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2-Fluorohistidine—Effects on protein synthesis in cell-free systems and in mouse L cells

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2-Fluoro-L-histidine (2-FHis) possesses a number of interesting biological properties: it induces a reversible bone marrow depression and atrophy of the spleen, thymus and lymph nodes in mice [1]; it prolongs the survival time of mice infected with P388 leukemia [2]; it inhibits the cytopathogenicity of several different RNA and DNA viruses [3]; it blocks the induction of interferon by poly(I).poly(C) [3], and inhibits the induction of several other enzymes such as steroid-induced tyrosine aminotransferase [4], benz[a]anthracene-induced aryl hydrocarbon hydroxylase [4] and the isoproterenol-stimulated pineal *N*-acetyl-transferase [4]. Although the mechanism(s) of action of 2-FHis has not been established yet, evidence for its *in vivo* incorporation into murine proteins has been gathered (C. R. Creveling, L. A. Cohen and K. L. Kirk, unpublished observations).

In order to learn more about the effects of 2-FHis on protein synthesis and the role this may play in the action of 2-FHis, we have studied its effects on protein synthesis in cell-free extracts of mouse L cells, Krebs II ascites cells, *Escherichia coli B* and cultures of mouse L cells.

2- and 4-Fluoro-L-histidine, and [4-^{3}H]-2-fluoro-L-histidine (sp. = 24.2 mCi/m-mole) were prepared as described previously [5–8]. L-[4,5-^{3}H]leucine (sp. act. = 58 Ci/m-mole) and L-[3-^{3}H]histidine (sp. act. = 11.3 Ci/m-mole) were obtained from Amersham–Searle (Arlington Heights, IL) and New England Nuclear (Boston, MA), respectively. Cycloheximide was obtained from the Sigma Chemical Co. (St. Louis, MO), and poly(U) and poly(A,C) from P. L. Biochemicals (Milwaukee, WI). Encephalomyocarditis (EMC) virus RNA was prepared according to the procedure of Kerr and Martin [9]. Krebs II cells were propagated by i.p. injection into NIH general purpose, white female mice. Mouse L cells (Ly) were a clone of L929 developed by Dr. Julius Younger, and maintained in Eagle's minimal essential media (MEM) [10] with 10% fetal calf serum. Cultures of *E. coli B* were grown in L broth.

In studies with mouse L cells, confluent cultures (in either 25 cm^2 or 75 cm^2 plastic T flasks) were exposed to the appropriately labeled amino acid in Eagle's minimal essential media with 2% fetal calf serum minus the isotopically labeled amino acid. At the end of the incubation period, the monolayers were washed (3 times) with phosphate buffered saline, pH 7.2 (PBS), and then removed from the flask with PBS containing 0.2% EDTA. the cells were sedimented by centrifugation, washed with PBS (2 times) and then homogenized with an equal volume of water in a Potter–Elvehjem homogenizer. Hot trichloroacetic acid (TCA) (5%) insoluble radioactivity was determined as described below.

S_{30} extracts from *E coli B* or Krebs II ascites cells were prepared according to the procedures of Gesteland [11] and Mathews and Korner [12] respectively. S_1 extracts from Ly cells were prepared after the method of Friedman *et al.* [13]. Assays for amino acid incorporation were performed as described elsewhere [14].

For polyacrylamide electrophoresis studies, washed (above) mouse L cells were suspended in 0.3 ml PBS, subjected to direct probe sonication for 15 sec (Sonifier model W185, Ultrasonics, Inc. Plainview, NY), and resedimented (15,000 *g*, 20 min). Aliquots of this supernatant fraction were mixed with equal volumes of a solution containing 10 M urea, 15 mM EDTA, and 2% sodium dodecylsulfate (SDS) in 0.01 M phosphate buffer (pH 7.2), and the resulting mixture was incubated at 37° for 30 min. Aliquots of the *in vitro* reaction mixtures were mixed directly with equal volumes of this solution. The SDS–urea-treated extracts were chromatographed on 7%, 5 cm, polyacrylamide disc gels with a 3% stacking gel, according to the method of Neville [15]. After fixation with 7% acetic acid, the gels were stained with Coomassie brilliant blue and destained against activated charcoal with a mixture of acetic acid, methanol and water (70:75:900). Gels were cut into 1 mm sections, and the radioisotope content was determined as described previously [16].

As shown in Table 1, neither 2-FHis nor 4-FHis inhibited the incorporation of radiolabeled leucine into protein synthesized with the Ly cell-free, endogenous mRNA-directed system, even when the ratio of histidine analog to histidine was as high as 7500 to 1. However, when, histidine was used as the labeled amino acid (0.01 mM), 2-FHis (3.0 mM) blocked completely the incorporation of histidine into the hot TCA insoluble material (results not shown). 4-FHis, at the same concentration, had no effect on the incorporation of histidine. The inhibitory effect of 2-FHis could be detected when the ratio of 2-FHis/His was as low as 10/1. A 50 per cent

Table 1. Effects of fluorohistidines on protein synthesis in cell-free extracts*

System	Expt. No.	Addition	Concn (mM)	Hot TCA insoluble (nCi)	[His. analog]+ [His]
Control	1	None	—	54	—
[^{3}H]Leucine	2	2-FHis	1.0	54	75
L cell–S_{10} extract (endogenous RNA)		4-FHis	1.0	56.3	75
	4	2-FHis	1.0	51.8	75000
	5	4-FHis	1.0	54	75000
[^{3}H]Phenylalanine:	1	None	—	540	—
E. coli S_{30} extract Poly (U) stimulated‡	2	2-FHis	1.0	631	300
	3	4-FHis	1.0	586	300

* Incubation time, 45 min (~80 per cent synthesis in controls).
+ Ratio of added histidine analog to histidine already present in reaction mixture.
‡ Poly(U) gave a 12-fold stimulation of [^{3}H]phenylalanine incorporation.

Table 2. Incorporation of [^{3}H]-2-fluoro-L-histidine into protein in mouse L cell cultures—Comparison with histidine incorporation*

Labeled amino acid	Concn (μCi/ml)	Specific activity (Ci/mole)	Hot TCA insoluble (nCi)	Incorporation† (%)
[^{3}H]-2-FHis	6.8	24.2	105.8	0.24‡
[^{3}H]His	6.8	24.2	675.7	1.4

* 1.25 Cells 1.25 10^7 were exposed, in duplicate, to labeled 2-FHis or His for 18 hr at 37° in Eagle's MEM plus 2% fetal calf serum. For experiments with [^{3}H]His, the medium contained no cold histidine.

† Calculated as 2-FHis or His present in hot TCA insoluble material divided by total amino acid added.

‡ Addition of histidine (6 mM), unlabeled 2-FHis (2 mM) or cycloheximide (0.04 mM) reduced the incorporation of labeled 2-FHis by 97, 86 and 87% per cent respectively.

inhibition of histidine incorporation occurred at a ratio of 2-FHis/His of 30/1 (results not shown).

When protein synthesis in the Ly cell-free extract was directed by encephalomyocarditis virus mRNA, the same effects of 2-FHis were observed: the incorporation of labeled leucine was uninhibited while incorporation of labeled histidine was blocked completely by 2-FHis (1.0 mM) (results not shown). EMC mRNA-directed synthesis in the Krebs II–S_{30} system behaved in a similar manner (results not shown).

For the *E. coli*–S_{30}, synthetic mRNA-directed system, the effects of 2-FHis were also similar to those obtained with the L cell extract. Poly(U)-directed incorporation of [^{3}H]phenylalanine into hot TCA insoluble material was not diminished by high concentrations of 2-FHis (Table 1), but the incorporation of [^{3}H]histidine directed by poly(A,C) was blocked by 2-FHis. Unlike the other systems studied, in the *E. coli*–S_{30} system, 4-FHis showed a small, but significant inhibition of poly(A,C)-directed [^{3}H]histidine incorporation at a ratio of 2-FHis/his of 100/1 but was without effect on poly(U)-stimulated incorporation of [^{3}H]phenylalanine (results not shown).

Leucine incorporation in the cell-free systems described above proceeded to the same extent in the presence and absence of 2-FHis. The incorporation of histidine, on the other hand, was blocked effectively by the presence of 2-FHis. A premature termination of polypeptide formation due to incorporation of 2-FHis might provide a rationale for this effect of 2-FHis. To examine this possibility, the nature of the polypeptide products formed in response to ENC RNA-directed synthesis in the Krebs II–S_{30} system was compared in the presence and absence of 2-FHis. [^{3}H]leucine-labeled viral polypeptides synthesized in the presence of 1.0 mM 2-FHis gave the same electrophoretic pattern as the polypeptides synthesized in the absence of 2-FHis (results not shown). The relative mobilities of the viral polypeptides synthesized in both cases are in agreement with the values reported by Kerr *et al.* [17]. Thus, 2-FHis does not appear to result in premature termination of polypeptides ranging in size from 16,000 to 200,000 daltons.

The above results indicate strongly that 2-FHis does not inhibit protein synthesis, as monitored by the incorporation of either [^{3}H]leucine or [^{3}H]phenylalanine in the cell-free sys-

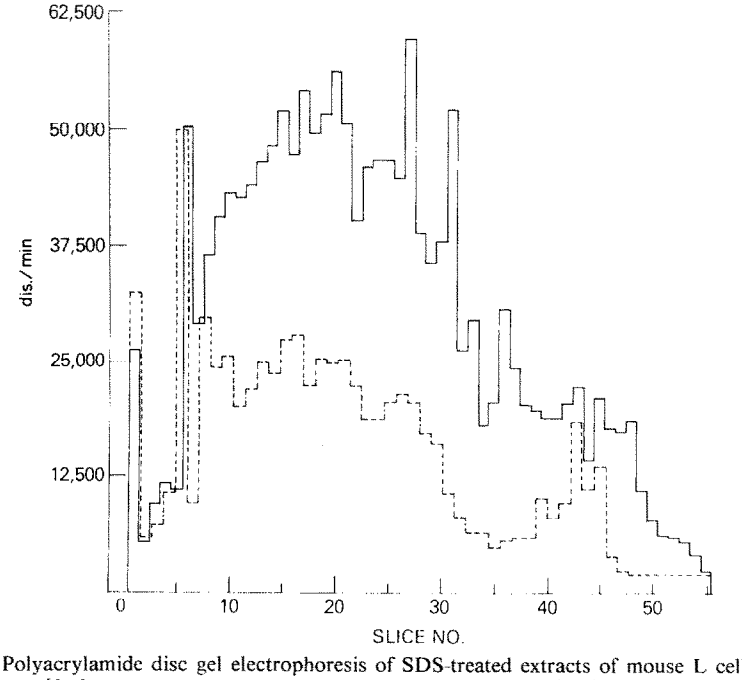

Fig. 1. Polyacrylamide disc gel electrophoresis of SDS-treated extracts of mouse L cells from (a) cells exposed to [^{3}H]His for 16 hr (solid line) and (b) cells exposed to [^{3}H]-2-FHis for 16 hr (dotted line).

tems described above. However, the present evidence demonstrates clearly that 2-FHis blocks the incorporation of [^{3}H]histidine into the polypeptide products of these cell-free systems. these results can be explained best by competition of 2-FHis with histidine for histidine tRNA-histidine aminoacyl synthetase, resulting in the formation of 2-FHis-tRNA and subsequent incorporation of 2-FHis into the polypeptide chain in place of histidine. Two factors precluded a direct test of 2-FHis incorporation into peptides synthesized by the cell-free systems: (a) due to the relative infrequency of histidine in proteins, the stimulation of [^{3}H]histidine incorporation, itself, is only two to three times greater in the EMC RNA-directed system than in the endogenous mRNA-directed system, and (b) the relatively low specific activity of the available [^{3}H]-2-FHis, together with its inefficiency of incorporation, resulted in measurements of [^{3}H]-2-FHis incorporation which could not be regarded as significant.

To circumvent the limitations of the cell-free systems mentioned above, the incorporation of [^{3}H]-2-FHis was studied in cultures of intact mouse Ly cells. Exposure of confluent monolayers of Ly cells to [^{3}H]-2-FHis for 18 hr in histidine-free media resulted in a significant incorporation of radioactivity into hot TCA insoluble material (Table 2). The incorporation of [^{3}H]-2-FHis was approximately 17% of that obtained with [^{3}H]His under identical conditions. The incorporation of [^{3}H]-2-FHis could be reversed when either unlabeled 2-FHis or unlabeled histidine was present during the exposure of the culture to [^{3}H]-2-FHis. Incorporation was also blocked if cycloheximide was present during the incubation period. Examination of the soluble proteins extracted from these cultures by polyacrylamide disc gel electrophoresis indicated that the [^{3}H]-2-FHis was associated with proteins of varying molecular size. The pattern obtained was qualitatively similar to, but not identical with that obtained with [^{3}H]His (Fig. 1).

In conclusion, it is evident that 2-FHis mimics its parent amino acid, histidine, with regard to incorporation into protein either *in vitro* (cell-free protein synthesis) or *in vivo* (mouse L cells). 4-FHis, on the other hand, had little, if any, discernible effects in these systems. The inactivity of 4-FHis in this regard, is consistent with its lack of biological activity in a variety of other systems [3, 4]. The behavior of 2-FHis, blocking incorporation of His into protein but not a effecting leucine incorporation in the cell-free systems, can be explained best by its replacement of histidine in conversion to the amino-acylated tRNA and subsequent incorporation into protein. The latter result is in agreement with earlier studies on the incorporation of 2-FHis into TCA insoluble material in organ cultures of rat pineal glands [8] and into murine proteins *in vivo* [2]. Furthermore, polypeptide products synthesized in the Krebs II cell-free system in the presence of 2-FHis appear to be identical in size and number to those synthesized in the presence of His, although it remains to be established if 2-FHis replaces histidine exclusively and if 2-FHis containing proteins are still functional. In certain respects, at least, 2-FHis behaves similarly to the histidine antagonist 1,2,4-triazolealanine [18], which may also be incorporated into protein.

Laboratories of Bio-organic Chemistry, Chemistry, and Experimental Pathology, National Institute of Arthritis, Metabolism, and Digestive Disease, National Institutes of Health, Bethesda, MD 20014, U.S.A.

PAUL F. TORRENCE
ROBERT M. FRIEDMAN
KENNETH L. KIRK
LOUIS A. COHEN
CYRUS R. CREVELING

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