Mechanism of Histidine Decarboxylase Inhibition by NSD-1055 and Related Hydroxylamines

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SUMMARY

The kinetics of inhibition by 4-bromo-3-hydroxybenzyloxyamine and several other O-substituted hydroxylamines were studied using partially purified histidine decarboxylase. Hydroxylamine and O-substituted derivatives were competitive inhibitors with respect to the substrate. This type of inhibition was ascribed to competition of the oxyamino group of the inhibitor and the amino group of the substrate for a carbonyl site of the holoenzyme. Interaction of O-substituted hydroxylamines and pyridoxal phosphate, leading to the formation of oximes, was observed by following characteristic changes in absorbance and fluorescence spectra of pyridoxal phosphate oximes. Several such oximes were prepared and shown to be effective competitive inhibitors with respect to the coenzyme. Two such oximes, lacking the phosphate ester group, were substantially less inhibitory than their analogs containing the phosphate ester group.

INTRODUCTION

Several recent reports have indicated a variety of pharmacologic effects following administration of 4-bromo-3-hydroxybenzyloxyamine (NSD-1055). After injection into rats the histamine content in heart, stomach, and urine was markedly lowered (1). Gastric acid secretion in the rat was drastically lowered after injection of NSD-1055 (2). Human subjects suffering from mastocytosis, a disease involving abnormal proliferation of mast cells and excessive histamine production, have been successfully treated by oral administration of NSD-1055 (3). Anti-inflammatory effects of NSD-1055 were reported in a histologic study on experimentally induced granuloma and eczema, caused by contact with 2,4-dinitrochlorobenzene (4). The pharmacologic effects of NSD-1055 have been attributed to its ability to inhibit histidine decarboxylase (EC 4.1.1.22) (1, 5); histamine, the product of the reaction catalyzed by this enzyme, is believed to regulate acid secretion (2, 6).

Although its physiological role is far from clear, it has been proposed that histamine plays a role as regulator of the microcirculation (7) and as mediator of the secondary inflammatory response (7, 8). Histamine, furthermore, appears to be implicated in the manifestation of allergic conditions (cf. review, ref. 9). In view of the diversity of the reported pharmacologic effects of NSD-1055, a kinetic analysis of histidine decarboxylase inhibition by this drug was carried out in an attempt to elucidate its mechanism of action.

METHODS

Purification of enzyme. Whole rat fetus (day 19 or 20) was homogenized with 2 volumes of 0.1 M sodium acetate, pH 5.5. The extract was centrifuged for 90 min at 78,000 *g*. The supernatant fluid was fractionated using a saturated solution of ammonium sulfate, pH 6.7, when measured after diluting 1:10. The protein that precipitated between 25 and 45% saturation was dissolved in 0.05 M potassium phosphate buffer, pH 7.0, and dialyzed at 8° overnight. This protein fraction was di-

luted to 10 mg/ml and again precipitated by addition of a saturated solution of ammonium sulfate. The fraction which precipitated between 25 and 43% was redissolved and dialyzed against 0.05 M potassium phosphate buffer, pH 7.0. Storage at —15° resulted in no loss of activity over several months. The maximal velocity of a typical batch of enzyme thus prepared (preparation type A), determined under conditions of Fig. 3A, was 0.12 mμmole min^{-1} mg^{-1}. Stimulation by added pyridoxal phosphate (pyridoxal-P) was 4-fold.

Enzyme prepared by a purification procedure, described by Håkanson (10), but employing the following modifications, was used in certain experiments. The sodium acetate solution used for the preparation of cell-free extracts had a pH of 5.5. Aliquots of extract (70 ml) were immersed in a water bath at 52° for 5 min with occasional stirring. Subsequent fractionation with ammonium sulfate according to Håkanson (10) and final dialysis against 0.05 M potassium phosphate buffer, pH 7.0, provided a preparation (preparation type B) that was stable for at least 3 weeks. All experiments involving type B enzyme were done with one batch of enzyme. The maximal velocity of this preparation, determined under the conditions of Fig. 7, was 0.065 mμmole min^{-1} mg^{-1}. Stimulation by added pyridoxal-P was 5-fold. After storing enzyme preparations of type B at —15° no loss in activity was found after 11 weeks.

Preliminary experiments showed that 0.1 mM pyridoxal-P protects histidine decarboxylase against heat inactivation. The heating step (10) was, therefore, carried out at 55° in the presence of 0.1 mM pyridoxal-P by immersing for 5 min 14-ml samples of crude extract in a water bath maintained at 55°. Subsequent centrifugation and fractionation with ammonium sulfate (10) provided a preparation which was stimulated 3-fold by added pyridoxal-P (preparation type C). The maximal velocity was 0.18 mμmole min^{-1} mg^{-1}.

Protein was estimated by the method of Lowry *et al.* (11).

Assay of histidine decarboxylase. The typical reaction mixture contained: 0.1 M potassium phosphate, pH 6.9; 0.01 mM pyridoxal-P; 0.75 mM L-histidine, 0.375 μC DL-histidine (carboxyl-^{14}C). The reaction was initiated by adding enzyme without preincubation. The amounts of enzyme were such as to produce 2000–10,000 cpm as $^{14}CO_2$. The final volume was 0.5 ml. Time of incubation was 30 min, unless stated otherwise. Estimation of $^{14}C_2$ evolved was as described (12).

The rate of $^{14}CO_2$ evolution was constant for 2 hr at histidine concentrations of 0.25 mM and 1.0 mM. The rate of $^{14}CO_2$ evolution was proportional to the protein concentration; the largest amount of protein employed produced 45,000 cpm, equivalent to 54 mμmoles of $^{14}CO_2$, during 2 hr of incubation.

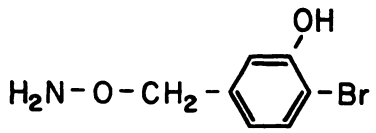
In experiments employing different histidine concentrations the choice of proper blanks can be critical. It was noted that there is no direct linear relationship between nonenzymatic $^{14}CO_2$ evolution and histidine concentration, the time of incubation or the time period during which the samples are kept acidified to collect the $^{14}CO_2$. Individual blank determinations were, therefore, carried out for each histidine concentration. Errors in the enzymatic assay were further minimized by employing enzyme in sufficient amounts to maintain blanks at 2–5% of the total amount of $^{14}CO_2$ formed.

Materials. Pyridoxal hydrochloride, pyridoxal-5-phosphate and L-histidine were obtained from Sigma Chemical Company. *O*-Benzyloxyamine hydrochloride was purchased from Aldrich Chemical Company, aminooxyacetic acid from Eastman Organic Chemicals, and ^{14}C-carboxyl-labeled DL-histidine from New England Nuclear Corp. or from Calbiochem.

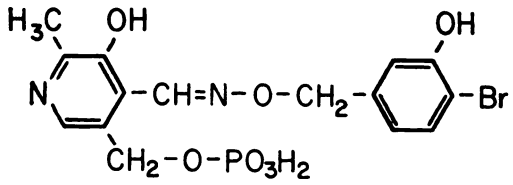
NSD-1024 (3-hydroxybenzyloxyamine dihydrogen phosphate) and NSD-1055 (4-bromo-3-hydroxybenzyloxyamine dihydrogen phosphate) were kindly donated by Smith and Nephew Research Ltd. The structure of NSD-1055 is shown in Fig. 1.

The animals used were Wistar rats, obtained from Carworth Farms.

Analytical methods. The hydroxylamine content of the oxime of pyridoxal-P and



NSD-1055



[NSD-1055]-[pyridoxal-P]

FIG. 1. *Structure of NSD-1055 (free base) and of the oxime formed between NSD-1055 and pyridoxal-P*

hydroxylamine was measured colorimetrically after oxidation of hydroxylamine to nitrous acid in the presence of sulfanilic acid; addition of α-naphthylamine gives rise to a red azo dye (13). It was noted that benzyloxyamine and NSD-1055 react in this test provided the duration of the oxidation step with iodine is increased to 30 min. The degree of color formation, however, from benzyloxyamine or NSD-1055 did not exceed 25% of that obtained with hydroxylamine or nitrite. No color formation was detectable with aminooxyacetate.

Separation of the oximes from pyridoxal-P was carried out by ascending paper chromatography (Table 1), using freshly prepared solvents; A: *n*-butanol–distilled water–90% formic acid (70:15:15); B: *n*-butanol–distilled water (86:14). Solutions of oximes were in water or *N,N'*-dimethylformamide; 0.2 μmole each were streaked in a 10-cm band on Whatman No. 1 filter paper. After developing and drying, the papers were examined under UV light, and the different zones were cut out and eluted with 0.1 M potassium phosphate, pH 6.9. Oximes thus eluted were identified by measuring the peaks of excitation and fluorescence. Efforts to quantitize the extent of pyridoxal-P contamination after chromatographic separation were unsuccessful. Peak and intensity of fluorescence of pyridoxal-P were found to vary greatly with concentration and age of the solution; utilizing the native fluorescence of pyridoxal-P was thus not practical. It was, therefore, attempted to measure pyridoxal-P after conversion to its cyanohydrin analog (14) or as its phenylhydrazone (15).

TABLE 1
R_F of pyridoxal-P oximes
Explanations under "*Analytical methods.*"

Compound	Solvent A	Solvent B
[NH_2OH] · [pyridoxal-P]	0.31	0.06
[aminooxyacetate] · [pyridoxal-P]	0.28	0
[aminooxyacetate] · [pyridoxal]	0.55	0.31
[benzyloxyamine] · [pyridoxal-P]	0.62	0.22
[NSD-1055] · [pyridoxal-P]	0.60	0.23
Pyridoxal	0.56	0.56
Pyridoxal-P	0.27	0

Known pyridoxal-P eluted from chromatograms failed to react in either test. Pyridoxal-P contamination of the oximes was, therefore, estimated (15) without prior separation. This test was found inapplicable to the measurement of pyridoxal; the required heating step (15) apparently causes oximes to react with phenylhydrazine, resulting in excessive "blanks."

Oxime of pyridoxal-5-phosphate and NSD-1055. To a solution of 123 mg of pyridoxal-P in 50 ml of water, pH 4, was added dropwise and with stirring a solution of 158 mg of NSD-1055 in 25 ml of water. A precipitate formed instantly. The crystals were collected on a filter and washed with water and ethanol. After careful drying the sample weighed 139 mg. Melting was noted below 166° (dec.). This fraction was contaminated by 5% NSD-1055 and 2.9% pyridoxal-P. Results of kinetic experiments discussed later are in agreement with the finding of a high content of unreacted NSD-1055.

To the mother liquor a few milliliters of ethanol were added. The resulting crystals

were washed and dried; weight 46 mg. Melting point 189–190° (dec.). Calculated for $C_{15}H_{16}O_7N_2PBr$: C 40.29; H 3.61; N 6.26%. Found C 39.78; H 3.81; N 6.14%. This fraction contained 0.5% NSD-1055 and 10.8% pyridoxal-P.

Oximes of either hydroxylamine or *O*-substituted hydroxylamines and pyridoxal-P were prepared in similar fashion. Only the precipitate that formed on combining the reactants was collected; the mother liquor of each reaction mixture was discarded. The oximes thus obtained were used without further purification. Solutions were usually made in 0.1 M or 0.5 M potassium phosphate, pH 6.9. For the sake of simplicity, the proper chemical names of the oximes prepared will not be used. Instead, abbreviated symbols denoting the parent compounds have been chosen. The oxime obtained from NSD-1055 and pyridoxal-P (4-[(bromo-3-hydroxybenzyloxyamino) methyl]-5-hydroxy-6-methyl-3-pyridine methyl phosphate) will thus be abbreviated [NSD-1055]·[pyridoxal-P]. The structure of this oxime is shown in Fig. 1.

Oxime of pyridoxal-5-phosphate and hydroxylamine, [NH_2OH]·[pyridoxal-P]

Melting point 221° (dec.); reported 229–230° (ref. 16).

Calculated: N 10.69%. Found 10.32%.

Contaminants were 0.6% NH_2OH and 3.8% pyridoxal-P.

Oxime of pyridoxal-5-phosphate and aminooxyacetate, [aminooxyacetate]·[pyridoxal-P]

Melting point 209° (dec.).

Calculated for $C_{10}H_{13}O_8N_2P$: C 37.51; H 4.09; N 8.75; P 9.67%. Found: C37.37; H 4.09; N 8.74; P 9.74%.

Contaminant: 0.2% pyridoxal-P.

Oxime of pyridoxal-5-phosphate and benzyloxyamine, [benzyloxyamine]·[pyridoxal-P]

Melting point 177–179° (dec.).

Calculated for $C_{15}H_{17}O_6N_2P$: C 51.14; H 4.86; N 7.95; P 8.79%. Found C 51.02; H 4.87; N 8.21; P 8.92%.

Contaminants: 2% benzyloxyamine and 1.0% pyridoxal-P.

Oxime of pyridoxal and aminooxyacetate, [aminooxyacetate]·[pyridoxal]

Melting point 207° (dec.).

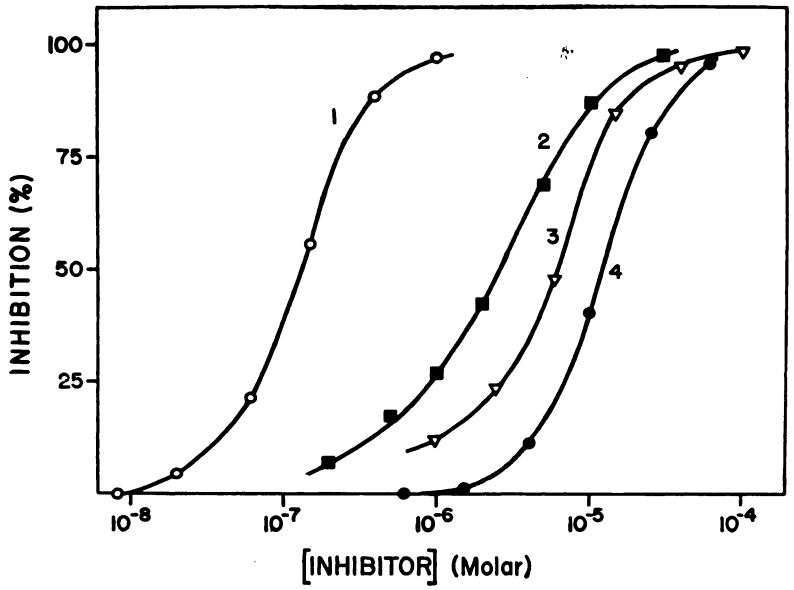


FIG. 2. *Inhibition of histidine decarboxylase as function of molar concentration of O-substituted hydroxylamines*

Assay as described under Methods but at 1 × 10^{-3} M L-histidine, using 1.3 or 2 mg each of enzyme type C. Incubation was for 90 min. Curves: *1*, NSD-1055; *2*, *O*-benzyloxyamine; *3*, aminooxyacetate; *4*, NH_2OH.

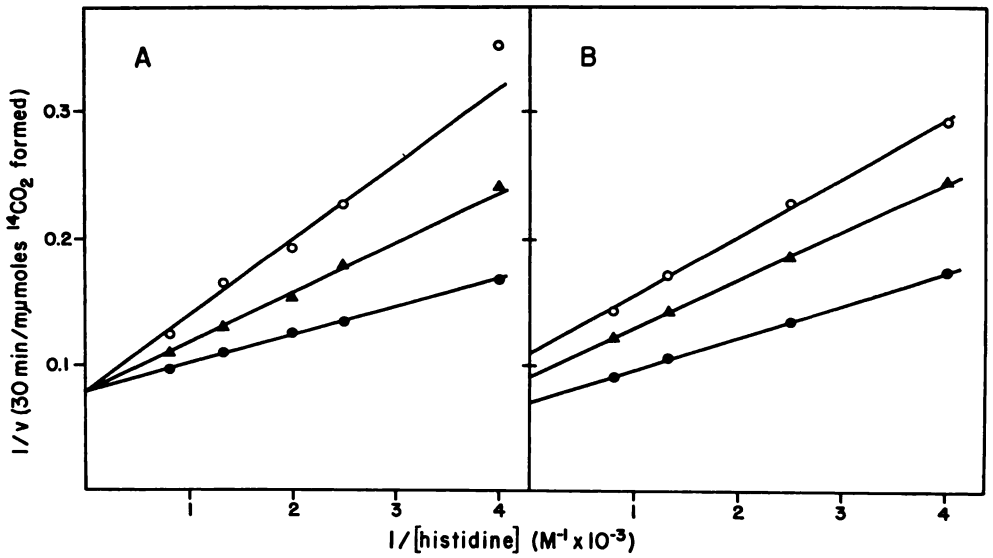


FIG. 3. *Inhibition of histidine decarboxylase by two concentrations of NSD-1055 (A) and the oxime of NSD-1055 and pyridoxal-P (B)*

Histidine concentrations varied from 2.5×10^{-4} to 1.25×10^{-3} M. Enzyme (4 mg of protein, type A) was added last to initiate the reaction. Pyridoxal-P was introduced together with the enzyme; final concentration 1×10^{-5} M. Incubation was for 30 min. (A) NSD-1055 concentration 4×10^{-6} (▲———▲) and 8×10^{-6} M (O——O). (B) [NSD-1055] · [pyridoxal-P] concentration 4.5×16^{-6} M (▲—▲) and 9×10^{-6} M (O———O). No inhibitor (●———●).

Calculated for $C_{10}H_{12}O_5N_2$: N 11.66%. Found: 11.75%.

The extent of contamination by the starting materials was unknown. Chromatograms suggested homogeneity (UV light).

RESULTS AND DISCUSSION

Based on structural considerations it was anticipated that inhibition of histidine decarboxylase by NSD-1055 might be due to the presence of the -O-NH_2 group in that molecule. In the present study the inhibition by several O-substituted hydroxylamines was, therefore, compared to that of NSD-1055. As shown in Fig. 2 the concentration of NSD-1055 required for complete inhibition of the enzyme was much lower than the concentration of benzyloxyamine, aminooxyacetate, and hydroxylamine. Kinetic experiments indicated that NSD-1055 is a competitive inhibitor relative to histidine (Fig. 3A). This finding would indicate that the apoenzyme (E) reacts first with the coenzyme (A):

$$E + A \rightleftharpoons EA$$

Competition would then involve interaction of a carbonyl group of the holoenzyme (EA), either with the amino group of the substrate (S):

$$EA + S \rightleftharpoons EAS$$

or with the oxyamino group of the inhibitor (I)

$$EA + I \rightleftharpoons EAI$$

The competitive relation of Fig. 3A suggests a free equilibrium between NSD-1055 and holoenzyme. The potency of the inhibitor is remarkable. The presence of excess coenzyme in relation to inhibitor (125- and 250-fold, respectively, in the experiments shown in Fig. 3A) indicated that a mere removal of pyridoxal-P by reaction with NSD-1055 cannot account for the inhibition. The affinity of the catalytic site of the holoenzyme for NSD-1055 is apparently much greater than that of pyridoxal-P. The study of hydroxylamine and several other O-substituted derivatives (R-O-NH_2) showed that these compounds

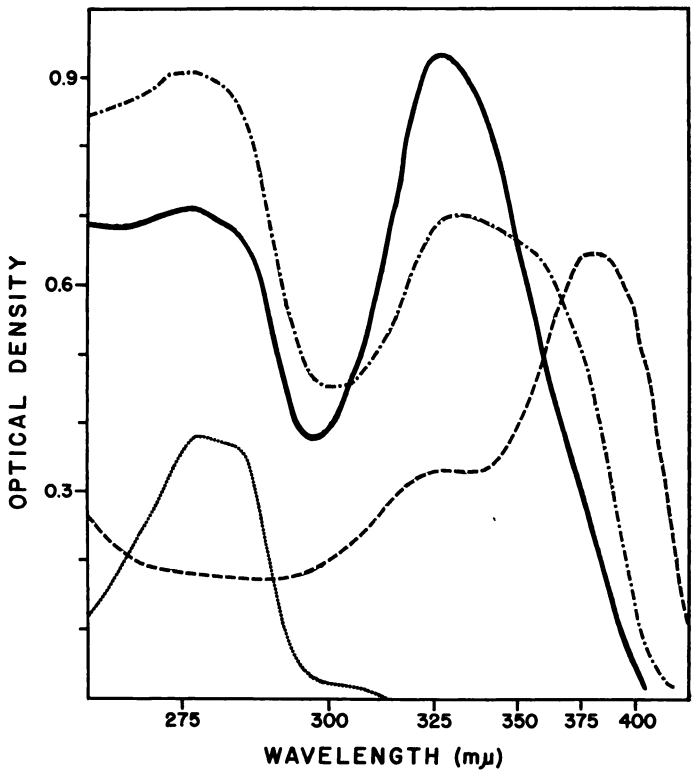


FIG. 4. *Absorption spectra of NSD-1055* (·····), *pyridoxal-P* (— — — —), *the reaction mixture of NSD-1055 and pyridoxal-P* (· — · — · — · —), *recorded 80 min after combining the reactants, and the oxime prepared from NSD-1055 and pyridoxal-P* (————)

Compounds were assayed at 1.5×10^{-4} M in 0.1 M potassium phosphate, pH 6.9. Recordings were done with a Beckman DK-2 recording spectrophotometer, UV light source.

inhibit in the same fashion as NSD-1055. The compounds and their inhibitor dissociation constants are listed in Table 2.

The kinetics of inhibition and the values of the inhibitor dissociation constants of hydroxylamine, benzyloxamine, and NSD-1055 were determined in a separate experiment employing standard conditions, but during an incubation period of 10 min. The results were in good agreement with those summarized in Table 2. Inhibition experiments with hydroxylamine and benzyloxyamine using a 90-min incubation period showed that inhibition was no longer competitive with respect to the substrate. In the case of hydroxylamine the inhibition appeared to be noncompetitive and with benzyloxyamine inhibition was of the mixed type (17). This finding prompted a study of the interaction of pyridoxal-P and the various hydroxylamines. An earlier report indicated that the interaction of NSD-1055 with pyridoxal-P was accompanied by spectral changes (18). This observation was confirmed in the present study. When equimolar amounts of NSD-1055 and pyridoxal-P were combined at pH 6.9 the main absorbance peak of pyridoxal-P, seen at 380 mμ, disappeared gradually; a new peak appeared near 328 mμ (Fig. 4). NSD-1055, on the other hand, absorbed in the region near 280 mμ. The reaction of NSD-1055 with pyridoxal-P did not change the absorption peak at 280 mμ; the extent of absorbance, however, increased. A solution of the oxime, formed between NSD-1055 and pyridoxal-P at pH 3–4 ([NSD-1055] · [pyridoxal-P], see under *Materials*), showed the identical absorption spectrum (Fig. 4). Several other hydroxyl-

TABLE 2

Inhibition of histidine decarboxylase by O-substituted hydroxylamines and pyridoxal-P oximes

Assay conditions as described under Methods; see also legend of Fig. 3. An asterisk denotes conditions employed as those shown in the legend of Fig. 7. The inhibitor dissociation constants (K_i) listed in Table 1 were obtained from plotting $i[\alpha/(1-\alpha)]$ against s, where i is the inhibitor concentration, s the substrate concentration, and α the ratio of the (velocity in the presence of inhibitor)/(velocity in the absence of inhibitor) with the same substrate concentration (17). Only values are included that agree satisfactorily with those obtained from other types of plots (17).

Inhibitor	Variable	K_i (M)	Type of inhibition
NH_2OH	Histidine	6.3×10^{-6}	Competitive
[NH_2OH] · [pyridoxal-P]	Histidine	9.7×10^{-5}	Noncompetitive
	Pyridoxal-P*	—	Competitive
Aminooxyacetate	Histidine	1.6×10^{-6}	Competitive
[Aminooxyacetate] · [pyridoxal-P]	Histidine	3.6×10^{-4}	Mixed competitive
	Pyridoxal-P*	4×10^{-4}	Noncompetitive
Benzyloxyamine	Histidine	6.2×10^{-7}	Competitive
[Benzyloxyamine] · [pyridoxal-P]	Histidine	3.0×10^{-5}	Noncompetitive
	Pyridoxal-P*	—	Competitive
NSD-1024	Histidine	1.4×10^{-7}	Competitive
NSD-1055	Histidine	4.2×10^{-8}	Competitive
[NSD-1055] · [pyridoxal-P]	Histidine	1.3×10^{-5}	Noncompetitive
	Pyridoxal-P*	1.4×10^{-7}	Competitive

amines, combined with the enzyme at pH 6.9, similarly abolished the absorption peak of pyridoxal-P at 380 mμ. New absorbance peaks emerged in the region near 328 mμ. Absorbance in that spectral region, thus, appears to be common among oximes of pyridoxal-P (Table 3).

Pyridoxal-P exhibits strong fluorescence (14). It was found that the chemical reaction between pyridoxal-P and the hydroxylamines was accompanied by changes in the fluorescence spectrum of the reaction mixture. A comparison was made of the excitation and fluorescence spectra of reaction mixtures between pyridoxal-P and various hydroxylamines and the different oximes that had been prepared; excitation (near 370 mμ) and fluorescence peaks (near 450 mμ) agreed in every case. The excitation and fluorescence spectrum for [NSD-1055] · [pyridoxal-P] is shown in Fig. 5. Data for the oximes are summarized in Table 3. The rate of oxime formation with the various hydroxylamines, as judged by the increase in fluorescence at 453 mμ (uncorr.) at pH 6.9 varies to a considerable degree (Fig. 6). Based on approximately equal relative fluorescence intensities for [aminooxyacetate] · [pyridoxal - P] and [benzyloxyamine] · [pyridoxal-P] (Table 3) it appears that the initial rate of formation of the former oxime proceeds at twice the rate. The reaction of pyridoxal-P with hydroxylamine in the experiment shown in Fig. 6 no longer proceeds to an appreciable degree after 30 min; the reaction involving other hydroxylamines continues for much longer. This observation offers an explanation as to why hydroxylamine inhibition is competitive only in short-term incubation experiments. The concentration of hydroxylamine needed to demonstrate competitive inhibition was 10^{-5} M, the same concentration as that of pyridoxal-P. With prolonged incubation most of the hydroxylamine initially present thus reacts to form the oxime: the oximes formed with pyridoxal-P are also inhibitory. The absence of competitive inhibition relative to the substrate was noted with each of the oximes studied. An experiment using [NSD-1055] · [pyridoxal-P] is shown in Fig. 3B, indicating noncompetitive inhibition. The inhibitor dissociation constants calculated for the various oximes are listed in Table 2. The experiment shown

TABLE 3

Absorbance and fluorescence characteristics of pyridoxal-5-phosphate oximes

Absorbance measurements: 1.5×10^{-4} M solutions in 0.1 M potassium phosphate, pH 6.9, were used. Quinine sulfate was used as reference at 3 or 100 μg/ml of 0.1 N H_2SO_4. Fluorescence measurements: all compounds were assayed at 5×10^{-5} M in 0.1 M potassium phosphate, pH 6.9, except [aminooxyacetate] · [pyridoxal] which was diluted to 1×10^{-5} M. Excitation peaks (uncorr.) were determined by scanning at a constant fluorescence setting of 453 mμ. Fluorescence peaks (uncorr.) were determined by scanning at a constant excitation setting of 370 mμ, except for pyridoxal and pyridoxal-P (322 mμ). Quinine sulfate: 1 μg/ml of 0.1 N H_2SO_4. Fluorescence scanning was done using an Aminco-Bowman spectrophotofluorometer in conjunction with a 500 XY Recorder (Electro Instruments, Inc., San Diego, California).

Compound	Absorbance peaks (mμ)	Molar extinction coefficient (M^{-1} cm^{-1} at 330 mμ)	Excitation peak (mμ)	Fluorescence peak (mμ)	Relative fluorescence intensity at 453 mμ
[NH_2OH] · [pyridoxal-P]	326	7200	365	450	131
[Aminooxyacetate] · [pyridoxal-P]	328	4600	370	454	147
[Aminooxyacetate] · [pyridoxal]	332	4600	371	455	487
[Benzyloxyamine] · [pyridoxal-P]	327	5200	370	451	152
[NSD-1055] · [pyridoxal-P]	276, 332	6800	368	453	100
Pyridoxal-P	221, (329), 380	2100	325	388	0.4
Pyridoxal	217, 249, 312	—	323	380	4
NSD-1055	277	0	—	None	—
Quinine sulfate	249, 317, 346	—	350	450	770

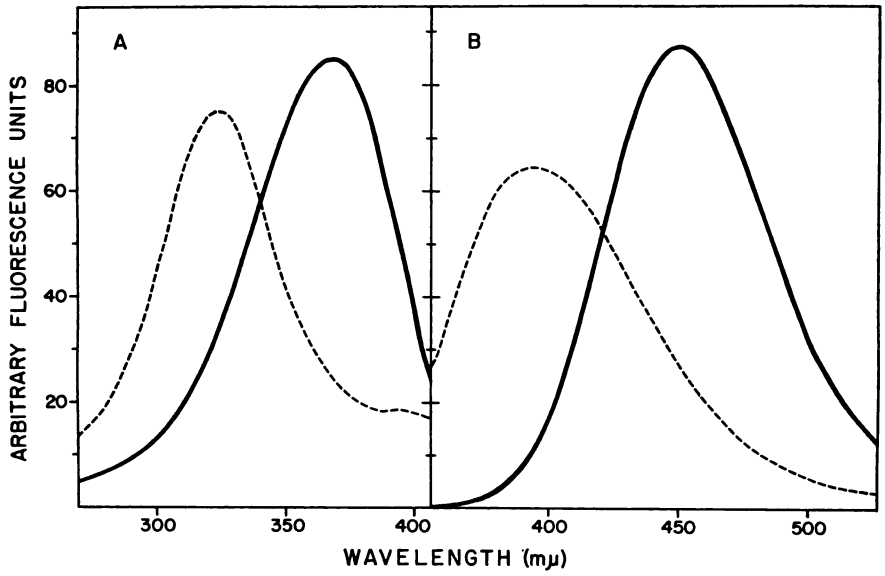


FIG. 5. *Excitation (A) and fluorescence spectra (B) of* 5×10^{-5} M *[NSD-1055] · [pyridoxal-P]* (———) *and* 5×10^{-5} M *pyridoxal-P* (— — —) *in 0.1 M potassium phosphate, pH 6.9*

For recording spectra of pyridoxal-P the sensitivity of the fluorometer was increased 50 and 100 times, respectively. Excitation was at 322 mμ for pyridoxal-P and at 370 mμ for [NSD-1055] · [pyridoxal-P]; fluorescence at 453 mμ.

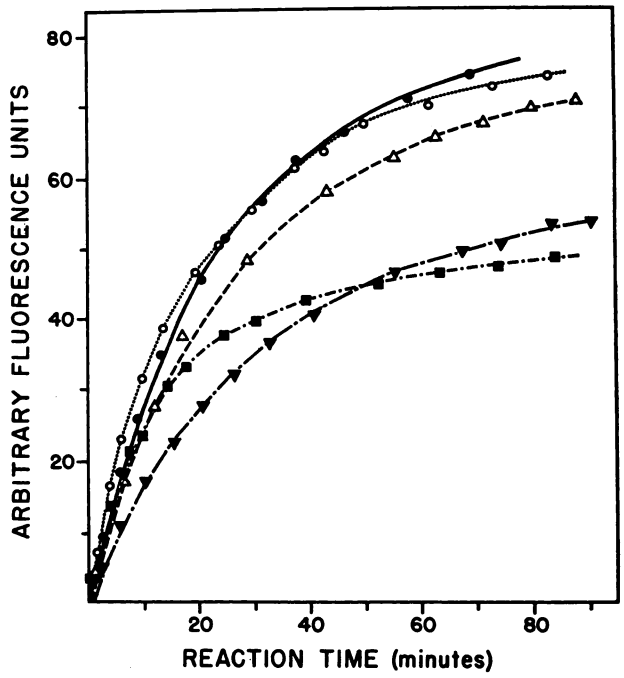


FIG. 6. *Relative rate of formation of oxime of pyridoxal-P* (1×10^{-5} M) *and several hydroxylamines* (1×10^{-5} M) *in 0.1* M *potassium phosphate buffer, pH 6.9, at 37°*

Excitation 370 mμ, fluorescence 453 mμ. Hydroxylamine (■——■), aminooxyacetate (O--------O), benzyloxyamine (▼- — - —▼), NSD-1024 (△— — —△), NSD-1055 (●———●).

in Fig. 3B was carried out with [NSD-1055]·[pyridoxal-P] that had a melting point of 189–190° (see under *Materials*). The fraction of this oxime that melted below 166° inhibited the enzyme competitively, suggesting the presence of unreacted NSD-1055. This interpretation was supported by an experiment in which the reaction mixture (complete system less enzyme) was preincubated for several hours; preincubation abolished the competitive nature of inhibition by either the impure fraction of the oxime or NSD-1055. The two fractions of [NSD-1055]·[pyridoxal-P] behaved very similarly with respect to absorbance and fluorescence characteristics.

The effectiveness as inhibitors of several of the oximes prepared from pyridoxal-P and hydroxylamines was remarkable. Several of these oximes were found to be competitive inhibitors with respect to the coenzyme, as demonstrated for [NSD-1055]·[pyridoxal-P] (Fig. 7), the most active inhibitor among the oximes (Table 2) studied. The absence of a perfect straight-line fit at low concentrations of pyridoxal-P in the presence of [NSD-1055]·[pyridoxal-P] may be noted (Fig. 7). Such deviations were also noted with the other oximes under these conditions. This observation appears to indicate contamination of the oxime by unreacted pyridoxal-P.

If inhibition of histidine decarboxylase by pyridoxal-P oximes is due to their competition with the coenzyme for the apoenzyme, one would expect far less inhibition by these compounds after dephosphorylation. This expectation was borne out in studying the oxime of aminooxyacetate and pyridoxal; only 12% inhibition was found with this derivative at 2×10^{-3} M. A similar study was attempted with the oxime prepared from NSD-1055 and pyridoxal. This compound also proved to be a competitive inhibitor with respect to pyridoxal-P, and its K_i increased 15 times compared to that of [NSD-1055]·[pyridoxal-P]. This finding has to be viewed

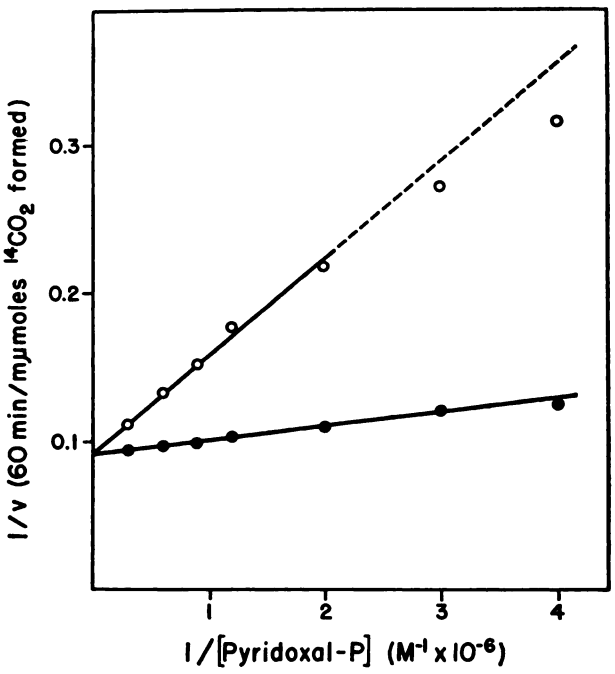


FIG. 7. *Inhibition of histidine decarboxylase by the oxime of NSD-1055 and pyridoxal-P*

The concentration of histidine was 7.5×10^{-4} M, of [NSD-1055] · [pyridoxal-P] (O——O) 5×10^{-7} M; pyridoxal-P concentrations varied from 1.5×10^{-7} to 1×10^{-5} M. Reaction was initiated by the addition 2.8 mg of protein, type B. Incubation was for 60 min at 37°.

as tentative since the elemental analysis of [NSD-1055] · [pyridoxal] proved unsatisfactory. Its spectral and fluorescent characteristics were, however, very similar to those of the oximes shown in Table 3.

The present kinetic study showed that hydroxylamine and O-substituted derivatives are competitive inhibitors relative to the substrate. Such a relation was reported in a study of mouse brain glutamate decarboxylase (19). An earlier study on the effect of NSD-1055 on histidine decarboxylase suggested irreversible inhibition (20). Several of the present experiments showed that after 30 or 90 min of incubation inhibition by NSD-1055 is competitive relative to the substrate. The data for NSD-1055 inhibition, shown in Fig. 2, were, therefore, analyzed as proposed by Reiner (21), by plotting the velocity of the reaction as a function of the total inhibitor concentration. In the case of irreversible inhibition a straight line would be obtained: the plot showed a sharply bending line. The experiment was repeated, incubating 4 mg of type A enzyme for 30 min. The result is shown in Fig. 8. An "irreversible" inhibitor might be expected to remain bound to the enzyme after dilution or dialysis. An experiment, summarized in Table 4, indicated that NSD-1055 inhibition can be reversed by dilution. Histidine decarboxylase, when preincubated with NSD-1055 at a concentration that resulted in 92% inhibition without dilution, was much less inhibited if addition of other reactants resulted in dilution of NSD-1055. Dilution reversed the inhibition to a smaller degree when the preincubation included the presence of pyridoxal-P, as was also noted in a different experiment. Enzyme (6 mg protein, type C) was preincubated for 20 min at 25° in the presence of 1×10^{-6} M NSD-1055 and 4×10^{-5} M pyridoxal-P. After passage over a Sephadex G-25 column (equilibrated with 0.1 M potassium phosphate, pH 6.75) the specific activity of the eluted enzyme was 50% as compared to the untreated enzyme.

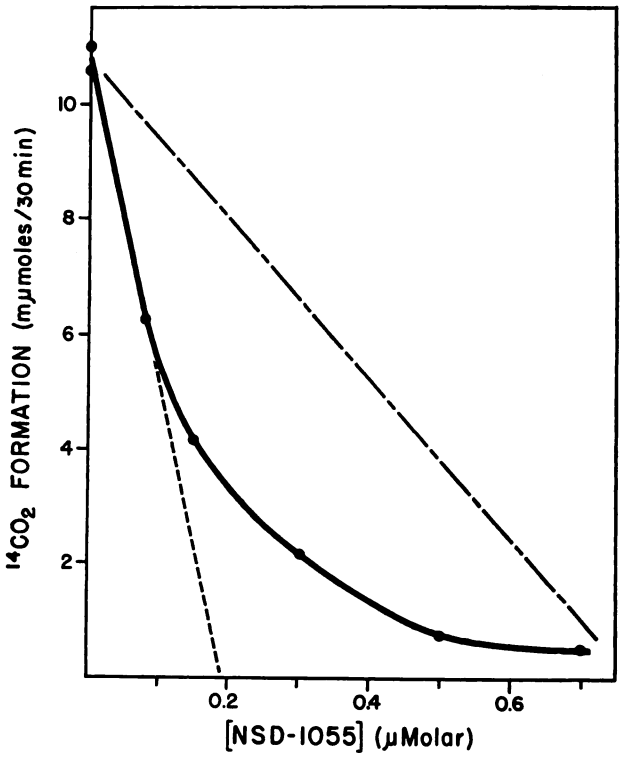


FIG. 8. *Rate of $^{14}CO_2$ formation as function of concentration of NSD-1055*

Conditions of assay as described under Methods. The dashed lines are hypothetical, as expected in the case of "irreversible" inhibition; the slope of these hypothetical lines is arbitrary. The actual slope equals $-k_2/k$ (ref. 21), a term containing the rate constants of the reaction. The intercept of the hypothetical line with the abscissa equals the amount of enzyme.

TABLE 4

Effect of preincubating histidine decarboxylase with NSD-1055 on the rate of $^{14}CO_2$ formation

To 50 μl of enzyme (1.4 mg of protein, type A), 2μl of 2.7×10^{-4} M pyridoxal-P and 2μl of 1.35×10^{-5} M NSD-1055 were added. After 20 min of preincubation at 24°, the reaction was initiated by adding 446 μl containing 100 μmoles of potassium phosphate, pH 6.9, 0.005 μmole of pyridoxal-P, and 0.5 μmole of ^{14}C-L-histidine. Incubation was for 90 min at 37°. Values in parentheses under "Expected inhibition" indicate the degree of inhibition expected (Fig. 2) if the inhibitor concentration employed during incubation had equaled the inhibitor concentration during preincubation.

Present during preincubation				
Pyridoxal-P	10^{-5} M	10^{-5} M	10^{-5} M	—
NSD-1055	—	—	5×10^{-7} M	5×10^{-7} M
Present during incubation				
Pyridoxal-P	10^{-5} M	10^{-5} M	10^{-5} M	10^{-5} M
NSD-1055	—	5.4×10^{-7} M	5.4×10^{-8} M	5.4×10^{-8} M
$^{14}CO_2$ formation (cpm)	7119	801	1933	4520
	6807	—	2320	4009
Observed inhibition (%)	0	88	69	39
Expected inhibition (%)	0	92	19 (92)	19 (92)

An aliquot of protein preincubated with NSD-1055 without added pyridoxal-P exhibited a specific activity of 71% relative to the untreated enzyme. The present evidence is, therefore, incompatible with "irreversible" inhibition of histidine decarboxylase by NSD-1055.

Preincubation of hydroxylamines with excess coenzyme or prolonging the incubation periods will change the kinetics of inhibition. Only unreacted hydroxylamines compete with the substrate for a common site on the enzyme. Oxime formation generates derivatives of the coenzyme, several of which possess remarkable affinity for the enzyme. Among four of the pyridoxal-P oximes studied, three were competitive inhibitors with respect to the coenzyme. Chemical interaction between hydroxylamines and the coenzyme, thus, is insufficient to abolish the inhibitory properties of hydroxylamines. Further modification of that molecule, such as dephosphorylation, would be required to abolish the inhibition of histamine formation by pyridoxal-P oximes.

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