814. *The Oxidation of Proline, Hydroxyproline, and N-Methylglycine with Periodate.*

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Proline, hydroxyproline, and *N*-methylglycine react with periodate and undergo oxidative decarboxylation, proline giving Δ^1-pyrroline which is further oxidised to pyrrolid-2-one.

Structural investigations of the carbohydrate components of ovomucoid and ovalbumin have revealed that the mucoproteins react with more sodium metaperiodate than can be accounted for by the carbohydrate alone.[1] Similar results were obtained with starch–protein mixtures containing more than 23% of protein.[2] The protein of the jelly-coat substance of *Echinocardium cylindrica* is oxidised by periodate at the cysteine, cystine, tryptophan, and tyrosine residues.[3] Ovalbumin was oxidised with similar results.[4] The reactions of various amino-acids with periodate have been examined and at room temperature serine, threonine, methionine, and cystine were rapidly oxidised.[5] At 100°, other amino-acids reacted with the formation of aldehydes by processes involving oxidative decarboxylation and deamination, but secondary reactions were also significant.[6] Examination of mucoprotein hydrolysates on paper chromatograms using a periodate–*p*-anidisine method for the detection of the carbohydrates, revealed that hydroxyproline was rapidly oxidised by periodate. The periodate–*p*-anisidine method of detection was devised as an alternative to the periodate–benzidine [7] and periodate–starch iodide [8] spray reagents.

The reaction of hydroxyproline with periodate prompted a quantitative study of this and other amino-acids with sodium metaperiodate at room temperature and in the dark. Glycine, alanine, phenylalanine, *N*-acetylglycine, and glycylglycine showed no significant reaction: histidine, proline, and hydroxyproline were rapidly oxidised and *N*-methylglycine (sarcosine) also consumed periodate at a slow but significant rate. The pK values for proline, hydroxyproline, and *N*-methylglycine (10·01, 10·60, and 9·73 respectively) reveal that they are more basic than the other common amino-acids,[9] suggesting that the imino-groups are involved in the reaction and that the rate of oxidation of the amino-acid depends upon the basicity of this group.

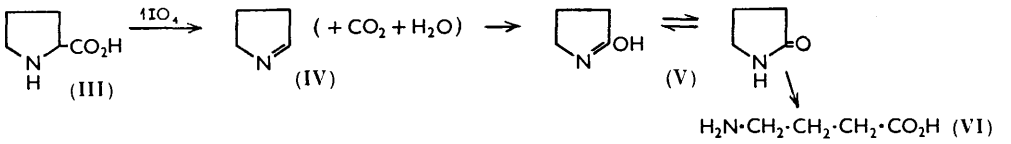
N-Methylglycine (I) reacted with 1 mol. of periodate in *ca.* 70 hr. with the formation of carbon dioxide and formaldehyde, in agreement with oxidation to the *N*-methylene derivative (II) and carbon dioxide, followed by hydrolysis to formaldehyde and methylamine.

$$\underset{\text{(I)}}{Me{\cdot}NH{\cdot}CH_2{\cdot}CO_2H} \xrightarrow{{}_1IO_4} \underset{\text{(II)}}{Me{\cdot}N{:}CH_2} (+ CO_2 + H_2O) \longrightarrow Me{\cdot}NH_2 + CH_2O$$

The reaction of proline (III) with periodate (2 mol.) occurred in two stages. The first mol. was consumed rapidly (3 hr.) with concomitant release of 1 mol. of carbon dioxide, whereas the second mol. of oxidant reacted slowly (24 hr.); no acid was liberated. The second stage was pH-dependent since at pH 2·2 only 1 mol. of oxidant was consumed, whereas 2 mol. were consumed at pH 10·2. Ether-extraction of an equimolar mixture of proline and periodate afforded Δ^1-pyrroline (IV). This product was characterised by the formation of a complex with mercuric chloride in dilute aqueous solution [10] and by catalytic

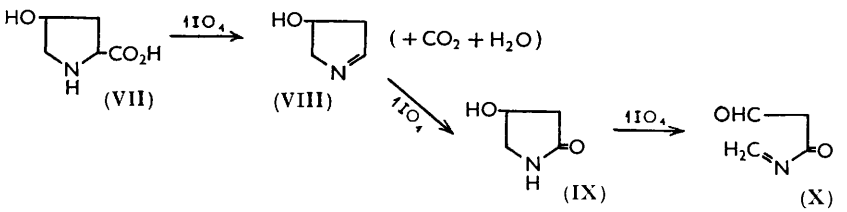
[1] Bragg and Hough, unpublished results.
[2] Anderson, Greenwood, and Hirst, *J.*, 1955, 225.
[3] Vasseur, *Acta Chem. Scand.*, 1952, **6**, 376.
[4] Maekawa and Kushibe, *Bull. Chem. Soc. Japan*, 1954, **27**, 277; *Chem. Abs.*, 1955, **49**, 9698.
[5] Nicholet and Shinn, *J. Amer. Chem. Soc.*, 1939, **61**, 1615; *J. Biol. Chem.*, 1941, **139**, 687.
[6] Fleury, Courtois, and Grandchamp, *Bull. Soc. chim. France*, 1949, **16**, 88.
[7] Cifonelli and Smith, *Analyt. Chem.*, 1954, **26**, 1132.
[8] Metzenberg and Mitchell, *J. Amer. Chem. Soc.*, 1954, **76**, 4187.
[9] "Amino Acids and Proteins," ed. D. M. Greenberg, Charles C. Thomas, Springfield, Illinois, U.S.A., 1951, p. 430.
[10] Langheld, *Ber.*, 1909, **42**, 2360; Krimm, Ph.D. Thesis, Darmstadt, 1950.

hydrogenation to pyrrolidine which was identified as the characteristic *N*-toluene-*p*-sulphonyl derivative.[11] The pyrroline readily polymerised, a property typical[10] of Δ^1-pyrroline (IV). Contrary to previous belief, Murray and Cloke[12] have shown that Δ^1- and Δ^2-pyrrolines are not in tautomeric equilibrium. Since glycylproline was not oxidised by periodate it follows that the imino-nitrogen atom of proline must be unsubstituted for oxidation to occur. Thus the first stage in the reaction of periodate with proline involves oxidative decarboxylation to Δ^1-pyrroline (IV). The subsequent product from the oxidation of Δ^1-pyrroline (IV) was identified as pyrrolid-2-one (V) by isolation in good yield as the hydrochloride and by acid-hydrolysis to γ-aminobutyric acid[13] (VI). At pH 10·2 pyrrolid-2-one (V) appeared to be the sole product after the consumption of 2 mol. of periodate, whereas at pH 2·2 only a trace (<1%) was found. Pyrrolid-2-one



and pyrrolidine were not oxidised by periodate. The conversion of Δ^1-pyrroline (IV) into pyrrolid-2-one (V) represents a novel type of periodate oxidation and, since the reaction was impeded in acid, quaternary ammonium salt formation was probably responsible.

In unbuffered solution hydroxyproline (VII) was extensively oxidised by 4 mol. of periodate (24 hr.) in agreement with the results of Carter and Loo,[14] with the formation of 1·33 equivalents of acid, 0·44 mol. of ammonia, 0·83 mol. of formaldehyde and 1·9 mol. of carbon dioxide. The first mol. of carbon dioxide appeared early in the reaction (2 hr.), suggesting that the first step, as with proline, was oxidative decarboxylation to hydroxypyrroline (VIII). At pH 2, only 3 mol. of periodate reacted with the liberation of 0·95 mol. of carbon dioxide, but no formaldehyde was detected. An acid-stable *N*-methylene derivative may have been formed by oxidative cleavage of 4-hydroxypyrrolid-2-one (IX).



Various routes could be suggested for the oxidation of hydroxyproline, but further evidence is required to elucidate the pathway subsequent to oxidative decarboxylation. The periodate reaction differs, however, from the action on hydroxyproline of either hydrogen peroxide in the presence of alkaline copper sulphate or catalase and amino-acid oxidase, both of which led to the formation of pyrrole-2-carboxylic acid.[15] This acid did not react with periodate and was not detected in the periodate oxidation mixtures by the sensitive paper-chromatographic technique.

Experimental

Quantitative Measurements.—The amino-acid (*ca.* 20 mg.; accurately weighed) was dissolved in water (50 ml.), then dilute sulphuric acid (50 ml.) or borate buffer (50 ml.; pH 10·2) and 0·3M-sodium metaperiodate solution (3 ml.) were added. Aliquot portions (5 ml.) were removed

[11] Mann and Smithies, *Biochem. J.*, 1955, **61**, 89.
[12] Murray and Cloke, *J. Amer. Chem. Soc.*, 1946, **68**, 126.
[13] Tafel and Stern, *Ber.*, 1900, **33**, 2224.
[14] Carter and Loo, *J. Biol. Chem.*, 1948, **174**, 723.
[15] Radhakrishnan and Meister, *Fed. Proc.*, 1956, **15**, 333; *J. Biol. Chem.*, 1957, **226**, 559; Witkop and Beiler, *J. Amer. Chem. Soc.*, 1956, **78**, 2882.

TABLE 1. *Periodate uptake (moles of periodate per mole of substance).*

Time of oxidn. (hr.)	Proline			Hydroxyproline			Histidine hydrochloride		*N*-Methylglycine
	NB *	pH 2·2	pH 10·2	NB *	pH 2·0	pH 8·5	NB *	pH 2·0	NB *
0·5	0·33	0·26	0·26	1·3	1·0	0·7	0·19	0·19	0·11
1	0·42	0·31	0·51	1·5	1·15	0·99	0·38	—	0·12
1·5	—	—	—	—	—	1·33	—	—	—
2·5	—	0·75	1·04	—	1·73	1·84	—	—	—
3	1·03	—	—	2·2	—	—	0·95	0·49	—
5·8	—	—	—	—	2·68	—	—	—	—
6·4	—	—	—	3·5	—	—	—	—	—
7·5	—	1·08	1·77	—	—	—	—	—	—
16·5	—	—	—	—	—	—	—	—	0·47
22	—	—	—	—	—	—	—	0·83	—
24	1·95	0·98	1·97	4·1	2·98	—	2·18	—	—
43	—	—	—	—	—	—	—	—	0·79
48	2·01	—	—	4·1	2·98	—	4·05	—	—
67	—	—	—	—	—	—	—	1·0	0·97
89	—	—	—	—	—	—	—	—	1·09
96	—	—	—	—	—	—	—	1·07	—

* NB = not buffered.

TABLE 2. *Carbon dioxide produced (moles of CO_2 per mole of substance).*

Time of oxidn. (hr.)	*N*-Methylglycine NB,* 37°	Proline		Hydroxyproline		
		NB * (25°)	In 0·2N-H_2SO_4 (25°)	NB * (20°)	In 0·01N-H_2SO_4 (25°)	NB * (37°)
0·25	—	0·18	0·04	0·20	0·13	—
0·5	0·07	0·28	0·08	0·39	0·31	—
1	0·13	0·48	0·16	0·63	0·55	—
1·5	0·16	—	—	0·80	—	—
2	0·18	0·66	0·29	0·95	0·79	—
2·5	0·19	—	—	—	0·83	—
3	0·22	0·77	—	1·17	—	—
3·5	0·26	—	—	—	0·89	—
4	0·33	0·83	0·50	1·25	—	1·9
4·5	0·38	—	—	—	0·93	—
5	—	—	0·61	1·33	0·95	1·9
6	—	—	0·66	1·36	—	—
7	—	—	0·71	1·39	0·95	—
13	—	0·99	—	—	—	—

* NB = not buffered.

for the determination of the periodate consumption [16] and in the case of unbuffered solution for the estimation of acid liberated.[2] Results are in Table 1.

Glycine, alanine, phenylalanine, *N*-acetylglycine, glycylglycine, glycylproline, pyrrolid-2-one, pyrrole-2-carboxylic acid and pyrrolidine did not react with sodium metaperiodate. Histidine hydrochloride yielded 0·27 equivalent of acid after oxidation for 48 hr.

N-Methylglycine (20 mg.), proline (0·36 mg.), and hydroxyproline (0·53 mg.) were separately oxidised with 0·03M-sodium metaperiodate (0·5 ml.), and the liberated carbon dioxide was determined in a Warburg apparatus [17] (see Table 2).

A solution of hydroxyproline (10 mg.) in 0·3M-sodium metaperiodate solution (6 ml.) was made up with water to 50 ml. and kept for 18 hr. in the dark. Aliquot portions (0·5 ml.) were removed and the ammonia contents determined by the Conway method.[18] After 18 hr., 0·44 mole of ammonia per mole of hydroxyproline was found.

An aqueous solution of hydroxyproline (0·53 mg.), 0·1N-*p*-hydroxybenzaldehyde (1 ml.), and 0·3M-sodium metaperiodate (2 ml.) was made up to 25 ml. and set aside for 17 hr. Aliquot portions (1 ml.) were taken for formaldehyde estimation by the chromotropic acid method.[19] After 17 hr., 0·83 mole of formaldehyde per mole of hydroxyproline was found. An oxidation was carried out at pH 2·0 for 15 hr.; after removal of periodate formaldehyde was absent as shown by Schryver's method (phenylhydrazine–ferricyanide reagents [20]). Another sample was

[16] Neumüller and Vasseur, *Arkiv Kemi, Min., Geol.*, 1953, **5**, 235.
[17] Dixon, "Manometric Methods," Cambridge University Press, 1953.
[18] Conway, "Micro-diffusion Analysis and Volumetric Error," Crosby Lockwood, London, 1950.
[19] O'Dea and Gibbons, *Biochem. J.*, 1953, **55**, 580.
[20] Hough, Powell, and Woods, *J.*, 1956, 4799.

treated with a solution saturated with barium chloride and sodium hydrogen carbonate (4 : 1 respectively) to remove periodate,[20] and the filtrate kept at pH 7·5 for 17 hr.: formaldehyde was again absent.

Oxidation of Proline.—(1) *Characterisation of pyrroline.* An aqueous solution (30 ml.) of L-proline (0·25 g.) was mixed with 0·3M-sodium metaperiodate solution (7 ml.) and kept in the dark for 2·5 hr. After saturation with potassium carbonate, the solution was continuously extracted with ether for 24 hr. The ethereal extract was concentrated to *ca.* 5 ml., then mixed with ethanol (15 ml.) and Adams catalyst (135 mg.), and the mixture shaken in hydrogen for 8 hr. at room temperature and atmospheric pressure. Consumption of hydrogen by the catalyst being excluded, *ca.* 10 ml. of hydrogen were consumed during the reaction. The catalyst was filtered off, and the filtrate acidified with concentrated hydrochloric acid (1 ml.) and evaporated under reduced pressure to a syrup (170 mg.). The toluene-*p*-sulphonyl derivative was then prepared and crystallised from light petroleum–methanol as needles (22 mg.), m. p. and mixed m. p. with *N*-toluene-*p*-sulphonylpyrrolidine, 120° (Found: C, 58·9; H, 6·5; N, 5·6. Calc. for $C_{11}H_{15}O_2NS$: C, 58·7; H, 6·7; N, 6·2%).

0·3N-Sodium metaperiodate (35 ml.) was added to proline (0·5 g.) in sulphuric acid (pH 2·0; 40 ml.) and the mixture kept for 24 hr. in the dark. Excess of periodate was precipitated by the addition of a solution saturated with barium chloride and sodium hydrogen carbonate (4 : 1).[20] The filtrate was saturated with potassium carbonate and continuously extracted with ether for 24 hr. The ethereal extract was mixed with aqueous methanol and concentrated under reduced pressure to *ca.* 10 ml. The resulting aqueous solution was exactly neutralised (2N-hydrochloric acid), and an equal volume of saturated aqueous mercuric chloride solution was added: a white complex (*ca.* 75 mg.) was precipitated. After being washed with ethanol and ether, the product was dried (P_4O_{10}) under reduced pressure (Found: C, 13·5; H, 2·2; N, 3·4. Calc. for $C_4H_7NCl_2Hg$: C, 14·0; H, 2·1; N, 4·1%).

(2) *Characterisation of pyrrolid-2-one.* (*a*) Unbuffered solution. A mixture of L-proline (0·26 g.) in water (10 ml.) and 0·3M-sodium metaperiodate solution (30 ml.) was set aside for 24 hr. in the dark. An ether extract, prepared as above, was mixed with concentrated hydrochloric acid (1 ml.) and concentrated under reduced pressure. The residue was then evaporated several times with water (*ca.* 5 ml. portions) to remove excess of hydrochloric acid, and finally with methanol (10 ml.). The product (0·16 g.) was sublimed under reduced pressure at 100° to give white needles, m. p. 88°, which gave an *X*-ray powder photograph identical with that of authentic pyrrolid-2-one hydrochloride (Found: C, 46·0; H, 7·4; N, 13·2; Cl, 16·8. Calc. for $C_8H_{15}N_2O_2Cl$: C, 46·5; H, 7·3; N, 13·6; Cl, 17·2%).

A mixture of L-proline (0·25 g.) and 0·3M-sodium metaperiodate solution (30 ml.) was kept for 5 hr., and after near-saturation with potassium carbonate the solution was continuously extracted with ether. The ether extract was evaporated almost to dryness, then the residue was heated at 100° under reflux for 2·5 hr. with either 50% sulphuric acid (5 ml.) or concentrated hydrochloric acid (5 ml.). The neutralised solution (barium or silver carbonate respectively) was concentrated to a syrup (90 mg.) which readily crystallised, having m. p. and mixed m. p. with γ-aminobutyric acid, 195°. An *X*-ray powder photograph of the product was identical with that of an authentic specimen.

(*b*) At pH 10·2. A mixture of L-proline (0·25 g.), 0·1M-boric acid (10 ml.), 0·1N-sodium hydroxide (10 ml.), and 0·3M-sodium metaperiodate (30 ml.) was kept in the dark for 20 hr. Pyrrolid-2-one hydrochloride (0·21 g.) was isolated as above (Found: C, 46·6; H, 7·5; N, 13·8%).

(*c*) At pH 2·1. The above procedure when repeated with proline (0·25 g.) at pH 2·1 (adjusted with 0·01N-sulphuric acid) yielded a syrup which on sublimation gave pyrrolid-2-one hydrochloride (*ca.* 1 mg.).

Periodate–p-Anisidine Spray Reagents.—Substances reacting rapidly with sodium metaperiodate were detected by thinly spraying the chromatograms with 0·01M-sodium metaperiodate,[7,8] followed after 2 min. by *p*-ansidine or *p*-anisidine hydrochloride (1% solution in butan-1-ol). White spots on a pink background appeared within 1—2 min. if periodate-reacting substances were present. This method will detect polyols at a concentration of 25×10^{-6} g.

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