MOLECULAR PHARMACOLOGY, 13, 1105–1110

Incorporation of 2-Fluoro-L-histidine into Cellular Protein

DAVID C. KLEIN AND JOAN L. WELLER

Neuroendocrinology Unit, Behavioral Biology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014

KENNETH L. KIRK

Section on Biochemical Mechanisms, Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

ROBERT W. HARTLEY

Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

(Received May 3, 1977)

(Accepted July 15, 1977)

SUMMARY

KLEIN, DAVID C., WELLER, JOAN L., KIRK, KENNETH L. & HARTLEY, ROBERT W. (1977) Incorporation of 2-fluoro-L-histidine into cellular protein. *Mol. Pharmacol.*, 13, 1105–1110.

The purpose of this study was to determine whether mammalian cells can utilize 2-fluoro-L-histidine in protein synthesis. Rat pineal glands were incubated in organ culture in medium containing 2-fluoro-L-[4-^{3}H]histidine (3 mM); trichloracetic acid-insoluble material from these glands contained radioactivity. Incorporation of radioactivity was decreased in the presence of histidine (3 mM). After the ^{3}H-labeled macromolecules were treated with a protease, over 75% of the radioactivity was soluble in trichloracetic acid. Amino acid analysis of the trichloracetic acid-soluble material indicated that the majority of the liberated radioactivity was 2-fluoro-L-[^{3}H]histidine. These findings demonstrate that mammalian cells can incorporate 2-fluoro-L-histidine into newly synthesized proteins. In view of this, it would appear likely that the reported inhibitory effects of 2-fluoro-L-histidine on enzyme induction could result, in part, from the incorporation of the analogue into proteins.

INTRODUCTION

2-Fluoro-L-histidine (1), a new and potentially useful analogue of histidine, is of special interest because of the unique properties imparted to the imidazole ring by fluorine substitution. While this substitution does not have a significant steric effect, it has a profound influence on the electronic configuration of the imidazole ring. As a result of the strong electron-withdrawing effect of fluorine, the fluoro derivative lacks the characteristic nucleophilic and catalytic properties that are necessary for histidine to function in enzyme action and protein structure (2, 3).

A wide range of biological effects have been described for 2-F-His[1] (1, 2), includ-

[1] The abbreviations used are: 2-F-His, 2-fluoro-L-histidine; 2-F-[^{3}H]His, 2-F-[4-^{3}H]His; [^{14}C]His, [*U*-^{14}C]His; *N*-acetyltransferase, acetyl-CoA:arylamine *N*-acetyltransferase (EC 2.3.1.5); TCA, trichloracetic acid.

ISSN 0026-895X

ing our report of inhibition of the induction of several enzymes (4). In addition, we found that 2-F-His inhibition of the isoproterenol induction of pineal acetyl-CoA: arylamine *N*-acetyltransferase (5) is concomitant with inhibition of [^{14}C]histidine incorporation into protein. In contrast, under the same conditions, 2-F-His does not significantly inhibit the incorporation of [^{3}H]leucine into protein, the incorporation of [^{3}H]uridine into RNA, or the activity of at least one other pineal enzyme (4), indicating that under these conditions 2-F-His is not generally toxic to the cell.

We have speculated that the inhibitory effects of 2-F-His on enzyme induction could result primarily from incorporation of the analogue into newly synthesized proteins. The question whether intact cells can incorporate 2-F-His into proteins is examined in the present report. Pineal glands were used because of our general interest in the mechanism of *N*-acetyltransferase induction in this tissue.

MATERIALS AND METHODS

The following were purchased: [^{3}H]water, [1-^{14}C]acetyl-CoA, [^{14}C]histidine, and [^{14}C]leucine (New England Nuclear); [^{14}C]serine (ICN); Pronase (B grade, lot 201305, Calbiochem); coated thin-layer chromatography plates (E. M. Laboratories); isoproterenol and acetyl-CoA (Schwarz/Mann); and cycloheximide (Nutritional Biochemicals). 2-F-His was synthesized (1). Histidine-free BGJ^{b} culture medium, Fitton-Jackson modification, was made by the National Institutes of Health Media Unit. Sprague-Dawley rats were purchased from the Zivic-Miller Company.

2-F-[^{3}H]His (specific activity, 24.2 Ci/mole) was synthesized by tritium exchange.[2] 2-F-His (75 mg) was dissolved in 1.1 ml of a mixture of [^{3}H]water (specific activity, 90 Ci/mole) and triethylamine (10:1, v/v); the resulting solution was stirred for 4.5 days at room temperature. Excess [^{3}H]water was removed by lyophilization; the residual solid was redissolved in 1.0 ml of water, and the solution was taken to dryness by lyophilization. The crude product was triturated with cold methanol and filtered, yielding 32.5 mg of white crystals. Purity was demonstrated by thin-layer chromatography on coated silica gel F-254 plates, using a solvent made of equal parts of ethyl acetate, acetic acid, water, and butanol-1. One peak of radioactivity (R_F 0.4), isographic with authentic 2-F-His, was detected.

NMR analysis[3] indicates that 2-fluoro-4-alkylimidazoles, including 2-F-His, exchange deuterium at position 4 under the conditions described above for the preparation of 2-F-[^{3}H]His. Based on this, we assume that the ^{3}H label is on the 4-position carbon:

$$\underline{\text{NHCF=NC[}^{3}\text{H]=C}}\text{CH}_2\text{CH(NH}_2\text{)COOH}$$

Racemization of 2-F-His does not occur under similar conditions.

Culture of pineal glands. Pineal glands (approximately 0.1 mg of protein) obtained from 100–125-g male Sprague-Dawley rats were cultured as described (5, 6), using histidine-free culture medium.

Trichloracetic acid precipitation of pineal macromolecules. Individual rat pineal glands were sonicated in 100 μl of 0.1 M sodium phosphate buffer, pH 6.8. A 50-μl sample of the sonicate was added to 1 ml of a 10% TCA solution containing 0.25% phosphotungstic acid (4°). The precipitate was collected on a glass fiber filter and washed sequentially with three 10-ml volumes each of (a) the TCA solution, (b) ether, and (c) chloroform–methanol (2:1, v/v); radioactivity was then determined.

Assay of N-acetyltransferase activity. *N*-Acetyltransferase activity was measured using our modification (7) of the procedure

[2] It has been determined that although 2-F-His is relatively stable to tritium exchange at low and neutral pH values, the rate of exchange increases linearly between pH 7 and 10. No further increase in the rate of exchange is observed above pH 10. It has also been determined that exchange is altered greatly by the nature of 2-F-His; i.e., free 2-F-His exchanges at a greater rate than 2-F-His incorporated into protein. In addition, it has been found that exchange is altered by buffers (Y. Takeuchi, K. L. Kirk, and L. A. Cohen, manuscript in preparation).

[3] Y. Takeuchi, K. L. Kirk, and L. A. Cohen, manuscript in preparation.

of Deguchi and Axelrod (8). A 25-μl sample of gland sonicate, prepared as described above, was added to a reaction tube containing 75 μl of the 0.1 M phosphate buffer, 1 μmole of tryptamine HCl, and 0.05 μmole of [^{14}C]acetyl-CoA (specific activity, 1 Ci/mole). The product *N*-[^{14}C]acetyltryptamine was extracted into chloroform. Other details have been described (7).

Proteolytic hydrolysis of ^{3}H-labeled macromolecules. A group of 24 pineal glands was cultured for 8 hr in histidine-free culture medium and then transferred to medium containing 3 mM 2-F-[^{3}H]His for an 11-hr incubation period. After this the glands were sonicated together in 150 μl of 0.01 M sodium phosphate buffer, pH 7.4. The resulting preparation was centrifuged at 17,000 × *g* for 2 min at 4°; the supernatant fraction was applied to a Sephadex G-15 column (0.5 × 7 cm) and eluted with 0.01 M sodium phosphate buffer, pH 6.8. Fractions containing the bulk of the protein were pooled and lyophilized; 250 μl of H_2O were added to the tube to dissolve the residue. A 90-μl volume of this solution, containing about 60 μg of protein, was mixed with 10 μl of a Pronase solution (50 μg/10 μl) or of water alone (controls) and then incubated for 120 min at 37°.

Amino acid analysis. Amino acid analysis was performed on TCA-soluble preparations. Samples subjected to enzymatic proteolysis as described above, as well as control samples, were mixed with equal volumes of 10% TCA and centrifuged. The supernatant was analyzed by conventional automated methods; amino acids were detected fluorometrically as *o*-phthalaldehyde condensation products (9).

Protein determination. Protein was determined by the method of Lowry *et al.* (10).

RESULTS

N-Acetyltransferase activity was measured to provide a reference of pineal function. Pineal glands were incubated for 12 hr, at which time *N*-acetyltransferase activity in control glands was 0.1 ± 0.05 nmole/min/mg of protein. Glands treated with *l*-isoproterenol for the last 8 hr of incubation had 20-fold higher enzyme values (Table 1); *N*-acetyltransferase values in glands incubated with 2-F-[^{3}H]His and in control glands were not significantly different.

2-F-[^{3}H]His-treated glands contained about 24 nCi in TCA-insoluble material. The appearance of radioactivity in TCA-insoluble material was reduced by 30 μM cycloheximide and by 3 mM histidine.[4] Approximately 21 nCi were incorporated into TCA-insoluble material of glands treated with both isoproterenol and 2-F-[^{3}H]His. The *N*-acetyltransferase activity in these glands, however, was less than 40% of that in isoproterenol-treated glands not receiving 2-F-[^{3}H]His. The inhibitory effect of 2-F-[^{3}H]His on the *l*-isoproterenol induction of *N*-acetyltransferase activity and the observation that this effect of 2-F-His is antagonized by an equimolar concentration of histidine confirm previous studies using 2-F-His (4).

Assuming that all the radioactivity incorporated into protein represented 2-F-His and that no loss of label by exchange occurred during isolation of TCA-insoluble material, we calculate from the data in Table 1 that in a 12-hr period each pineal gland incorporated about 10 nmoles of this analogue per milligram of protein.

In a similar experiment we substituted 3 mM [^{14}C]histidine (specific activity, 25 Ci/mole) for 3 mM 2-F-[^{3}H]His and found that during the same period each pineal gland incorporated about 19 nmoles of this amino acid per milligram of protein into TCA-insoluble material.

The last series of experiments was directed at determining whether the radioactivity in TCA-insoluble material indeed represented incorporation of 2-F-[^{3}H]His into protein. For this it was necessary to demonstrate that the radioactivity could be released by enzymatic proteolysis as 2-F-[^{3}H]His. Glands were incubated for 8 hr in histidine-free medium prior to an 11-hr incubation with the labeled analogue (in-

[4] Previous studies in the same experimental system have shown that cycloheximide (10 or 100 μM) blocks more than 91% of the incorporation of radiolabeled leucine into pineal protein (11).

TABLE 1

Incorporation of radioactivity into TCA-insoluble material in pineal glands cultured with 2-F-[^{3}H]His

Pineal glands were removed from 6-week-old male Sprague-Dawley rats at 10:00 a.m. and placed in organ culture as previously described (5, 6). Except where indicated, histidine was deleted from the culture medium. The incubation period was 11 hr. Isoproterenol was added after 3 hr; the other compounds were present for the entire period. Each pineal gland contained about 100 μg of protein. The specific activity of 2-F-[^{3}H]His was 24.2 Ci/mole. Data are the means ± standard errors of four determinations on replicate gland preparations. Radioactivity is presented on the basis of total protein in the sample prior to TCA precipitation.

Additions to culture medium				*N*-Acetyltransferase activity	Radioactivity in TCA-insoluble material
2-F-[^{3}H]His (3 mM)	Cycloheximide (30 μM)	Isoproterenol (10 μM)	Histidine (3 mM)		
				nmoles/min/mg protein	*nCi/mg protein*
0	0	0	0	0.10 ± 0.05	
+	0	0	0	0.08 ± 0.07	242 ± 39.5
+	+	0	0	0.07 ± 0.05	26 ± 1.5
0	0	+	0	2.20 ± 0.69	
+	0	+	0	0.79 ± 0.07	219 ± 9.9
+	0	+	+	1.72 ± 0.24	49 ± 7.1

cubation in histidine-free medium did not enhance net incorporation of radioactivity into protein). Glands were homogenized, and the supernatant was subjected to gel filtration to remove free 2-F-[^{3}H]His from the macromolecules as described in MATERIALS AND METHODS; the resulting protein fraction was incubated with Pronase for 2 hr (Table 2). Proteolysis decreased radioactivity in the TCA-insoluble material and increased the activity in TCA-soluble material as compared with controls. TCA-soluble material was subjected to ion-exchange chromatography using an automated amino acid analyzer (Fig. 1). Small amounts of [^{14}C]leucine and [^{14}C]serine were added to the samples to provide chromatographic reference standards. Preliminary experiments determined the elution position of 2-F-His to be between those of alanine and valine.[5] Pronase treatment increased the peak height of all amino acids and produced a peak in the 2-F-His position. Analysis of radioactivity in the eluate indicated that proteolysis also produced a peak of ^{3}H in the 2-F-His position, which was about 20 times higher than in the control; this peak, which spanned three fractions, accounted for about 30% of the ^{3}H applied to and eluted from the column; the remaining radioactivity was recovered in the remaining 77 fractions (approximately 1% each). The identity of any minor ^{3}H peak in the eluate is unknown; the last eight fractions contained those compounds eluted by a 0.2 N NaOH wash, and would include histidine and peptides. This broad, nonspecific distribution of radioactivity is probably a reflection of the tritium exchange of 2-F-[^{3}H]-His.[2]

[5] The elution position of 2-F-His among neutral amino acids, rather than near histidine and other basic amino acids, results from the lower pK_a value of the imidazole ring of 2-F-His (1.2) as compared with that of histidine (6.0) (12).

TABLE 2

Effect of proteolytic hydrolysis on radioactivity in homogenates of pineal glands incubated with 3 mM 2-F-[^{3}H]-His

Glands were incubated for 8 hr in histidine-free medium and then transferred to medium containing 3 mM 2-F-[^{3}H]His (specific activity, 24.2 Ci/mole) for an 11-hr incubation period. The glands were then sonicated. Free 2-F-[^{3}H]His was removed by gel filtration, and the resulting macromolecules were treated with Pronase for 120 min at 37° as described in MATERIALS AND METHODS. Data are the means of duplicate determinations. Radioactivity is presented on the basis of total protein present in the sample prior to incubation.

Pronase (500 μg/ml)	Radioactivity	
	TCA-soluble	TCA-insoluble
	nCi/mg protein	
0	18.0	156.5
+	175.4	39.7

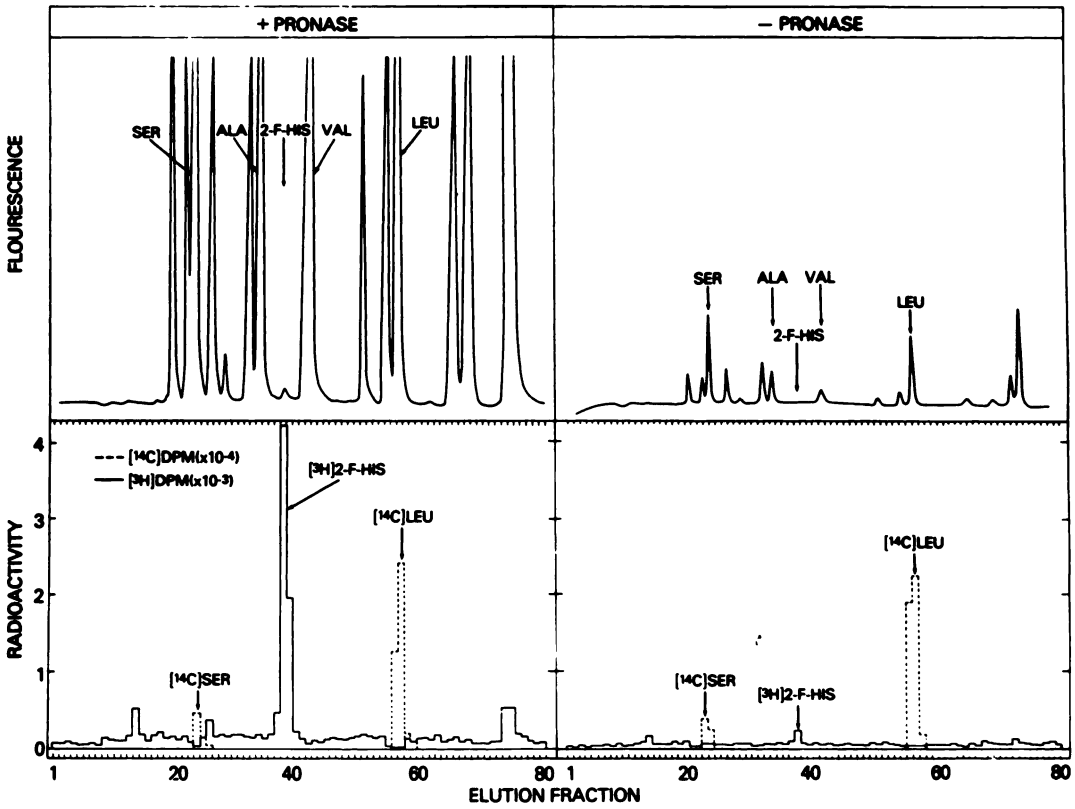

FIG. 1. *Amino acid analysis of TCA-soluble material from homogenates of pineal glands incubated with 3 mM 2-F-[^{3}H]His (specific activity, 24.2 Ci/mole)*

A 60-μl sample of a homogenate that had been incubated (120 min, 37°) with or without Pronase was mixed with an equal volume of 10% TCA. To 90 μl of this was added about 0.1 nmole each of [^{14}C]leucine (specific activity, 314 Ci/mole) and [^{14}C]serine (specific activity, 56.9 Ci/mole) in a volume of 10 μl. The fluorescent detection of amino acids in the eluate has been described (9). Full-scale deflection represents 0.1–0.3 nmole of amino acid per 17 μl of sample. The eluate was collected in eighty 2.0-min fractions (approximately 150 μl each); the radioactivity in 100-μl samples of each fraction is presented.

DISCUSSION

We have speculated that the inhibition of the induction of several enzymes by 2-F-His may result from the incorporation of this analogue into newly formed protein directly involved in the induction of specific enzymes (4). Prior to the present report, however, direct evidence indicating that 2-F-His could be incorporated into protein was not available. The work described here demonstrates that at least one mammalian tissue can utilize 2-F-His in protein synthesis *de novo*. This conclusion is based on our observations that 2-F-[^{3}H]His can be recovered after Pronase digestion of protein obtained from glands incubated with the compound. That this incorporation of 2-F-[^{3}H]His into protein can be blocked by cycloheximide also supports this conclusion.

The dramatic electronic effects resulting from fluorine substitution could have caused the fluoro analogue to be recognized in the process of protein synthesis as a neutral amino acid. We have evidence, however, that the incorporation of 2-F-His into protein occurs at the expense of histidine, rather than of another amino acid. Previously we had found that [^{14}C]histidine incorporation was inhibited by 3 mM 2-F-His; in the present study we found that 2-F-[^{3}H]His incorporation was inhibited by 3 mM histidine. This relationship suggests competition between histi-

dine and 2-F-His for incorporation into protein. Furthermore, analysis of the total amount of radiolabeled histidine and 2-F-His incorporated into protein indicates that incorporation of both during a 12-hr period is in the range of 10–20 nmoles/mg of protein, with [^{14}C]histidine apparently being incorporated at about twice the net rate of 2-F-His. The observation of greater incorporation of [^{14}C]histidine may be due to one or a combination of several factors, including the loss of ^{3}H from 2-F-[^{3}H]His by exchange during preparation of TCA-insoluble material, the incorporation of unlabeled intracellular histidine by glands incubated with 2-F-[^{3}H]His, and a greater rate of uptake of the natural amino acid. In any case, we are impressed by the similar magnitude of the incorporation.

Our results suggest to us that 2-F-His effectively competes with and substitutes for histidine in protein synthesis by intact cells. Definitive proof of this substitution will ultimately depend upon the isolation of known peptide sequences and the demonstration that 2-F-His does replace histidine.

REFERENCES

1. Kirk, K. L., Nagai, W. & Cohen, L. A. (1973) *J. Am. Chem. Soc.*, 95, 8389–8392.
2. Kirk, K. L. & Cohen, L. A. (1976) in *Biochemistry of the Carbon-Fluorine Bond, Am. Chem. Soc. Symp. Ser.* (Filler, R., ed.), pp. 23–36, American Chemical Society, Washington, D. C.
3. Klein, D. C. & Kirk, K. L. (1976) in *Biochemistry of the Carbon-Fluorine Bond, Am. Chem. Soc. Symp. Ser.* (Filler, R., ed.), pp. 37–56, American Chemical Society, Washington, D. C.
4. Klein, D. C., Kirk, K. L., Weller, J. L., Oka, T., Parfitt, A. J. & Owens, I. A. (1976) *Mol. Pharmacol.*, 12, 720–730.
5. Klein, D. C. & Weller, J. L. (1973) *J. Pharmacol. Exp. Ther.*, 186, 516–527.
6. Parfitt, A. P., Weller, J. L. & Klein, D. C. (1976) *Neuropharmacology*, 15, 353–358.
7. Parfitt, A. P., Weller, J. L., Klein, D. C., Sakai, K. & Marks, B. (1975) *Mol. Pharmacol.*, 11, 241–255.
8. Deguchi, T. & Axelrod, J. (1972) *Anal. Biochem.*, 50, 174–179.
9. Benson, J. R. & Hare, P. E. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, 72, 619–622.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, 193, 265–275.
11. Klein, D. C., Yuwiler, A., Weller, J. L. & Plotkin, S. (1973) *J. Neurochem.*, 21, 1261–1271.
12. Yeh, H. J. C., Kirk, K. L., Cohen, L. A. & Cohen, J. S. (1975) *J. Chem. Soc. Perkin Trans. II*, 928–934.