Reversible Inhibition by Histidinol of Protein Synthesis in Human Cells at the Activation of Histidine*

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SUMMARY

L-Histidinol has been found to be a potent, reversible inhibitor of protein synthesis in cultured human cells. When the culture medium contains 0.005 mM histidine, protein synthesis is inhibited 50% by 0.1 mM histidinol. Histidinol does not significantly affect the transport of histidine into cells or the amount of histidine accumulated in the free amino acid pool.

Histidinol probably inhibits protein synthesis by decreasing the activation of histidine since it competitively inhibits the pyrophosphate-ATP exchange reaction promoted by crude histidyl-tRNA synthetase. In this case, histidinol does not support the exchange reaction. The inhibition by histidinol of the over-all histidine charging reaction with tRNA is also competitive. The apparent $K_i$ of histidinol for the charging reaction, under different reaction conditions than the exchange reaction, is $3 \times 10^{-4}$ M. Histidinol appears to be a useful inhibitor for studying the regulation of macromolecule synthesis in cultured human cells.

Studies of the regulation of macromolecular synthesis in cultured mammalian cells currently employ a variety of inhibitors of nucleic acid and protein synthesis. Two compounds, 5-methyltryptophan (1) and O-methylthreonine (2), have been shown to competitively inhibit, respectively, the activation of tryptophan and isoleucine by the corresponding aminoacyl synthetases of mammalian cells. It has been demonstrated that O-methylthreonine is a reversible inhibitor of protein synthesis in intact mammalian cells (3-5), while we have found that 5-methyltryptophan is not an effective inhibitor of protein synthesis in intact HeLa cells, presumably because of failure to enter the cells. In searching for additional competitive inhibitors of activation of amino acids essential to human cells, which would not be charged onto tRNA, we have found that histidinol, a normal precursor of histidine in procaryotes and some eucaryotes, competitively inhibits histidine activation in HeLa cells. While histidinol has not previously been observed to be an inhibitor of protein synthesis, others have shown that histidinol (6) and the amino alcohols derived from methionine (7), tyrosine (8), isoleucine (7), and leucine (9) competitively inhibit in vitro the activation of these amino acids by the aminoacyl synthetases of bacteria.

EXPERIMENTAL PROCEDURE

Cell Growth—Suspension cultures of HeLa S3 cells were grown in Eagle's medium (10) with 5% calf serum, except where noted.

Cell Labeling—Cells were collected by centrifugation and suspended in modified Eagle's medium, containing 0.04 mM leucine and 0.005 mM histidine, supplemented with 5% dialyzed calf serum, at a cell concentration of $4 \times 10^4$ cells per ml. After a 30-min preliminary incubation with continuous stirring at 37°C, histidinol (Mann Research Laboratories) was added to the stated concentrations in experimental cultures, no addition being made to the control culture. [14C]Leucine (New England Nuclear Corp.) was added 10 min later to each culture, to a final specific activity of 9.6 mCi per mmole. Duplicate 0.5-ml samples were removed at intervals and processed for determination of 14C incorporation into protein as previously described (11, 12).

Preparation of Crude Aminoacyl-tRNA Synthetase—A crude aminoacyl-tRNA synthetase preparation was made from HeLa cell cytoplasmic extract by a modification of the method of Yang and Novelli (13). Cytoplasmic extract of HeLa cells (107 x 10^5 cells) was made by homogenizing washed cells in 12 ml of hypotonic buffer (0.01 M NaCl, 0.0015 M MgCl₂, 0.01 M Tris, pH 7.4) in a Dounce homogenizer and removing nuclei by centrifugation for 15 min at 10,000 rpm in the Sorvall RC-2B centrifuge. The supernatant was then centrifuged for 45 min at 50,000 rpm in the A321 rotor in the International Equipment Co. PR-6 centrifuge, as previously described (14). Mitochondria and membranes were removed by centrifugation for 15 min at 10,000 rpm in the Sorvall RC-2B centrifuge. The supernatant was then centrifuged for 45 min at 50,000 rpm in the A321 rotor in the International Equipment Co. B60 centrifuge, and the entire supernatant down to the ribosomal pellet was removed. This supernatant was applied to a DEAE-cellulose column, 1.5 x 6 cm, equilibrated with 20 mM Tris, pH 7.6, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM EDTA. The column was rinsed with 40 ml of the equilibration buffer and then eluted with this buffer with NaCl raised to 0.8 M. The elution of histidine-activating enzyme was followed by the exchange of [32P]pyrophosphate into ATP as described below.
The active material was pooled and precipitated with (NH₄)₂SO₄ at 50% of saturation. The precipitate was dissolved in a minimal amount of 0.1 M diethanolamine, 2 mM MgCl₂, 0.15% glycerol, pH 7.4, and was desalted and cleansed of free amino acids by passage through a column of coarse Sephadex G-25, 1.3 X 31 cm, equilibrated with this solution. Active fractions were pooled and stored at -70°C. All operations were at 4°C.

Pyrophosphate Exchange—A modification of the method of Calendar and Berg (8) was used. The reaction mixture, 100 μl to 1 ml, contained 3 mM ATP; 20 mM MgCl₂; 100 mM Tris, pH 7.5; 10 mM β-mercaptoethanol; 2 mM [³²P]pyrophosphate, 2 μCi per ml; histidine and histidinol as specified; and crude enzyme, 0.003 mg per 100 μl of reaction mixture. All dilutions of enzyme were made into 100 μM Tris, 20 mM MgCl₂, 0.01% bovine serum albumin, pH 7.5. Reaction was initiated by addition of enzyme to reaction mixture previously incubated to 37°C. Aliquots, 50 or 100 μl, were removed and added to 1 ml of 10% trichloroacetic acid containing 0.2 mM sodium pyrophosphate and 10 mg of activated charcoal (Norit). The sample was filtered onto a Millipore filter, rinsed 20 times with 3 ml of cold water, and dried for counting with a gas flow counter. Blank incorporation was determined from reaction mixtures with enzyme, without addition of histidine. Incorporation was linear for at least 20 min and did not exceed 1% of the added [³²P]pyrophosphate. Initial experiments showed that under the conditions described the reaction had a magnesium concentration optimum around 20 mM; no exchange occurred below 3 mM. Under the conditions adopted for the assay the apparent Kₐ values, at 1 mM histidine, were found to be 1.3 X 10⁻³ M for ATP and 1.4 X 10⁻³ M for pyrophosphate.

Determination of Histidine Binding to tRNA—Transfer RNA was isolated from whole HeLa cells or their cytoplasmic extract by a hot phenol-sodium dodecyl sulfate procedure at 60°C as previously described (15). After ethanol precipitation the RNA was dissolved in 0.1 M NaCl, 0.01 M Tris, 0.01 M EDTA, 0.5% sodium dodecyl sulfate, pH 7.4, and sedimented for 17 hours at 26,000 rpm on 15 to 30% sucrose gradients, in the latter buffer, in the SW 40 rotor of the Beckman centrifuge. The ultraviolet absorption profile of the gradients was monitored, and the peak containing tRNA was collected and precipitated with ethanol. The tRNA was dissolved in 1.8 M Tris, pH 8.0, and incubated 75 min at 37°C, to remove endogenous amino acids, before reprecipitation with ethanol. The tRNA was redissolved in 50 mM Tris, pH 7.6, 0.1 mM EDTA, 2 mM MgCl₂, and stored at -70°C.

The assay mixture (modified from Reference 16) for histidine binding contained in 100 μl: 2.5 mM ATP; 0.25 mM CTP; [³⁵S]histidine (278 mCi per mmole) and histidinol as specified; the other 19 amino acids at 0.013 mM each, nonradioactive; 0.32 A₂₆₀ unit of tRNA, and crude enzyme, 0.024 mg. Reactions were initiated by addition of enzyme to reaction mixture previously incubated to 37°C. Aliquots of 20 μl were applied to 1-inch filter paper discs (Whatman 340), according to the method of Bollum (17), and were immediately placed in 10% trichloroacetic acid containing 1 mg per ml of nonradioactive histidine. The filters were rinsed twice through 5% trichloroacetic acid, once through 95% ethanol, and were dried for counting in a toluene-based scintillator fluid ( Omnifluor, New England Nuclear Corp.) in a Packard counter.

Since the observed inhibitions of ATP-pyrophosphate exchange and of histidine charging by histidinol are competitive, as judged from Lineweaver-Burk plots of initial velocities versus substrate concentration, the values of apparent Kₐ were determined from plots of the concentration of histidinol versus the reciprocals of initial velocities (18).

RESULTS

Inhibition of Protein Synthesis by Histidinol—As shown in Fig. 1, histidinol is an inhibitor of protein synthesis in HeLa cells. The inhibition requires some 30 min to become maximal, presumably due to a relatively slow entry of histidinol into the cells. Addition of excess histidine rapidly and completely restores the normal rate of protein synthesis, as shown in Fig. 1. The dependence of the inhibition on histidinol concentration is shown in Fig. 2.

As indicated in Table I histidinol has no significant effect on either the rapid transport of histidine into HeLa cells, as shown by entry into the acid-soluble pool during a 2-min incubation with labeled histidine, or the level of free histidine in the cells, as shown by incorporation into that pool after a 24-min incubation. This result excludes the possibility that the observed inhibition of protein synthesis is due to a histidinol-mediated starvation of the cells for histidine.

Inhibition by Histidinol of Pyrophosphate-ATP Exchange Promoted by Histidine—The possibility that histidinol may interfere with histidine activation was tested by examining its effects on histidine-promoted exchange of [³²P]pyrophosphate into ATP. Fig. 3 shows that this reaction can be considered linear within the first 20 min under the conditions described under "Experimental Procedure." A low background level of exchange in the absence of added histidine, probably due to remaining amino acid contaminants of crude enzyme, is shown in the figure. Histidinol alone at concentrations up to at least 1 mM does not promote exchange. No lag is observed in the inhibition by histidinol of exchange promoted by histidine.
Table I

Entry of L-[14C]histidine into acid-soluble pool of HeLa cells, with and without L-histidinol

Cells at a concentration of 2 x 10^6 per ml were incubated 10 min at 37\(^\circ\) in complete medium containing 0.005 mM histidine, with and without 0.2 mM L-histidinol. L-[14C]Histidine (45 mCi per mmole, final specific activity) was then added, and 2-ml samples of cells were taken 2 and 24 min later for centrifugation and rinsing with ice-cold Earle's salts solution. The free amino acids were extracted with cold 10% trichloroacetic acid from the cell pellets, and the insoluble debris was removed by centrifugation. After extraction with ether to remove trichloroacetic acid aliquots of the supernatant solution were taken for determination of radioactivity by scintillation counting.

| L-Histidinol     | Amount of L-[14C]histidine incorporated at |
|-----------------|-----------------------------------------|
|                 | 2 min | 24 min |
| Present         | 3820  | 4850   |
| Absent          | 4320  | 4220   |

Fig. 3 (left). Effect of L-histidinol on [32P]pyrophosphate-ATP exchange promoted by histidine. The details of the assay are given under "Experimental Procedure"; 50-μl aliquots were taken. A, 1 mM histidine; B, 1 mM histidine, 0.02 mM L-histidinol; C, 1 mM histidine, 0.05 mM L-histidinol; D, no amino acids added.

Fig. 4 (right). Inhibition by L-histidinol of [32P]pyrophosphate-ATP exchange promoted by histidine. Double reciprocal plots of velocities against histidine concentration. Velocities, measured as in Fig. 3, have been normalized by division into V\(_{\text{max}}\) which was indistinguishable from the velocity at 1 mM histidine. A, 0.05 mM L-histidinol; B, 0.02 mM L-histidinol; C, 0.01 mM L-histidinol; D, no histidinol.

The double reciprocal plot of velocities versus histidine concentration at differing histidinol concentrations, Fig. 4, indicates that histidinol is a competitive inhibitor of histidine activation. The apparent K\(_{\text{s}}\) for histidine is 1.0 x 10^-5 M for the exchange reaction and apparent K\(_{\text{i}}\) for histidinol is 4 x 10^-3 M.

Inhibition by Histidinol of Histidine Charging onto tRNA—In view of the competitive inhibition of histidine-promoted pyrophosphate exchange by histidinol, this compound would be expected to competitively inhibit the charging of tRNA with histidine. As shown in Fig. 5 this is the case. The addition of histidinol to charging assays was also found not to affect the final level of charging of tRNA with histidine. The apparent

![Figure 5](https://example.com/figure5.png)

**Fig. 5.** Inhibition by L-histidinol of histidyl-tRNA synthesis. The charging of L-[14C]histidine onto tRNA was assayed as described under "Experimental Procedure." Double reciprocal plots are shown of initial velocities versus histidine concentration. A, 0.0152 mM L-histidinol; B, 0.0057 mM L-histidinol; C, no L-histidinol.

K\(_{\text{s}}\) for histidine for charging was 7 x 10^-6 M and apparent K\(_{\text{i}}\) for histidinol was 3 x 10^-6 M. It is interesting to note that although the K\(_{\text{s}}\) for histidine is similar for both pyrophosphate exchange and the over-all charging reaction, the K\(_{\text{i}}\) of histidinol is 8 times lower for the exchange than for the over-all reaction. Since the enzyme used was not purified, the significance of this observation is at present unclear.

**Discussion**

Our results establish that histidinol is a potent, reversible inhibitor of protein synthesis in human cells. The data indicate that histidinol acts at the level of histidine charging to tRNA by competitively inhibiting histidine activation. The shape of the dose-inhibition dependence curve for protein synthesis inhibition and the prompt, full reversal of the inhibition by excess histidine are consistent with this explanation. The fact that histidinol takes some time to exert maximal inhibition of protein synthesis in intact cells is probably due to a comparatively slow entry of the inhibitor into the cell. We have not studied histidinol transport, but we have observed that the compound does not appear to significantly interfere with histidine transport into the cell.

The observation that histidinol will not support pyrophosphate-ATP exchange and does not affect the final level of histidine charging effectively rules out the possibility that histidinol is activated. There is thus no reason to suppose that histidinol interferes with the charging capacity of the histidine acceptor tRNA species. Since mammals cannot carry out the de novo synthesis of histidine, histidinol probably does not occur in human cells. However, histidinol is synthesized during histi-
dine formation in the procaryotes and lower eucaryotes (19, 20), and it has been reported (6) that it competitively inhibits the activation of histidine by Salmonella typhimurium histidyl-tRNA synthetase, with a $K_i$ of $5 \times 10^{-5}$ M.

Another competitive inhibitor of amino acid activation, O-methylthreonine (2), which competes with isoleucine, has been used in several studies of protein synthesis and its regulation in mammalian cells (4, 5, 21). Histidinol and O-methylthreonine are useful compounds for the study of regulation of macromolecule synthesis in cultured human cells because they are effective inhibitors at low concentrations with intact cells. Their effects are rapidly, specifically reversible by the corresponding amino acid and neither compound is attached to tRNA. Both of these inhibitors can be used to rapidly put cultured cells into a condition similar to that achieved by depriving the cells for the corresponding amino acid for several hours (2, 12). Furthermore, because of endogenous protein turnover, the decrease in the absolute rate of protein synthesis obtained by amino acid deprivation is limited to about 80% (12), while these inhibitors can virtually abolish protein synthesis at high concentrations relative to the amino acids with which they compete.

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