chem. Biophys. Res. Commun. 13, 49.
Robertson, A. V., Katz, E., and Witkop, B. (1962), *J. Org. Chem. 27*, 2676.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Witkop, B. (1955), Special Publication No. 3, London, The Chemical Society, p. 60.
Witkop, B. (1956), *Experientia 12*, 372.
Witkop, B., and Beiler, T. (1956), *J. Am. Chem. Soc. 78*, 2882.
Witkop, B., and Foltz, C. M. (1957), *J. Am. Chem. Soc. 79*, 192.

Incorporation of *cis*- and *trans*-4-Fluoro-L-prolines into Proteins and Hydroxylation of the *trans* Isomer During Collagen Biosynthesis*

A. Arthur Gottlieb,† Yoshimasa Fujita, Sidney Udenfriend, and Bernhard Witkop‡

ABSTRACT: *cis*- and *trans*-4-fluoro-L-prolines (IV and XI), synthesized *via* the tosyloxy-L-proline derivatives I and VIII by S$_N$2 displacement with fluoride ion, were tritiated catalytically to the ^{3}H derivatives V and XII. Both tritiated fluoroprolines V and XII were incorporated into protein and unhydroxylated precursor of collagen. The latter was degradable by collagenase. Enzymatic hydroxylation of bound tritiated *trans*-4-fluoro-L-proline led to bound 4-hydroxy-L-proline which after hydrolysis was isolated and identified.

Substituted amino acids have frequently been useful in elucidating mechanisms of amino acid metabolism. *p*-Fluorophenylalanine was shown to serve as a substrate of phenylalanine hydroxylase (Kaufman, 1962) and halogen-substituted tyrosines have recently proved useful as inhibitors of tyrosine hydroxylase (Udenfriend *et al.*, 1965). The hydroxylation of proline was shown to take place after incorporation into protein (Peterkofsky and Udenfriend, 1963). Two questions of interest emerged: is fluoroproline incorporated into proteins and, if so, is the fluoroproline bound in the collagen precursor oxidatively defluorinated to yield bound hydroxyproline?

The present report describes the preparation of the tritium-labeled *cis*- and *trans*-4-fluoro-L-prolines, their use as substrates for protein synthesis in *Escherichia coli* extracts and guinea pig granuloma minces, and as substrates for hydroxylation in the latter system.

Experimental Section

The ascorbic acid deficient diet came from Nutritional Biochemical Corp. Carrageenan was obtained from Marine Colloids Inc. Commercial L-proline-3,4-^{3}H (320 μc/μmole) (New England Nuclear Corp., Boston, Mass.) was purified by chromatography on a Dowex 50-X8 column (H^+ form) with 1.0 N HCl as the eluent. Purified collagenase was obtained from the Worthington Biochemical Corp. This material gave no evidence of proteolysis with the following substrates (1 mg/ml): albumin, casein, fibrin, and elastin. Optimal digestion of hot trichloroacetic acid extractable material from microsomes was achieved by treatment of 15.0-μg quantities of collagenase substrate with 10 μg of purified collagenase for 90 min in a 1-ml volume containing 0.025 M Tris–HCl buffer (pH 7.2) and 0.33 M calcium acetate.

Induction of Granuloma. Male guinea pigs were maintained on regular diets, which included cabbage, or on an ascorbic acid deficient diet for 10 days prior to injection. Granulomata were induced by subcutaneous injection of 8 ml of a 1% solution of carrageenan. The granulomata were excised 6 days after injection, placed in ice-cold 0.01 M glucose, washed once with cold 0.01 M glucose, and then minced thoroughly. Five grams of tissue was incubated in 10 ml of medium containing 25 μc of 3,4-tritiated L-proline-^{3}H or 100–140 μc of *cis*- or *trans*-4-fluoro-L-proline-^{3}H for 2 hr at 37° under O_2–CO_2 (95:5). The medium also contained NaCl (0.022 M), KCl (0.003 M), $MgSO_4$ (0.012 M), $CaCl_2$ (0.0013 M), KH_2PO_4 (0.0004 M), $NaHCO_3$ (0.025 M), and D-glucose (0.01 M). At the end of the incubation the mixture was centrifuged at 3°, the supernatant was discarded, and the tissue washed once with 5 ml of cold 0.25 M sucrose solution. Another 5 ml of 0.25 M sucrose

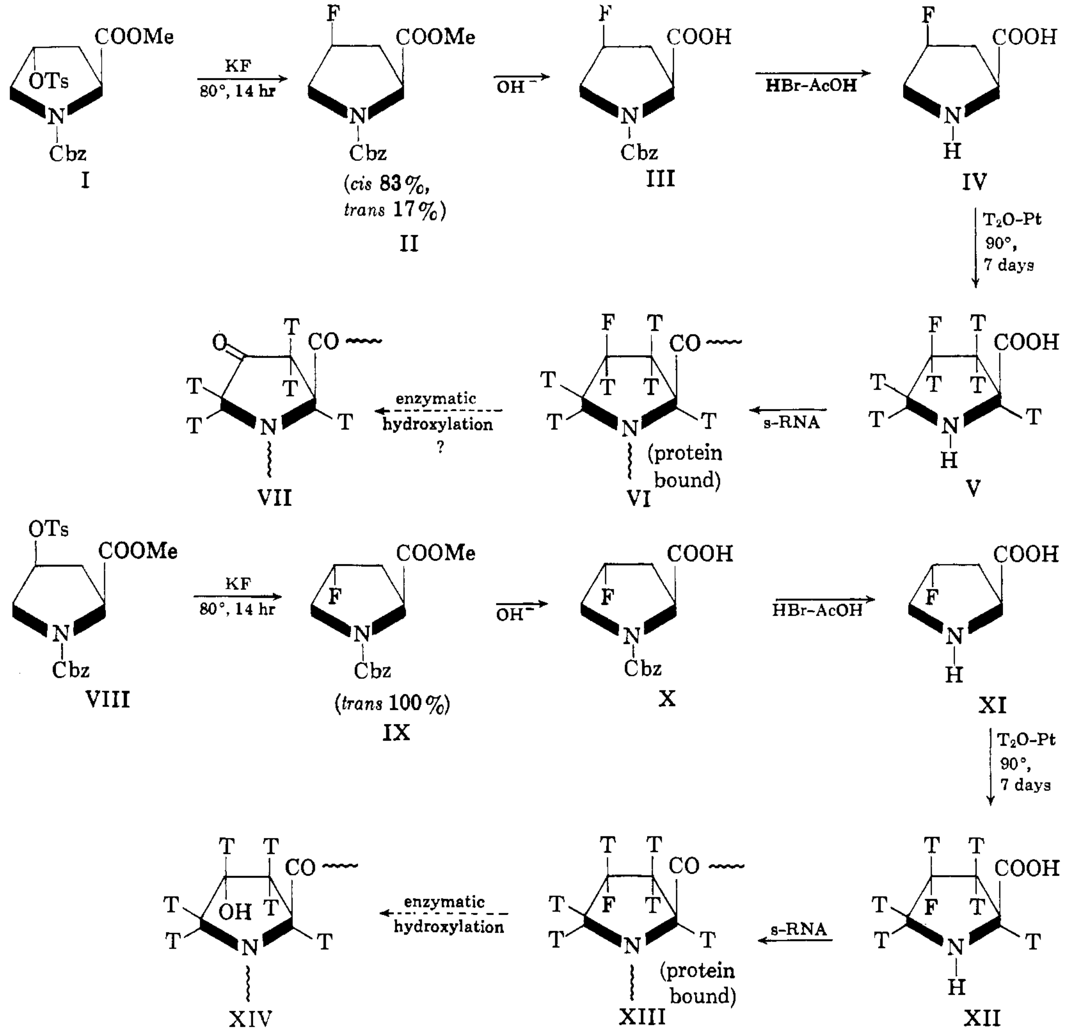
---

[*] From the National Heart Institute and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Md. *Received June 7, 1965.*

[†] Present address: Department of Chemistry, Harvard University, Cambridge, Mass.

[‡] Requests for reprints should be sent to the Laboratory of Chemistry, National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

SCHEME I: Synthesis and Incorporation of Tritiated *cis*-and *trans*-4-Fluoroproline into Protein (Procollagen).



solution was then added to the centrifuge tube and the precipitated tissue was resuspended by homogenization in a Servall omni-mixer. Aliquots of the homogenate were treated with equal volumes of 10% trichloroacetic acid and, after centrifugation, the supernatant fluid was decanted. The precipitates were washed with cold 5% trichloroacetic acid, extracted into hot trichloroacetic acid (gelatinization), and dialyzed, as described previously (Peterkofsky and Udenfriend, 1963). The extracts were then lyophilized and made up to a volume of 2.0 ml, 0.05 ml of which was taken for protein assay (Lowry *et al.*, 1951); 0.95 ml was subjected to hydrolysis in 6.0 N HCl and the remaining 1.0 ml was treated with collagenase as described above. After this digestion the proteins were precipitated by the addition of an equal volume of 20% tannic acid prepared as previously described (Gottlieb *et al.*, 1965). The tannic acid precipitate was hydrolyzed in 1 ml of 6.0 N HCl; tannic acid was extracted from the supernatant solution (Gottlieb *et al.*, 1965), and an equal volume of concentrated HCl was added. Hydrolysis was carried out in an autoclave for 2.5 hr at 18 psi and 124°. Proline and hydroxyproline were isolated and their specific activities assayed as previously described (Peterkofsky and Prockop, 1962).

The incorporation of fluoroprolines and proline into cold trichloroacetic acid precipitable protein of *E. coli* S-30 fractions was determined on material made available by Dr. M. Nirenberg. Samples were incubated for 20 min with 8 mμmoles of isotopically labeled *cis*- and *trans*-fluoroproline in the manner described by Nirenberg (1964), but with omission of exogenous messenger ribonucleic acid (m-RNA). At the end of the incubation trichloroacetic acid was added to a final concentration of 10% and the mixture heated at 94° for 20 min. The suspension was then poured onto a Millipore filter and the precipitate was washed four times with 5-ml portions of cold 5% trichloroacetic acid, twice with 5 ml of ethanol–ether (4:1), and twice with absolute ether. The filters were dried thoroughly in an oven at 70° for 1 hr, then placed in a fluorophor liquid scintillation medium (Prockop *et al.*, 1961). "Zero-time" blanks

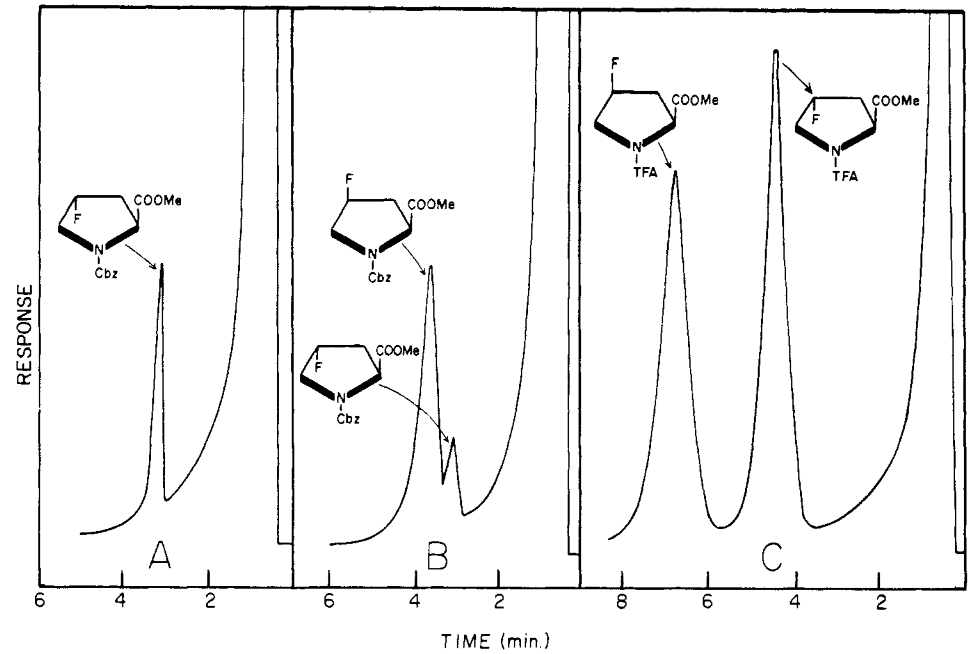
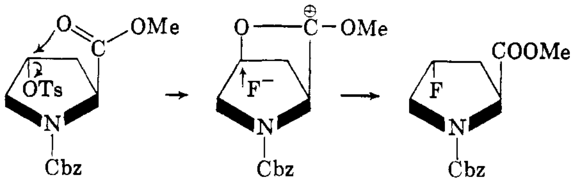


FIGURE 1: Gas chromatographic characterization and separation of 4-fluoroprolines. A, pure *trans*-4-fluorocarbobenzyloxy-L-proline methyl ester (IX) obtained by fluoride displacement on the *cis*-tosylate VIII; B, mixture of 83% *cis*- and 17% *trans*-fluoroprolines II resulting from fluoride displacement on the *trans*-tosylate I; C, separation of the *cis*- and *trans*-4-fluoroproline methyl esters in the form of their *N*-trifluoroacetyl derivatives.

were obtained by treating the incubation mixture with cold trichloroacetic acid in a final concentration of 10%, prior to adding the S-30 enzyme fraction. Radioactivity was measured in a liquid scintillation spectrometer with an efficiency of 18% for ^{3}H in toluene.

Preparation and Characterization of the Labeled Fluoroprolines (Scheme I). Displacement of sulfonic esters of alcohols by anionic fluoride species has been successfully applied to the introduction of fluorine into organic compounds (*cf.* Hudlicky, 1962; Djerassi, 1963). In the present study *cis*- and *trans*-4-fluoro-L-prolines were prepared by the displacement of the *O*-*p*-toluenesulfonyl esters derived from hydroxy-L-proline and its allo diastereoisomer by fluoride. *N*-Carbobenzyloxy-*O*-tosylallohydroxy-L-proline methyl ester (VIII) was heated with anhydrous potassium fluoride in diethylene glycol. *trans*-*N*-Carbobenzyloxy-4-fluoro-L-proline methyl ester (IX) was obtained as a colorless oil and its homogeneity was proved by gas chromatography (Morita *et al.*, 1963) (Figure 1A). *N*-Carbobenzyloxy-*O*-tosylhydroxy-L-proline methyl ester (I) was fluorinated in the same way to produce a mixture of 83% *cis*- and 17% *trans*-4-fluoro derivatives (II and IX, Figure 1B).

The previously reported analogous replacement of *O*-tosyl by iodide ion was complicated by secondary replacement reactions on the 4-iodoprolines by excess iodide ion. The reaction product invariably consisted of a mixture of *cis*- and *trans*-4-iodoprolines (Fujita *et al.*, 1964a). Since attack of excess fluoride ion on the *cis*- or *trans*-4-fluoroprolines would not be expected to happen, the formation of 17% *trans*-fluoroproline from the *trans*-tosylate I is better explained by intramolecular participation of the ester carbonyl in the displacement process as follows.



trans-*N*-Carbobenzyloxy-4-fluoro-L-proline methyl ester (IX) was saponified to X which was then decarbobenzoxylated to XI by hydrogen bromide in glacial acetic acid (Ben-Ishai and Berger, 1952). The crude amino acid XI was purified by repeated recrystallization. Pure *cis*-4-fluoro-L-proline IV was obtained in analogous fashion.

cis- and *trans*-4-fluoro-L-prolines failed to separate by analytical column chromatography under conditions which easily separated the diastereoisomers of 3- or 4-hydroxyprolines and 4- or 5-hydroxypipecolic acids (Fujita *et al.*, 1964b). However, the *N*-trifluoroacetylprolines were separated by gas chromatography on a 4% NGS column (Cruickshank and Sheehan, 1964) (Figure 1C). By this technique the steric homogeneity of both isomers was ascertained.

cis- and *trans*-4-fluoroprolines were tritiated catalytically under slightly basic conditions (Rothchild, 1963). The crude *cis*- and *trans*-4-fluoro-L-proline-^{3}H (V and XII) were purified by ion-exchange column chromatography.

In the course of the present study, we found that the

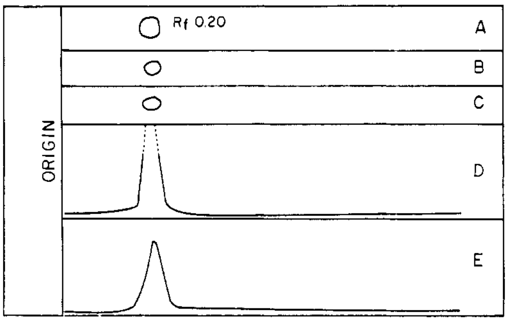


FIGURE 2: Paper chromatography and radiography of *cis*- and *trans*-4-fluoro-L-proline-U-^{3}H. Solvent used was 1-butanol–acetic acid–pyridine–water (40:10:10:20), one-dimensional, ascending. The paper was sprayed with Ehrlich's reagent after oxidation with H_2O_2. A, authentic *trans*-4-fluoro-L-proline; B and C, *cis*- and *trans*-4-fluoro-L-proline-U-^{3}H; D and E, radioautograms of *cis*- and *trans*-4-fluoro-L-proline-U-^{3}H, respectively.

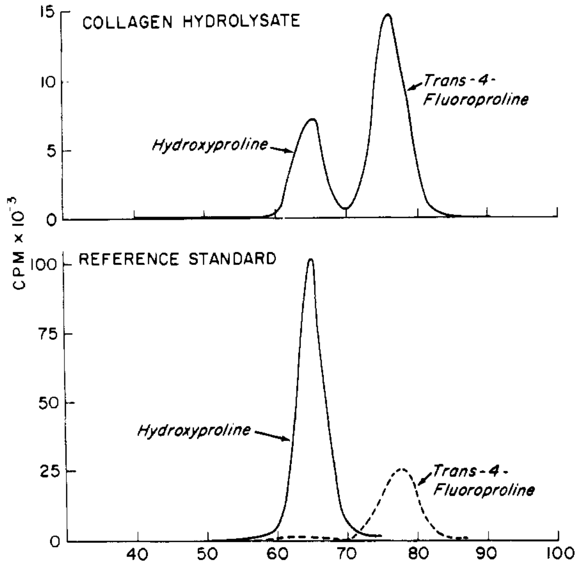


FIGURE 3: DL-Hydroxyproline-^{14}C (1.1 × 10^6 dpm) and 1.3 × 10^6 dpm of 4-fluoroproline-^{3}H chromatographed on a Dowex 50-X8 column (NH_4^+ form) buffered at pH 6.8 using 0.2 M NH_4OAc in 40% ethanol as eluent (Robertson *et al.*, 1962). Fractions (1 ml) were collected at 6 ml/hr and added to 10 ml of Bray's solution before counting in a liquid scintillation spectrometer. Trichloroacetic acid extractable material prepared from granuloma which had been incubated with *trans*-4-fluoroproline was chromatographed in a similar manner.

fluoroprolines have a surprisingly low color yield with ninhydrin reagent. A more sensitive detection method for the determination of the fluoroprolines was oxidation and decarboxylation under conditions which convert hydroxyproline to pyrrole. Addition of *p*-dimethylaminobenzaldehyde (Dahl and Persson, 1963) easily made the fluoroprolines visible on paper chromatograms as bluish purple spots. The minimum detectable amount was 0.1 μg.

trans-4-Fluoro-L-*proline* (*XI*). To a solution of *N*-carbobenzyloxy-*O*-*p*-toluenesulfonylallohydroxy-L-proline methyl ester (VIII) (Patchett and Witkop, 1957) (866 mg, 2 mmoles) in diethylene glycol (8 ml) was added anhydrous potassium fluoride (800 mg), and the mixture was heated at 80° for 14 hr under stirring. The solution was diluted with 8 ml of water and extracted with two 15-ml portions of ethyl acetate. The extract was washed with saturated salt solution and dried over sodium sulfate. The solvent was evaporated *in vacuo* and the residual oil was repeatedly evaporated after addition of methanol. The crude preparation of *N*-carbobenzyloxy-*trans*-4-fluoro-L-proline methyl ester (IX), though it failed to crystallize, was homogeneous as shown by gas (Figure 1A) and thin layer chromatography.

The oily ester IX was dissolved in methanol (5 ml) and cooled to 0°. To the solution was added 2.0 N NaOH (1.2 ml, 2.4 mmoles) and the mixture was left at 0° for 1 hr, then at room temperature overnight. The solution was diluted with 10 ml of water and extracted with ether. The aqueous layer was acidified with concentrated hydrochloric acid and extracted with two 5-ml portions of ethyl acetate. The extract was washed with saturated salt solution and dried over sodium sulfate. After removal of the solvent the oily residue of X was dissolved in 32% hydrogen bromide in glacial acetic acid (2 ml) and the solution was left at room temperature for 1 hr. Ether (20 ml) was added to the reaction mixture and the oily precipitate was washed with ether by decantation. The residue was dissolved in water (5 ml) and passed through a column of Dowex 1-X8 (acetate form). The effluent was evaporated and the colorless crystals were collected and washed with aqueous ethanol to yield 149 mg (56%), mp 230–232° dec. Two recrystallizations from 90% ethanol raised the melting point to 243–245° dec, $[\alpha]^{20}_D$ −87.6 ± 1.0° (*c* 1, water).

Anal. Calcd for $C_5H_8FNO_2$: C, 45.11; H, 6.06; F, 14.27; N, 10.53. Found: C, 45.29; H, 5.93; F, 14.49; N, 10.51.

Further recrystallizations failed to raise the melting point. The purity of the preparation was ascertained by paper and gas chromatography (Figure 2).

cis-4-Fluoro-L-*proline* (*IV*) was synthesized starting with *N*-carbobenzyloxy-*O*-*p*-toluenesulfonylhydroxy-L-proline (I, 1.731 g, 4 mmoles) in the same way as described above. There was obtained 330 mg (62%) of colorless crystals, mp 251° dec. Two recrystallizations from 90% ethanol raised the melting point to 271° dec, $[\alpha]^{20}_D$ −40.2 ± 1.0° (*c* 1, water).

Anal. Calcd for $C_5H_8FNO_2$: C, 45.11; H, 6.06; F, 14.27; N, 10.53. Found: C, 45.21; H, 5.89; F, 14.25; N, 10.67.

The homogeneity of the amino acid was ascertained by paper and gas chromatography.

TABLE I: Incorporation of Fluoroproline into *E. coli* Proteins.[a]

		Incorporated into Protein		
Imino Acid Added	Total cpm	Cpm Corrected for Controls	mμc	mμmole
cis-4-Fluoroproline	311	115	0.29	0.0053
cis-4-Fluoroproline	333	137	0.35	0.0064
Zero-time control	196			
trans-4-Fluoroproline	1018	716	1.81	0.024
trans-4-Fluoroproline	1222	924	2.33	0.030
Zero-time control	298			
Proline	768	657	1.66	0.0052
Zero-time control	111			

[a] Eight mμmoles of imino acid was used in each incubation. The specific activities of the compounds studied were *cis*-4-fluoroproline 55 μc/μmole, *trans*-4-fluoroproline 77 μc/μmole, and L-^{3}H-proline 320 μc/μmole. Incorporation was due to endogenous m-RNA. A zero-time control was run by adding the *E. coli* extract to the incubation flask after addition of trichloroacetic acid.

*trans-4-Fluoro-*L*-proline-^{3}H (Generally Labeled).* *trans*-4-Fluoro-L-proline (XI, 17 mg) was dissolved in 0.2 ml of pH 9.0 buffer solution containing 10 c of tritiated water and 25 mg of prereduced platinum catalyst. The mixture was heated at 90° for 7 days and stirred magnetically. After separation of the catalyst, exchangeable tritium was removed by the addition of 10 ml of water and subsequent evaporation (50°) to dryness *in vacuo.*[1] The residue was dissolved in 1 ml of 0.2 M ammonium acetate solution in water–ethanol (40:60 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; 2.4-ml fractions were collected. Tubes 17–22 contained fluoroproline. These fractions were combined and desalted by passage through a column of Dowex 50-X8 (H^+ form; 1 × 11 cm) and elution with 7.0 N ammonium hydroxide. The eluate was evaporated *in vacuo* and the crystalline residue was collected and washed with the aid of ethanol. The yield of pure *trans*-4-fluoro-L-proline-U-^{3}H (XII) was 11.0 mg (65%), and the specific activity was 77 mc/mmole. The tritiated fluoroproline showed a single spot with Ehrlich's reagent after oxidation with hydrogen peroxide on a paper chromatogram, and a sharp single peak at the location of the color spot, when a paper chromatogram was scanned by the Vanguard automatic scanner (Figure 2). The purity of the tritiated material was further confirmed by ion-exchange chromatography (Figure 3). One radioactive peak corresponding in retention volume to authentic *trans*-4-fluoro-L-proline was obtained.

*cis-4-Fluoro-*L*-proline-^{3}H (Generally Labeled) (V).* *cis*-4-Fluoro-L-proline (IV, 20 mg) was tritiated and purified in the same way as described above. The yield of pure *cis*-4-fluoro-L-proline-U-^{3}H (V) was 9.1 mg (45%) and the specific activity was 55 mc/mmole. The purity of the preparation was confirmed by paper and ion-exchange chromatography (Figure 2).

Results

As shown in Table I, both *cis*- and *trans*-fluoroproline were incorporated into the proteins of *E. coli.* The *trans* isomer appeared to be incorporated to an even greater extent than proline itself. However, it should be pointed out that *E. coli* preparations contain endogenous proline which causes substantial dilution of the added material. No such dilution can occur with the fluoroprolines.

When guinea pig granuloma minces were incubated with the fluoroprolines, both imino acids were incorporated into proteins (Table II). With this tissue more of the *trans* isomer was incorporated than the *cis* isomer. Incorporation of *trans*-4-fluoroproline appeared to be as efficient as that of proline. However, exact comparisons are not possible because the imino acids were not added in comparable amounts and because appreciable amounts of free proline were present in the minces, thereby diluting the proline and competing with the fluoroprolines. The hydrolysates of the gelatinized hot trichloroacetic acid extracts were also assayed for

TABLE II: Fluoroproline and Hydroxyproline Appearing in Proteins of Granuloma Minces Following Incubation with Fluoroproline-^{3}H.[a]

Imino Acid in Incubation Flask	Expt	Imino Acids Incorporated (cpm) Pro[b]	Hypro	Pro[b]/Hypro
trans-4-Fluoroproline	1	51,742	12,109	4.3
	2	17,695	7,903	2.2
cis-4-Fluoroproline	1	3,097	167	19
	2	628	0	∞
L-Proline	1	10,323	1,534	6.7
	2	16,558	—	—

[a] One-hundred and forty microcuries (1.9 μmoles) of *cis*- and *trans*-fluoro-L-proline-^{3}H was used in expt 1 while 100 μc (1.14 μmoles) was used in expt 2; 25 μc (0.1 μmole) of L-proline-^{3}H was used. [b] Represents fluoroproline in those studies where the fluoroprolines were used as labeled precursors.

---

[1] The tritiation procedure was carried out by the New England Nuclear Corp., Boston, Mass.

TABLE III: Labeled Fluoroproline and Hydroxyproline Released by Collagenase from Tissues Incubated with Fluoroproline-^{3}H.[a]

Labeled Imino Acid Added to Incubation Flask	Expt	Imino Acid Incorporated (cpm)				
		Collagenase–Trichloroacetic Acid Precipitate		Collagenase–Trichloroacetic Acid Supernatant		Supernatant
		Pro[b]	Hypro	Pro[b]	Hypro	Pro[b]/Hypro
trans-4-Fluoroproline	1	22,116	5594	16,800	5921	2.8
	2	9,555	2686	2,746	2705	1.0
cis-4-Fluoroproline	1	1,365	68	2,620	78	34
	2	353	0	186	0	∞
Proline	1	7,068	1095	2,430	935	2.6
	2	9,630	1096	1,015	661	1.5

[a] These experiments represent aliquots from the same incubation flasks shown in Table II. [b] Represents fluoroproline in those studies where the fluoroprolines were used as labeled precursors.

hydroxyproline radioactivity. As shown in Table II the hydroxyproline isolated following incubation with either labeled proline or *trans*-fluoroproline was highly radioactive. The *trans* isomer is therefore not only incorporated into proteins, but is hydroxylated to yield 4-hydroxyproline. The labeled 4-hydroxyproline obtained on incubation with *trans*-4-fluoroproline-^{3}H was further identified by column chromatography. As shown in Figure 3, two peaks were obtained with the reference standards, as well as with the material obtained from the incubation mixture.

Table III presents the results of two experiments in which the hot trichloroacetic acid extractable material was treated with collagenase. In each experiment, both isomers were incorporated into a fraction which could be degraded by collagenase, although the incorporation of the *cis*-fluoroproline was considerably less than that of the *trans* isomer. Moreover, little of the *cis*-fluoroproline appeared to be hydroxylated, whereas substantial amounts of the *trans* isomer were converted to hydroxyproline. Hydroxylation of the *cis* isomer, if it had occurred, would have led to the formation of 4-ketoproline. Our methods of analysis and radioassay would not have detected 4-ketoproline if it had survived the conditions of hydrolysis.

Since fluoroproline could be oxidized to pyrrole compounds under certain conditions (see above), it was appropriate to determine if any of the *trans*-4-fluoro-L-proline-^{3}H was converted to pyrrole by the procedure used for radioassay (Peterkofsky and Prockop, 1962). Accordingly, 0.82 μc of *trans*-4-fluoro-L-proline-^{3}H was taken through the radioassay. In two experiments, 198 and 124 cpm, and 193 and 903 cpm were recovered in the proline fraction, whereas the hydroxyproline fraction contained no radioactivity when corrected for the controls which are run with each determination.

Discussion

Enzymatic hydroxylation of L-proline to *trans*-4-hydroxy-L-proline involves a direct displacement of the *trans* hydrogen in the 4-position (Fujita *et al.*, 1964a) Systems which hydroxylate phenylalanine to tyrosine (Kaufman, 1962) and aniline to *p*-aminophenol (Renson, *et al.*, 1965) can utilize the corresponding *p*-fluoro derivatives as substrates and form the corresponding phenols by oxidative displacement of the fluorine atom. The formation of *trans*-4-hydroxyproline from *trans*-4-fluoroproline offers further proof that proline hydroxylase resembles other (aromatic) hydroxylases in its mode of action.

Since proline hydroxylase acts on protein-bound proline (Peterkofsky and Udenfriend, 1963, 1965; Prockop and Juva, 1965; Lukens, 1965), the present findings provide additional evidence that *trans*-4-fluoroproline is incorporated into the unhydroxylated precursor of collagen. The findings with collagenase show that both the *cis* and *trans* isomer are incorporated into the collagen precursor. It should be noted that the ratios FluoroPro/Hypro in the acid hydrolysates of the 4-fluoroproline-^{3}H experiments were much lower than the ratios of Pro/Hypro obtained with proline-^{14}C (Table II). In other experiments with *trans*-fluoroproline ratios were even lower than shown in Table II. With proline-^{14}C, ratios were most frequently closer to 10. These findings suggest that *trans*-4-fluoroproline may be more readily incorporated into the collagen precursor protein than into other proteins of the guinea pig granuloma minces. Nevertheless, both *cis*- and *trans*-fluoroproline can be incorporated into other proteins, not only in granuloma but in *E. coli* as well. The fluoroprolines should prove of interest in elucidating the specificity of proline incorporation into protein-synthesizing systems, and more specifically into the collagen-synthesizing system. Fluoroproline may offer certain advantages over proline in coding experiments since it is not diluted with endogenous amino acid present in biological preparations.

References

Ben-Ishai, D., and Berger, A. (1952), *J. Org. Chem. 17*, 1564.

Cruickshank, P. A., and Sheehan, J. C. (1964), *Anal. Chem. 36*, 1191.

Dahl, O., and Persson, K. (1963), *Acta Chem. Scand. 17*, 2499.

Djerassi, C. (1963), *in* Steroid Reactions, San Francisco, Holden-Day, p. 170.

Fujita, Y., Gottlieb, A., Peterkofsky, B., Udenfriend, S., and Witkop, B. (1964a), *J. Am. Chem. Soc. 86*, 4709.

Fujita, Y., Irreverre, F., and Witkop, B. (1964b), *J. Am. Chem. Soc. 86*, 1844.

Gottlieb, A. A., Peterkofsky, B., and Udenfriend, S. (1965), *J. Biol. Chem.* (in press).

Hudlicky, M. (1962), *in* Chemistry of Organic Fluorine Compounds, New York, MacMillan, p. 112.

Kaufman, S. (1962), *in* Oxygenases, Hayaishi, O., ed., New York, Academic, pp. 151–156.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Lukens, L. N. (1965), *J. Biol. Chem. 240*, 1661.

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

Nirenberg, M. (1964), *Methods Enzymol. 6*, 17.

Patchett, A. A., and Witkop, B. (1957), *J. Am. Chem. Soc. 79*, 185.

Peterkofsky, B., and Prockop, D. J. (1962), *Anal. Biochem. 4*, 400.

Peterkofsky, B., and Udenfriend, S. (1963), *J. Biol. Chem. 238*, 3966.

Peterkofsky, B., and Udenfriend, S. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 335.

Prockop, D. J., and Juva, K. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 661.

Prockop, D. J., Udenfriend, S., and Lindstedt, S. (1961), *J. Biol. Chem. 236*, 1395.

Renson, J., Weissbach, H., and Udenfriend, S. (1965), *Mol. Pharmacol. 1*, 145.

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

Rothchild, S. (1963), *Advan. Tracer Methodology 1*, 50.

Udenfriend, S., Zaltzman-Nirenberg, P., and Nagatsu, T. (1965), *Biochem. Pharmacol.* (in press).

Synthesis and Metabolism of 6-Hydroxycatecholamines*

J. W. Daly,† J. Benigni,‡ R. Minnis,‡ Y. Kanaoka,§ and B. Witkop†

ABSTRACT: The following 6-hydroxycatecholamines (2,4,5-trihydroxyphenethylamines or -phenethanolamines), potential metabolites of catecholamines, were synthesized (Charts I–III): 3-*O*-methyl-6-hydroxydopamine, 6-hydroxynorepinephrine, 3-*O*-methyl-6-hydroxynorepinephrine, 6-hydroxyepinephrine, and 3-*O*-methyl-6-hydroxyepinephrine. Enzymatic *O*-methylation with catechol *O*-methyltransferase with *S*-adenosylmethionine-^{14}C as donor of $^{14}CH_3$ gave radioactive ^{14}C-labeled 3-*O*-methyl-6-hydroxycatecholamines which were used for metabolic studies in the rat. The corresponding phenylacetic (**20a**) and mandelic acids (**23a**) as well as the phenylglycol (**30a**) were identified as metabolites by comparison with synthetic compounds (Table I, Chart IV). Eleven new potential metabolites were compared and characterized (Table II). The relative substrate activity of some of these 3-*O*-methyl-6-hydroxycatecholamines with monoamine oxidase (Table III) was much lower than that of 3-*O*-methyldopamine or normetanephrine.

Alternate pathways for the metabolism of catecholamines, such as dopamine, norepinephrine, and epinephrine, have received intensive scrutiny in attempts to define normal metabolism and in the hope of detecting metabolic aberrations in congenital and chronic dyscrasias (Daly and Witkop, 1963). One such possible pathway is the demonstrated nuclear hydroxylation of dopamine *in vivo* (Senoh *et al.*, 1959a,c) and *in vitro* (Senoh and Witkop, 1959a,b; Senoh *et al.*, 1959a,c) which leads to 2,4,5-trihydroxyphenethylamine (6-hydroxydop-

---

* *Received June 7, 1965.*

† National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Md.

‡ Regis Chemical Co., Chicago, Ill. Performed under Research Contract SA-43-ph-3021 of the Psychopharmacology Service Center, National Institute of Mental Health, Bethesda, Md.

§ Visiting Scientist of the U. S. Public Health Service, 1960–1961. Present address: Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan.