

107. *The Stereochemistry of Hydroxyproline.*

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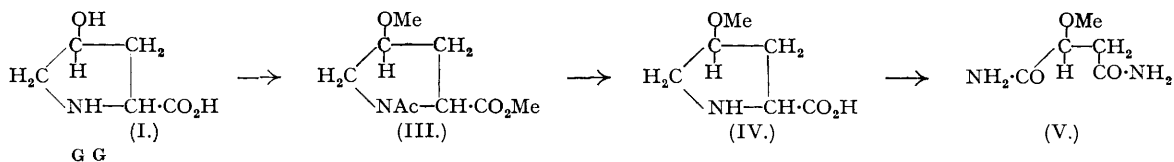
l-(-)-Hydroxyproline (I) was converted into its *O*-methyl derivative, which was oxidised to *d*-(+)-methoxysuccinic acid. The absolute configurations of both asymmetric carbon atoms in *l*-(-)- and *d*-(+)-hydroxyproline are now established. The configuration of C₄ in (I) has been inverted through saponification of the *O*-toluenesulphonyl ester, and *l*-(-)-allohydroxyproline (VIII) thus produced. This transformation permits absolute configurations to be also assigned to both asymmetric carbon atoms of allohydroxyproline.

HYDROXYPROLINE was first isolated from gelatin by Fischer (*Ber.*, 1902, **35**, 2660) and later from several other proteins. Fischer showed that the new imino-acid was structurally related to proline by reducing it thereto with hydriodic acid and phosphorus in a sealed tube. Under these drastic conditions only racemic proline was obtained and a configurative relationship between the two compounds could therefore not be established. The position of the hydroxyl group was ascertained by Leuchs and Brewster (*Ber.*, 1913, **46**, 986), who resolved one of the two racemic 4-hydroxyprolines prepared earlier by Leuchs (*Ber.*, 1905, **38**, 1907); these authors showed that the laevorotatory isomeride was identical with the imino-acid obtained by Fischer from proteins. The second racemic product, which was called "oxy-proline b" by Leuchs but may be more conveniently named allohydroxyproline, was also resolved by Leuchs and Bormann (*Ber.*, 1919, **52**, 2086) and thus all the four optically active and the two racemic forms of 4-hydroxyproline have been synthesised. 4-*allo*-Hydroxyproline was recently isolated from phalloidine, the highly toxic peptide obtained from *Amanita phalloides* (Wieland and Witkop, *Annalen*, 1940, **543**, 171), though it appears to be absent from proteins.

It is believed that all amino-acids obtained from proteins have the same absolute configuration on the carbon atom carrying the amino- or imino-group and that this configuration is identical with that of *l*-(+)-lactic acid or *l*-(-)-glycerose. The first statement is based on the many chemical transformations which have been performed between different optically active amino-acids through reactions not involving the asymmetric carbon atom, and also on the fact that changes in rotation occurring on ionisation (Lutz and Jirgensons, *Ber.*, 1930, **63**, 448), acylation, and esterification (Clough, J., 1918, **113**, 526) are in the same direction for all amino- or imino-acids isolated from proteins. The relationship to lactic acid is based on a similar comparison of rotational displacements brought about by different substituents in alanine and lactic acid (Freudenberg and Rhino, *Ber.*, 1924, **57**, 1547; Freudenberg and Meister, *Annalen*, 1935, **518**, 86). (-)-Proline and (-)-hydroxyproline are configuratively related, as shown by Kaneko (*J. Chem. Soc. Japan*, 1940, **61**, 207; *Chem. Abstr.*, 1943, **37**, 117), who converted (I) into 4-chloroproline, which could be reduced to (-)-proline. That both these laevorotatory imino-acids are also stereochemically related to the *l*-amino-acids found in proteins follows from a number of observations. For instance, *d*-amino-acid oxidase, which is known to be stereochemically specific, attacks only (+)proline (Krebs, *Enzymologia*, 1939, **7**, 53), whereas the *l*-amino-acid oxidase from *Proteus vulgaris* (Stumpf and Green, *J. Biol. Chem.*, 1944, **153**, 387) and a similar enzyme present in animal tissue oxidise *l*-(-)proline only (Blanchard, Green, Nocito, and Ratner, *ibid.*, **155**, 421). Also, the changes in rotation which occur on addition of acid and alkali to (I) and (-)proline are analogous to those observed with other *l*-amino-acids (Lutz and Jirgensons, *Ber.*, 1931, **64**, 1221). Moreover, Kaneko (*loc. cit.*) claimed to have oxidised *l*-(-)hydroxy-*N*-acetylproline to *l*-(+)-aspartic acid, an observation which could not, however, be repeated in this laboratory.

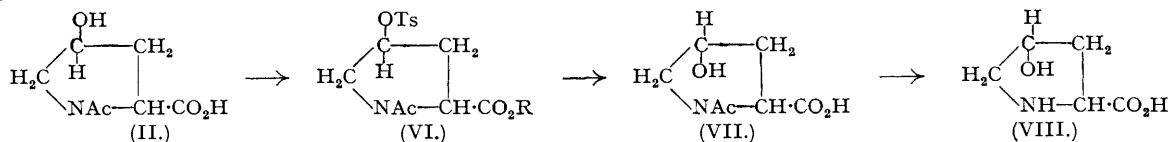
The configuration of C₄ in (I) is still doubtful. Kaneko (*loc. cit.*) oxidised 4-chloroproline obtained from (I) to (-)chlorosuccinic acid, which is assumed to have the *l*-configuration (Freudenberg and Luchs, *Ber.*, 1928, **61**, 1083; Kuhn and Wagner-Jauregg, *ibid.*, p. 508). Kaneko assumed that a Walden inversion had taken place during the treatment of (I) with phosphorus pentachloride and that C₄ had a *d*-configuration. The supposition that replacement of a secondary alcoholic hydroxyl group by chlorine through treatment with phosphorus pentachloride invariably involves an inversion of configuration is unwarranted, as the varying results found with thionyl chloride indicate. Whether or not inversion is associated with chlorination will depend on the mechanism or kinetics of the reaction (Cowdrey, Hughes, Ingold, Masterman, and Scott, *J.*, 1937, 1252).

In the work now described the *d*-configuration of C₄ in (I) is shown by a series of reactions not involving this asymmetric carbon atom and therefore not likely to cause inversion. (I) was acetylated on the imino-group by Kolb and Toennies's method (*J. Biol. Chem.*, 1942, **144**, 193) to yield *l*-(-)-hydroxy-*N*-acetylproline (II); this compound was methylated as described by Syngé (*Biochem. J.*, 1939, **33**, 1931) and *l*-(-)-hydroxy-*N*-acetyl-*O*-methylproline methyl ester obtained. This ester was hydrolysed first with alkali and then with acid to yield 4-methoxyproline (IV), which resembles proline in its general properties. This was oxidised with permanganate to *d*-(+)-methoxysuccinic acid, which was converted successively into the dimethyl ester and diamide (V).



The diamide (V) has a specific rotation equal in magnitude but opposite in sign to that reported for (–)methoxysuccinamide (McKenzie and Smith, J., 1922, 121, 1348) obtained from (–)malic acid. The identity of (V) was further demonstrated by preparing the levorotatory amide from natural (–)malic acid and crystallising equal quantities of this and the dextrorotatory compound from alcohol. An optically inactive compound of m. p. 177° was obtained. Purdie and Marshall (J., 1891, 59, 470) give m. p. 175°. Now, (–)malic acid has been shown by Freudenberg and Brauns (*Ber.*, 1922, 55, 1339) to have the *l*-configuration. It follows, therefore, that the asymmetric carbon atoms in (V) and C₄ in *l*-(–)hydroxyproline have the *d*-configuration. *d*-(+)Hydroxyproline must have the *d*-configuration at C₂ and the *l*- at C₄.

The configuration of the two asymmetric centres in *allo*hydroxyproline was ascertained in the following manner. The *N*-acetyl derivative (II) was methylated with diazomethane, and the resulting *ester* treated with toluene-*p*-sulphonyl chloride and pyridine to give *l*-(–)hydroxy-*N*-acetyl-*O*-toluene-*p*-sulphonylproline methyl ester (VI; R = Me). The methoxy group was removed by saponification with 1 equiv. of sodium hydroxide in the cold and the free acid (VI; R = H) was obtained; this acid on 20 mins.' heating with 2 equivs. of alkali at 100° gave an *acetimino-acid* (VII) which differed from (II) by its m. p., specific rotation, and the fact that it crystallised in the anhydrous state. On boiling with dilute acid (–)*allo*hydroxyproline (VIII) was obtained; its specific rotation was identical with that of the compound isolated by Wieland and Witkop (*loc. cit.*) from phalloidine and also with that found by Leuchs and Bormann (*loc. cit.*) for one of the two isomerides obtained by resolution. It appears, therefore, that in one of these reactions an inversion of one of two asymmetric centres has taken place. It cannot have occurred in the acetylation and sulphonation, since these reactions do not involve the asymmetric centres and have never been shown in analogous cases to involve inversion; also the last reaction, *i.e.*, acid hydrolysis of the acetimino-acid, must also be presumed to proceed with retention of configuration. It follows that inversion must have occurred during the alkaline fission of the toluenesulphonyl group and it is assumed that it has taken place on C₄. The alternative explanation that the configuration of C₂ has been inverted is unlikely, since *l*-(–)hydroxy-*N*-acetylproline exposed to identical conditions—heating with 2 equivs. of alkali—is recovered unchanged in good yield. The assumption that the treatment of (VI) with alkali causes inversion on C₄ is in accordance with other observations; *e.g.*, Houssa, Kenyon, and Phillips (J., 1929, 1700), and Kenyon, Phillips, and Taylor (J., 1933, 173) have shown that anionic reagents acting on toluenesulphonates of active alcohols may cause an inversion, although this is not invariably the case (Kenyon, Phillips, and Turley, J., 1925, 127, 399). This interpretation which assumes an *l*-configuration at C₂ for (VIII) is also supported by the observation that the specific rotation of (VIII) becomes more positive on addition of acid; this behaviour is typical for *l*-amino-acids as shown by Lutz and Jirgensons (*loc. cit.*).



The absolute configurations of all four optically active isomerides are thus established and are set out in this table; the prefix *l* or *d* refers to the configuration of C₂, since the configuration of this carbon atom appears to be the more important in biological reactions.

Compound.	[α] _D .	Configuration.		Compound.	[α] _D .	Configuration.	
		C ₂ .	C ₄ .			C ₂ .	C ₄ .
<i>l</i> -(–)Hydroxyproline	–76.3°	<i>l</i>	<i>d</i>	<i>l</i> -(–) <i>allo</i> Hydroxyproline	–58°	<i>l</i>	<i>l</i>
<i>d</i> -(+)Hydroxyproline	+75.2	<i>d</i>	<i>l</i>	<i>d</i> -(+) <i>allo</i> Hydroxyproline	+58.6	<i>d</i>	<i>d</i>

The relative positions of the carboxyl and hydroxyl groups to each other cannot be easily deduced from their configurations. Wieland and Witkop (*loc. cit.*) found that, whilst (I) gave a colour reaction with isatin in glacial acetic acid, the *allo*-compound did not react under these conditions. These authors deduced from this observation that (VIII) is transformed into a lactone in the acid medium used and does not therefore react as an imino-acid. A similar difference in behaviour, however, is found if the reaction is carried out at pH 7.4, where lactone formation is highly improbable. Moreover, neither the acetyl compound (VII) nor the hydrochloride of (VIII) shows any tendency to form a lactone; for instance, (VII) is recovered unchanged if heated just below its melting point in a vacuum or if kept at 100° in glacial acetic acid. The difference in the behaviour of the two compounds must therefore be ascribed to other factors.

EXPERIMENTAL.

Preparation of l-(–)Hydroxyproline.—Kapfhammer and Eck's method (*Z. physiol. Chem.*, 1927, 170, 294) was modified in that histidine and lysine were removed before precipitation of proline and (I) as reineckates, for unless this was done the mixed reineckates were dirty and contaminated by the insoluble salts of histidine and lysine (*cf.* Kapfhammer and Spoerer, *Z. physiol. Chem.*, 1928, 173, 245; Grassmann and Land, *Biochem. Z.*, 1934, 269, 223). In the modified procedure gelatin is hydrolysed with hydrochloric acid, and arginine removed with flavianic acid as described by Kapfhammer and Eck (*loc. cit.*); the filtrate is then diluted in such a manner that the equivalent of 100 g. of gelatin is contained in 2.5 l. of solution; a concentrated solution of 100 g. of phosphotungstic acid (A.R.) per 100 g. of protein is then added slowly at 70°, and the mixture left for 18 hrs. at room temperature. The solid is then collected and used for the preparation of lysine and histidine. Excess phosphotungstic and flavianic acids are then extracted by a mixture of aqueous

ammonia and ether. The resulting colourless solution is concentrated to small volume, and proline and (I) are precipitated with ammonium reineckate as described by Kapfhammer and Eck (*loc. cit.*): The dilution of the solution before the phosphotungstic acid precipitation is necessary in order to prevent precipitation of proline, which forms a fairly insoluble phosphotungstate (van Slyke, Hiller, and Dillon, *J. Biol. Chem.*, 1942, **146**, 137). The decomposition of the reineckates is carried out as described by the German workers, but their separation of (I) from proline leads to a product which is contaminated with proline. The crude (I) (alcohol-insoluble fraction) is taken up in a small quantity of hot water and precipitated by addition of 5 vols. of absolute alcohol. This recrystallisation is repeated once more. The yield of pure (I) is about 65 g. per kg. of air-dry gelatin. The specific rotation of the product varied between -76.0° and -76.5° ($c = 2.5$; $l = 2.0$). Most values in the literature are higher: Fischer (*loc. cit.*) gives $[\alpha]_D - 81.04^\circ$, Klabunde (*J. Biol. Chem.*, 1931, **90**, 293) $[\alpha]_D - 80.5^\circ$, and Kapfhammer and Eck (*loc. cit.*) -80.6° . On the other hand, Leuchs and Bormann found for their synthetic product $[\alpha]_D - 75.7^\circ$ and Dunn ("Handbook of Chemistry and Physics," 28th ed., 1944, p. 1373) reports a value of -75.2° . It is believed that the higher figures obtained are due to contamination with proline. Proline was recovered from the mother-liquors as its picrate.

Oxidation of l(-)Proline to Succinic Acid.—To a solution of *l(-)*proline (2.3 g.) in water (20 ml.) was added during 20 mins. a solution of potassium permanganate (8.43 g.) in water (200 ml.), whilst the mixture was stirred mechanically. The solution was then heated to 70° and left at that temperature for 10 mins., cooled, and filtered. The precipitate was well washed with boiling water, the combined filtrates made acid with 2*N*-hydrochloric acid (40 ml.), and concentrated to dryness. The residue was taken up in alcohol, insoluble material removed, the solution concentrated to dryness in a vacuum, and the residue dissolved in ether. On concentration of the ethereal solution and addition of light petroleum (b. p. $30-40^\circ$) a crystalline precipitate was obtained which was filtered off, dried, and shown to be succinic acid (m. p. and mixed m. p. 185°) (Found: C, 40.8; H, 4.9; equiv., 60.4. Calc. for $C_4H_6O_4$: C, 40.7; H, 5.1%; equiv., 59); yield 30%.

4-Methoxy-l(-)proline (IV).—The acid (III) (Synge, *loc. cit.*) (3.55 g.) was suspended in 0.33*N*-baryta (175 ml.), and the mixture kept for 3 hrs. at 18° . Barium was then removed exactly with sulphuric acid, and the solution concentrated in a vacuum to about 40 ml. 5*N*-Sulphuric acid (25 ml.) was then added, and the solution heated under reflux for 3 hrs. Sulphuric acid was removed, and the solution evaporated to dryness. The crystalline residue was dissolved in anhydrous alcohol and crystallised by addition of anhydrous ether. The product (Found: C, 49.5; H, 7.7; N, 9.5. $C_6H_{11}O_3N$ requires C, 49.7; H, 7.6; N, 9.7%) (yield 74%) had, after recrystallisation from alcohol-ether, m. p. 202° , $[\alpha]_D 56.0^\circ$ ($c = 1.0$; $l = 2.0$); it was very hygroscopic, easily soluble in water and methyl and ethyl alcohol; but insoluble in ether; on heating with ninhydrin in the presence of a trace of pyridine a deep purple colour was obtained.

Oxidation of 4-Methoxy-l(-)proline to d(+)-Methoxysuccinic Acid.—1.31 G. of (IV) were dissolved in 25 ml. of water and treated with potassium permanganate (3.8 g.) as described above for proline. The solution was concentrated to dryness after acidification with 2*N*-hydrochloric acid (15 ml.), and the residue repeatedly extracted with ether. The combined ethereal extracts were dried and evaporated to dryness; the oily residue failed to crystallise. It was taken up in ether and treated at -10° with an ethereal solution of diazomethane, prepared from 4.2 g. of nitrosomethylurea; the solution was left at 0° for 18 hrs., concentrated under reduced pressure, and the residue distilled. The fraction which distilled at $110/20$ mm. was collected, and dissolved in methyl alcohol which had been saturated with ammonia (20 ml.). The solution was left for 3 days and then evaporated to dryness. The residue was crystallised and recrystallised from a small quantity of boiling alcohol; it had m. p. 180° , $[\alpha]_D 40.7^\circ$ ($c = 2.0$ in water), $[\alpha]_D + 55.9^\circ$ ($c = 1.0$ in methyl alcohol) (Found: C, 40.9; H, 6.6; N, 19.2. Calc. for $C_5H_{10}O_3N_2$: C, 41.1; H, 6.85; N, 19.2%); yield 35%. Purdie and Neave (*J.*, 1910, **97**, 1517) give the following values for *l(-)*methoxysuccinamide: m. p. 178° ; $[M]_D$ (water) -58.8° , $[M]_D$ (methyl alcohol) -83° , whence $[\alpha]_D - 40.3^\circ$ and -56.8° , respectively.

dl-Methoxysuccinamide.—*l(-)*Malic acid (Merck) was recrystallised from ether by addition of light petroleum (b. p. $30-40^\circ$). Its optical purity was estimated by measuring the specific rotation of its molybdate complex. The rotation of a solution containing 0.2233 g. of *l(-)*malic acid and 0.5152 g. of ammonium molybdate was $[\alpha]_D + 564^\circ$. McKenzie and Plenderleith (*J.*, 1923, **123**, 1090) give for malic acid under identical conditions $[\alpha]_D + 568^\circ$.

4.0 G. of *l(-)*malic acid were esterified in ether with diazomethane prepared from 10 g. of nitrosomethylurea, and the ester distilled in a vacuum. It was then methylated with methyl iodide and silver oxide and 3 g. of this ester were treated with methyl-alcoholic ammonia, and the amide crystallised and purified as described above. It had m. p. 180° , $[\alpha]_D - 56.9^\circ$ (in methyl alcohol).

100 Mg. each of *l(-)*methoxysuccinamide and of (V) were dissolved in a small quantity of boiling alcohol. On cooling, a crystalline substance was obtained, which was filtered off and dried. It had m. p. 177° and was optically inactive. Purdie and Marshall (*loc. cit.*) give m. p. 175° for *dl*-methoxysuccinamide.

l(-)Hydroxy-N-acetylproline Methyl Ester.—5.5 G. of the acetimino-acid (II) were dissolved in dry dioxan (100 ml.) and treated at -10° with a dry, freshly distilled ethereal solution of diazomethane prepared from 12.5 g. of nitrosomethylurea. The mixture was left at 0° for 4 hrs. and then evaporated in a vacuum to dryness. Traces of dioxan were completely removed by drying in a desiccator over phosphoric oxide; the oil slowly crystallised (yield, 95%). The substance was recrystallised by careful addition of dry ether to a solution of the ester in alcohol and had m. p. 78° (Found: 51.1; H, 6.95; N, 7.4. $C_8H_{13}O_4N$ requires C, 51.3; H, 6.95; N, 7.5%).

l-Hydroxy-N-acetyl-O-toluene-p-sulphonylproline Methyl Ester (VI; R = Me).—9.5 G. of the above ester were dissolved in dry pyridine (15 ml.), and the solution cooled to -10° . Toluene-*p*-sulphonyl chloride (10.1 g.) was then added, the mixture kept at 0° for 18 hrs., poured into ice-cold *N*-hydrochloric acid (145 ml.), and left for 20 hrs. at 0° . The crystalline material was collected and dried (yield 78%). The substance, which was easily soluble in alcohol and chloroform, was recrystallised from ether and had m. p. 60° .

l-Hydroxy-N-acetyl-O-toluene-p-sulphonylproline (VI; R = H).—13 G. of the above ester were dissolved in methyl alcohol (25 ml.), and *N*-sodium hydroxide (38.5 ml.) added. The solution was kept at 0° for 18 hrs., and *N*-hydrochloric acid (38.5 ml.) then added. The precipitate was filtered off, and the mother-liquor, on removal of methyl alcohol, gave a further crop of crystals (total yield 90%). The acid is fairly soluble in boiling but almost insoluble in cold water, soluble in alcohol and ethyl acetate, but insoluble in ether. It had m. p. $181-182^\circ$ (Found: C, 51.7; H, 5.4; N, 4.4; equiv. (cresol-red), 321. $C_{14}H_{17}O_6NS$ requires C, 51.4; H, 5.5; N, 4.3%; equiv., 327).

l(-)alloHydroxy-N-acetylproline (VII).—9.8 G. of the acid (VI; R = H) and 120 ml. of 0.5*N*-sodium hydroxide (2 equivs.) were heated at 100° for 20 mins. The solution was then cooled, and 30 ml. of *N*-hydrochloric acid (1 equiv.) added; the pH was then about 2.1 and toluene-*p*-sulphonic acid was still mainly present as its sodium salt. The solution was then evaporated to dryness at reduced pressure, the bath temperature being kept between 40° and 45° . The residue was extracted with three 100-ml. portions of a mixture of absolute alcohol and dry ethyl acetate (1:1). The combined filtered extracts were evaporated to dryness, and the residue again extracted with several portions of boiling dry ethyl acetate, 600 ml. altogether being required. On cooling, crystalline material was obtained; a further crop was obtained on concentrating the mother-liquors. The substance on recrystallisation from ethyl acetate had m. p. $144-145^\circ$ (yield 35%) (Found: C, 48.2; H, 6.15; N, 7.9; equiv., 175. $C_7H_{11}O_3N$ requires C, 48.0; H, 6.35; N, 8.1%; equiv., 173) $[\alpha]_D - 91.5^\circ$ ($c = 2.0$; $l = 2.0$ in water). It was very soluble in water, alcohol, sodium hydroxide, and dioxan, fairly

insoluble in ethyl acetate and chloroform, and almost insoluble in ether. It was recovered unchanged by heating to 140° in a vacuum for 1 hr. or after prolonged boiling in acetic acid.

1-(—)allo*Hydroxyproline*.—A solution of the acetyl compound (1.4 g.) in 3*N*-hydrochloric acid (25 ml.) was boiled under reflux for 4 hrs. and then evaporated in a vacuum to dryness. The crystalline residue was suspended in alcohol-ether and filtered. The hydrochloride, which was dried and freed from excess of acid over potassium hydroxide in a desiccator at 1 mm. pressure, was not a derivative of a lactone (which should be almost neutral), but of the imino-acid (VIII), as shown by the following properties: A solution in water had a pH of about 1.8, a value to be expected for the acid salt of (VIII). 33.5 Mg. of the hydrochloride required 1.92 ml. of 0.1*N*-sodium hydroxide for neutralisation to pH 5 (methyl-red) (Calc. : 2.00 ml.); the hydrochloride of the lactone should use up only a trace of alkali under these conditions.

The hydrochloride was then dissolved in a small quantity of water, the pH adjusted to 5.0 with lithium hydroxide, and alcohol added. The crystalline *base* was filtered off and recrystallised from aqueous alcohol (yield 85%); m. p. 248° (decomp.), $[\alpha]_D - 58.0^\circ$ ($c = 2.0$; $l = 2.0$ in water); $[\alpha]_D - 15.7^\circ$ ($c = 1.4$; $l = 2$ in 6% HCl) (Found: C, 45.7; H, 6.8; N, 10.5. $C_8H_9O_3N$ requires C, 45.7; H, 6.9; N, 10.7%).

Both (I) and (VIII), if heated with ninhydrin at their isoelectric points, give only a yellow colour, as found for (I) by Grassmann and von Arnim (*Annalen*, 1934, **509**, 288; 1935, **519**, 192); however, if pyridine is added, or if the reaction is carried out in a phosphate buffer of pH 7.4, both compounds give a transient red colour turning into a dirty brown. With isatin at pH 7.4, (I) gives a transient dark red colour, whereas (VIII) gives a much less intense red colour changing to green. If the reaction is carried out in acetic acid, (I) gives a transient red colour turning to a fluorescent olive green, but (VIII) gives an uncharacteristic dark orange colour.

Attempted Inversion of 1-(—)Hydroxy-N-acetylproline with Alkali.—(II) (0.9 g.) was dissolved in 10 ml. of water, and 2 equivs. of *N*-sodium hydroxide were added. The solution was heated at 100° for 30 mins., cooled, and exactly neutralised with hydrochloric acid; it was then evaporated in a vacuum to dryness, and the residue extracted with hot moist ethyl acetate. On cooling and concentration, 0.55 g. of material was obtained, which was dried over phosphoric oxide in a desiccator. It then had m. p. 135°, $[\alpha]_D - 118^\circ$ ($c = 1.5$; $l = 2.0$), and was unchanged (II).

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