361. *β-Hydroxyglutamic Acid.*

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Dakin (*Biochem. J.*, 1918, **12**, 290; 1919, **13**, 388) first isolated hydroxyglutamic acid (I) in a yield of about 10% from the products of the hydrolysis of caseinogen, in smaller amounts from glutenin and gliadin, and then from those of zein (*Z. physiol. Chem.*, 1923, **130**, 159). He had apparently no difficulty in obtaining it crystalline, and amongst a number of properties and reactions by far the most characteristic was the oxidation by chloramine-T to malic semialdehyde (II), which was isolated almost quantitatively as the *p*-nitrophenylosazone.

$$\underset{\text{(I.)}}{CO_2H\cdot CH_2\cdot CH(OH)\cdot CH(NH_2)\cdot CO_2H} \longrightarrow \underset{\text{(II.)}}{CO_2H\cdot CH_2\cdot CH(OH)\cdot CHO}$$

Jones and Johns (*J. Biol. Chem.*, 1921, **48**, 347), Jones and Wilson (*Cereal Chem.*, 1928, **5**, 473), and Calvery (*J. Biol. Chem.*, 1932, **94**, 626) isolated from lactalbumin, gliadin, and crystallised ovalbumin, respectively, amorphous powders of the correct analytical composition, but did not confirm the identity with β-hydroxyglutamic acid by oxidation to malic semialdehyde. We are not convinced that their materials were pure hydroxyglutamic acid, for their method of isolation, namely, extraction with glacial acetic acid, followed by precipitation by alcohol as silver salts and as complex mercury derivatives, was not such as to remove glutamic and aspartic acids. Our experience is that, although these two acids, when pure, are relatively insoluble in acetic acid, they dissolve in considerable quantities when mixed with other amino-acids.

Harington and Randall (*Biochem. J.*, 1931, **25**, 1917) concluded that β-hydroxyglutamic acid occurs as a result of the hydrolysis of caseinogen, since some fractions of their hydrolytic products yielded not inconsiderable amounts of malic semialdehyde when oxidised by chloramine-T. They were, however, unable to isolate hydroxyglutamic acid, and were of the opinion that the separation from other dicarboxylic acids is not so straightforward a process as the original description by Dakin might lead one to suppose. Their fraction which should have consisted of hydroxyglutamic acid was always heavily contaminated by glutamic and aspartic acids.

Other workers in this country have informed us that they also have been unable to isolate hydroxyglutamic acid from protein hydrolysates.

Our repetitions of Dakin's process and a complicated fractional crystallisation of the strychnine salts prepared from the fraction which should have been hydroxyglutamic acid showed that in our experiments also this amorphous material was a complex mixture, which contained little or no hydroxyglutamic acid.

When caseinogen is hydrolysed by sulphuric acid and the sulphate ions are removed with barium hydroxide, considerable quantities of amino-acids and humin-like substances are adsorbed on the resulting barium sulphate and render it easily filterable. Exhaustive washing with water, the usual treatment, has failed in all our experiments to remove the amino-acids thus adsorbed. Elution with 1% hydrochloric acid, on the other hand, readily liberates all the adsorbed nitrogenous material. The observations were put into practice in a number of hydrolyses of caseinogen, and the selected details here described are sufficient to illustrate the processes by which crystalline hydroxyglutamic acid has again been isolated.

It was soon found that hydroxyglutamic acid occurred only in the barium sulphate precipitate obtained by neutralising the solution (Congo-red) with baryta, and in subsequent work, therefore, the other products of hydrolysis were discarded. Elution of the amino-acids from this precipitate was followed by treatment of the resulting solution to remove glutamic acid, aspartic acid, monoamino-monocarboxylic acids, proline, hydroxyproline, and basic amino-acids. Decomposition of the silver salts precipitated from the residual solution yielded a crude gum; this stage corresponded to that at which Dakin obtained relatively pure hydroxyglutamic acid. Extraction with acetic acid and then with alcohol divided this gum into (a) an alcohol-insoluble powder consisting of glutamic acid, pyrrolidonecarboxylic acid, and an impure sulphur-containing nitrogenous acid, and (b) an alcohol-soluble gum which contained about 10% of hydroxyglutamic acid, as shown by the amount of malic semialdehyde *p*-nitrophenylosazone isolated after oxidation. After the gum had been hydrolysed by acid, however, the yield of osazone rose to 17%, showing that part of the hydroxyglutamic acid was present as hydroxypyrrolidonecarboxylic acid, which is probably not oxidised by chloramine-T. The solution obtained by hydrolysing the gum was freed as far as possible from glutamic and aspartic acids and from a small amount of *l*-leucine, which had evidently escaped the prolonged butyl alcohol extraction of the initial routine fractionation. The material resulting from this partial purification still contained glutamic acid, and oxidation with chloramine-T and precipitation of malic semialdehyde as *p*-nitrophenylosazone indicated that only 22% of it was hydroxyglutamic acid. It was clear that crystallisation and the customary methods of precipitation had become valueless at this stage, and resort was therefore made to a process in which advantage was taken of the presence of the hydroxyl group in hydroxyglutamic acid and its absence in the accompanying amino-acids. Phosphorylation by means of phosphoric acid and phosphoric oxide (Manaka, *J. Biochem. Japan*, 1931, **14**, 191) yielded a crystalline barium salt; this decomposed during recrystallisation into barium phosphate and hydroxyglutamic acid, which separated in characteristically shaped needles. The analyses of this acid and its properties, so far as the small amount of material (20 mg.) has allowed them to be determined, agree closely with those recorded by Dakin, and an excellent yield of malic semialdehyde *p*-nitrophenylosazone was obtained in a chloramine-T oxidation. This incomplete description is made now, because some time must necessarily elapse before sufficient of the amino-acid can be prepared for a reinvestigation of its properties and reactions.

The view is adopted in this paper that the only safe criterion of the presence of β-hydroxyglutamic acid in mixtures is the oxidation to malic semialdehyde, and it has been assumed that this aldehyde can only arise from hydroxyglutamic acid. No malic semialdehyde is formed when an artificial mixture of glutamic and aspartic acids and leucine is oxidised by chloramine-T. Further, the *p*-nitrophenylhydrazone of succinic semialdehyde (the oxidation product of glutamic acid) undergoes no change when heated for some hours with excess of *p*-nitrophenylhydrazine under the conditions employed

for osazone formation. It may be assumed, therefore, that the weight of malic semialdehyde *p*-nitrophenylosazone obtained from a mixture is a measure of its content of β-hydroxyglutamic acid.

The tedious and wasteful procedure described above was clearly unsuitable for the determination of the actual amount of hydroxyglutamic acid in the caseinogen used in this work, but the following method is rapid and should prove useful in the case of other proteins. The barium sulphate precipitate formed by neutralisation of a caseinogen hydrolysate to Congo-red with baryta was eluted with acetic acid, and the amino-acids of this solution were submitted to electrodialysis in a three-cell apparatus under conditions in which the dicarboxylic acids migrated to the anode (Foster and Schmidt, *J. Amer. Chem. Soc.*, 1926, **48**, 1709). Technical improvements of this process have been devised. The liquid from the anode compartment was used as described below for the estimation of hydroxyglutamic acid.

The sulphur-containing amino-acid mentioned on p. 1645 contained neither sulphydryl nor disulphide groups and the sulphur was labile and tended to be split off in the elemental form during attempted purification; it might be a glutamic acid substituted in the β-position by a sulphur-containing group which becomes replaced by hydroxyl with consequent formation of hydroxyglutamic acid. No evidence of the presence of such an acid was found in the liquid from the anode compartment; methionine was the only sulphur-containing amino-acid isolated, and its amount was naturally very small. After its removal, oxidation of the solution and isolation of malic semialdehyde *p*-nitrophenylosazone showed that the caseinogen contained 0·33% of hydroxyglutamic acid. The exactness of this method of estimation cannot be tested until a supply of the pure acid is available.

The combined processes of adsorption on barium sulphate, electrodialysis, and phosphorylation offer a relatively simple means of isolating pure hydroxyglutamic acid. Investigations along these lines are in progress.

Experimental.

Elutions of Barium Sulphate Precipitates.—The precipitate formed by neutralising to Congo-red with solid milled baryta the hydrolysate from caseinogen (500 g.) and 25% sulphuric acid (2 l.) was collected, pressed in a filter-press, and dried in air at 40° (Found: N, 5·28 mg. per g. of $BaSO_4$). The procedure throughout was to seal in a glass ampoule weighed amounts of the barium sulphate precipitates and known volumes of the eluting liquids. The mixtures were shaken mechanically at 25° for the required time, the contents were centrifuged, and the total nitrogen of the clear supernatant liquids was estimated in aliquot portions by the micro-Kjeldahl method. Each elution was performed in duplicate.

(i) *By hydrochloric acid.* (a) Barium sulphate precipitate (3·0 g., containing 5·28 mg. N per g.) eluted with 1% hydrochloric acid (6·0 c.c.).

Duration of elution, hrs.	N eluted, mg.	% of total N.
½ (*X*)	10·5, 10·4	66
1	10·4, 10·4	66
4	11·2, 11·1	71

(b) Barium sulphate precipitate (2·0 g.) from ½-hour elution (*X*) eluted with 1% hydrochloric acid (4·0 c.c.).

½	4·8, 4·8	
4	5·4, 5·4	30

Thus all the adsorbed amino-acids were eluted by 1% hydrochloric acid in two successive treatments of 4 hours each.

(ii) *By acetic acid.* Portions (3·0 g. each) of the barium sulphate precipitate (5·28 mg. N per g.) were eluted four successive times with glacial acetic acid (6·0 c.c.). (Table on p. 1647.)

Hydrolysis and Fractionation.—The description of one representative experiment is sufficient to illustrate the procedure. All concentrations by evaporation were carried out under reduced pressure below 45°, or at lower temperatures where stated.

	Duration of elution, hrs.	N eluted, mg.	% of total N.
(a)	½	6·48, 6·44	40
	1	6·32, 6·26	
	4	6·24, 6·26	
(b)	½	2·65, 2·65	17·4
	1	2·56, 2·57	
(c)	½	1·47, 1·51	9·5
(d)	½	1·11, 1·12	7·0
			73·9

Caseinogen (1·5 kg.) was hydrolysed for 30 hours with boiling 25% sulphuric acid (6 l.); samples taken after 25 and 30 hours contained the same amount of amino-nitrogen (van Slyke), indicating that hydrolysis was complete. The hot solution was diluted with water (9 l.), stirred mechanically, and neutralised to Congo-red by the addition of finely-milled barium hydroxide. Next day the barium sulphate (fraction 1) was collected in Buchner funnels and freed from liquid by means of a filter-press. The combined sulphuric and amino-acids of the filtrate were determined by titrating an aliquot part in 90% alcohol with alcoholic potassium hydroxide (Willstätter and Waldschmidt-Leitz, *Ber.*, 1921, **54**, 2988), and the required amount of milled baryta was added to the well-stirred solution. The barium sulphate (fraction 2) thus formed was collected and washed with water. The filtrate was poured slowly into vigorously stirred 96% alcohol (3 vols.), and the mixture was preserved in closed vessels for 4 days while the barium salts of the dicarboxylic acids separated (fraction 3) (Kingston and Schryver, *Biochem. J.*, 1924, **18**, 1070).

Fraction 1 was eluted three times by shaking it mechanically for 4 hours with twice its weight of 1% hydrochloric acid in each case. The solution was concentrated to small volume, cooled to 0°, and saturated with hydrogen chloride. Several days later glutamic acid hydrochloride was collected, and the saturation at 0° was repeated to precipitate a further small amount. The precipitates were washed with concentrated hydrochloric acid, and the filtrate and washings were evaporated to dryness, and the residue dissolved in hot water (1·5 l.) and mixed with excess of A.R. lead oxide. The mixture was heated for 2 hours at 100°, chilled, and the lead aspartate, tyrosinate, and chloride collected. The excess of lead was removed as sulphide, and the filtrate concentrated and extracted for 60 hours with butyl alcohol in a continuous-flow apparatus. When no more material was extracted, the aqueous layer was freed from butyl alcohol by evaporation, mixed with the requisite amount of sulphuric acid to make the concentration 5%, and then with phosphotungstic acid in 5% sulphuric acid until no further precipitate of the hexone base phosphotungstates separated. The solid was collected, the combined filtrate and washings mixed with saturated baryta till no further precipitation occurred, and the slight excess of barium accurately removed with dilute sulphuric acid. The filtrate and washings from this precipitate were just acidified with nitric acid, and the chloride was removed by means of dilute silver nitrate (no heating). The chloride-free solution was made just alkaline to litmus, and the silver salts were precipitated by additions of 3*N*-silver nitrate and 2*N*-sodium hydroxide solutions until no further white precipitate separated; a large excess of alkali was avoided. The silver salts were washed with water, suspended in water, and decomposed with hydrogen sulphide, and the filtrate evaporated to dryness. The residue was extracted several times with glacial acetic acid at room temperature, the insoluble aspartic and glutamic acids collected, and the filtrate concentrated over solid sodium hydroxide in a vacuum desiccator at 37° to remove all acetic acid. The resulting clear gum was extracted three times with absolute alcohol at 50°, leaving a residue (*X*). The solution was evaporated and left a clear, pale yellow gum (*Y*) which contained hydroxyglutamic acid.

The material (*X*) was submitted to fractionation in aqueous alcohol, and yielded (i) glutamic acid, m. p. 198° alone or mixed with an authentic specimen (Found: C, 40·1; H, 6·1; N, 9·7. Calc. for $C_5H_9O_4N$: C, 40·8; H, 6·1; N, 9·5%); (ii) an inseparable mixture, m. p. 184°, of glutamic and pyrrolidonecarboxylic acids (Found: C, 34·5; H, 5·6; N, 9·6%), which gave the pine-splinter pyrrole reaction when heated with zinc dust, but yielded no malic semialdehyde when oxidised with chloramine-T; and (iii) an impure sulphur-containing substance, which was insoluble in all solvents except water and gave negative Millon, Pauly, sulphydryl and disulphide reactions. In one sample, which did not melt, the ninhydrin reaction was negative (Found: C, 34·0; H, 5·9; N, 12·0; S, 13·0%), in another, m. p. 150°, it was positive (Found: C, 31·3; H, 5·5; N, 10·6; S, 6·7%).

Fraction 2 was eluted three times for 4 hours with 1% hydrochloric acid, the solution

concentrated and made alkaline to litmus with baryta, and next day barium *i*-aspartate (Kingston and Schryver, *Biochem. J.*, 1924, **18**, 1070) was collected. The excess of barium was removed from the filtrate by means of sulphuric acid, the precipitate eluted with hydrochloric acid, and the elute and filtrate were added to the solution from fraction 1 at the appropriate stage.

Fraction 3 was submitted to the routine fractionation already described in the case of fraction 1, and finally a negligible amount of impure glutamic acid was obtained by decomposing the silver salts.

Examination of the Alcohol-soluble Gum (Y).—*Oxidation.* Chloramine-T (1·5 g.) was added to a solution of the gum (1 g.) in water (5 c.c.) neutralised with *N*-sodium hydroxide (7 c.c.). Toluenesulphonamide was immediately precipitated, and after 30 minutes the solution was heated at 60° for 30 minutes till no chlorine was detectable, cooled to 0°, filtered, and heated for 1 hour at 100° with *p*-nitrophenylhydrazine (2 g.) in warm glacial acetic acid. The dark red-brown precipitate was collected while the solution was still hot, washed repeatedly with boiling alcohol, dried (0·07 g.), and crystallised from nitrobenzene, forming dark red needles, m. p. 297°, identical with authentic malic semialdehyde *p*-nitrophenylosazone (Found : N, 21·6. Calc. for $C_{16}H_{14}O_6N_6$: N, 21·7%*). A minute trace gave very intensely the blue coloration with alcoholic sodium hydroxide characteristic of *p*-nitrophenylosazones.

In another experiment the gum (0·13 g.) was hydrolysed with 15% hydrochloric acid (2 c.c.) for 1 hour at 100° and the product was freed from hydrochloric acid by distillation, dissolved in water (2 c.c.), neutralised with sodium carbonate, and oxidised with chloramine-T (0·15 g.) in water (1·5 c.c.) as before. The yield of *p*-nitrophenylosazone was 14 mg., indicating that 17% of the gum consisted of hydroxyglutamic and hydroxypyrrolidonecarboxylic acids. Calculations of amounts of hydroxyglutamic acid are based on the yield of malic semialdehyde *p*-nitrophenylosazone obtained by Dakin from the pure amino-acid.

Hydrolysis.—The gum (30 g.) which contained traces of sulphate ion, was dissolved in 2*N*-hydrochloric acid (200 c.c.), the sulphate removed exactly by means of barium chloride, the barium sulphate removed, concentrated hydrochloric acid (20 c.c.) added, and the solution boiled under reflux for 5 hours. The chloride was eliminated with silver acetate, and the excess of silver with hydrogen sulphide. The filtrate was concentrated by distillation, and then to dryness over phosphoric oxide, and the resulting solid was extracted several times with glacial acetic acid (80 c.c. in all). The insoluble material consisted chiefly of aspartic acid, which was purified as the characteristic copper salt. The acid crystallised in plates from aqueous alcohol and showed the usual variations of m. p. with rate of heating (Found : C, 36·1; H, 5·3; N, 10·5. Calc. for $C_4H_7O_4N$: C, 36·1; H, 5·3; N, 10·5%).

The acetic acid solution was distilled to dryness, the residue dissolved in water at 60°, mixed with excess of zinc oxide to precipitate zinc glutamate, the excess of zinc removed with hydrogen sulphide, and the filtrate concentrated to small volume and placed in a flat dish over absolute alcohol (renewed as necessary) in a desiccator evacuated to a pressure of 30 mm. of mercury. The gradual absorption of alcohol by the aqueous solution depressed the solubility of the amino-acids, which slowly separated in crystalline form. Fine needles, m. p. 270°, separated first (Found : C, 54·6; H, 9·6; N, 10·9. Calc. for $C_6H_{13}O_2N$: C, 54·9; H, 9·9; N, 10·7%). They gave the ninhydrin reaction and had $[\alpha]_D = -10·2°$ in water and $+14·7°$ in 20% hydrochloric acid. These facts are sufficient to establish the identity of this substance with *l*-leucine ($[\alpha]_D = -10·8°$ in water and $+15·9°$ in 20% hydrochloric acid), since in the case of it alone of the five isomeric leucines does the change of solvent cause a reversal of the sign of the rotation.

A mixture of aspartic and glutamic acids then separated, and as it was obviously too complex to be separated by the usual methods, the aqueous alcohol was evaporated, and the residue (1·0 g.) dried thoroughly. When a portion was oxidised with chloramine-T in the usual way, it yielded an amount of malic semialdehyde *p*-nitrophenylosazone corresponding to the presence of 22% of hydroxyglutamic acid.

β-*Hydroxyglutamic Acid.*—The preceding solid (0·52 g.) was stirred into an ice-cold mixture of glacial phosphoric acid (14 g.) and phosphoric oxide (0·6 g.). After this mixture had remained over phosphoric oxide in a desiccator for 40 hours, it was cooled in a freezing mixture, and crushed ice was cautiously added, followed by excess of barium carbonate until no further evolution of carbon dioxide occurred. The reaction of the solution was then at p_H 6. When the solution had been filtered from barium phosphate and carbonate, it was evaporated under as highly reduced pressure as possible at 30°, poured into about 15 volumes of absolute alcohol, and the

* The theoretical percentage of nitrogen in this substance is as stated, and not 22·7 as quoted by Dakin and by Harington and Randall.

solution concentrated for 24 hours over phosphoric oxide in a vacuum desiccator. The crystalline precipitate which separated contained barium and phosphorus, but after three crystallisations from aqueous alcohol, all the barium had been precipitated as free phosphate. The nature of this unstable compound of amino-acid, barium and phosphoric acid is unknown.

β-Hydroxyglutamic acid, which separated from the aqueous alcoholic solution, formed thick colourless needles, which appeared to lose water at about 110° and became transformed into a glassy solid which finally melted at 130—135° (Found: C, 33·4; H, 7·3; N, 7·8. Calc. for $C_5H_9O_5N,H_2O$: C, 33·2; H, 6·1; N, 7·7%). In aqueous solution it was either optically inactive or had a slight dextrorotation; owing to the small amount of material available the specific rotation could not be accurately determined. It gave a green fluorescent solution with α-naphthol and concentrated sulphuric acid, the fluorescence being equal to or more intense than that given by an equal weight of pure glutamic acid under the same conditions.

The behaviour of Dakin's β-hydroxyglutamic acid when heated is described as follows: "In the neighbourhood of 100° the acid becomes pasty and appears to part with water. Eventually on raising the temperature the whole of the acid is converted into a clear glassy mass. The change is brought about rapidly at a temperature of about 140—150°. . . ." Dakin's acid showed a low dextrorotation in aqueous solution and gave a clear fluorescent green colour with α-naphthol and concentrated sulphuric acid.

When the acid (10 mg.) was oxidised with chloramine-T (15 mg.) in the usual way and the product was treated with *p*-nitrophenylhydrazine (20 mg.) in alcoholic acetic acid, crystalline malic semialdehyde *p*-nitrophenylosazone, m. p. 280°, separated in amount (4·2 mg.) corresponding to 57% of Dakin's yield. It gave the blue colour reaction with sodium hydroxide, and when crystallised from nitrobenzene melted at 298°. The lower yield as compared with that of Dakin was naturally due to the difficulties of working with very small quantities.

Electrodialysis.—The apparatus closely resembled that of Cox, King, and Berg (*J. Biol. Chem.*, 1929, **81**, 755). The only details which need be mentioned are the present use of platinum, instead of carbon, electrodes to avoid the formation of colloidal carbon; the use of cellophane no. 400, 0·00126 in. thick, instead of parchmentised paper; the waterproofing of the cell walls with four coats of cellulose varnish; and mechanical stirring of the centre compartment. The cellophane membranes were soaked in distilled water over-night and lasted during 30 hours of dialysis before they became less permeable. During the whole dialysis the temperature was maintained below 30° by the use of efficient cooling coils. The liquid in the centre compartment was kept at p_H 5·5 by the addition of 5*N*-sodium hydroxide or glacial acetic acid as required. The electrical arrangements consisted essentially of a potential divider across the 200 v. D.C. mains, by means of which the required *E.M.F.* was applied to the circuit containing the cell, an ammeter and a 50-ohm variable resistance for fine adjustments. A uniform current of 1·5 amps. was maintained. In addition, a third electrode B was placed in the centre compartment, so that the inter-compartment resistances could be determined by measuring the potential differences between the anode (A) and B, and between the cathode (C) and B. At the beginning, the resistances AB and BC were equal, but as the dialysis proceeded, AB increased relatively to BC, and when AB was equal to 10BC electrodialysis was stopped.

Hydroxyglutamic Acid Content of Caseinogen.—Caseinogen (500 g.) was hydrolysed with 25% sulphuric acid (2·5 l.) as before, and water (3·7 l.) added. The barium sulphate obtained by neutralisation to Congo-red with baryta was collected, pressed, and eluted with twice its weight of 90% acetic acid for four periods of 1 hour each, and the combined solutions were concentrated, diluted with water, and electrodialysed as described above. The anode liquor was freed from methionine by butyl alcohol extraction or by precipitation with mercuric chloride (Pirie, *Biochem. J.*, 1932, **26**, 1270), the methionine being identified by analysis, m. p., specific rotation, and conversion into the derivative with α-naphthyl *iso*cyanate. After removal of the butyl alcohol by evaporation, or precipitation of the mercury and chloride by hydrogen sulphide and silver acetate, the solution was concentrated to 50·0 c.c., and 2 c.c. were neutralised, oxidised with chloramine-T (0·5 g.), and the products treated with *p*-nitrophenylhydrazine (0·3 g.) in alcoholic acetic acid in the usual way. The precipitate thus obtained was extracted repeatedly with boiling absolute alcohol to dissolve glyoxal *p*-nitrophenylosazone and *p*-nitrophenylhydrazones. The weight of the residual malic semialdehyde *p*-nitrophenylosazone (48·6 mg.) corresponded to the presence of 0·33% of β-hydroxyglutamic acid in the caseinogen, on the assumption that this amino-acid is totally adsorbed on the barium sulphate; this assumption is based on the preceding experiments.

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