THE ISOLATION OF THE 2, 4-DINITROPHENYLHYDRAZONE OF 'GLUTAMIC ACID SEMIALDEHYDE' FROM THE ACID HYDROLYSATE OF PERIODATE-TREATED GELATIN

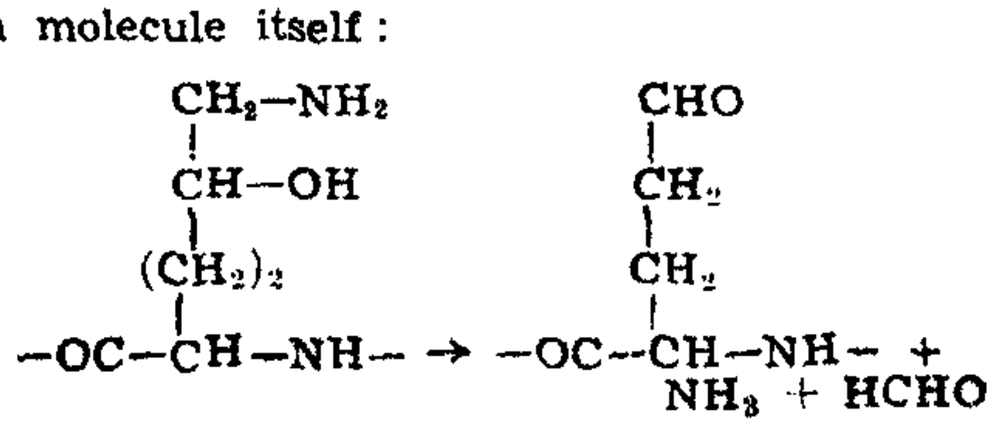
L. K. RAMACHANDRAN AND P. S. SARMA

(University Biochemical Laboratory, Madras.)

PERIODATE oxidation of serine results in the formation of one mole of ammonia, one mole of formaldehyde and one mole glyoxalic acid per mole of serine.[1] In the case of threonine, however, one mole of ammonia and one mole of formaldehyde result, the other reaction product being apparently glyoxalic acid.[2] It is thought that malonic semialdehyde, resulting from the oxidation of β-hydroxyglutamic acid, being a β-aldehyde, is likely to be decarboxylated yielding acetaldehyde.[3] However, β-hydroxyglutamic acid is an unusual protein constituent. Speaking generally, the aldehydo acids arising from the periodate oxidation of hydroxyamino acids, have not so far been much studied.[4]

In the case of hydroxylysine (1, 6-diamino-5-hydroxy caproic acid) periodate oxidation was found to yield one mole of ammonia and one mole of formaldehyde per mole of the amino acid.[5] This has been confirmed by various workers.[6-11] The other reaction product is expected to be glutamic semialdehyde, $OHC-CH_2-CH_2-CH(NH_2)-COOH$, whose existence may be doubted since it would cyclise easily. The compound is not, in fact, listed in literature even though it has been recently suggested as an intermediate in protein metabolism. For some time, we have tried to isolate this aldehyde as a derivative, under favourable experimental conditions.

The technique consists in the treatment of intact gelatin in aqueous solution with periodic acid under optimum conditions, when one mole of ammonia and one mole of formaldehyde would be split off, leaving the glutamic semialdehyde bound in peptide linkage in the protein molecule itself :



This scheme was based on our observation[11] that all the functional groups of hydroxylysine in gelatin are free, at least as far as the reaction with periodate was concerned, based on analyses of the hydroxylysine content of the native protein and its hydrolysate, and on the further observation that a sulphuric acid hydrolysate of periodate-treated gelatin does not contain any hydroxylysine as analysed by the method of Van Slyke[5] and Macpherson.[6] In the face of such evidence, the recent suggestion[12] that the amino-acid may be bound to the protein molecule by an amide linkage at the ε-position lacks experimental support. The treated protein was hydrolysed under reducing conditions ($SnCl_2$-HCl), the hydrochloric acid and the stannous ion removed, treated with 2, 4-dinitrophenylhydrazine and the hydrazone formed extracted with ethyl acetate and recrystallised.

A neutralised sulphuric acid hydrolysate of the treated gelatin contained no hydroxylysine, possibly due to oxidation of the aldehyde to glutamic acid under the experimental conditions of autoclaving for effecting the hydrolysis, and yielded no crystalline semicarbazone after suitable treatment. It was further found that from a sample of a $SnCl_2$-HCl hydrolysate, answering the phenylhydrazone colour test, the aldehydo-acid could be extracted fairly efficiently at a pH of 7-8 with ether. But on the large-scale the extraction at pH 7·5 with ether was extremely difficult due to absence of a clear separation of the layers in the presence of the small amounts of precipitated $Sn(OH)_2$. Even after the removal of the tin as stannous sulphide the ether was not found to extract the aldehydo acid efficiently. Where the 2, 4-dinitrophenylhydrazine treatment was carried out prior to the removal of the tin the hydrazone was found to be strongly absorbed and carried down by the precipitate of stannous sulphide and could not be extracted.

The experimental details which yielded finally the pure crystalline hydrazone are indicated below.

EXPERIMENTAL

200 g. gelatin (Kodak brand, moist. Analysing 1·08% hydroxylysine as percentage of the total nitrogen by the periodate-NH_3 method applied to the intact protein[11]) were dissolved in 1,200 c.c. warm water (60° C.), cooled to R.T. and the following reagents added in order with thorough mixing during and after each addition : 100 c.c. 2 N NaOH, 100 c.c. 0·15 M HIO_4 and 100 c.c. saturated potassium carbonate solution. The

mass was well stirred. During the addition of the potassium carbonate solution the mass turned highly viscous. It was left to stand at 37° C. for 4 hrs., during which period, there was a definite fall in viscosity. It was now placed in the centre compartment of a three-compartment wax-coated wooden cell, the compartments being separated by parchment paper and the side compartments being filled with distilled water to the same level. Carbon electrodes were placed one in each of these and connected through a proper resistance to a 220 volts D.C. main so as to get a final current strength of 0·5 amp. The electrodialysis was carried out for a period of 68-92 hrs. until the treated gelatin in the centre compartment was absolutely free of all electrolytes. The contents of the side compartments were replaced by fresh distilled water at intervals. The gelatin solution was found to have regained its original reaction at the end of this period.

1 litre of concentrated hydrochloric acid containing 10 g. stannous chloride was added to the electrodialysed gelatin the mixture placed in a 3 litre bolt head flask and gently boiled under reflux on a Babo air-bath for 16 hrs. After the hydrolysis the whole was concentrated to a volume of 250 c.c. under reduced pressure and diluted to 4 litre with water and treated with H_2S. The precipitated tin sulphide was removed by filtration and the filtrate and washings concentrated to 2 litres. To this was added, 3 g. 2, 4-dinitrophenylhydrazine and the whole heated at 100° C. on a steam-bath for 15 min. Afterwards it was cooled down and concentrated under reduced pressure to a volume of 500 c.c. and the hydrazone present extracted with ethyl acetate. The combined extracts were dried with anhydrous sodium sulphate. Most of the extract was evaporated at room temperature to remove the ethyl acetate and the crude residue recrystallised from ethanol after norit treatment. The reddish orange highly crystalline hydrazone weighed nearly 400 mg. Previous trials had shown that most other organic solvents used in the recrystallisation of 2, 4-dinitrophenylhydrazones were not helpful in the present case. The hydrazone had the m.p. 119-20° (Uncorr.) and under the microscope the crystals appeared needle-shaped and in clusters.

Analysis.—N content determined by Pregl's micro method—22·56% ; Do. calculated for $C_6H_4N_4O_4C_5H_9NO_2$—22·5%. Molecular weight by Rast's method was found to be 308, while the value calculated for the above formula is 311·2.

Another small portion of the ethyl acetate extract was applied to an alumina column (30 × 2 cm.) packed with the help of ethyl acetate. The column was developed by further addition of ethyl acetate and eluted with the same solvent under a pressure head of 25 mm. Hg. There were observed a fast moving yellow diffuse zone corresponding to the unreacted reagent and a slower moving but sharply defined single reddish brown band. The latter was eluted out and the eluate on evaporation again yielded the hydrazone with the m.p. 119-20°.

Thus the 2, 4-dinitrophenylhydrazone isolated, and analysed as above, appears to correspond to that of glutamic semialdehyde, the melting points of the 2, 4-dinitrophenylhydrazones of most of the other commoner aldehydes being quite different. It is to be expected that further confirmation regarding the identity of this third periodate oxidation product from hydroxylysine would become available since four methods have recently been reported for the synthesis of hydroxylysine.[12-14] Further, the isolation and characterisation of this compound would be easier with pure synthetic hydroxylysine.

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