% amino-N).

The free amino-acids of this fraction crystallized fairly readily on addition of alcohol to an aqueous solution. (Tyrosine was first obtained from fibrin and wool by direct crystallization from water.) The crystalline products were obviously mixtures, of which the N content usually lay between the theoretical values for serine and threonine. At the suggestion of Mr J. H. Humphrey, mechanical separation by "float-and-sink" analysis was attempted. A mixture of benzene and carbon tetrachloride was found which gave good flotation separation of a finely ground powder of the crystals. Recrystallization of the "top" fraction resulted, however, in a product which again gave two fractions when subjected to analysis in the same density liquid. This suggested that mixed crystals might be present, and further attempts at isolation by crystallization were abandoned.

In paper IV of this series a possible approach to the problem of separating mixtures of hydroxyamino-acids is discussed.

In the case of gelatin, the low amino-N content of the hydroxyamino-acid fraction suggests the presence of hydroxyproline, which gelatin is known to contain in large quantities. *L*-Hydroxyproline was readily isolated from this fraction by precipitation with phosphotungstic acid [cf. Kapfhammer & Eck, 1927].

It was later observed that after acetylating and benzoylating a gelatin hydrolysate from which arginine had been removed by precipitation with phosphotungstic or flavianic acids, *N*-acetyl-*O*-benzoyl-*L*-hydroxyproline crystallized out directly on acidification. This substance can readily be purified by recrystallization, and can be converted into *L*-hydroxyproline by alkaline followed by acid hydrolysis. This affords a method for the preparation of hydroxyproline which may prove of practical value as the existing methods leave much to be desired. Although the yield of hydroxyproline is only about 5% of the weight of gelatin used, no expensive reagents are required for the preparation. Full details are given in the experimental section of the paper.

EXPERIMENTAL

N-Acetyl-*O*-benzoyl-dl-serine (I)

1 g. *dl*-serine (Hofmann-La Roche) was dissolved in 5 ml. 2*N* NaOH and acetylated with 26 ml. 2*N* NaOH and 2.6 ml. of acetic anhydride added in portions at 0°. A Van Slyke amino-N determination on the resulting mixture indicated 94% *N*-acetylation. The mixture was kept at 0° overnight, and was then neutralized to phenol red with acetic acid. It was then placed on ice in the shaker, and treated with 37 ml. 2*N* NaOH and 4.6 ml. of benzoyl chloride, added in 10 equal portions at 30 min. intervals. The solution was made neutral to

phenol red with acetic acid before each addition of the reagents. The solution was then acidified to thymol blue with 6*N* H₂SO₄. The resulting precipitate was found to contain 80 % of the N present. It was dried, and extracted with ligroin at 100°. The insoluble residue was recrystallized from alcohol. Yield 1.6 g. *N*-acetyl-*O*-benzoyl-dl-serine has m.p. 192–194° (Found: C, 56.7; H, 5.25; N (Kjeldahl), 5.62, 5.66 %. C₁₂H₁₃O₅N requires C, 57.3; H, 5.18; N, 5.57 %.)

The compound has a solubility in water at 20° of 0.15 mg. per ml. Its solubility in chloroform at the same temperature is slightly greater. Its partition coefficient between chloroform and water is therefore approximately 1.

An attempt to use NaHCO₃ in excess instead of NaOH in the benzoylation led to a complete failure to prepare the compound. The yield obtained is significantly less if the benzoylation mixture is not thoroughly cooled in ice.

N-Acetyl-dl-serine (II)

Compound (I) was completely debenzoylated by saponification for 4 hr. in excess of 0.1*N* NaOH at room temperature. A Van Slyke amino-N determination on the resulting solution was completely negative. Chloroform extraction of the solution after acidification yielded benzoic acid; the chloroform extract was completely free from N and contained no material not volatile from an evaporating dish maintained in air at 100°. In order to isolate *N*-acetyl-dl-serine, Ba(OH)₂ was employed instead of NaOH; after saponification, H₂SO₄ exactly equivalent to the Ba(OH)₂ used was added, and the resulting BaSO₄ and benzoic acid were filtered off. The filtrate was extracted with chloroform to remove last traces of benzoic acid, and was then evaporated to dryness *in vacuo*. The product was a resin, soluble in water and alcohol, slightly soluble in ethyl acetate and acetone, and insoluble in other organic solvents. No attempt was made to characterize the compound further, as all attempts to make it crystallize were unsuccessful. The preparation from it of a crystalline derivative is described in Paper IV of this series.

The isolation of (II) through its benzoyl derivative is more satisfactory than its direct isolation from the acetylation mixture, since in the latter preparation it is difficult to ensure separation of the compound from serine that has escaped acetylation.

N-acetyl-*O*-benzoyl-*l*-hydroxyproline (III)

l-Hydroxyproline (Hofmann-La Roche) was treated with the same molecular proportions of reagents as employed in the preparation of (I) from serine. The product, isolated in the same manner, was recrystallized from water containing 20 % of alcohol, to aid solution. m.p. 185–186°. $[\alpha]_D^{20} - 42.9^\circ$ (alcohol, *l* = 2, *c* = 1.1). (Found: C, 61.2; H, 5.46; N (Kjeldahl), 4.84, 4.96 %. C₁₄H₁₅O₅N requires C, 60.6; H, 5.42; N, 5.05 %.)

The compound is readily extractable from water into chloroform. It may be recrystallized from alcohol by the addition of ether, in which it is sparingly soluble.

N-acetyl-*l*-hydroxyproline (IV)

This compound was prepared from (III) in exactly the same manner as (II) was prepared from (I). It crystallized from moist ethyl acetate as the *monohydrate*, m.p. 74–76°. This readily lost water in a vacuum desiccator at room temperature, to give the anhydrous compound, m.p. 133–134°. $[\alpha]_D^{20} - 116.5^\circ$ (water, *l* = 2, *c* = 3.2). (Found: C, 48.25; H, 6.71; N (Kjeldahl), 7.6 %. C₇H₁₁O₄N requires C, 48.5; H, 6.36; N, 8.1 %.)

On exposure to an atmosphere saturated with water vapour, a sample of the anhydrous product increased in weight by 9.65%. The theoretical increase for formation of a monohydrate is 10.4%.

Regeneration of l-hydroxyproline from (III)

For this purpose no attempt was made to work up the intermediate (IV). (III) was saponified for 4 hr. in excess of $N/3$ $Ba(OH)_2$ at room temperature; H_2SO_4 was added to make the mixture $2N$, the mixture was filtered, and was refluxed for 3 hr. It was then cooled, benzoic acid was removed by chloroform extraction, and SO_4^{2-} was removed exactly from the aqueous layer by $Ba(OH)_2$. The filtrate from $BaSO_4$ was concentrated to a very small volume *in vacuo* and, on addition of alcohol, *l*-hydroxyproline crystallized immediately. The overall yield from (III) was 80%.

Acid hydrolysis of (III): O-benzoyl-l-hydroxyproline (V)

An attempt was made to shorten the procedure for regenerating *l*-hydroxyproline from (III) by using acid hydrolysis alone. 5.3 g. of (III) were refluxed for 3 hr. with 200 ml. $2N$ H_2SO_4 , by which time, as judged from a preliminary hydrolysis using a lower concentration of (III), the optical rotation of the mixture had reached a constant value. On cooling, the mixture was extracted with chloroform and was worked up for hydroxyproline as above. The resulting crystalline product had $[\alpha]_D^{20} - 58^\circ$ (water, $l=2$, $c=4$), and tasted bitter as well as sweet. Lutz & Jirgensons [1931] give the optical rotation of *l*-hydroxyproline as $[\alpha]_D^{20} - 76.3^\circ$ (water, $c=6.5$). *l*-hydroxyproline tastes sweet.

On recrystallization of the product from a minimum of hot water, precipitate A and mother-liquor B resulted. Recrystallization of A from water to constant m.p. gave 0.35 g. of *O*-benzoyl-*l*-hydroxyproline monohydrate. The compound does not lose water readily over H_2SO_4 *in vacuo* at room temperature. On heating, it loses water first, and then decomposes at 220° . $[\alpha]_D^{20} - 5.4^\circ$ (water, $l=2$, $c=1.2$). (Found: C, 57.0; H, 5.83; N (Dumas), 5.20%. $C_{12}H_{13}O_4N$, H_2O requires C, 57.0; H, 5.93; N, 5.53%.) On drying at 100° *in vacuo* over P_2O_5 the compound lost 6.2% of its weight. (Calc. for monohydrate, 7.1%). The compound tasted bitter.

On addition of alcohol to mother-liquor B, crystallization of 0.95 g. of *l*-hydroxyproline resulted. $[\alpha]_D^{20} - 75.2^\circ$ (water, $l=2$, $c=5$).

O-benzoyl-*dl*-serine (VI)

0.37 g. of (I) was refluxed for 1.5 hr. with 10 ml. N H_2SO_4 and sufficient alcohol to effect solution. The mixture was cooled and extracted with chloroform. The aqueous layer was added to a strong solution of hydrated sodium acetate (2.7 g.), and the resulting mixture was evaporated slowly in a desiccator. Crystallization soon set in and, when crystals of hydrated Na_2SO_4 began also to appear, the mixture was warmed to 30° to dissolve these, and filtered. The crystals so obtained were recrystallized once from water. Yield 0.045 g. of *O*-benzoyl-*dl*-serine, m.p. (decomp.) 149° . Bergmann & Miekeley [1924] give m.p. (decomp.) $149-150^\circ$. (Found: C, 57.2; H, 5.59; N (Dumas), 6.57%. Calc. for $C_{10}H_{11}O_4N$: C, 57.4; H, 5.26; N, 6.70%.)

The isolation of a hydroxyamino-acid fraction from protein hydrolysates

20–25 g. of protein were hydrolysed by refluxing with 200 ml. $6N$ H_2SO_4 . The mixture was cooled, diluted and treated with a strong aqueous solution of phosphotungstic acid (75 g.). The final volume was 11. After keeping overnight

in the ice chest the precipitate was filtered off, and the filtrate was treated with $\text{Ba}(\text{OH})_2$ at 100° until permanently alkaline to thymol blue. The resulting precipitate was thoroughly washed, and the combined filtrate and washings were concentrated *in vacuo* to a thin syrup, and acetylated as above with 200 ml. 2N NaOH and 20 ml. of acetic anhydride. (This usually produced about 95% acetylation of the amino-groups present.) The mixture was kept at 0° overnight, and was then neutralized to phenol red with acetic acid. Benzoylation was then carried out exactly as described for the preparation of (I), using in all 185 ml. 2N NaOH and 23 ml. of benzoyl chloride. The resulting mixture was acidified to thymol blue with 10N HCl, and filtered. The precipitate was dried in a vacuum desiccator; the filtrate was subjected to extraction by about 5 vol. of chloroform in the Neuberger extractor. When the precipitate was dry, it was exhaustively extracted with ligroin at 100° , and the insoluble residue was suspended in water in a separating funnel, and extracted with several volumes of chloroform. This extract was combined with the first chloroform extract, and evaporated to dryness *in vacuo*. The product was dissolved in 200 ml. of sat. aqueous $\text{Ba}(\text{OH})_2$ at room temperature and kept overnight. The resulting solution was practically free from amino-N. It was acidified to thymol blue with H_2SO_4 and filtered. The filtrate was extracted with about 50 vol. of chloroform in the Neuberger extractor. The aqueous layer was then concentrated *in vacuo* to about 100 ml. and treated with sufficient H_2SO_4 to render it 2N. It was then refluxed until the Van Slyke amino-N figure reached a constant value (3 hr.). SO_4^{--} was then removed exactly by $\text{Ba}(\text{OH})_2$, and the filtrate from BaSO_4 was concentrated until crystallization (first of tyrosine) began. The amounts of N and amino-N obtained in this fraction from various proteins and the attempted isolation from it of pure compounds have been described above.

Preparation of N-acetyl-O-benzoyl-l-hydroxyproline from gelatin directly

30 g. of air-dry Coignet "Gold Label" gelatin were refluxed for 6 hr. with 300 ml. 6N HCl. Excess HCl was removed by repeated evaporation with water *in vacuo*, and the product was then treated with 11 g. of flavianic acid at a volume of 150 ml. After seeding with arginine flavianate the solution was well stirred, and was kept at 0° overnight. The resulting precipitate was filtered off, and the filtrate was freed from flavianic acid by boiling with 10 g. of charcoal. The filtrate from this was made alkaline to thymol blue with NaOH, and was concentrated to a small volume *in vacuo*. Successive acetylation and benzoylation were carried out as described in the previous section. For the acetylation 400 ml. 2N NaOH and 40 ml. of acetic anhydride were used, and for the benzoylation 185 ml. 2N NaOH and 23 ml. of benzoyl chloride. The mixture was then treated with 50 ml. 10N HCl, and the resulting precipitate of benzoic acid was filtered off. On further gradual acidification of the filtrate with 10N HCl no precipitation occurred with the first additions of the acid. Later an oil appeared, which crystallized fairly rapidly. Acidification of the mixture was continued until it was acid to thymol blue. In some preparations it was advisable to seed at this stage with (III). Crystallization was allowed to continue at 0° overnight. The mixture was then filtered, and the precipitate was dried and extracted with hot ligroin. The product was then recrystallized from water containing 20% of alcohol. Yield 2.8-3.5 g. m.p. 185° . No depression of m.p. was obtained on admixture with (III). $[\alpha]_D^{20} - 42.7^\circ$ (alcohol, $l=2$, $c=1.1$). (Found: C, 60.4; H, 5.22; N (Kjeldahl), 5.0, 5.3%. Calc. for $\text{C}_{14}\text{H}_{15}\text{O}_5\text{N}$: C, 60.6; H, 5.42; N, 5.05%.)

The preparation of *l*-hydroxyproline from (III) by alkaline followed by acid hydrolysis is described above.

If arginine is not removed from the gelatin hydrolysate before acetylation, a gummy oil is obtained as final product, in which crystals of (III) can be detected, but from which (III) can be isolated only with difficulty and in low yield.

Phosphotungstic acid can alternatively be used to remove arginine, but flavianic acid seems to lead to a better product, and is also cheaper. In some cases the final product is slightly gummy. Recrystallization from alcohol-ether is then indicated.

SUMMARY

1. The preparation and properties of the *N*-acetyl-*O*-benzoyl derivatives of *dl*-serine and *l*-hydroxyproline are described. These compounds are converted on treatment with dilute alkali into the corresponding *N*-acetyl derivatives. Acid hydrolysis yields the *O*-benzoyl derivatives.

2. On the basis of these properties, a general method is outlined for the isolation of a hydroxyamino-acid fraction from protein hydrolysates.

3. The preliminary results of such a fractionation of fibrin, wool, and gelatin hydrolysates are described.

4. A new and improved method for the preparation of *l*-hydroxyproline from gelatin has been evolved.

I am indebted to Mr B. H. Wilsdon, Director of Research, Wool Industries' Research Association, Leeds, for a supply of wool.

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