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Effect of the α-Hydrazino Analogue of Histidine on Histidine Transport and Arginine Biosynthesis

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SUMMARY

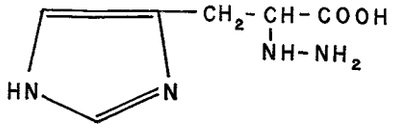
The α-hydrazino analogue of histidine was found to be an effective inhibitor of the growth of *Salmonella typhimurium* wild type as well as *Escherichia coli* W and *E. coli* K-12.

A mutant, *hisP1650*, which is resistant to the inhibitory action of the compound was isolated and found to be defective in the histidine-specific transport system.

The hydrazino analogue reacts rapidly with pyridoxal phosphate in a nonenzymatic reaction to give a product which does not have the spectral properties expected of a hydrazone.

The analogue inactivates several pyridoxal phosphate enzymes. Evidence is presented suggesting that the primary point of inhibition in the cell is the pyridoxal phosphate moiety of acetylornithine transaminase in the pathway of arginine biosynthesis.

This communication deals with the mechanism by which the α-hydrazino analogue of histidine (Formula I) inhibits the growth of *Salmonella typhimurium*. A mutant resistant to the inhibitory action of this analogue was isolated and was found to be defective in the histidine-specific transport system, and therefore not able to take up 2-hydrazino-3-(4-imidazolyl)propionic acid. The data suggest that the inhibitory action of HIPA[1] is



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not on the pathway of histidine biosynthesis but results from its rapid reaction with the pyridoxal phosphate of acetylornithine δ-transaminase, an enzyme of arginine biosynthesis.

MATERIALS AND METHODS

Chemicals—2-Hydrazino-3-(4-imidazolyl)propionic acid hydrochloride[2] and 2-hydrazino-*n*-caproic acid were generously supplied by Dr. Lewis Sarett and Dr. Frederick A. Kuehl, Jr., of Merck Sharp and Dohme. Freshly prepared solutions of the histidine analogue were colorless but gradually turned yellow after several days whether kept at room temperature (25°) or at 4°. Keeping the solution protected from light and at −20° delayed the discoloration. Whereas the freshly prepared solutions have negligible absorption above 240 mμ, the yellow material has a very broad absorption band with an apparent maximum at 280 mμ (ε ~ 1000). Solutions of HIPA were freshly prepared prior to use.

Pyridoxal 5-phosphate, *N*-acetylornithine, and all of the unlabeled amino acids were purchased from Calbiochem. Histidinol dihydrochloride was obtained from Cyclo Chemical Company, and *o*-aminobenzaldehyde from K and K Laboratories. Uniformly labeled ^{14}C-L-histidine (223 μC per μmole) was purchased from New England Nuclear Corporation, and ^{14}C-L-arginine (200 μC per μmole) was obtained from Schwarz BioResearch.

Growth of Bacterial Strains—*S. typhimurium* wild type strain LT-2 and mutant *hisP1650* were grown in Medium E (1) containing a mixture of trace elements (2) and 0.5% glucose as carbon source in a New Brunswick rotary shaker at 37°. Bacterial growth was measured by following the turbidity in a Zeiss PMQ II spectrophotometer at 650 mμ. On our spectrophotometer an absorbance of 0.500 corresponds to a bacterial density of 4×10^8 cells per ml and to 235 μg of cells, dry weight, per ml.

Inhibition by HIPA—When the absorbance of the growing culture reached a value between 0.150 and 0.500, 10-ml aliquots of the culture were withdrawn and added to an appropriate volume of 100 mM HIPA, and the shaking at 37° was continued. To test the reversibility of the inhibition, the amino acid or other test substance was added at the same time as HIPA.

Uptake of Radioactive Amino Acids—The uptake of ^{14}C-L-his-

[1] The abbreviation used is: HIPA, 2-hydrazino-3-(4-imidazolyl)propionic acid.

[2] Two samples of HIPA were generously supplied, one labeled D(+) and one labeled L(−). Very peculiarly, both samples were equally effective as growth inhibitors although both were reversed only by L-histidine and not by D-histidine. This could be explained if there were some racemization of the inactive form (or both forms), presumably during their chemical synthesis although possibly by the bacteria. The rotations were found to be opposite in sign but not numerically equal. With the sodium D-line at 25°, and in 0.1 N HCl solution, the (+) form had a rotation of +16.8° and the (−) form a rotation of −10.0°. The D and L configurations were assigned by the supplier on the basis of the synthesis from L- and D-histidine, respectively, through an α-chloroimidazolepropionic acid intermediate.

tidine and ^{14}C-L-arginine was determined by the methods described by FerroLuzzi-Ames (2).

Enzyme Assays—Histidinol dehydrogenase was assayed on toluenized cells (3). Imidazolylacetol phosphate transaminase was assayed as previously described (4).

N-Acetylornithine δ-transaminase was assayed by a modification of the procedure described by Albrecht and Vogel (5). Bacterial sonic extracts of *S. typhimurium* or *E. coli* W were preincubated in 0.1 M phosphate buffer, pH 7.1, containing 10^{-3} M glutathione, with varying quantities of HIPA prior to the addition of either pyridoxal phosphate or α-ketoglutarate.

The effect of HIPA on the activity of purified acetylornithine transaminase and argininosuccinase was examined by Dr. Evan Jones of Rutgers University.

Spectrophotometric Studies—Absorption spectra were taken on a Cary recording spectrophotometer, model 15. The rate of reaction of pyridoxal phosphate with HIPA was followed in a Zeiss PMQ II spectrophotometer equipped with a temperature-controlled cuvette holder.

Pseudo-first order rate constants were determined as follows. Pyridoxal phosphate (6.56×10^{-5} M), in the appropriate buffer, was kept in the thermostated cuvette holder (25.0° ± 0.5°) for 10 min prior to the addition of a 100-fold excess of HIPA. The fraction of the product then formed, x, was obtained from the following expression: $x = (A_t - A_0)/(A_f - A_0)$, where A_t is the absorbance at time t, A_0 is the initial absorbance, and A_f is the final value.

Quantitative Determination of HIPA—The amount of HIPA present in the medium of a bacterial culture was determined as follows. An aliquot of the bacterial culture which had been initially treated with 0.02 ml of 100 mM HIPA was filtered through a Millipore filter (pore size, 0.45 μ) to remove the bacteria. One milliliter of the filtrate was placed in a 3-ml volumetric flask to which 5 μl of 100 mM pyridoxal phosphate were added. Water was added to bring the volume of solution near the 3-ml mark, and the pH of the solution was adjusted to 4.8 by the addition of 10 μl of 6 N HCl. After the total volume was brought to the mark, the absorbance was read at 359 mμ, where the extinction coefficient of the complex was 22,000 M^{-1} cm^{-1}.

RESULTS

Inhibition of Bacterial Growth—The effect of several concentrations of HIPA on the growth of wild type *S. typhimurium* is shown in Fig. 1. Bacterial growth slows down immediately upon the addition of HIPA. The culture resumes its normal doubling time of 48 min after a lag, the duration of which depends upon the concentration of the analogue. Similar inhibition curves were obtained with *E. coli* W and *E. coli* K-12.

The inhibition of growth was completely abolished by addition of histidine at the same time as HIPA. The reversal of the inhibition suggests a connection between histidine and HIPA. The following data indicate that one possible connection is at the level of the histidine transport system.

Isolation and Characterization of HIPA-resistant Mutant—A spontaneous mutant, resistant to HIPA, was isolated from a zone of inhibition created by HIPA on a lawn of *S. typhimurium*. This mutant, *hisP1650*, grows with a normal doubling time (48 min) on minimal medium and is not inhibited at all by HIPA up to a concentration of 2×10^{-4} M.

The resistance to HIPA in this mutant has been traced to a defective histidine transport system, which takes up L-histidine

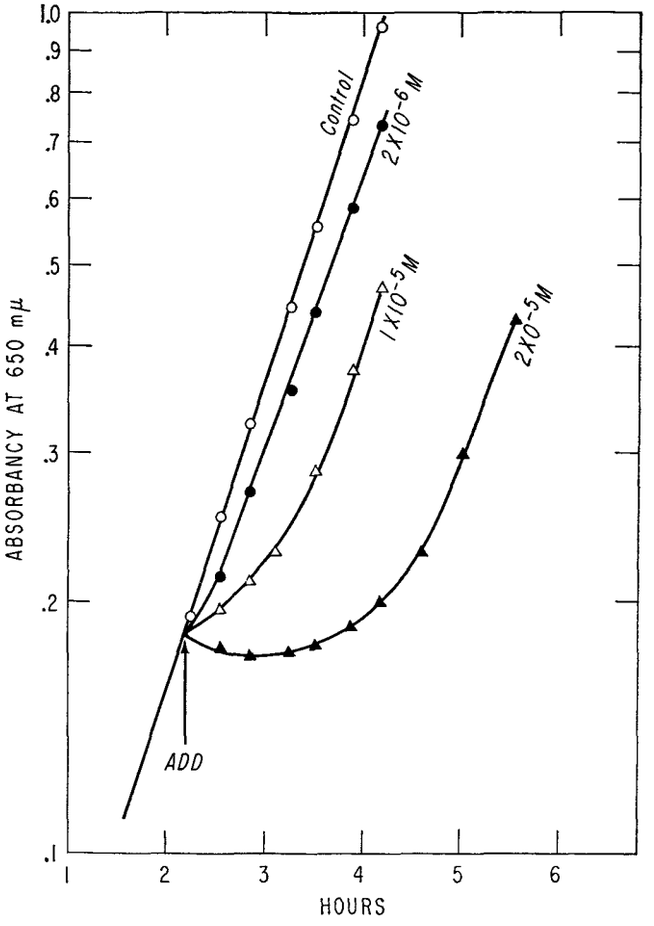

FIG. 1. Inhibition of growth of *S. typhimurium* wild type. The exponentially growing culture of bacteria was added to concentrated solutions of HIPA at the point shown by the *arrow*. The final concentrations of the inhibitor are given alongside the appropriate growth curve.

very poorly and HIPA not at all. The rate of uptake of uniformly labeled ^{14}C-L-histidine was measured for the wild type (LT-2) and for *hisP1650* both in growing and in starved cells as previously described (2). The results are shown in Figs. 2 (starved cells) and 3 (growing cells). The rate of uptake of histidine in *hisP1650* is 19% of that of the wild type when assayed in starved cells (Fig. 2) and 9% when assayed in growing cells (Fig. 3). This difference in residual rates, which was found when either of the two assay methods was used, can be accounted for by a slight excretion of histidine by the *hisP* mutant during growth (about 0.3 μmole per min per g of cells, dry weight).

The excretion of histidine cannot account for the resistance of *hisP1650* to HIPA. This is shown in an isotope dilution experiment (Fig. 3) in which the amount of histidine excreted by this mutant was measured. Addition of the growth medium of the mutant to the wild type culture caused only a slight decrease of ^{14}C-histidine incorporation. However, a high rate of histidine excretion can cause partial resistance to HIPA as observed in a class of mutants different from *hisP* mutants. Mutant *hisG1102*, which excretes a very large amount of histidine (2 μmoles per min per g of cells, dry weight), because of the lack of feedback inhibition by histidine (6), is partially HIPA-

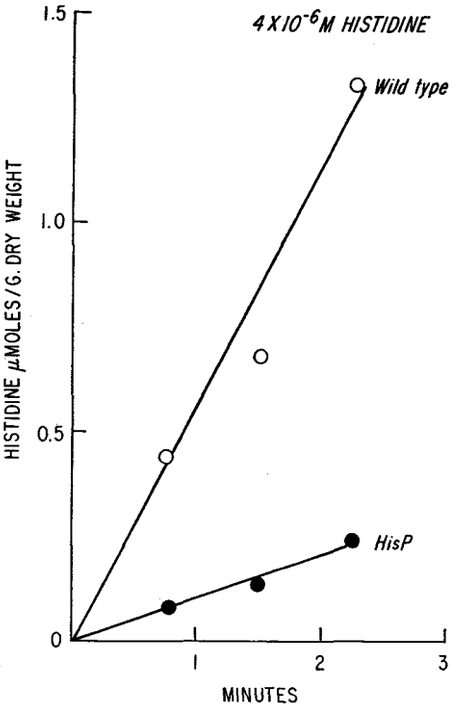

FIG. 2. Rate of uptake of ^{14}C-L-histidine (4×10^{-6} M) by wild type (○) and by *hisP1650* (●) measured in starved cells (2). At this histidine concentration, only the histidine-specific permease is active. The rates obtained are: for the wild type, 0.56 μmole per g (dry weight) per min; for *hisP1650*, 0.11 μmole per g (dry weight) per min.

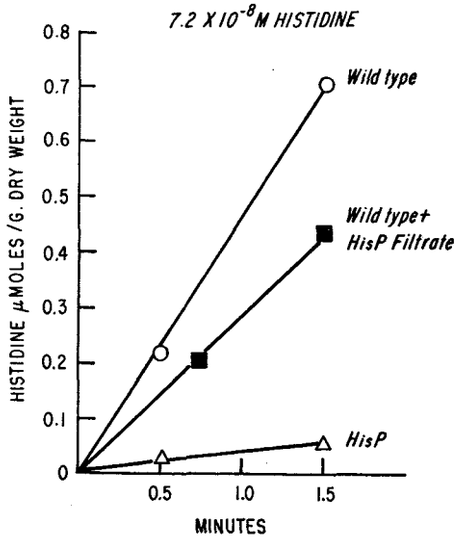

FIG. 3. Rate of uptake of ^{14}C-L-histidine (7.2×10^{-8} M) by wild type (○) and by *hisP1650* (△) measured in growing cells, as determined by protein incorporation (2). The rate obtained for the wild type is 0.46 μmole per g (dry weight) per min. The apparent rate for *hisP1650* (*i.e.* no correction was made for the dilution of the specific activity of the ^{14}C-histidine by the unlabeled excreted histidine) was 0.04 μmole per g (dry weight) per min. The histidine excretion (■) was measured (2) by filtering a portion of the *hisP1650* culture immediately after assaying its uptake rate, and adding an appropriate amount of the filtrate to the wild type assay mixture in place of the usual amount of fresh Medium E used (○ = control).

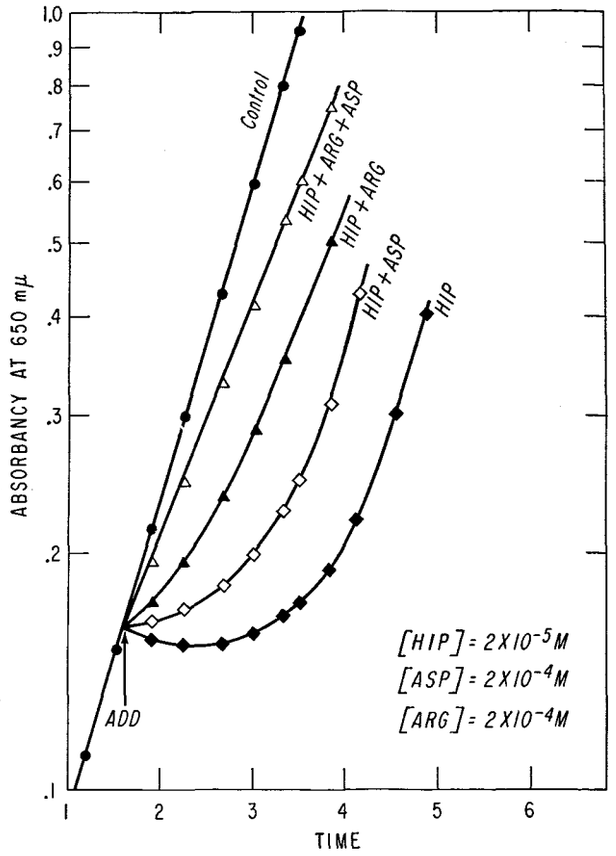

FIG. 4. Growth curves illustrating the reversal of 2-hydrazino-3-(4-imidazolyl)propionic acid (*HIP*) inhibition. All of the test solutions contained the analogue at a final concentration of 2×10^{-5} M. The amino acids were incubated with the analogue prior to the addition of the growing culture.

resistant. The wild type begins to be inhibited at 2×10^{-6} M HIPA, *hisG1102* at 7×10^{-5} M HIPA, and *hisP1650* at 2×10^{-4} M HIPA.

The reversal of HIPA toxicity by histidine presumably results from competition with, and exclusion of, HIPA because of its much weaker affinity for the histidine-specific transport system: a 100-fold excess of HIPA over histidine does not inhibit bacterial growth.

The mutation in the histidine transport system does not affect the rate of growth of *hisP1650*, which also was indistinguishable from the wild type in a number of other respects. These included measurements of the specific activities of the arginine transport system, the histidine-activating enzyme, and some of the histidine-biosynthetic enzymes. Therefore, as the transport system itself does not appear essential for bacterial growth (except in a histidine-requiring mutant), the finding that a histidine transport mutant is HIPA-resistant still does not explain the mechanism of the inhibition of wild type bacteria by HIPA.

Site of Action of HIPA—The possibility existed that HIPA, once carried inside the cell by the histidine transport system, inhibited histidine biosynthesis or the formation of histidyl transfer RNA. This possibility appears unlikely for the following reasons.

1. If HIPA inhibited bacterial growth by interfering with the biosynthesis of histidine, the decreased production of this amino acid should result in derepression, *i.e.* increased rates of synthesis

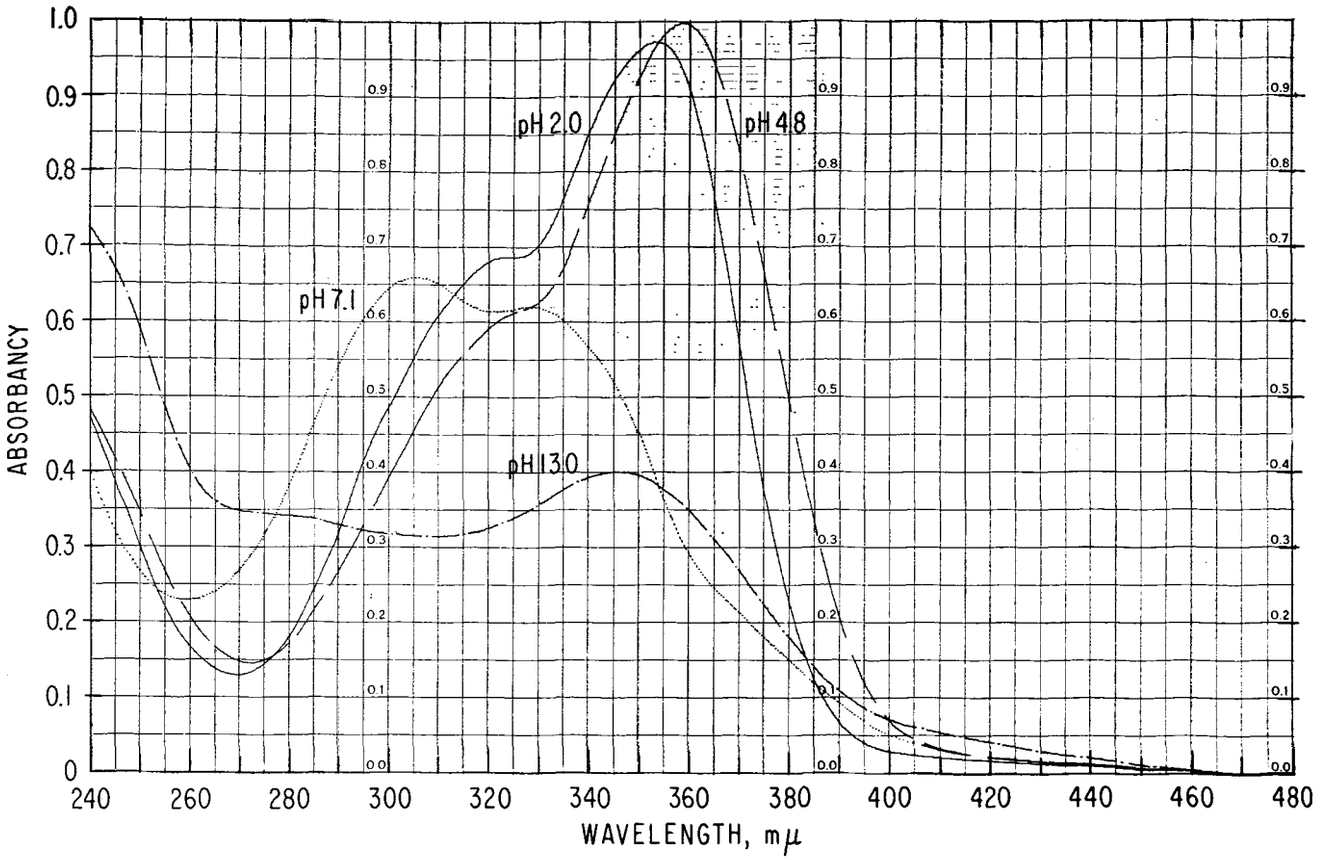

FIG. 5. Absorption spectrum of the product of HIPA and pyridoxal phosphate at several pH values. The concentration of coenzyme was 4.54×10^{-5} M, and HIPA was in a slight excess at 6.5×10^{-5} M. The blank contained the equivalent amount of HIPA in the appropriate buffer.

of the histidine-biosynthetic enzymes triggered by a shortage of histidine. This is not the case with HIPA inhibition. The activity of histidinol dehydrogenase was assayed in the wild type and in *hisP1650* before the addition of 2×10^{-5} M HIPA, during the period of growth arrest, and after the cultures recovered from the inhibition and resumed normal doubling times. In all of the samples the specific activity of the enzyme was essentially identical. Other histidine analogues, such as thiazolealanine, which inhibit the biosynthesis cause derepression (3, 6, 7)).

2. The first enzyme of histidine biosynthesis, phosphoribosyl-ATP pyrophosphorylase, was not inhibited by 3×10^{-3} M HIPA.[3] This enzyme is 50% inhibited by 5×10^{-5} M histidine (8) and is a likely site for inhibition by a histidine analogue (*e.g.* thiazolealanine, a false feedback inhibitor (6, 7)).

3. HIPA is a good inhibitor of a histidine-requiring mutant growing on histidinol as a histidine source. Since histidinol dehydrogenase is the last step of the pathway, this would rule out a site of inhibition before this step.

4. The rate of formation of ^{14}C-histidyl transfer RNA, as assayed by the method of Berg *et al.* (9), was not inhibited by 3×10^{-3} M HIPA at a histidine concentration of 3×10^{-5} M.

The HIPA did, however, inhibit imidazolylacetol phosphate transaminase, an enzyme of histidine biosynthesis which has a pyridoxal phosphate coenzyme. A concentration of 1×10^{-4} M HIPA inhibited the enzyme 50%. Because of the above arguments, however, and the following experiments, it is felt that this is not the primary site of toxicity in the cell.

Reversal of HIPA Inhibition by Other Amino Acids—A survey of the remaining amino acids for their effectiveness in reversing HIPA inhibition revealed that aspartate and arginine were active when added singly, and were even more effective when added together (Fig. 4). High concentrations of arginine alone (5×10^{-3} M) could completely reverse the inhibition by 2×10^{-5} M HIPA. Two other intermediates in the arginine-biosynthetic pathway, L-citrulline and L-ornithine, at 5×10^{-3} M, were also active in completely preventing the inhibition of bacterial growth, a suggestion that an inhibition of arginine biosynthesis occurs prior to ornithine synthesis.

Inhibition of Acetylornithine δ-Transaminase—Since hydrazines have been reported to be inhibitors of some vitamin B_6-dependent enzymes by forming hydrazones (10), it appeared likely that HIPA might prevent the biosynthesis of arginine by reacting with the active site of acetylornithine δ-transaminase. The activity of the partially purified transaminase from *E. coli* or of a bacterial sonic extract of the wild type from both *Salmonella* and *E. coli* was inhibited 50% by 5×10^{-5} M HIPA and completely by 5×10^{-4} M HIPA. The fact that ornithine and citrulline, which reverse HIPA toxicity, occur later in the biosynthetic pathway in which acetylornithine δ-transaminase participates is also consistent with its being the sensitive site.

Inhibition of Bacterial Growth by α-Hydrazino-n-caproic Acid—Another α-hydrazinocarboxylic acid, α-hydrazinocaproic acid,

[3] M. J. Voll, personal communication.

TABLE I

Absorption maxima and extinction coefficients of pyridoxal 5-phosphate and its product with hydrazino analogue of histidine at several pH values

Compound	pH	λ_{max}	ϵ_{max}
		mμ	M^{-1} *cm*$^{-1}$
Pyridoxal phosphate	7.1	388	5,000
		330	2,400
Reaction product	2.0	354	21,000
	4.8	358.5	22,000
	7.0	305	14,300
		325	13,000
	8.6	300	6,700
	13.0	346	8,700

TABLE II

Pseudo-first order rate constants for reaction of pyridoxal 5-phosphate with 100-fold excess of hydrazino analogue of histidine at 25°

pH	Pseudo-first order rate constant
	min^{-1}
2.0	0.720
4.8	5.70
7.1	5.34
8.6	1.86
13.0	1.02

the carbon skeleton of which is unrelated to HIPA, was found to be about as inhibitory as HIPA for the growth of the wild type bacteria. The *hisP1650* mutant, which is resistant to HIPA, was not resistant to the hydrazinocaproic acid, an indication that this analogue does not enter through the histidine permease.

The enzymatic activity of acetylornithine δ-transaminase was 85% inhibited by 1×10^{-4} M hydrazinocaproic acid and was completely inhibited when the concentration was increased to 5×10^{-4} M. That hydrazinocaproic acid is as effective as HIPA as an inhibitor of the transaminase suggests that the carbon skeleton of the inhibitor is of minor importance compared to the hydrazino group.

If acetylornithine δ-transaminase is the target in the growing bacteria for hydrazinocaproic acid as well as for HIPA, inhibition of growth by the caproic acid derivative should be reversed by arginine. Surprisingly, inhibition by hydrazinocaproic acid was not reversed by arginine. A survey of the amino acids indicated that methionine completely reversed the effect of the caproic acid derivative and that norleucine was partially effective. Both of these amino acids are isosteres of hydrazinocaproic acid.

Although both hydrazinocarboxylic acids are equally effective as inhibitors of the isolated acetylornithine δ-transaminase *in vitro*, it is possible that hydrazinocaproic acid, once transported into the bacterial cell, reacts most readily with the pyridoxal phosphate moiety of one of the several B_6-dependent enzymes involved in methionine biosynthesis.

Nonenzymatic Reaction of HIPA with Pyridoxal Phosphate—It has been postulated that the mechanism by which hydrazines inhibit B_6-dependent enzyme systems is hydrazone formation with the 4-formyl group of pyridoxal phosphate (10–12). When equimolar amounts of HIPA and pyridoxal phosphate were mixed in 0.1 M phosphate buffer, pH 7.1, there was an immediate loss of the characteristic absorption band of the coenzyme at 388 mμ. There was no evidence of increased absorption above 388 mμ within several seconds after the reactants were mixed, as would have been anticipated if a Schiff base were formed (13). The absorption spectra of the reaction product at several pH values are shown in Fig. 5, and the maxima together with the corresponding extinction coefficients are summarized in Table I.

The rate of formation of the reaction product was followed spectrophotometrically at several pH values at the wave length of maximum absorption. The pseudo-first order rate constants with a 100-fold excess of HIPA are summarized in Table II.

Rate of Uptake of HIPA—The high extinction coefficient of the product at pH 4.8, coupled with the rapid rate of its formation, has enabled direct evaluation of the rate of HIPA uptake by growing cells. The inhibitor, at a concentration of 5×10^{-5} M, was taken up at a rate of 0.56 μmole g^{-1} min^{-1} by the wild type strain and was not taken up at all by *hisP1650*.

The HIPA-pyridoxal phosphate reaction product was not inhibitory to the wild type strain, nor was it taken up by the logarithmically growing culture.

DISCUSSION

Our data indicate that HIPA enters the bacterial cell through the histidine transport system, and that after entry it inhibits a pyridoxal phosphate enzyme. Since the toxicity is reversed by arginine, histidine, and to some extent by aspartate, the biosynthetic pathways of these amino acids are likely points of inhibition by HIPA. Our evidence suggests that it is the arginine pathway that is the most sensitive to the analogue, though it is difficult to determine this point with certainty.

Since the histidine-biosynthetic pathway contains a pyridoxal phosphate-dependent transaminase, the imidazolylacetol phosphate transaminase, it is of interest to speculate why HIPA should inhibit a transaminase involved in arginine biosynthesis rather than the imidazolylacetol phosphate transaminase, which acts on a substrate structurally more similar to HIPA. The simplest explanation would be a greater sensitivity of acetylornithine transaminase toward HIPA *in vivo* compared with imidazolylacetol phosphate transaminase. The greater sensitivity may result from the accessibility of the pyridoxal phosphate moiety, which is readily removed from the acetylornithine transaminase with ammonium sulfate at acid pH.[4] Full enzymatic activity is regained when the coenzyme is added back to the apoenzyme. The pyridoxal phosphate moiety of imidazolylacetol phosphate transaminase, on the other hand, cannot be removed without extensive irreversible denaturation of the enzyme.[5] It is of interest in this regard that there are reports of the reversibility by arginine (14) of the antibacterial action of hydroxylamine (which also would react with pyridoxal phosphate) and the protective action of arginine against the toxicity of hydrazine (15).

Levine, Sato, and Sjoerdsma (16) have reported that intraperitoneal injection of the α-hydrazino analogue of histidine prevents the formation of histamine in rats, presumably as a result of inhibition of histidine decarboxylase, a pyridoxal phos-

[4] E. Jones, personal communication.

[5] R. G. Martin, personal communication.

phate-dependent enzyme. This decarboxylase is presumably of no great importance in *S. typhimurium*, if it is present at all.

The reaction of HIPA with pyridoxal phosphate without the transient formation of a Schiff base is reminiscent of the reaction of histidine with this coenzyme to give an imidazole-tetrahydropyridine derivative (17, 18). The formation of the cyclic product accounts for the inhibition of histidine decarboxylase by histidine (18). The rate of formation of the product from histidine and pyridoxal phosphate (18) is considerably slower than the rate of formation of the HIPA complex.

The precautions which must be taken to classify a mutant as being defective in a transport system have been discussed previously (2). *HisP1650* has been demonstrated to have a defective histidine-specific transport system, and the small amount of histidine which is excreted by the mutant cannot account for the very low incorporation of ^{14}C-histidine into *hisP1650* protein. It was previously demonstrated (2) that histidine is transported into the bacterial cell either by the histidine-specific transport system ($K_m = 1.7 \times 10^{-7}$ M) or by the nonspecific aromatic transport system ($K_m = 1.1 \times 10^{-4}$ M). The structural similarity between HIPA and histidine permits the inhibitor to enter the bacterial cell via the histidine-specific system, but once inside the bacterium its structural similarity to the natural amino acid is overshadowed by the high reactivity of the hydrazine function. A further study of *hisP* mutants will be reported in a separate communication.[6]

[6] G. FerroLuzzi-Ames, manuscript in preparation. These results show that a double mutant containing a hisP mutation and a deletion, lacking histidine-biosynthetic enzymes, is inhibited in its growth on high concentrations of histidine by the aromatic amino acids. This was expected, as some histidine can be taken up into the cell by the aromatic permease in addition to the specific histidine permease (2). Also as expected, this double mutant appears to be derepressed for the histidine-biosynthetic enzymes when growing on histidine, indicating that insufficient histidine is being incorporated into protein. The hisP gene is closely linked (50%) to purF on the side opposite from the hisT regulator gene.

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