[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES AND THE NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE, BETHESDA, MARYLAND 20014]

The Preparation of *cis*- and *trans*-4-H^3-L-Prolines and Their Use in Studying the Mechanism of Enzymatic Hydroxylation in Chick Embryos

BY Y. FUJITA,[1] A. GOTTLIEB, B. PETERKOFSKY, S. UDENFRIEND, AND B. WITKOP

RECEIVED JUNE 20, 1964

While iodide exchange reactions did not permit the preparation of sterically homogeneous *cis*- and *trans*-4-iodoproline derivatives as starting materials for subsequent hydrogenolysis experiments, pure *cis*-4-H^2(H^3)-(XI, XIV) and *trans*-4-H^2(H^3)-L-proline derivatives (XXI, XXIV) were prepared by SN2 displacements on the tetrahydropyranyl ethers of *trans*- and *cis*-4-tosyloxy-N-tosyl-L-prolinols, (X, XX) followed by cleavage of the ethers, oxidation of the N-tosylprolinols (XII, XXII) to the N-tosylprolines (XIII, XXIII), and detosylation with hydrogen bromide in acetic acid. The steric homogeneity of *cis*- and *trans*-4-H^2(H^3)-N-tosyl-L-prolinols (XI, XXI) was ascertained by n.m.r. spectrometry and by enzymatic methods. Enzymatic hydroxylation *in vivo* with chick embryos demonstrated 94% retention of tritum in *cis*-4-H^3-L-proline (XIV → XV) and 98% loss of tritium in *trans*-4-H^3-L-proline (XXIV → XXV). The hydroxylating enzyme system converts bound proline to hydroxyproline in a front-side displacement with complete retention of configuration at C-4 comparable to other enzymes which directly use molecular oxygen in the formation of the hydroxyl function. The tritium studies in conjunction with the O^{18} studies confirm that enzymatic conversion to collagen-bound hydroxyproline does not proceed *via* 3,4-dehydro-L-proline or 4-keto-L-proline as intermediates.

Introduction

Although proline is the precursor of hydroxyproline, it is now generally agreed that the introduction of oxygen occurs after the proline has been converted to some intermediate of protein biosynthesis,[2] most likely a ribosome-bound peptide.[3] Although the exact nature of the substrate for hydroxylation is not known, isotopic methods have provided information concerning the chemical mechanism. It has been found that the hydroxyl oxygen of hydroxyproline in avian and plant tissues is derived from $^{18}O_2$ and that the oxygen from H_2O^{18} is not incorporated.[4-7] In this respect proline hydroxylation resembles the mechanism of hydroxylation by other oxygenases.[8] However, if the mechanism is, in fact, comparable to that of steroidal hydroxylation, direct displacement of a hydrogen atom would be expected to occur at carbon 4 of proline. The availability of catalytically prepared 3,4-tritiated proline[9] seemed to offer an excellent tool for studying the displacement of tritium, and several groups have used this as a substrate in a number of biological systems.[10-13] These studies showed that in the process of enzymatic hydroxylation tritium was lost from positions 3 and 4 to an extent dependent on the mode of preparation of the respective commercial sample. Furthermore, the use of 3,4-tritiated proline did not permit conclusions to be drawn concerning the stereospecificity of the hydrogen atom displacement. There are even more disadvantages in using uniformly tritiated proline for such studies.[14] The present report describes the synthesis of the diastereoisomeric *cis*- and *trans*-4-H^3-L-prolines and their use in the determination of the stereochemistry of the enzymatic displacement of one hydrogen from carbon 4 during the conversion of proline to hydroxyproline in the intact chick embryo.[15] The first attempts to arrive at selectively tritiated prolines were directed toward the preparation of sterically homogeneous 4-iodoprolines and subsequent hydrogenolysis as described in the following.

A. Direct Nucleophilic Displacement Reactions on Tosyloxyprolines with Iodide Ion.—Displacements of *cis*- and *trans*-tosyloxy-L-proline derivatives by sulfhydryl and methyl mercaptide groups have led smoothly to 4-thioproline derivatives.[16] Iodide, as a much weaker nucleophilic group, should displace the tosyloxy group at a much lower rate than HS^- or CH_3S^-.[17] The five-membered ring should not affect the rate of displacement, because cyclopentyl systems are about as fast in this respect as isopropyl systems.[17] However, a second displacement reaction to be considered is the attack of excess iodide ion on the *cis*- or *trans*-4-iodoprolines (III and IV). The easy racemization or epimerization of optically active iodo derivatives is a well known phenomenon.[18] Only if $K_{I \rightarrow III} >> K_{III \rightarrow IV}$ or $K_{II \rightarrow IV} >> K_{IV \rightarrow III}$ will it be possible to obtain the pure iodoprolines (III and IV). To test the homogeneity of the expected iodoprolines a gas chromatographic assay was developed.[19] Figure 1 shows the separation and Table I gives the semiquantitative data of a number of iodide displacements on the tosylates I and II.

(1) Associate in the Visiting Program, United States Public Health Service, 1963-1965.

(2) M. R. Stetten, *J. Biol. Chem.*, **181**, 3 (1949).

(3) B. Peterkofsky and S. Udenfriend, *Biochem. Biophys. Res. Commun.*, **12**, 257 (1963).

(4) D. Fujimoto and N. Tamiya, *Biochem. J.*, **84**, 333 (1962).

(5) D. Fujimoto and N. Tamiya, *Biochim. Biophys. Acta*, **69**, 559 (1963).

(6) D. J. Prockop, A. Kaplan, and S. Udenfriend, *Biochem. Biophys. Res. Commun.*, **9**, 162 (1962); D. J. Prockop, A. Kaplan, and S. Udenfriend, *Arch. Biochem. Biophys.*, **101**, 499 (1963).

(7) D. T. A. Lamport, *J. Biol. Chem.*, **238**, 1438 (1963).

(8) *Cf.* "Oxygenases," O. Hayaishi, Ed., Academic Press, Inc., New York, N. Y., London, England, 1962.

(9) A. V. Robertson and B. Witkop, *J. Am. Chem. Soc.*, **84**, 1697 (1962).

(10) N. Stone and A. Meister, *Nature*, **194**, 555 (1962); *Federation Proc.*, **21**, 414 (1962).

(11) A. Meister, N. Stone, and J. M. Manning, Abstracts, 143rd National Meeting of the American Chemical Society, Atlantic City, N. J., Sept., 1962, p. 37.

(12) P. S. Ebert and D. J. Prockop, *Biochem. Biophys. Res. Commun.*, **8**, 305 (1962); D. J. Prockop, P. S. Ebert, and B. M. Shaoiro, *Arch. Biochem. Biophys.*, **106**, 112 (1964).

(13) D. T. A. Lamport, *Federation Proc.*, **22**, 647 (1963); *Nature*, **202**, 293 (1964).

(14) K. Konno and T. Tetsuka, *J. Biochem.* (Tokyo), **53**, 231 (1963).

(15) *Cf.* D. J. Prockop, B. Peterkofsky, and S. Udenfriend, *J. Biol. Chem.*, **237**, 1581 (1962).

(16) A. A. Patchett and B. Witkop, *J. Am. Chem. Soc.*, **79**, 185 (1957), *cf.* K. J. Ryan, H. Arzoumanian, E. M. Acton, and L. Goodman, *ibid.*, **86**, 2497 (1964).

(17) A. Streitwieser, Jr., "Solvolytic Displacement Reactions," McGraw-Hill Book Co., Inc., New York, N. Y., 1962.

(18) *Cf.* E. D. Hughes, F. Juliusberger, S. Masterman, B. Topley, and T. Weiss, *J. Chem. Soc.*, 1525 (1935).

(19) *Cf.* K. Morita, F. Irreverre, F. Sakiyama, and B. Witkop, *J. Am. Chem. Soc.*, **85**, 2832 (1963).

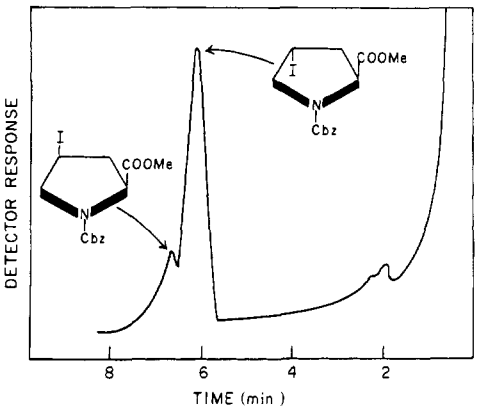
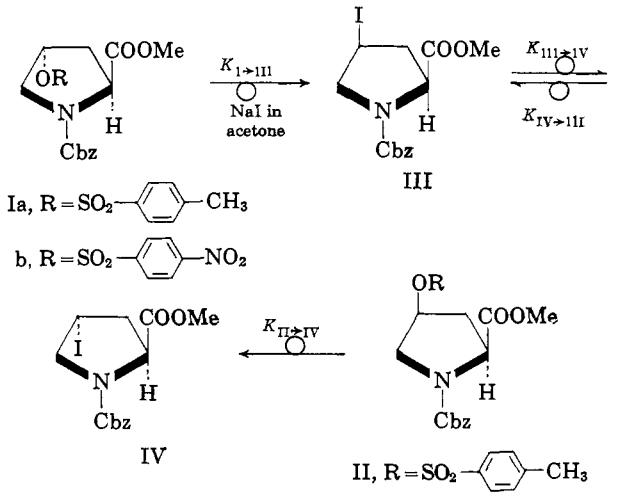


Fig. 1.—Gas chromatographic separation of *trans*-4-iodo-N-carbobenzyloxy-L-proline methyl ester (IV) from the *cis*-diastereoisomer III after a typical iodide displacement of N-carbobenzyloxy-O-tosylhydroxy-L-proline methyl ester (I).

Whenever the group to be displaced is *trans*, as in I and IV, the attacking nucleophile, iodide ion in both cases, is hindered in its approach by the carbomethoxy group on the same side. This is



clearly reflected in the rates of formation of III from I and III from IV. On the basis of these semiquantitative data, it appears that $K_{II \rightarrow IV} \geqq K_{IV \rightarrow III}$, because in the initial phase of the displacement reaction the *trans*-4-iodoproline derivative IV accumulates in 95–100% purity.

TABLE I[a]

Time, hr.	Starting material	Yield of iodo compound, %	Ratio of *cis* and *trans* products, % *trans*	*cis*
2	*trans*	Trace	8	92
	cis	10	100	0
5	*trans*	20	13	87
	cis	30	95	5
10	*trans*	30	22	78
	cis	50	78	22
24	*trans*	50	42	58
	cis	100	72	28

[a] Yields and ratios of 4-iodoprolines (III, IV) resulting from direct displacements on the tosylates I and II by iodide. In a typical run a solution of N-carbobenzyloxy-O-tosyl(*allo*)-hydroxy-L-proline methyl ester (10 mg.) and sodium iodide (20 mg.) in acetone (0.5 ml.) was heated at 50–55° for different time intervals. The yields were estimated by thin layer chomatography, and the ratios by gas chromatography.

In order to minimize the iodide exchange reaction, the displacement was carried out using an equimolar amount (10 mg., 0.023 mmole) of I and of sodium iodide (3.5 mg., 0.023 mmole) in 0.5 ml. of acetone at 100°. The reaction mixtures after 1 and 2 hr. were analyzed by thin layer and gas chromatography (Table II). The results show ratios of *cis*:*trans* 1:1 and 1:2, exceeding the ratio presented in Table I because of the higher temperature.

TABLE II[a]

Time, hr.	Yield of 4-iodoprolines, %	Ratio of products, % *trans*	*cis*
1	30	47	53
2	40	62	38

[a] Yields and ratios of 4-iodoproline derivatives III and IV with equimolar quantities of sodium iodide in the displacement reactions (acetone, 100°) on the *trans*-tosylate I.

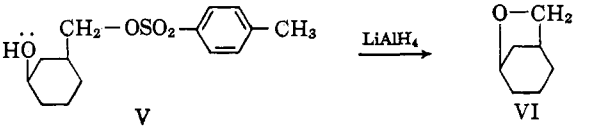
In order to increase the rate of displacement on I, the O-*p*-nitrobenzenesulfonate ("nisylate") Ia was prepared. Nisyl, as a leaving group, is up to 20 times faster than the tosyl group.[20] The results in Table III show that the yield of iodoprolines after 2 hr. is 70% compared with a trace under the conditions given in Table I. However the *cis*:*trans* ratio has not improved.

TABLE III[a]

Time, hr.	Yield of 4-iodoprolines, %	Ratio of products, % *trans*	*cis*
1	50	45	55
2	70	55	45
4	100	75	25

[a] Yields and ratios of 4-iodoprolines III and IV after displacement reactions on N-carbobenzyloxy-O-*p*-nitrobenzenesulfonylhydroxy-L-proline methyl ester (10 mg.) with sodium iodide (20 mg.) in 0.5 ml. of acetone at 50–55° for 1, 2, and 4 hr.

B. Hydrogenolysis of O-Tosyl and O-Nisylhydroxyprolines with Lithium Aluminum Deuteride and Tritide.—Attempts to hydrogenolyze the O-tosyl group of N-tosyl- or N-carbobenzyloxy-O-tosylhydroxy-L-proline or its methyl ester with lithium aluminum hydride under different reaction conditions always led to mixtures of several products. One of these products had the properties of a bicyclic ether. Such participation of a hydroxyl group in an intramolecular displacement reaction has precedents: the monotosylate of *cis*-3-hydroxycyclohexylmethanol (V) is not reduced to 3-methylcyclohexanol with lithium aluminum hydride, but instead to the *endo*-methyleneoxy compound VI.[21]



The N-protecting group of choice for hydrogenolysis of hydroxyproline derivatives is the tosyl group whose stability to lithium aluminum hydride is well known.[22]

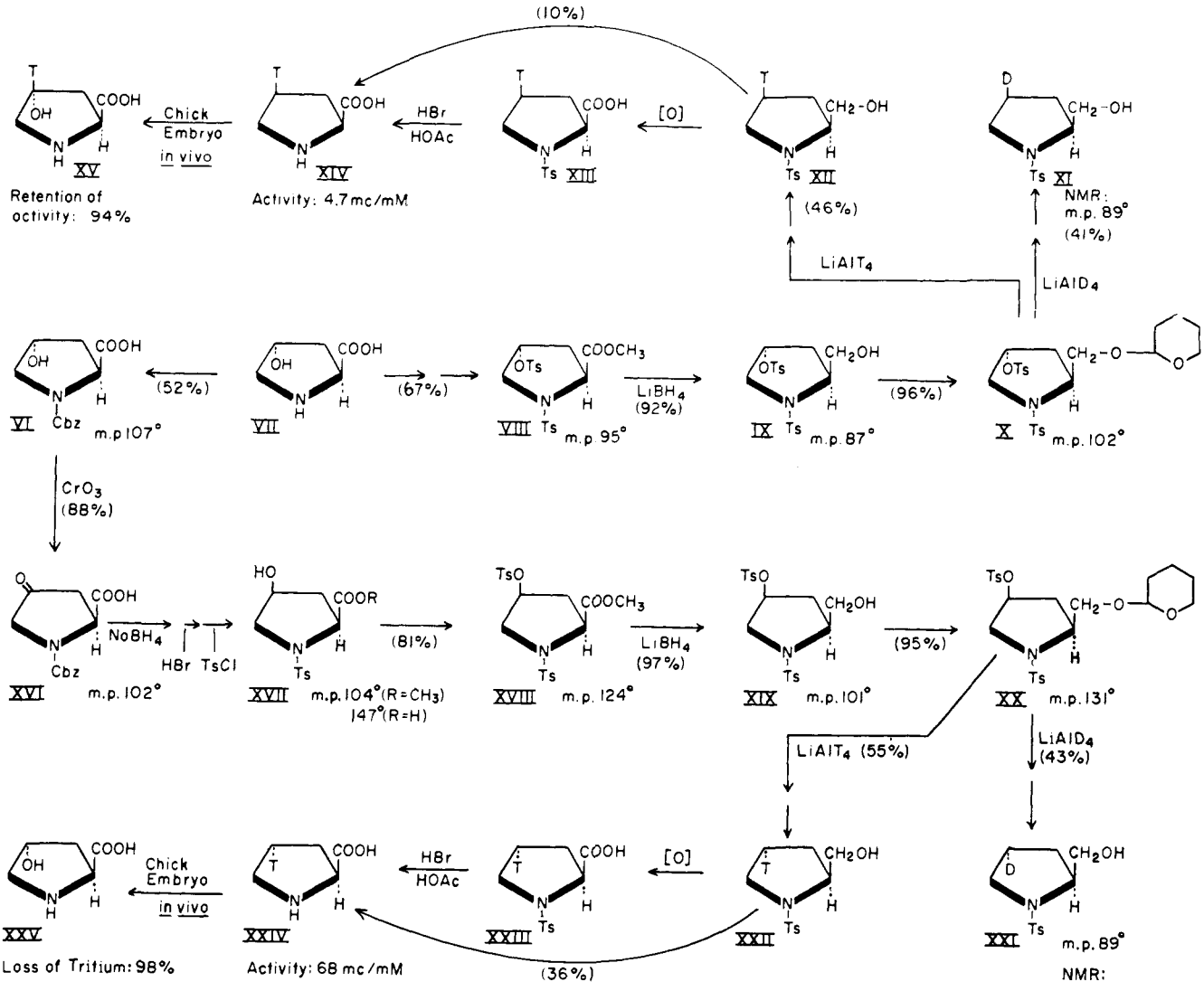
Lithium borohydride was used instead of lithium aluminum hydride for the selective reduction of the ester function of N,O-ditosylhydroxy-L-proline methyl ester (VIII, Scheme I). The O-tosyl (or nisyl) group remained intact during this reduction. The N,O-ditosyl-

(20) *Cf.* S. Winstein, E. Gruenwald, and H. W. Jones, *J. Am. Chem. Soc.*, **73**, 2077 (1951).

(21) H. L. Goering and C. Serres, Jr., *ibid.*, **74**, 5908 (1952).

(22) P. Karrer and K. Ehrhardt, *Helv. Chim. Acta*, **34**, 2202 (1951).

SCHEME I



hydroxy-L-prolinol (IX) was converted to the tetrahydropyranyl ether (X).[21,23,24] Initially, because of its better leaving character,[20] the N-tosyl-O-nisyl derivative was used as starting material. However, the great reactivity of the nisyl group introduced difficulties for the purification of the starting material as well as the products of hydrogenolysis. Eventually only the O-tosyl derivatives were used. The tetrahydropyranyl ethers of both *cis*- (XX) and *trans*-N,O-ditosylhydroxy-L-prolinol (X) were obtained pure in crystalline form.

SN2 displacements by hydride ions are stereospecific,[25] whereas reductive cleavages of asymmetric sulfonate esters by catalytic agents, such as Raney nickel, or by sodium in liquid ammonia, are not stereospecific and may not involve 100% inversion.[26] Under special conditions methanesulfonate esters are hydrolyzed without inversion.[27]

In order to ascertain the formation of one sterically homogeneous product the displacement reactions were first studied with deuteride ions, *i.e.*, lithium aluminum deuteride. In this case displacement of the tosyloxy group by deuteride ion showed a distinct isotope effect in that the time of refluxing with $LiAlD_4$ had to be more than doubled for complete reaction. The two diastereoisomers, *cis*-4-H²-N-tosyl-L-prolinol (XI) and *trans*-4-H²-N-tosyl-L-prolinol (XXI), both crystalline compounds, were characterized by n.m.r. spectrometry. Although a detailed interpretation of their spectra (Fig. 2) is not possible at this stage, two conclusions could be drawn: (i) each diastereoisomer had a characteristic n.m.r. spectrum; (ii) there was no indication of the presence of *cis*-4-H²-prolinol (XI) in the spectrum of the *trans*-H² isomer (XXI), and *vice versa*.

In analogous fashion the tetrahydropyranyl ethers of *cis*- (XX) and *trans*-4-tosyloxy-N-tosyl-L-prolinol (X) were hydrogenolyzed with lithium aluminum tritide. The tritiated prolinols (XII, XXII)[28] were oxidized with chromium trioxide followed by detosylation with hydrogen bromide in acetic acid.[29] The

(23) W. G. Dauben and H. L. Bradlow, *J. Am. Chem. Soc.*, **74**, 559 (1952).
(24) A. C. Ott, M. F. Murray, and R. L. Pederson, *ibid.*, **74**, 1239 (1952).
(25) G. J. Schroepfer, Jr., Abstracts, 146th National Meeting of the American Chemical Society, Denver, Colo., Jan., 1964, p. 18A.
(26) G. W. Kenner and M. A. Murray, *J. Chem. Soc.*, S178 (1949); D. B. Denney and B. Goldstein, *J. Org. Chem.*, **21**, 479 (1956); *cf.* S. Mitsui, Y. Senda, and K. Konno, *Chem. Ind.* (London), 1354 (1964).
(27) F. C. Chang, *Tetrahedron Letters*, 305 (1964); *cf.* D. H. Ball, E. D. M. Eades, and L. Long, Jr., *J. Am. Chem. Soc.*, **86**, 3579 (1964).
(28) L-Prolinol, first prepared by P. Karrer and P. Portmann [*Helv. Chim. Acta*, **31**, 2088 (1948)], occurs naturally as a constituent of the antibiotic actinonin: W. D. Ollis, A. J. East, J. J. Gordon, and I. O. Sutherland, *Symp. Microbiol.*, **6**, 204 (1964) (The Institute of Applied Microbiology, University of Tokyo).
(29) D. I. Weisblat, B. J. Magerlein, and D. R. Myers, *J. Am. Chem. Soc.*, **75**, 3630 (1953).

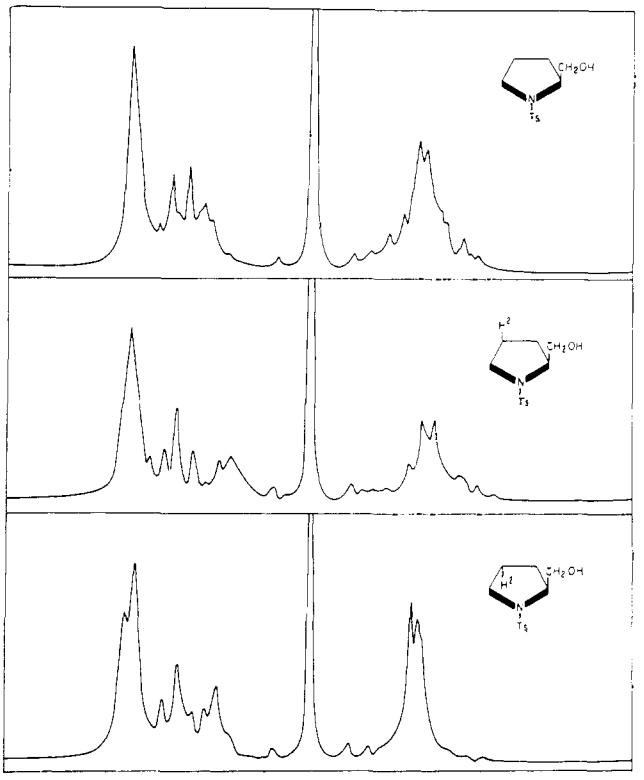


Fig. 2.—N.m.r. spectra of N-tosyl-L-prolinol, *cis*-4-H²-N-tosyl-L-prolinol, and *trans*-4-H²-N-tosyl-L-prolinol in $CDCl_3$.

crude *cis*- and *trans*-4-H³-L-prolines (XIV, XXIV) were purified by ion-exchange column chromatography.

Experimental

Preparation of Substrates. Displacement Reactions on N-Carbobenzyloxy-O-tosylhydroxy-L-proline Methyl Ester (I) by Iodide Ion.—A solution of 1 g. of carbobenzyloxy-O-tosylhydroxy-L-proline methyl ester[16] and 2 g. of sodium iodide in 30 ml. of acetone was heated at 100° for 4 hr. in a pressure bottle. After cooling the crystals of sodium *p*-toluenesulfonate which were collected, washed with acetone, and dried, weighed 436 mg. (95% of theory). The filtrate was evaporated to dryness and the residue was extracted with a mixture of ethyl acetate (10 ml.) and benzene (40 ml.). The solution, free from excess sodium iodide, was evaporated to dryness *in vacuo* and the resulting oil (898 mg.) dried in a vacuum desiccator. The oily ester (870 mg.) was dissolved in 8 ml. of methanol. To the solution was added 2.1 ml. of 1.0 *N* alkali in small portions and the temperature kept at 0°. The mixture was kept stirring at this temperature for 1 hr. and then allowed to stand overnight at room temperature. The reaction mixture was diluted with water and extracted with ether. The aqueous layer was acidified with dilute hydrochloric acid, and extracted with ether. The ether extract was washed with water, dried over sodium sulfate, and evaporated *in vacuo* to yield a colorless oil (665 mg.). This oily residue (446 mg.) was dissolved in 8 ml. of acetone, treated with excess *t*-butylamine, and allowed to stand for crystallization. The colorless crystals of the *t*-butylammonium salt of N-carbobenzyloxy-4-iodo-L-proline (about 90% *cis*- and 10% *trans*-4-iodo derivatives III and IV, as assayed by gas chromatography, *cf.* Fig. 1) were not hygroscopic and showed m.p. 142–143°.[30]

Anal. Calcd. for $C_{17}H_{25}IN_2O_4$: C, 45.54; H, 5.62; I, 28.30; N, 6.25; Found: C, 45.17; H, 5.97; I, 26.72; N, 6.10.

In analogous fashion 10 mg. of N-carbobenzyloxy-O-tosyl-*allo*-hydroxy-L-proline methyl ester (II)[16] and 20 mg. of sodium iodide, dissolved in 0.5 ml. of acetone, was heated at 100° for 4 hr. in a sealed tube. The reaction conditions were varied as indicated in Tables I and II and the reaction products were analyzed by gas chromatography.

N-Carbobenzyloxy-O-*p*-nitrobenzenesulfonylhydroxy-L-proline Methyl Ester (Ia).—A solution of 530 mg. (2 moles) of N-carbobenzyloxy-4-hydroxy-L-proline in 3 ml. of dry dioxane was treated with ethereal diazomethane at 0° until the yellow color persisted. The solution was dried over sodium sulfate, evaporated to dryness *in vacuo*, and the residue was dissolved in dry pyridine (1.3 ml.) and cooled in an ice–salt bath. A solution of *p*-nitrobenzenesulfonyl chloride (487 mg., 2.2 mmoles) in dry pyridine (0.7 ml.) was added to this cooled solution and the mixture was left at −8° for 4 hr. Ice-cold 2.0 *N* hydrochloric acid (12.5 ml.) was added to the mixture and the oily precipitate was extracted two times with 10 ml. of ethyl acetate. The extract was washed with saturated sodium chloride solution and dried over sodium sulfate. The solvent was removed *in vacuo* and the oily residue was purified by adsorption on a silicic acid column (3 × 50 cm.) and elution with a mixture of ethyl acetate-benzene (1:4). The buff crystals (377 mg.) were recrystallized from ethyl acetate and petroleum ether to yield 349 mg. (37%), m.p. 97–98°.

Anal. Calcd. for $C_{20}H_{20}N_2O_9S$: C, 51.72; H, 4.34; N, 6.03. Found: C, 51.64; H, 4.68; N, 5.78.

N-Nisylhydroxy-L-proline.—To an ice-cooled solution of 1.311 g. (10 mmoles) of hydroxy-L-proline in 10 ml. of 1.0 *N* alkali was added 2.658 g. (12 mmoles) of *p*-nitrobenzenesulfonyl chloride with vigorous stirring for 10 min. and for 1 additional hr. at room temperature. During the course of the reaction the pH of the solution was maintained at 10 by the constant addition of 2.0 *N* alkali. A small amount of insoluble material was removed by filtration and the filtrate acidified to congo blue with concentrated hydrochloric acid at 0°. The colorless crystalline precipitate was collected, washed with water, and dried in a vacuum desiccator to yield 2.715 g. (86%), m.p. 176–179°. The compound was recrystallized from acetone-petroleum ether (b.p. 40–60°) to yield 2.101 g. (66%), m.p. 179–181°.

Anal. Calcd. for $C_{11}H_{12}N_2O_7S$: C, 41.77; H, 3.82; N, 8.86. Found: C, 41.94; H, 4.15; N, 8.87.

N-Tosyl-O-nisylhydroxy-L-proline Methyl Ester.—A solution of 5.71 g. (20 mmoles) of N-tosylhydroxy-L-proline[31] in 30 ml. of dry dioxane at 0° was treated with ethereal diazomethane until the yellow color persisted. The reaction mixture was dried over magnesium sulfate, the solvent was evaporated, and the residue was dissolved in 12.8 ml. of dry pyridine and chilled in an ice–salt bath. A solution of *p*-nitrobenzenesulfonyl chloride (5.312 g., 24 mmoles) in 6.4 ml. of dry pyridine was added and the mixture was left at −8° for 3 days.

Ice-cold 2.0 *N* hydrochloric acid (108 ml.) was added to the mixture. The crystalline precipitate was collected, washed with water, and redissolved in 150 ml. of ethyl acetate. The ethyl acetate solution was washed with 100 ml. of 4% sodium bicarbonate solution and dried over sodium sulfate. The solution was concentrated to a small volume, and ether was added to the crystalline mush which was left in a refrigerator overnight. The crystals were collected, washed with ether, and dried to yield 6.515 g. (67%), m.p. 141–142°. After recrystallization from ethyl acetate–ether, there was obtained 6.058 g. of colorless crystals, m.p. 141–142°, $[\alpha]^{20}D$ −53.8 ±1.0° (*c* 1, chloroform).

Anal. Calcd. for $C_{19}H_{20}N_2O_9S_2$: C, 47.10; H, 4.16; N, 5.78. Found: C, 47.06; H, 3.91; N, 5.86.

N-Tosyl-O-nisylhydroxy-L-prolinol.—To an ice-cooled solution of 4.845 g. (10 mmoles) of N-tosyl-O-nisylhydroxy-L-proline methyl ester in 20 ml. of dry dimethoxyethane was added a solution of 916 mg. (30 mmoles) of lithium borohydride in 30 ml. of dimethoxyethane, and the mixture was stirred at 0° for 2 hr. Excess lithium borohydride was decomposed with acetic acid (4 ml.) and the mixture was diluted with water (10 ml.) and ethyl acetate (50 ml.). The organic layer was separated, washed several times with saturated sodium chloride solution, twice with 4% sodium bicarbonate solution, and dried over sodium sulfate. The solvent was evaporated *in vacuo* and the residue was dissolved in a mixture of ethyl acetate (5 ml.) and benzene (5 ml.). The solution was put on a column of silicic acid (3 × 50 cm., Mallinckrodt, 100 mesh) and eluted with a mixture of ethyl acetate and benzene (1:1). The eluate was collected in 5-ml. fractions and fractions 51 to 73 were pooled. The solvent was concentrated *in vacuo* and the crystalline residue was collected and washed with petroleum ether to yield 3.032 g. (66%) of colorless crystals, m.p. 142–143°. After recrystallization from ethyl acetate–ether there was obtained 2.885 g., m.p. 142–143°, $[\alpha]^{20}D$ −7.8 ± 1.0° (*c* 1, chloroform).

Anal. Calcd. for $C_{18}H_{20}N_2O_8S_2$: C, 47.35; H, 4.42; N, 6.14. Found: C, 47.05; H, 4.44; N, 5.98.

(30) This experiment and some of the initial investigations on iodide displacements were carried out by Dr. K. Morita, Associate in the Visiting Program of the United States Public Health Service, 1962–1963.

(31) E. W. McChesney and W. K. Swann, *J. Am. Chem. Soc.*, **59**, 1116 (1937).

N-Tosyl-L-proline Methyl Ester.—N-Tosyl-L-proline[32] (8.08 g., 30 mmoles) dissolved in 45 ml. of dioxane was treated with excess ethereal diazomethane at 0°. The solution was dried over magnesium sulfate for several hours, and the solvent was evaporated *in vacuo*. The oily residue crystallized after storage in a refrigerator overnight with petroleum ether. The colorless crystals were collected, washed with petroleum ether, and dried to yield 8.15 g. (96%), m.p. 70–73°. They were recrystallized from ethyl acetate–ether–petroleum ether to yield 7.39 g. (87%), m.p. 75–76°, $[\alpha]^{20}D$ −93.3° ± 1.0° (*c* 1.5, chloroform).

Anal. Calcd. for $C_{13}H_{17}NO_4S$: C, 55.10; H, 6.05; N, 4.94. Found: C, 55.17; H, 6.06; N, 4.95.

N-Tosyl-L-prolinol.—N-Tosyl-L-proline methyl ester (5.67 g., 20 mmoles) was dissolved in 30 ml. of dry tetrahydrofuran. The solution was cooled to 0° and 1.53 g. of 82% lithium borohydride (50 mmoles) was added with stirring. Stirring was continued for 2 hr. at room temperature. Excess lithium borohydride was destroyed with dilute hydrochloric acid and the solution was diluted with water. The mixture was extracted with ethyl acetate (two 50-ml. portions) and the organic layer was washed with saturated sodium chloride solution and dried over sodium sulfate. After evaporation of the solvent the product crystallized in a refrigerator overnight. The colorless crystals were filtered with the aid of petroleum ether to yield 4.99 g. (98%), m.p. 79–83°. An analytical sample was recrystallized from ether, m.p. 87–88°, $[\alpha]^{20}D$ −62.7° ± 0.5° (*c* 1, chloroform).

Anal. Calcd. for $C_{12}H_{17}NO_3S$: C, 56.44; H, 6.71; N, 5.49. Found: C, 56.66; H, 6.47; N, 5.38.

***allo*-Hydroxy-L-proline.**—A solution of 13.74 g. (51.8 mmoles) of N-carbobenzyloxy-*allo*-hydroxy-L-proline, prepared from N-carbobenzyloxyhydroxy-L-proline,[16] in 50% acetic acid (100 ml.) was hydrogenated in the presence of 5% palladium–carbon (2 g.) at room temperature. After the completion of hydrogenolysis, the catalyst was filtered off and washed with water. The combined filtrate and washings were concentrated to a small volume *in vacuo*. Ethanol was added to precipitate the crystalline *allo*-hydroxy-L-proline which was collected, washed with ethanol and ether, and dried. There was obtained 6.506 g. (96%) of colorless crystals, m.p. 238–241° (dec.), $[\alpha]^{20}D$ −59.6° (*c* 1, water).

N-Tosyl-*allo*-hydroxy-L-proline (XVII, R = H).—To a solution of 6.56 g. (50 mmoles) of *allo*-hydroxy-L-proline in 50 ml. of 1.0 *N* sodium hydroxide was added *p*-toluenesulfonyl chloride (10.0 g., 52.5 mmoles) with vigorous stirring. Stirring was continued for 3 hr. at room temperature. The pH of the reaction mixture was kept at 8–9 by the constant addition of 1.0 *N* sodium hydroxide. A small amount of insoluble material was filtered off and the filtrate adjusted to pH 3–4 by the addition of concentrated hydrochloric acid at 0°. The resulting oily precipitate was extracted three times with 100 ml. of ethyl acetate and the ethyl acetate extract was washed with sodium chloride solution and dried over sodium sulfate. The solvent was removed *in vacuo* until crystallization began. The crystals were triturated with ether and the mixture was left in a refrigerator overnight. The crystals were collected, washed with ether, and dried to yield 9.92 g. (70%), m.p. 140–145°. An analytical sample, obtained by recrystallization from ethanol–ether, had m.p. 146–147°, $[\alpha]^{20}D$ −43.9 ± 1.0° (*c* 1, ethanol).

Anal. Calcd. for $C_{12}H_{15}NO_5S$: C, 50.51; H, 5.30; N, 4.91. Found: C, 50.57; H, 5.23; N, 4.96.

N-Tosyl-*allo*-hydroxy-L-proline Methyl Ester (XVII, R = CH_3).—A solution of N-tosyl-*allo*-hydroxy-L-proline (285 mg., 1 mmole) was dissolved in 1 ml. of dioxane and was treated with an excess of ethereal diazomethane at 0°. The solution was dried over magnesium sulfate for several hours and the solvent removed *in vacuo*. Petroleum ether was added to the oily residue which crystallized when kept in a refrigerator overnight. The crystals were collected, washed with petroleum ether, and dried to yield 287 mg. (96%), m.p. 100–101°. After recrystallization from ethyl acetate–ether–petroleum ether there was obtained 265 mg. (89%) of colorless crystals, m.p. 103–104°, $[\alpha]^{20}D$ −66.5 ± 1.0° (*c* 1, chloroform).

Anal. Calcd. for $C_{13}H_{17}NO_5S$: C, 52.16; H, 5.72; N, 4.68. Found: C, 52.41; H, 5.82; N, 4.57.

N,O-Ditosylhydroxy-L-proline Methyl Ester (VIII).[33]—N-Tosylhydroxy-L-proline (8.56 g., 30 mmoles), dissolved in 45 ml. of dioxane, was esterified with excess ethereal diazomethane at 0° as described above. After drying several hours over magnesium sulfate, the solvent was removed *in vacuo* and the residue was dissolved in dry pyridine (19.2 ml.) and cooled in an ice-salt bath. A solution of *p*-toluenesulfonyl chloride (6.95 g., 36 mmoles) in dry pyridine (9.6 ml.) was added to this solution, and the mixture was left at −8° for 3 days. Ice-cold 2.0 *N* hydrochloric acid (162 ml.) was added to the reaction mixture and the resulting crystalline precipitate was collected, washed with water, and dried to yield 9.05 g. (67%) of colorless crystals, m.p. 93–95°. After recrystallization from 20 ml. of ethyl acetate and 100 ml. of ether there was obtained 7.54 g. (55%), m.p. 94–95.5°, $[\alpha]^{20}D$ −54.1 ± 1.0° (*c* 1, chloroform).

Anal. Calcd. for $C_{20}H_{23}NO_7S_2$: C, 52.96; H, 5.11; N, 3.09. Found: C, 53.07; H, 5.37; N, 3.02.

N,O-Ditosyl-*allo*-hydroxy-L-proline Methyl Ester (XVIII).—This compound was synthesized from N-tosyl-*allo*-hydroxy-L-proline (8.56 g., 30 mmoles) in the same way as described above to yield 11.00 g. (81%) of colorless crystals, m.p. 92–94°. After recrystallization from 20 ml. of ethyl acetate and 100 ml. of ether there was obtained 9.65 g. (71%), m.p. 123–124°, $[\alpha]^{20}D$ −25.0 ± 1.0° (*c* 1, chloroform).

Anal. Calcd. for $C_{20}H_{23}NO_7S_2$: C, 52.96; H, 5.11; N, 3.09. Found: C, 52.91; H, 4.92; N, 3.15.

N,O-Ditosylhydroxy-L-prolinol (IX).—To a solution of 6.8 g. (15 mmoles) of N,O-ditosylhydroxy-L-proline methyl ester in dry dimethoxyethane (50 ml.) was added a solution of 1.53 g. (50 mmoles) of lithium borohydride in dry dimethoxyethane (50 ml.) at 0°, and the mixture was stirred at room temperature for 2 hr. Excess hydride was decomposed with 15 ml. of acetic acid at 0° and the solution was diluted with 50 ml. of water and extracted two times with 100 ml. of ethyl acetate. The ethyl acetate extract was washed with water and sodium bicarbonate solution and dried over sodium sulfate. The solvent was removed *in vacuo*. Ether (20 ml.) and petroleum ether (100 ml.) was added to the residue. The oily residue crystallized on standing in the refrigerator overnight. The crystals were collected, washed with petroleum ether, and dried to yield 5.87 g. (92%), m.p. 85–87°. Recrystallization from ethyl acetate–ether–petroleum ether gave colorless crystals, m.p. 86–87°, $[\alpha]^{20}D$ 0 ± 1.0° (*c* 1, chloroform).

Anal. Calcd. for $C_{19}H_{23}NO_6S_2$: C, 53.67; H, 5.45; N, 3.29. Found: C, 53.57; H, 5.18; N, 3.12.

N,O-Ditosyl-*allo*-hydroxy-L-prolinol (XIX).—The reduction of N,O-ditosyl-*allo*-hydroxy-L-proline methyl ester (4.535 g., 10 mmoles) with lithium borohydride was carried out in the same way as described above. There was obtained 4.123 g. (97%) of colorless crystals, m.p. 100–102°, and, after recrystallization from ethyl acetate–ether–petroleum ether, 4.072 g. (96%), m.p. 101–102°, $[\alpha]^{20}D$ +22.7 ± 1.0° (*c* 1, chloroform).

Anal. Calcd. for $C_{19}H_{23}NO_6S_2$: C, 53.67; H, 5.45, N, 3.29. Found: C, 53.44; H, 5.21; N, 3.13.

N,O-Ditosylhydroxy-L-prolinol Tetrahydropyranyl Ether (X).—To the solution of 2.13 g. (5 mmoles) of N,O-ditosylhydroxy-L-prolinol (IX) in dry tetrahydrofuran (10 ml.) was added dihydropyran (2.1 g., 25 mmoles) and 1 drop of concentrated hydrochloric acid and the resulting solution was left at room temperature for 20 hr. A few sodium hydroxide pellets were added to the solution and the flask was shaken for 5 min. The sodium hydroxide was filtered off and the filtrate was evaporated *in vacuo*. The oily residue was dissolved in 25 ml. of ether. A small amount of insoluble material was filtered off and the filtrate was concentrated to about 15 ml. To the concentrate was added petroleum ether (15 ml.) and the crystalline precipitate was collected after storage in a refrigerator for 2 hr. The crystals were washed with a mixture of ether and petroleum ether and dried to yield 2.45 g. (96%), m.p. 101–102°, and, after recrystallization from tetrahydrofuran–ether–petroleum ether, 2.413 g., m.p. 102–103°.

Anal. Calcd. for $C_{24}H_{31}NO_7S_2$: C, 56.55; H, 6.13; N, 2.75. Found: C, 56.65; H, 5.80; N, 2.68.

N,O-Ditosyl-*allo*-hydroxy-L-prolinol Tetrahydropyranyl Ether (XX).—N,O-Ditosyl-*allo*-hydroxy-L-prolinol (2.130 g., 5 mmoles) was converted to its tetrahydropyranyl ether in the same way as described above. The reaction mixture, after the removal of hydrochloric acid with sodium hydroxide, was concentrated *in vacuo* until crystals appeared. Petroleum ether was added and the mixture was left in a refrigerator for 2 hr. The crystals were collected, washed with petroleum ether, and dried to yield 2.540 g. (100%), m.p. 129–131°, and, after recrystallization from tetrahydrofuran–petroleum ether, 2.503 g. (98%), m.p. 129–131°

(32) N. Izumiya, *Bull. Chem. Soc. Japan*, **26**, 53 (1953); Z. Pravda and J. Rudinger, *Collection Czech. Chem. Commun.*, **20**, 1 (1955).

(33) This compound was synthesized by John E. Francis.

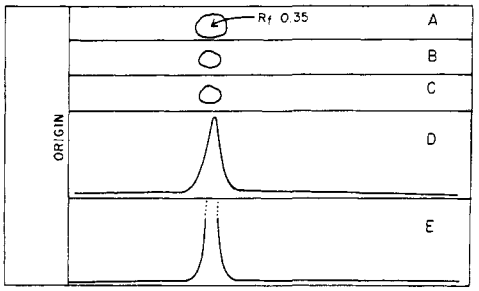


Fig. 3.—Paper chromatography of *cis*- (*trans*-) 4-H^3- and authentic L-proline. Solvent: 1-butanol–acetic acid–pyridine–water (40:10:10:20). One-dimensional ascending method. The paper was sprayed with isatin; A: authentic L-proline; B: *cis*-4-H^3-L-proline (XIV); C: *trans*-4-H^3-L-proline (XXIV); D and E: radioautograms of *cis*- and *trans*-4-H^3-L-proline, respectively.

Anal. Calcd. for $C_{24}H_{31}NO_7S_2$: C, 56.55; H, 6.13; N, 2.75. Found: C, 56.22; H, 6.22; N, 2.82.

***cis*-4-H^3-N-Tosyl-L-prolinol (XI).**—A solution of N,O-ditosylhydroxy-L-prolinol tetrahydropyranyl ether (X, 510 mg., 1 mmole) in a mixture of tetrahydrofuran (3 ml.) and ether (2 ml.) was added dropwise to a slurry of lithium aluminum deuteride (114 mg., 3 mmoles) in 5 ml. of ether. The mixture had to be refluxed for 14 hr., rather than 6 hr. as required for reduction with lithium aluminum hydride. The reaction mixture was then cooled to 0° and excess metal hydride was destroyed with water (10 ml.). A sufficient amount of concentrated hydrochloric acid was added to dissolve aluminum hydroxide. The organic material was extracted with ethyl acetate (10 ml.). The extract was washed with water and 4% sodium bicarbonate solution and dried over sodium sulfate. After the evaporation of the solvent, *cis*-4-H^3-N-tosyl-L-prolinol tetrahydropyranyl ether was obtained as a colorless oil which, without purification, was subjected to hydrolysis. The oily ether was dissolved in ethanol (10 ml.) and refluxed for 2 hr. with *p*-toluenesulfonic acid monohydrate (228 mg., 1.2 mmoles). Ethanol was removed *in vacuo* and the residue taken up in ethyl acetate (5 ml.), washed with water and 4% sodium bicarbonate solution, then dried over sodium sulfate. The solvent was removed *in vacuo* and the residue dissolved in ethyl acetate (1 ml.). This solution was purified by preparative thin layer chromatography on two plates of silica gel[34] (Research Specialties Co., 20 × 20 × 0.1 cm.) and developed with a mixture of ethyl acetate and benzene (1:2). The position of N-tosyl-L-prolinol was detected by spraying with 8.0 *N* chromic acid–sulfuric acid reagent.[35] The band was cut out and extracted with ethyl acetate. The extract was evaporated to a viscous oil and ether and petroleum ether were added. The colorless crystals which appeared on storage overnight in a refrigerator were collected, washed with petroleum ether, and dried to yield 105 mg. (41%), m.p. 88–89°.

Anal. Calcd. for $C_{12}H_{16}DNO_3S$: C, 56.22; H(D), 7.06; N, 5.47. Found: C, 56.40; H, 6.87; N, 5.43.

***trans*-4-H^3-N-Tosyl-L-prolinol (XXI).**—The *trans* isomer XXI was synthesized from N,O-ditosyl-*allo*-hydroxy-L-prolinol tetrahydropyranyl ether (XX, 510 mg., 1 mmole) in the same way as described above, to yield 109 mg. (43%) of colorless crystals, m.p. 88–89°.

Anal. Calcd. for $C_{12}H_{16}DNO_3S$: C, 56.22; H(D), 7.06; N, 5.47. Found: C, 56.01; H, 6.80; N, 5.37.

***cis*-4-H^3-L-Proline (XIV).**—A solution of N,O-ditosylhydroxy-L-prolinol tetrahydropyranyl ether (X, 510 mg., 1 mmole) in a mixture of tetrahydrofuran (3 ml.) and ether (2 ml.) was added dropwise to a slurry of lithium aluminum tritide[36] (114 mg., 3 mmoles, 100 mc.) in ether (5 ml.). The mixture was refluxed for 6 hr. The isolation and purification of *cis*-4-H^3-N-tosyl-L-prolinol was carried out in the same way as described for the deuterium analog. The yield of crystalline material was 118 mg. (46%). The crystalline *cis*-4-H^3-N-tosyl-L-prolinol (118 mg.) was dissolved in acetone (5 ml.). To the stirred solution was added in the course of 5 min. 8.0 *N* chromic acid in sulfuric acid solution (0.5 ml.) and the mixture stirred for an additional 30 min. Excess oxidant was destroyed with methanol and the chromium salt was filtered off and washed with chloroform (5 ml.). The combined filtrate and washings were washed several times with saturated sodium chloride solution and dried over sodium sulfate. The solvent was removed *in vacuo* and the oily residue further dried in a vacuum desiccator over phosphorus pentoxide. The colorless oil weighed 104 mg. The oily *cis*-4-H^3-N-tosyl-L-proline (104 mg.) and phenol (100 mg., 1 mmole) were dissolved in freshly prepared 36% hydrogen bromide in glacial acetic acid (1 ml.) and the mixture was left at room temperature for 24 hr. Ether (10 ml.) was added to the reaction mixture and the oily precipitate was washed with ether by decantation. The residue was dissolved in water (1 ml.) and put on a column of Dowex 50W–X8 (1 × 11 cm.). The column was washed with water and eluted with 2.0 *N* ammonium hydroxide. The eluate was evaporated to dryness *in vacuo* and the crystalline residue was collected with the aid of ether. The crude *cis*-4-H^3-L-proline weighed 13 mg.

Since the tritiated proline was not completely homogeneous by the criteria of paper chromatography (ninhydrin), it was further purified by ion-exchange column chromatography[37] in the following way.

The crude proline was dissolved in 1 ml. of 0.2 *M* ammonium acetate solution in water–ethanol (60:40, v./v.). The solution was put on a column of Dowex 50W–X8 (70 ml., 200–400 mesh) prepared and eluted with the same buffer solution, and 2.4-ml. fractions were collected. Aliquots (10 λ) from each tube were spotted on paper and sprayed with isatin reagent. Tubes 26–31 contained proline. These fractions were combined and the solvent was removed *in vacuo*. The residue was desalted by passing it over a column of Dowex 50W–X8 (1 × 11 cm.) and eluted with 3.0 *N* ammonium hydroxide. The eluate was evaporated *in vacuo* and the crystalline residue was collected with the aid of ether. The yield of pure *cis*-4-H^3-L-proline (XV) was 5.0 mg. (10% based on N-tosyl-L-prolinol), and the specific activity was 4.7 mc./mmole.

The tritiated proline XIV showed a single yellow spot with ninhydrin reagent, a single blue spot with isatin reagent on paper chromatograms, and a sharp single peak at the location of the color spot, when a paper chromatogram was scanned by the Vanguard automatic chromatogram scanner.

***trans*-4-H^3-L-Proline (XXIV).**—N,O-Ditosyl-*allo*-hydroxy-L-prolinol tetrahydropyranyl ether (XX, 510 mg., 1 mmole) was reduced with lithium aluminum titritide[38] (140 mg., 3.7 mmoles, 920 mc.), and the product, *trans*-4-H^3-N-tosyl-L-prolinol, crystallized in the same way as described above. The yield was 141 mg. (55%).

The compound was oxidized with chromium trioxide reagent giving an oily preparation of *trans*-4-H^3-N-tosyl-L-proline (XXIII 132 mg.) which was subjected to detosylation. The crude *trans*-4-H^3-L-proline (XXIV, 31 mg.) was purified by ion-exchange column chromatography as described above. The yield of pure crystalline *trans*-4-H^3-L-proline (XXIV) was 23 mg. (36% based on N-tosyl-L-prolinol), and the specific activity was 68 mc./mmole. The purity of the preparation was confirmed by the same criteria as described above and as shown in Fig. 3.

Biological Procedures.—Nine-day-old embryonated eggs were purchased from the Duckworth hatchery and kept in an incubator at 37° until used. Uniformly labeled L-proline-C^{14} (33 μc./μmole) was purchased from Nuclear-Chicago Corporation and purified on a Dowex-50 column to remove traces of hydroxyproline.[39] The proline-C^{14} was dissolved in deionized water and adjusted to a final concentration of 1.08 × 10^8 d.p.m./ml. The tritium-labeled compounds were dissolved and adjusted to concentrations of 4.76 × 10^8 d.p.m./ml. for the *trans* isomer and 5.28 × 10^8 d.p.m./ml. for the *cis* isomer. A small opening was made in each egg and 0.050 ml. of one of the radioactive solutions was placed on top of the membrane lining the bottom of the air sac. The eggs were then incubated for 6.5 hr. at 37°. The embryos were excised, placed in cold 0.15 *M* KCl, washed with cold 0.15 *M* KCl, and homogenized in 1.5 volumes of 0.15 *M* KCl. One-ninth volume of cold 50% trichloroacetic acid was added to the homogenates and the samples were centrifuged. The supernatant solutions were twice extracted with ether and the free amino acids partially purified by the use of a Dowex-50 (H^+) column as described previously.[15] The precipitates were ex-

(34) C. G. Honegger, *Helv. Chim. Acta*, **45**, 1409 (1962).

(35) P. Bladon, J. M. Fabian, H. B. Henbest, H. P. Koch, and G. W. Wood, *J. Chem. Soc.*, 2407 (1951).

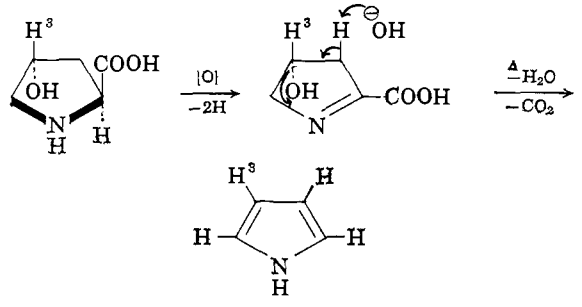
(36) Purchased from New England Nuclear Corporation as a solid.

(37) A. V. Robertson, E. Katz, and B. Witkop, *J. Org. Chem.*, **27**, 2676 (1962).

(38) Supplied by the New England Nuclear Corporation as ethereal solution.

(39) S. Lindstedt and D. J. Prockop, *J. Biol. Chem.*, **236**, 1399 (1961).

tracted with 5 ml. of hot 5% trichloroacetic acid and the gelatin solutions were hydrolyzed in 6.0 *N* HCl for 2.5 hr. in an autoclave at 124° and 18 lb. The hydolysates were decolorized with a mixture of Dowex-1 (H^-) and charcoal, filtered through a sintered glass funnel, and concentrated by evaporation under vacuum. Proline and hydroxyproline in the gelatin hydrolysates and in the mixture of partially purified free amino acids were oxidized and their oxidation products extracted into toluene as described previously.[40] Radioactivity was assayed in a scintillation spectrometer. The efficiency for C^{14} was 65%, for H^3 21%. In a separate experiment it was established that 4-H³-hydroxy-L-proline[37,41,42] retained its entire activity in its oxidative conversion to 3-H³-pyrrole.



Results

Two separate experiments were carried out with each tritiated proline isomer. In each experiment two eggs were injected, one with the appropriate tritiated isomer and the other with proline-C^{14}. In each case, radioactivity in both protein-bound and free imino acids were determined.

Ideally it would have been preferable to administer both C^{14}- and H^3-labeled prolines to the same embryo and measure directly the H^3/C^{14} ratio of each sample of isolated hydroxyproline. However, the residual C^{14} which is measured at the H^3 settings of the scintillation spectrometer would have made it impossible to demonstrate the almost complete loss of tritium which was obtained with the *trans* isomer.

The results of these experiments are shown in Tables I and II, respectively. When the tritiated *cis* isomer was used, there was complete retention of the tritium label in hydroxyproline when compared to proline.

TABLE IV

DISTRIBUTION OF LABEL IN PROTEIN-BOUND L-PROLINE AND HYDROXY-L-PROLINE OF CHICK EMBRYOS FOLLOWING ADMINISTRATION OF *cis*- AND *trans*-4-H³-L-PROLINE

4-H³-Proline isomer		Expt.	Radioactivity in protein-bound imino acids: H^3 (C.p.m × 10^{-5})	C^{14}	H^3/C^{14}	H^3 retention,[a] %
cis	1	Proline	3.77	1.56	2.41	
		Hydroxyproline	1.67	0.80	2.09	86.9
	2	Proline	8.35	2.31	3.61	
		Hydroxyproline	2.66	0.73	3.64	100
					Av.	93.5
trans	1	Proline	15.7	2.68	5.87	
		Hydroxyproline	0.067	0.61	0.11	1.88
	2	Proline	16.9	3.08	5.49	
		Hydroxyproline	0.087	0.55	0.16	2.91
					Av.	2.40

[a] Per cent retention $= \frac{(H^3/C^{14}) \times HP}{(H^3/C^{14}) \times P} \times 100.$

(40) Details of the counting procedures are presented in the following paper: B. Peterkofsky and D. J. Prockop, *Anal. Biochem.*, **4**, 400 (1962).

(41) I. Bergman, and R. Loxley, *Anal. Chem.*, **35**, 1961 (1963).

(42) E. Adams and A. Goldstone, *J. Biol. Chem.*, **235**, 3492 (1960).

TABLE V

DISTRIBUTION OF LABEL IN FREE L-PROLINE AND HYDROXY-L-PROLINE OF CHICK EMBRYOS FOLLOWING ADMINISTRATION OF *cis*- AND *trans*-4-H³-L-PROLINE

4-H³-Proline isomer		Expt.	Radioactivity in free imino acids: H^3 (C.p.m. × 10^{-6})	C^{14}	H^3/C^{14}	H^3 retention,[a] %
cis	1	Proline	2.25	0.499	4.51	
		Hydroxyproline	0.586	0.113	5.19	115
trans	1	Proline	3.07	0.461	6.65	
		Hydroxyproline	0.0031	0.120	0.02	0.39
	2	Proline	2.57	0.367	7.00	
		Hydroxyproline	0.0017	0.094	0.018	0.26
					Av.	0.38

[a] Calculated as shown in Table IV.

On the other hand, when 4-*trans*-H³-L-proline was administered, there was almost complete absence of the tritium label in hydroxyproline in both comparative studies. These findings prove unequivocally that the hydroxylation of 4-H³-proline involves a direct displacement of a single hydrogen atom from carbon atom 4 with retention of configuration.

Discussion

It must be emphasized that the methods for preparation of the two isomeric forms of 4-H³-proline and proof of their structures were key points of this study. Previous attempts to determine the fate of the hydrogen at position 4[10-13] made use of the product prepared by catalytic tritiation of 3,4-dehydroproline.[9] However, considerable randomization of tritium is known to occur in such catalytic tritiations of olefinic precursors, which may well explain the contradictory results obtained by the different investigators.

The related conversion of lysine into collagen-bound *erythro*-δ-hydroxy-L-lysine in chick embryos[43] does not utilize $^{18}O_2$ but $H^3{}_2O$,[43] and a sample of what was considered to be 4,5-H³-DL-lysine prepared by catalytic tritiation of an olefinic precursor apparently lost only one tritium atom in the process.[44] Here, too, it would be desirable to study the conversion with selectively tritiated *erythro*- and *threo*-5-H³-L-lysine.

The present studies, together with the previous findings on the incorporation of atmospheric $^{18}O_2$ in the hydroxylation of proline,[4-7] indicate that the process involves a displacement mechanism. The mechanism of hydroxyproline biosynthesis most closely resembles the mode of steroidal hydroxylation, *e.g.*, at the 11-α,[45] 11-β,[46] and 7-α positions.[47] A point of similarity between the two processes is that they occur without an isotope effect,[12] indicating that the rupture of the C–H (C–T) bond occurs after the rate-determining step of the hydroxylation or that a slow physical step, such as adsorption and alignment of the enzyme–substrate complex, precedes the chemical reaction. However, while steroidal hydroxylases attack both equatorial and axial positions,[48] 3- and 4-hydroxyprolines in col-

(43) D. Fujimoto and N. Tamiya, *Biochem. Biophys. Res. Commun.*, **10**, 498 (1963).

(44) E. A. Popenoe, R. B. Aronson, and D. D. Van Slyke, in preparation.

(45) E. J. Corey, G. A. Gregoriou, and D. H. Peterson, *J. Am. Chem. Soc.*, **80**, 2338 (1958).

(46) M. Hayano, M. Gut, R. I. Dorfman, O. K. Sebek, and D. H. Peterson, *ibid.*, **80**, 2336 (1958).

(47) S. Bergstrom, S. Lindstedt, B. Samuelson, E. J. Corey, and G. A. Gregoriou, *ibid.*, **80**, 2337 (1958).

(48) M. Hayano in "Oxygenases," edited by O. Hayaishi, Academic Press, Inc., New York, N. Y., and London, England, 1962, p. 182.

lagen are formed only as the *trans* diastereoisomers.[49,50]

Nonenzymatic displacements of a hydrogen at saturated carbon atoms with retention of configuration are relatively rare. One such case is the oxidation of *cis*-decalin to *cis*-9-hydroxydecalin, and of *trans*-decalin to *trans*-9-hydroxydecalin by the action of ozone.[51] We may deal here with an internal electrophilic displacement (SEI or "SEi") in which the ozone dipole acts simultaneously as acceptor of the proton and donor of the oxygen function in a concerted front-side displacement. The comparable autoxidation, with the diradicaloid oxygen as participant, should proceed by abstraction of a hydrogen radical from *trans*-decalin only to yield *trans*-9-decalyl hydroperoxide.[52] The stereoselectivity and retention of configuration is remarkable for a radical reaction.

The resonance spectrum of *trans*-4-hydroxy-L-proline in D_2O has been interpreted in favor of a nonplanar regular pentagon with hydroxyl in a *quasi-axial* position.[53] Nonenzymatic hydroxylation of proline[54] by the Udenfriend–Wieland system[55,56] is nonspecific and introduces hydroxyl to yield all four possible hydroxyprolines.[13] This hydroxylation has been considered radical rather than electrophilic in character.[57,57a] All these mechanistic and conformational considerations on the level of free proline, however, become a secondary point, if one considers that it is a microsomal RNA-bound polypeptide of considerable size[58] in which selected proline residues are stereospecifically hydroxylated in positions *trans* to the C-2 carboxyl function with full retention of the stereochemistry of C-4.

(49) F. Irreverre, K. Morita, A. V. Robertson, and B. Witkop, *J. Am. Chem. Soc.*, **85**, 2824 (1963).
(50) *Cf.* A. Kaplan, B. Witkop, and S. Udenfriend, *J. Biol. Chem.*, **239**, 2559 (1964).
(51) J. R. Durland and H. Adkins, *J. Am. Chem. Soc.*, **61**, 429 (1939).
(52) R. Criegee, *Ber.*, **77**, 722 (1944).
(53) R. J. Abraham and K. A. McLauchlan, *Mol. Phys.*, **5**, 513 (1962).
(54) M. Chvapil and J. Hurych, *Nature*, **184**, 1145 (1959).
(55) H. Wieland, "On the Mechanism of Oxidation," "Silliman Memorial Lectures," Vol. XXII, Yale University Press, New Haven, Conn., 1932, p. 86.
(56) S. Udenfriend, C. T. Clark, J. Axelrod, and B. B. Brodie, *J. Biol. Chem.*, **208**, 731 (1954).
(57) R. Breslow and L. N. Lukens, *ibid.*, **235**, 292 (1960).
(57a) NOTE ADDED IN PROOF.—A direct oxygen atom transfer has recently been suggested: G. A. Hamilton, *J. Am. Chem. Soc.*, **86**, 3391 (1964).
(58) B. Peterkofsky and S. Udenfriend, *ibid.*, **238**, 3966 (1963); *cf.* R. F. Lyndon and F. C. Steward, *J. Expll. Bot.*, **14**, 42 (1963).

[CONTRIBUTION FROM THE DEPARTMENTS OF BIOCHEMISTRY AND NEUROLOGY, COLUMBIA UNIVERSITY COLLEGE OF PHYSICIANS AND SURGEONS, NEW YORK 32, NEW YORK]

Factors Affecting the Competitive Formation of Oxazolines and Dehydroalanines from Serine Derivatives[1]

BY SARA GINSBURG AND IRWIN B. WILSON

RECEIVED APRIL 24, 1964

Several factors affecting the relative amounts of oxazolines and dehydroalanines formed from N-carbonyl-O-sulfonylserine derivatives were investigated. It was found that N-carbobenzoxy derivatives do not form oxazolines even under very favorable conditions while N-*p*-nitrobenzoyl compounds can form either product. Serine esters yield either product while the amide strongly favors the formation of oxazoline, but the dehydroalanine can be obtained if the N-carbonyl group is carbobenzoxy. Strong bases favor the formation of dehydroalanines. More polar solvents favor the formation of oxazolines. The application of these findings to the reactions of sulfonyl proteins is discussed.

Introduction

In the reaction of sulfonyl chlorides with proteins numerous sulfonyl groups may be readily introduced into the protein molecule. One protein site in chymotrypsin reacts faster than the others.[2] With sulfonyl fluorides the reaction is more specific and in the case of esterases only one group reacts readily. This group is introduced at the active site and the resulting sulfonyl enzyme derivative is analogous to the normal acyl enzyme formed during the hydrolysis of substrates.[3,4] The sulfonyl enzyme is inactive, of course, but activity can be restored by suitable nucleophilic agents in the case of acetylcholinesterase or by suitable changes in the medium in the case of chymotrypsin. Sulfonyl fluorides in their reaction with esterases behave quite similarly to the organophosphorus antiesterases and it would therefore be expected that serine is the amino acid residue that is sulfonylated. The reactions of O-substituted serine derivatives are therefore quite pertinent for inferring the chemical possibilities of sulfonylated esterases. The ability of some of these compounds to react to form oxazolines and the isomeric dehydroalanine derivatives is especially interesting. The reactions which are pertinent to the problem are indicated in Chart I.

Working with N-benzoyl-DL-serine methyl ester and thionyl chloride, Fry[5], using the method of Bergmann and co-workers,[6] obtained a "complex salt" with a composition approximating the O-chlorosulfinyl derivative. He prepared the oxazoline from this complex salt and also obtained the oxazoline and dehydroalanine from the chloro compound. Riley[7] obtained the dehydroalanine derivative from N-carbobenzoxy-O-diphenylphosphoryl-DL-serine ethyl ester. Attenburrow[8] prepared the oxazoline from an O-tosylthreonine derivative. Photaki's work[9] is most pertinent for our problem. She obtained *only* the dehydroalanine derivatives from N-carbobenzoxy-O-tosyl-L-serine methyl ester.

(1) This work was supported by the Division of Research Grants and Fellowships of the National Institutes of Health, Grant B-00573 (16) of the United States Public Health Service, and Research Career Award GM-K3-15,012, and by National Science Foundation Grant 18926.
(2) V. Massey, W. F. Harrington, and B. S. Hartley, *Discussions Faraday Soc.*, **20**, 24 (1955).
(3) R. Kitz and I. B. Wilson, *J. Biol. Chem.*, **237**, 3245 (1962); J. Alexander, I. B. Wilson, and R. Kitz, *ibid.*, **238**, 741 (1963).
(4) A. M. Gold and D. Fahrney, *Biochemistry*, **3**, 783 (1964).
(5) E. M. Fry, *J. Org. Chem.*, **14**, 887 (1949).
(6) M. Bergmann and A. Miekeley, *Z. Physiol. Chem.*, **140**, 128 (1924); **143**, 108 (1925); M. Bergmann and E. Brand, *Ber.*, **56**, 1280 (1923).
(7) G. Riley, J. Turnbull, and W. Wilson, *J. Chem. Soc.*, 1373 (1957).
(8) J. Attenburrow, D. F. Elliott, and G. F. Remy, *ibid.*, 310 (1948).
(9) I. Photaki, *J. Am. Chem. Soc.*, **85**, 1123 (1963).