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The Linkage of Glutamine in Proteins

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The excess of free amino-groups over the number of lysine molecules in different proteins has been attributed by Chibnall (1942) to the existence of several smaller peptide units within the protein molecule, each of them containing a free terminal α -amino-group. In lactoglobulin these peptide chains are supposed to be held together by non-peptide linkages involving carboxyl and not amino-groups; three possibilities were discussed: (1) ester linkages with the hydroxyamino acids; (2) imide linkages $R.CO.NH.CO.R'$ formed with the amide groups of asparagine or glutamine, and (3) thiol-ester linkages formed with cysteine.

The problem of chain-branching in the protein molecule being under investigation in our laboratory for some years, we have examined the possibility of imide linkages of the type suggested by Chibnall (1942) in formula (1):

Several proteins were deaminized and then hydrolyzed by boiling with acids. It was expected that the action of nitrous acid on the residue (1) would lead to the formation of α -hydroxyglutaramide residues (2), which after complete hydrolysis should yield free α -hydroxyglutaric acid (3):

The same acid would be formed if residues of glutamic acid were bound in the peptide chain by their two carboxyl groups, thus leaving the α -amino-group free (formula (4)):

The hydrolysate of deaminized proteins was tested for α -hydroxyglutaric acid by extraction with ether and oxidation of the ether-soluble fraction with permanganate. Under these conditions a considerable part of hydroxyglutaric acid is oxidized

to succinic acid, which can be determined manometrically with succinic dehydrogenase (Krebs, 1937).

If the excess of free amino-groups in the protein molecule were due to mono-amino-acids other than glutamic acid, the corresponding α -hydroxyacids would be formed by the action of nitrous acid, and these would also pass into the ether extract of the hydrolysate.

EXPERIMENTAL

Estimation of α -hydroxyglutaric acid in protein hydrolysates

Pure α -hydroxyglutaric acid was prepared according to Fischer & Moreschi (1912). 2 g. of casein with varying amounts of α -hydroxyglutaric acid, as indicated in Table 1, were boiled with 20 ml. of 23% (w/w) HCl or 8*N*-sulphuric acid. The hydrolysate was evaporated under reduced pressure, the residue dissolved in 10 ml. of water and continuously extracted with ether for 8 hr. The ether was removed by distillation, the residue dissolved in 3 ml. of water and filtered. The filtrate and washings, after evaporation to dryness, furnished much less than the expected amount of ether-soluble substances (Table 1), and prolonged extraction with ether did not give higher yields. Hence, it follows that a part of the hydroxyglutaric acid is lost during the boiling with acid. The ether-soluble residue containing α -hydroxyglutaric acid or its lactone (butyrolactone carbonic acid) was dissolved in 5 ml. of 0.1*N*-NaOH and the lactone hydrolyzed by keeping the solution in a boiling water-bath for 30 min. The solution was cooled to 25°, acidified with 2 ml. of 2*N*-sulphuric acid, mixed with 3 ml. of 0.1*N*-permanganate and heated slowly to 60°. Precipitated oxides of manganese were dissolved by the addition of sodium sulphite; then potassium sulphate was added and succinic acid was extracted as described by Goepfert (1940). The ether extract was evaporated, dried, weighed, dissolved in small amounts of water and neutralized by NaOH. Succinic acid was determined in the neutral solution by treatment with succinic dehydrogenase prepared from pigeon's breast muscle (Krebs, 1937). These experiments, as well as those in which α -hydroxyglutaric acid was treated

directly with permanganate, indicate that only 12–23 % of the hydroxyglutaric acid is converted to succinic acid (Table 1). Control experiments with pure casein, to which no hydroxyglutaric acid had been added, furnished insignificant amounts of succinic acid (Table 2). There is no doubt, therefore, that the succinic acid found in our experiments (Table 1) originates only from added α -hydroxyglutaric acid.

Table 1. *Yields of succinic acid from α -hydroxyglutaric acid, boiled with casein and hydrochloric acid (in the third experiment with sulphuric acid)*

α -Hydroxyglutaric acid (mg.)	Casein (g.)	Ether-soluble substances		Succinic acid	
		Before oxidation (mg.)	After oxidation (mg.)	Amount (mg.)	Yield (%)
20	2.0	10.2	3.2	2.9	18
20	2.0	—	3.4	3.0	19
20	2.0	12.6	2.0	2.0	12
20	2.0	15.6	7.4	3.0	19
20	0	10.0	3.8	3.8	23
5	2.0	2.0	0.6	0.54	13

Hydrolysis of deaminized proteins

Ovalbumin was prepared from hens' eggs (Kekwick & Cannan, 1936), pseudoglobulin from sheep serum (Haurowitz, Sarafyan & Schwerin, 1941), haemoglobin from horse blood (Zilzer, 1926); in addition, casein (Merck, 'nach Hammarsten') and edestin (Schuchardt) were used. Aqueous solutions containing 5–10 % (w/v) of the protein and, if casein or edestin were used, small amounts of NaOH required for the dissolution of these substances, were mixed in a flask with 2 g. of sodium nitrite for 1 g. of protein. The air was displaced by CO₂, the solutions cooled to 1–2° and 50 ml. of N-HCl/g. of protein were added drop by drop. The mixture was kept at room temperature for 12 hr. with occasional shaking and the nitrous gases were then driven out by a current of CO₂. The insoluble deaminized proteins were washed with water. They are intensely yellow substances, which become dark brown when dried at 110°.

Their yellow colour indicates that nitro-groups are formed during the deamination.

Dry deaminized protein (0.5–2.0 g.) was boiled with 20 ml. of 23 % (w/w) HCl or 8N-sulphuric acid for 24 hr. When HCl was used, the excess was evaporated *in vacuo* and the residue dissolved in water. The hydrolyzed solutions were extracted continuously with ether, the ether-soluble fraction oxidized, and succinic acid determined as described in the first section. Control experiments were run with the original proteins, without previous deamination.

In some experiments the ether-soluble fraction, extracted from the hydrolysate, was titrated with 0.1 N-NaOH; equivalent weights between 34 and 122 were observed. The microscopical examination of the ether-soluble acids showed a mixture of crystalline and liquid substances. They dissolved easily in water.

The percentage of glutamic acid in pseudoglobulin was determined according to Olcott (1944). 4.0 mg. of hydrolyzed globulin gave 0.495 mg. of amino-N before, and 0.408 mg. of amino-N after, autoclaving.

RESULTS

The experiments recorded in Table 1 demonstrate that 5–20 mg. of α -hydroxyglutaric acid, when mixed with 2 g. of casein and boiled with HCl, furnish 12–24 %, on an average 18 %, of the theoretical yield of succinic acid. The same method was applied for the approximate estimation of α -hydroxyglutaric acid in hydrolysates of natural and deaminized proteins. Table 2 shows the amount of succinic acid, determined manometrically, and the equivalent amount of α -hydroxyglutaric acid. Since the average yield of succinic acid from α -hydroxyglutaric acid was 18 %, the 'corrected' figures of Table 2 have been obtained by multiplying by the factor $100/18 = 5.5$. These values are slightly higher for deaminized than for the original proteins. The excess of α -hydroxyglutaric acid, recorded in the last column of Table 2 is negligibly small in comparison with the large amounts of glutamic acid

Table 2. *Estimation of α -hydroxyglutaric acid in the hydrolysates of natural and of deaminized proteins*

(In the first two experiments sulphuric acid was used as hydrolyzing agent, in the other experiments hydrochloric acid.)

Protein	Weight of protein (g.)	Ether-soluble substance		Succinic acid found (mg.)	α -Hydroxyglutaric acid		
		Before oxidation (mg.)	After oxidation (mg.)		Equiv. amount (mg.)	Corrected value (mg.)	Excess (mg.)
Casein (native)	2.0	10.0	0.6	0.14	0.18	0.96	—
„ (deaminized)	2.0	9.8	1.5	0.39	0.49	2.7	1.7
Casein (native)	2.0	—	0.8	0.02	0.025	0.14	—
„ (deaminized)	2.0	3.1	1.4	0.17	0.23	1.3	1.2
Globulin (native)	2.0	11.6	4.4	0.13	0.16	0.89	—
„ (deaminized)	2.0	6.3	1.0	0.23	0.29	1.6	0.7
Ovalbumin (native)	2.0	3.1	0.6	0.07	0.09	0.48	—
„ (deaminized)	2.0	5.7	2.1	0.50	0.62	3.4	2.9
Haemoglobin (deaminized)	0.5	—	1.1	0	0	0	0
Edestin (deaminized)	0.5	—	1.8	0.11	0.14	0.75	<0.75

present in the same proteins (Table 3). It corresponds to less than one molecule of hydroxyglutaric acid per protein molecule (Table 3, last column). We conclude, therefore, that *neither glutaminy residues containing free α -amino-groups (formula (1)) nor analogous glutamyl residues (4) exist in the examined proteins.*

Table 3. *Molecular ratio of glutamic acid and α -hydroxyglutaric acid per molecule of deaminized protein*

Protein	Assumed molecular weight	Glutamic acid		Molecules of α -hydroxyglutaric acid per protein molecule
		%	Molecules	
Casein	100,000*	22.0†	150	0.38
Ovalbumin	45,000†	16.1‡	47	0.44
Globulin	175,000	24.2§	279	0.41
Edestin	50,000‡	20.7‡	71	0.47
Haemoglobin	69,000†	6.75‡	32	0

* Svedberg, Carpenter & Carpenter (1930).

† Svedberg (1934).

‡ Chibnall (1942).

§ Determined in our laboratory by Mrs Vedia Göksu.

Remarks on Table 3. The molecular ratio of α -hydroxyglutaric acid per molecule of deaminized protein has been calculated according to the formula $E \times M/1000W$, where E =excess of hydroxyglutaric acid in mg. (last column of Table 2), M =molecular weight of the protein and W =weight of the analyzed protein sample. The values thus calculated are 'corrected' values; actually only 18% of these quantities have been determined analytically.

The excess of succinic acid obtained from deaminized proteins was observed regularly, although in very small quantities. It might originate from a molecule of glutamic acid or of glutamine bound terminally, by means of its α -carboxyl group, to

the long peptide chain forming the protein molecule. But the possibility cannot be excluded that these small amounts of succinic acid arise by the oxidizing action of the previously mentioned nitro-compounds on glutamic acid set free during the hydrolysis of the deaminized proteins.

Our experiments give little support then to the assumption of multiple-free α -amino-groups in the protein molecule. If such groups were present, the hydrolysis of deaminized proteins should furnish a larger amount of ether-soluble α -hydroxyacids. Table 2 indicates, however, that approximately the same quantities of ether-soluble substances were obtained from the deaminized and the original proteins. Although this is contradictory to the assumption of multiple free α -amino-groups in the protein molecule, no definite conclusion can be drawn concerning the existence of these groups, for account must be taken of the fact that some α -hydroxyacids are unstable and might be destroyed by the action of boiling acids.

SUMMARY

1. A method for the approximate estimation of small quantities of α -hydroxyglutaric acid has been described.

2. Casein, ovalbumin, serum globulin, edestin and haemoglobin have been deaminized and then hydrolyzed. Nearly the same amount of ether-soluble substances has been obtained from the hydrolysates of the deaminized and of the original proteins. The ether-soluble fractions contain less than one molecule of α -hydroxyglutaric acid per protein molecule, a fact hardly reconcilable with the assumption of imide linkages, involving glutamine residues within the molecules of the examined proteins.

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