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MECHANISM OF INHIBITION OF HISTIDINE DECARBOXYLASE BY RHODANINES*

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Abstract—The inhibition of rat gastric histidine decarboxylase *in vitro* by rhodanine and 26 of its 3- or 5-substituted derivatives has been investigated. Inhibitory activities of the 3-substituted derivatives of rhodanine ranged from I_{50} values of 2×10^{-6} M to 2×10^{-4} M. Eight 5-substituted derivatives were relatively inactive, yielding I_{50} values higher than 5×10^{-4} M. Studies performed with 3-*p*-chlorobenzylrhodanine ($I_{50} = 3 \times 10^{-6}$ M) on preparations of histidine decarboxylase holoenzyme and apoenzyme indicated that only the holoenzyme form of the decarboxylase is susceptible to inhibition on treatment with an excess of the inhibitor, and that this inhibition is irreversible. The results are consistent with a mechanism of inhibition that involves a condensation reaction between the 5-methylene carbon of the rhodanine ring and enzyme-bound pyridoxal phosphate.

SYNTHESIS of histamine in the gastric mucosa[1] and in other mammalian tissues[2,3] is considered to be catalyzed principally by decarboxylases (EC 4.1.1.22) specific for L-histidine. During screening of agents for the inhibition of gastric histidine decarboxylase *in vitro*, activity was observed in a previously unrecognized group of inhibitors, the rhodanines. The present study is an investigation of the relative inhibitory potencies of rhodanine and a number of its derivatives against gastric histidine decarboxylase of the rat. Specific investigations into the mechanism of the inhibition have been performed with 3-*p*-chlorobenzylrhodanine (PCBR), a relatively potent inhibitor from the group of compounds studied.

EXPERIMENTAL

Histidine decarboxylase preparations. The "crude histidine decarboxylase" employed for routine inhibition experiments was prepared by a procedure similar to that of Levine and Watts.[4] Female rats, 100–200 g, purchased from Carworth Farms, New City, N.Y., were employed as a source of the enzyme. The animals, after overnight access to food and water, were killed by decapitation. The glandular portions of the stomachs were separated from the forestomachs, rinsed in cold water and saline, and homogenized in 3 ml per stomach of 0·1 M sodium acetate buffer, pH 5·5. The homogenate was centrifuged at 30,000 *g* for 10 min, yielding a clear supernatant that was filtered through glass wool to remove fat droplets. Gel filtration was employed to

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separate buffer and low molecular weight contaminants from the crude enzyme. In the latter procedure, the enzyme, in approximately 30 ml supernatant derived from 12 rat stomachs, was eluted with either 0·05 M sodium phosphate buffer (pH 7·2) or water, from a 2 × 53 cm (165 ml) column of Sephadex G-25. Effluent from the column was monitored at 254 mμ by passage through the continuous flow cell of an ultraviolet absorption meter. The protein peak of approximately 40 ml was collected, diluted to 100 ml with the same solution used for elution of the peak, and frozen until use.

Histidine decarboxylase holoenzyme was prepared by the addition of pyridoxal phosphate (to a final concentration of 1×10^{-4} M) to the supernatant obtained from centrifugation of the rat stomach homogenate during the standard preparative procedure. After standing for at least 15 min at 0°, the mixture was subjected to gel filtration and subsequent operations as in the standard procedure. Excess pyridoxal phosphate was removed from the enzyme solution during the gel filtration step.

Apoenzyme of histidine decarboxylase was prepared by mixing, at 0°, 25 ml of crude histidine decarboxylase solution in 0·05 M sodium phosphate buffer, pH 7·2, with 75 ml of 0·1 M sodium acetate buffer, pH 4·5 (final pH = 4·7). The enzyme was precipitated from the solution by the addition of solid ammonium sulfate to 85 per cent of saturation. The precipitate was recovered by centrifugation for 10 min at 37,000 *g* and was redissolved in 10 ml of 0·05 M sodium phosphate buffer, pH 7·2. Gel filtration of the redissolved protein on a 70-ml Sephadex G-25 column removed residual ammonium sulfate. The protein peak, eluted in a volume of 18 ml with 0·05 M sodium phosphate buffer, pH 7·2, was diluted to a final volume of 25 ml with the phosphate buffer and frozen until use.

Histidine decarboxylase assay. Enzyme assays were conducted according to the procedure of Levine and Watts,[4] a method based on trapping in hyamine hydroxide and subsequently measuring the $^{14}CO_2$ formed during the enzymic decarboxylation of carboxyl-labeled histidine. Total assay volumes of 2 ml were employed, containing 1 ml or less of enzyme solution and 0·5 ml of 0·1 M sodium phosphate buffer, pH 7·2. Incubation mixtures also contained, unless otherwise noted, 1×10^{-4} M L-histidine labeled with ^{14}C in the carboxyl position (0·10 μc per incubation vial) and 1×10^{-5} M pyridoxal phosphate. Incubation mixtures were preincubated for 10 min at 37° prior to initiation of the enzyme reaction by the addition of 0·1 ml of the labeled substrate. Incubations, generally of 60 min at 37°, were terminated by injection of 1 ml of 1 M citric acid into the assay vials. After an additional 60-min equilibration, hyamine hydroxide-saturated papers containing trapped CO_2 were removed and counted by liquid scintillation. Each assay included appropriate blanks containing all additions plus 5×10^{-5} M 4-bromo-3-hydroxybenzyloxyamine (NSD-1055), a concentration of the inhibitor sufficient to produce total inhibition of decarboxylase activity. After subtraction of the radioactivities of blanks, decarboxylation rates were calculated from the fractions of $^{14}CO_2$ formed from the [^{14}C]L-histidine. Formation of $^{14}CO_2$ in control incubations was linear with time in incubations of up to 4 hr, or with enzyme concentrations over a range of at least 5–50 units (picomoles CO_2 formed/min) per assay vial.

For the calculation of enzyme specific activity, protein concentrations were determined by the procedure of Lowry *et al.*,[5] using bovine serum albumin as the standard.

Materials. [^{14}C]L-histidine labeled in the carboxyl position was purchased from New England Nuclear Corp., Boston, Mass. NSD-1055 was a gift from Smith &

Nephew Research, Ltd., Essex, England. Derivatives of rhodanine were prepared and generously donated by Drs. F. C. Brown and C. K. Bradsher, Duke University, Durham, N.C. Other compounds were obtained commercially.

RESULTS AND DISCUSSION

Relative potencies of histidine decarboxylase inhibitors. To compare the potencies of compounds as inhibitors of crude gastric histidine decarboxylase, I_{50} values were determined by interpolation to the concentration that would inhibit by 50 per cent the activity demonstrated by a control incubation. Rhodanine (structure shown in Fig. 1) demonstrated an I_{50} of 2×10^{-5} M. Table 1 compares the I_{50} of rhodanine with those

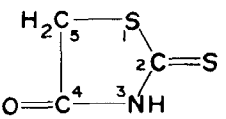

FIG. 1. Structural formula of rhodanine.

determined for a group of known histidine decarboxylase inhibitors. The group includes NSD-1055, the most potent known inhibitor of the enzyme, and three related compounds containing the oxyamine group. The I_{50} values found for these compounds are in agreement with values reported in the literature.[6,7] Many of the reported inhibitors of histidine decarboxylase contain the oxyamine or hydrazine moieties, allowing them to form oximes or hydrazones with pyridoxal phosphate, the cofactor for mammalian histidine decarboxylase. Rhodanine, in contrast, contained neither of these carbonyl-reactive groups, and thus introduced a new class of inhibitory compounds for investigation.

Table 2 summarizes the data obtained for a series of rhodanine derivatives. The first group of compounds, comprising 3-substituted rhodanines, showed a wide range of inhibitory activity. Although several of the derivatives were less active than rhodanine, the majority displayed inhibitory activity greater than that of the parent compound. The most active compound investigated was 3-*p*-chlorophenethylrhodanine, whose I_{50} (2×10^{-6} M) is within an order of magnitude of the potencies of the aliphatic oxyamine compounds and the less potent of the benzyloxyamines,[6,7] and also compares favorably with the potencies of inhibitory hydrazine derivatives such as *N'*-methyl-*N'*-(3-hydroxylbenzyl) hydrazine (NSD-1034)[8,9] and α-hydrazinohistidine (MK-785).[4]

TABLE 1. RELATIVE POTENCIES OF HISTIDINE DECARBOXYLASE INHIBITORS

Compound	I_{50} (M)
Rhodanine	2×10^{-5}
Hydroxylamine	7×10^{-6}
Aminooxyacetic acid	5×10^{-6}
Benzyloxyamine	3×10^{-7}
4-Bromo-3-hydroxybenzyloxyamine (NSD-1055)	6×10^{-9}

TABLE 2. RELATIVE INHIBITORY POTENCIES OF SUBSTITUTED RHODANINES

Compound	I_{50} (M)	Relative potency (%)
3-Substituted rhodanines		
3-*p*-Chlorophenethylrhodanine	2×10^{-6}	100
3-*p*-Chlorobenzylrhodanine	3×10^{-6}	67
3-*p*-Methylthiobenzylrhodanine	4×10^{-6}	50
3-*p*-Methylbenzylrhodanine	4×10^{-6}	50
3-*p*-Fluorobenzylrhodanine	5×10^{-6}	40
3-Aminorhodanine	5×10^{-6}	40
3-(3,4-Dichlorobenzyl)rhodanine	5×10^{-6}	40
3-*p*-Bromobenzylrhodanine	5×10^{-6}	40
3-*p*-Methoxybenzylrhodanine	6×10^{-6}	33
3-*p*-Bromoanilinorhodanine	9×10^{-6}	22
3-*p*-Iodoanilinorhodanine	9×10^{-6}	22
3-*p*-Chloroanilinorhodanine	1×10^{-5}	20
3-*p*-Toluidinorhodanine	2×10^{-5}	10
3-Anilinorhodanine	3×10^{-5}	6·7
3-(2,5-Dichloroanilino)rhodanine	4×10^{-5}	5·0
3-Dimethylaminorhodanine	5×10^{-5}	4·0
3-*p*-Methoxyphenylrhodanine	8×10^{-5}	2·5
3-*p*-Anisidinorhodanine	2×10^{-4}	1·0
5- and 3,5-Substituted rhodanines		
3-(2,4-Dichlorophenyl)-5-hydroxyiminorhodanine	6×10^{-4}	0·3
3-*p*-Chlorobenzyl-5-hydroxyiminorhodanine	9×10^{-4}	0·2
3-Benzyl-5-hydroxyiminorhodanine	1×10^{-3}	0·2
3-*p*-Chlorophenyl-5-ethylrhodanine	$>1 \times 10^{-3}$	<0·2
3-*p*-Chlorophenyl-5-rhodanineacetamide	$>1 \times 10^{-3}$	<0·2
3-Phenyl-5-rhodanineacetamide	$>1 \times 10^{-3}$	<0·2
5-Propylrhodanine	$>1 \times 10^{-3}$	<0·2
5-Rhodanineacetamide	$>1 \times 10^{-3}$	<0·2

A second group of compounds in Table 2, composed of 5- or 3,5-substituted rhodanines, was without exception inactive, as indicated by I_{50} values greater than 5×10^{-4} M. It thus appears that substitutions on the 5-carbon of the rhodanine ring destroy the inhibitory activity of the compound.

One of the more potent of the 3-substituted rhodanines, the 3-*p*-chlorobenzyl derivative (PCBR), was selected for use in further studies of the mechanism of inhibition.

Reversibility of inhibition by 3-p-*chlorobenzylrhodanine.* Preparations of histidine decarboxylase holoenzyme and apoenzyme, prepared as described above, were employed for examination of the reversibility of inhibition by PCBR. Figure 2 illustrates the pyridoxal phosphate requirements of the holoenzyme and apoenzyme preparations in comparison with the crude enzyme preparation from which both originated. Enzyme assays of the three preparations were performed in the presence and absence of 1×10^{-5} M pyridoxal phosphate. It is assumed that the activity observed in the absence of added pyridoxal phosphate represents holoenzyme, and that the increment of activity observed only in the presence of added cofactor reflects the apoenzyme content of the preparations. Thus, the crude enzyme contained 85 per cent holoenzyme. Studies with other similarly prepared batches revealed a range from 50 to 85 per cent of the activity present as holoenzyme. Levine and Watts,[4] using a

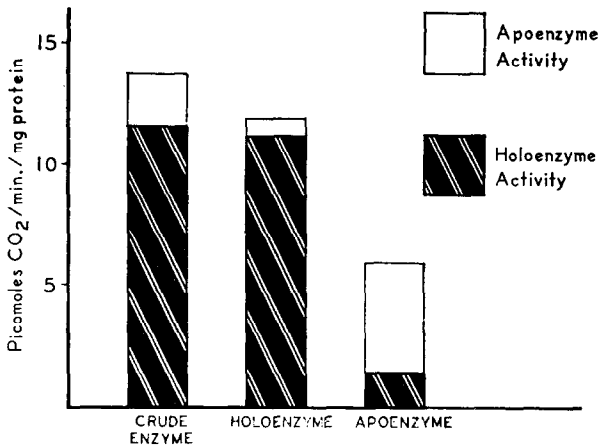

FIG. 2. Pyridoxal phosphate requirements of histidine decarboxylase preparations. Each value represents the average of activities calculated from duplicate incubations. The areas represented by hatched bars show histidine decarboxylase activities of enzyme preparations incubated in the absence of added pyridoxal phosphate, and are assumed to represent the holoenzyme activities of the preparations. Unhatched areas show the incremental activity observed in standard enzyme incubations containing 1×10^{-5} M pyridoxal phosphate. The incremental activity is assumed to represent the apoenzyme content of the enzyme preparations.

similar procedure for extracting histidine decarboxylase of fetal rats, obtained preparations containing only 3–17 per cent holoenzyme. In Fig. 2, the holoenzyme preparation showed a content of 94 per cent holoenzyme, whereas the apoenzyme preparation contained only 23 per cent holoenzyme.

For determination of reversibility of inhibition, solutions of histidine decarboxylase holoenzyme and apoenzyme, respectively, were incubated at room temperature for 15 min with 1×10^{-4} M PCBR, a concentration sufficient to produce greater than 95 per cent inhibition of crude histidine decarboxylase activity under routine assay conditions. After the incubation, excess inhibitor was removed from the enzyme solutions by gel filtration of 10-ml volumes of the enzyme–inhibitor mixtures through 70-ml columns of Sephadex G-25, employing 0·05 M sodium phosphate buffer, pH 7·2, as the eluent. Control preparations of holoenzyme and apoenzyme were treated in an identical manner, except that inhibitor was omitted during the incubation. To assure that all unbound PCBR was removed by the gel filtration step, aliquots of the inhibitor-treated, gel-filtered enzyme solutions were assayed alone and together with 0·5-ml aliquots of untreated crude histidine decarboxylase. The results of the assays are shown in Table 3. In the cases of both holoenzyme and apoenzyme, combining the inhibitor-treated preparations with untreated crude enzyme resulted in activities equivalent to the sums of the constituent activities, thus excluding the possibility that the inhibitor-treated enzyme preparations retained significant quantities of unbound inhibitor.

Results of histidine decarboxylase assays of the PCBR-treated holoenzyme and apoenzyme and their respective controls are shown in Fig. 3. Activities measured in the absence and presence of 1×10^{-5} M pyridoxal phosphate, as in Fig. 2, serve to indicate the actual holoenzyme and apoenzyme content of the preparations. It is evident that treatment of the holoenzyme with PCBR, followed by removal of the

TABLE 3. ACTIVITIES OF MIXTURES OF 3-*p*-CHLOROBENZYLRHODANINE-INHIBITED AND UNINHIBITED HISTIDINE DECARBOXYLASE

Enzyme additions	Volume (ml)	Net* counts/min	Theoretical† net counts/min
Expt. 1			
Crude enzyme	0·5	600	
Inhibitor-treated holoenzyme	1·0	32	
Crude enzyme +	0·5		
inhibitor-treated holoenzyme	0·5	650	616
Expt. 2			
Crude enzyme	0·5	442	
Inhibitor-treated apoenzyme	1·0	231	
Crude enzyme +	0·5		
inhibitor-treated apoenzyme	0·5	549	558

* Net counts/min values are averages from duplicate incubations minus the radioactivity of blanks prepared as described under Experimental.

† Theoretical net counts/min values are sums of values expected for 0·5-ml aliquots of crude and inhibited enzyme assayed individually.

unbound inhibitor, resulted in an irreversible loss of 98 per cent of the control holoenzyme activity. Identical inhibitor treatment of the apoenzyme preparation, in contrast, resulted in recovery of 79 per cent of the apoenzyme activity of the corresponding control. It may be concluded that inhibition by the PCBR is irreversible, and that it occurs via an interaction between the inhibitor and the holoenzyme form of the decarboxylase.

Kinetic studies of inhibition by 3-p-*chlorobenzylrhodanine.* The effect of L-histidine concentration on inhibition of histidine decarboxylase by PCBR was plotted by the double reciprocal method, yielding the results shown in Fig. 4. In the absence of the

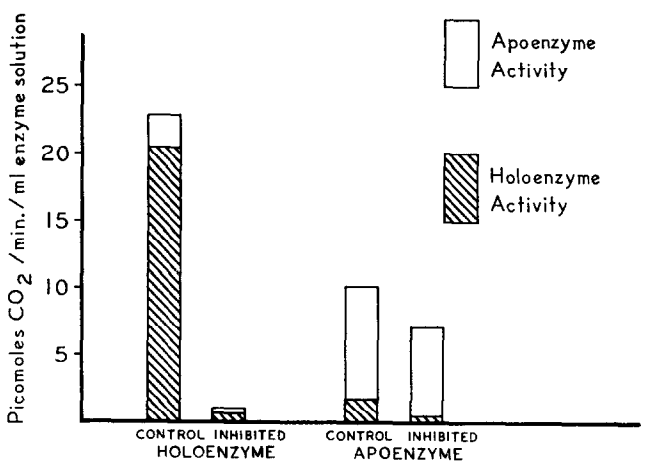

FIG. 3. Histidine decarboxylase activities of control vs. PCBR-inhibited enzyme preparations. Each value represents the average of activities calculated from duplicate incubations. Inhibition was measured by treating holoenzyme- and apoenzyme-enriched preparations with 1×10^{-4} M PCBR, removing the unbound inhibitor (see text), and assaying the PCBR-treated vs. control preparations. The hatched areas indicate activity measured during incubation in the absence of added pyridoxal phosphate (holoenzyme activity); unhatched areas represent incremental activity (apoenzyme) observed in standard incubations containing 1×10^{-5} M pyridoxal phosphate.

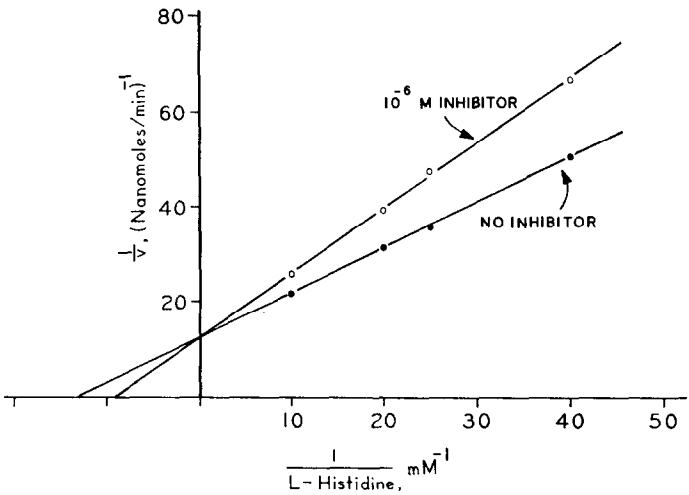

FIG. 4. Lineweaver–Burk plot of inhibition of histidine decarboxylase by PCBR. Each point represents an average activity calculated from duplicate incubations.

inhibitor, a K_m of 8×10^{-5} M was calculated for L-histidine, in approximate agreement with the value of 1×10^{-4} M observed at pH 7·0 for the rat gastric enzyme by Håkanson and Owman.[10] In the presence of 1×10^{-6} M PCBR, the maximum velocity was equivalent to that observed in the absence of inhibitor. In view of the irreversibility of inhibition by PCBR reported above, it is unlikely that the data indicate true competitive inhibition. If the inhibition is indeed irreversible during the 1-hr incubation interval employed in the assay, the results shown in Fig. 4 are probably indicative of binding of L-histidine or of the inhibitor at the same site on the enzyme, although not in the strictly competitive sense represented by reversible interactions of inhibitor and substrate with the binding site.

Our experimental results suggest a mechanism for the inhibition of gastric histidine decarboxylase by rhodanine and its derivatives. Rhodanine is able to undergo a condensation reaction between its 5-methylene carbon and carbonyl groups, particularly those of aromatic aldehydes.[11,12] It may be postulated that the inhibition occurs via such a reaction between rhodanine and enzyme-bound pyridoxal phosphate. This hypothesis is consistent with the lack of inhibitory activity observed with the 5-substituted rhodanines, and accounts for the failure of PCBR to react with the apoenzyme. It will be of interest to examine, in future studies, the activities of the rhodanines *in vivo*, and to compare their inhibitory activities toward other decarboxylating enzymes with those of known decarboxylase inhibitors.

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