RESEARCH PAPERS

THE INHIBITION OF THE L-HISTIDINE DECARBOXYLASES OF GUINEA-PIG KIDNEY AND RAT HEPATOMA

BY B. ROBINSON AND D. M. SHEPHERD

From the Department of Pharmacology and Therapeutics, University of St. Andrews, Queen's College, Dundee

Received October 26, 1961

The preparation of some potential inhibitors of L-histidine decarboxylase is described. These, and certain commercially available compounds, have been compared for their ability to inhibit *in vitro* the histidine decarboxylases of guinea-pig kidney and of the transplantable rat hepatoma (F-Hep). Structure-activity relationships of these inhibitors are discussed.

IN a recent paper (Mackay and Shepherd, 1960) several compounds were shown to be inhibitors of guinea-pig kidney L-histidine decarboxylase (GPHD), and it was suggested that such compounds might provide useful pharmacological tools. Later it was found that the transplantable rat hepatoma, F-Hep, contained an L-histidine decarboxylase (F-HepHD) which differed in its properties from GPHD (Mackay, Riley and Shepherd, 1961). Comparative studies on the inhibition of these two enzymes *in vitro* have now been made using several new inhibitors.

Enzyme Inhibition Studies

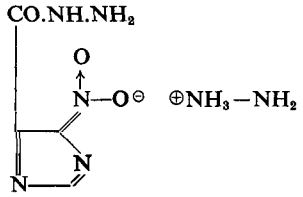
Enzyme activity was determined by assaying the histamine formed, using the isolated ileum of the guinea-pig. The amounts of histamine present initially in the extracts were very small compared with those formed during the incubations. The concentration, C50, of inhibitor required to reduce the initial rate of the uninhibited reaction by half was used as an index of inhibitory potency. The procedure with guinea-pig kidney extracts was as previously described (Mackay and Shepherd, 1960). With F-Hep extracts, incubations were at pH 6·8 and 36°, 1 μg. of pyridoxal-5′-phosphate was added per ml. of extract, and the L-histidine concentration was $6{\cdot}4 \times 10^{-4}$M.

Chemical Studies

While the chemical preparations are described in the experimental section, the properties of 4(5)-nitroimidazole-5(4)-carboxyhydrazide require further comment.

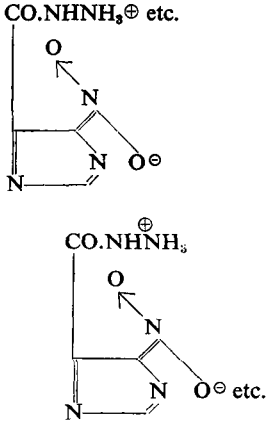
Owing to the influence of the two electron-attracting groups in ethyl 4(5)-nitroimidazole-5(4)-carboxylate, the *N*-hydrogen atom of the imidazole ring is more liable to be lost as a proton than in ethyl imidazole-4(5)-carboxylate, which contains only one electron-attracting group on the ring. Thus although ethyl imidazole-4(5)-carboxylate reacted with

hydrazine hydrate to give the corresponding hydrazide, ethyl 4(5)-nitroimidazole-5(4)-carboxylate and hydrazine hydrate gave the yellow hydrazonium salt, I, of the hydrazide.

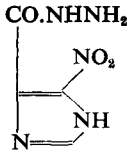


(I)

While this salt crystallised unchanged from concentrated aqueous solution, recrystallisation from dilute aqueous solution gave the free hydrazide as orange needles. The orange colour of this hydrazide in the solid phase and in concentrated aqueous solution is attributed to formation of an intermolecular salt, II, owing to the basic properties of the hydrazide grouping and the strong acidic properties of the imidazole ring containing two electron-attracting substituents. In dilute aqueous solution, however, the hydrazide exists in the unimolecular state, III, as is shown by examination of the ultra-violet spectrum of such solutions.



(II)



(III)

Discussion

The C50 values given below and in Table I have been multiplied by the factor 10^4 to facilitate comparisons. It should be noted that C50 values for GPHD are not directly comparable with those for F-HepHD, as the measurements on the two enzymes are necessarily made at different pH values and substrate concentrations.

It is known (Mackay and Shepherd, 1960) that 4(5)-methyl-5(4)-nitroimidazole (C50 = 34) is a better inhibitor of GPHD than is 4(5)-methylimidazole (C50 = 440). This may be due to the electron-attracting properties of the nitro-group increasing the electrostatic

attraction between the imidazole ring and the apo-enzyme. Compounds 1 and 2 (Table I), which contain electron-attracting groups, were therefore tested as inhibitors. As expected, compound 1 had approximately the same C50 value (C50 = 30) as 4(5)-methyl-5(4)-nitroimidazole on GPHD. Compound 2 (C50 = 65), though containing two electron-attracting groups, was a weaker inhibitor of the enzyme, possibly because in this instance the further increase in electrostatic attraction between the apo-enzyme and the imidazole ring of the inhibitor may be less than the accompanying decrease in hydrogen bonding between these entities. In the inhibition of F-HepHD also, compound 1 was more effective than compound 2.

TABLE I

CONCENTRATIONS OF VARIOUS COMPOUNDS REQUIRED TO PRODUCE 50 PER CENT INHIBITION (C50) OF L-HISTIDINE DECARBOXYLASES

No.	Compound	C50 × 10^4M	
		GPHD	F-HepHD
1	Imidazole-4(5)-carboxylic acid	30	150
2	4(5)-Nitroimidazole-5(4)-carboxylic acid	65	300
3	Imidazole-4(5)-carboxyhydrazide	20	15
4	4(5)-Nitroimidazole-4(5)-carboxyhydrazide	6·5	0·75
5	L-Histidine hydrazide $1\frac{1}{2}H_2SO_4$	0·85	2
6	Hydrazine salt of 4	0·2	0·15
7	Hydrazine hydrate	0·35	0·1
8	DL-5-HTP	0·65	75
9	DL-α-Methyl-5-HTP	0·075	1
10	DL-α-Methylhistidine dihydrochloride	150	15
11	L-DOPA	0·2*	7·5
12	DL-DOPA	0·2	4·5
13	DL-α-Methyl-DOPA	0·01*	70
14	Catechol	1·8*	65
15	Salicylic acid	35	30

* Quoted from the results of Mackay and Shepherd, 1960

The inhibitory effect of a series of hydrazides (compounds 3–6) bearing some structural relation to L-histidine was then studied. The inhibition of GPHD by compound 3 differed only slightly from its inhibition by compound 1, although F-HepHD was more sensitive to compound 3 (C50 = 15) than to compound 1 (C50 = 150). Compound 4 (C50 = 6·5) is more powerful as an inhibitor of GPHD than is compound 1 (C50 = 30), the nitro-group on the ring presumably increasing the interaction between the inhibitor and apo-enzyme; as an inhibitor of F-HepHD compound 4 (C50 = 0·75) is much more powerful than compound 1 (C50 = 150). Compound 5 is a good inhibitor of both enzymes (C50 = 0·85 and 2 for GPHD and F-HepHD respectively). The different effects of these inhibitors on the two enzymes may reflect a greater affinity and specificity of the F-HepHD for the imidazole ring. Support for this view is found in the fact that F-HepHD has no detectable DOPA- or 5-HTP-decarboxylase activity (Mackay, Riley and Shepherd, 1961); GPHD on the other hand is claimed to be non-specific, and to decarboxylate DOPA and 5-HTP more rapidly than L-histidine (Udenfriend, Lovenberg and Weissbach, 1960). The high inhibitory potency of compound 6, as compared with compound 4, against both enzymes, is consistent

with its free hydrazine content since hydrazine hydrate, compound 7, has C50 values similar to those of compound 6, and acts by direct combination with the co-enzyme, pyridoxal 5'-phosphate. Compounds 3–5 may not only react directly with the co-enzyme, but they are also capable of sorption to the apo-enzyme by means of their imidazole nucleus. The relative C50 values for compounds 4, 6 and 7 in both series are in agreement with the structures I and III assigned to compounds 4 and 6 on the basis of chemical evidence.

It has recently been shown (Weissbach, Lovenberg and Udenfriend, 1960) that in their rate of decarboxylation by a guinea-pig kidney preparation and in their ability to inhibit the decarboxylation of natural aromatic amino-acids, the α-methylamino-acids fall in the order α-methylDOPA > α-methyl-5-HTP > α-methyl-TP. The relative rates of decarboxylation of the natural amino-acids DOPA, 5-HTP, TP and histidine by this preparation are in the sequence DOPA > 5-HTP > TP > histidine. In accordance with these observations we have found that α-methylhistidine (compound 10), is a much weaker inhibitor of GPHD than are α-methylDOPA (compound 13) or α-methyl-5-HTP (compound 9).

While DL-α-methylDOPA is a more potent inhibitor of GPHD than is L-DOPA (C50 = 0·01 and 0·2 respectively), their relative inhibitory powers are reversed in the inhibition of F-HepHD (C50 = 50–150 and 6–12 respectively, Mackay and Shepherd, (1962)), DL-α-methylDOPA becoming a rather poor inhibitor; the C50 values of these two compounds with F-HepHD have been redetermined and confirmed. The apparent discrepancy is not due to the use of racemic α-methylDOPA in comparison with the L-isomer of DOPA, since DL-DOPA has similar C50 values to L-DOPA for both enzymes (Table I). No preferential destruction of the DL-α-methylDOPA by other enzymes in the F-HepHD extract was detected by paper chromatography of aliquots taken at various times as the incubation proceeded, and no preferential binding of the DL-α-methylDOPA by foreign protein present in the enzyme extract was found by equilibrium dialysis.

Catechol was a relatively poor inhibitor of F-HepHD (C50 = 65) whereas for GPHD it was very effective (C50 = 1·8, Mackay and Shepherd, 1960). Salicylic acid, however, had comparable C50 values for both enzymes (Table I). This further illustrates the difference between the apo-enzyme moieties of the two enzymes.

The reproducibility of the method for the measurement of C50 values was examined by making several determinations of the C50 values of imidazole-4(5)-carboxyhydrazide for GPHD: five incubations gave 17, 16, 24, 26 and 23, with a standard deviation of 4·5. The C50 values of imidazole-4(5)-carboxylic acid for F-HepHD were, for four incubations 151, 169, 142 and 188 with a standard deviation of 20·4.

EXPERIMENTAL

Ethyl 4(5)-nitroimidazole-5(4)-carboxylate. A solution of 5·1 g. 4(5)-nitroimidazole-5(4)-carboxylic acid (Windaus and Langenbeck, 1923) in 100 ml. dry ethanol, was protected from atmospheric moisture and

saturated with dry hydrogen chloride. After refluxing 1 hr., the solution was re-saturated with dry hydrogen chloride and refluxed a further 2 hr. Evaporation of the ethanol gave a pale-yellow solid which recrystallised from ethanol as white plates (4·88 g., 80 per cent), m.p. 200–203°. A further crop (0·5 g., 8 per cent), m.p. 195–201° was obtained by concentration of the mother liquors. After two recrystallisations from ethanol the ester formed white plates, m.p. 205–207° (Found: C, 38·85; H, 3·7. $C_6H_7N_3O_4$ requires C, 38·9; H, 3·8 per cent) λ_{max} 279–280 mμ (ϵ 4,540, in ethanol), unchanged on addition of hydrochloric acid, but changed on addition of aqueous sodium hydroxide to λ_{max} 345 mμ (ϵ 7850). The infra-red spectrum (in Nujol) showed a strong band at 1720 cm.$^{-1}$ (C = 0).

Reaction of ethyl 4(5)-nitroimidazole-5(4)-carboxylate with hydrazine hydrate. When a solution of the ester (3·0 g.) in 99–100 per cent hydrazine hydrate (10 ml.) was warmed on a steam bath for 2 hr., progressive darkening of the reaction mixture occurred; longer heating led to extensive decomposition. Ethanol (200 ml.) was added to the cooled dark-red solution and, after ½ hr. at room tempeature, the deposit was filtered off and washed with ethanol to give I as yellow crystals (2·9 g., 88 per cent), m.p. 186–188° (with decomposition). Evaporation of the ethanol from the filtrate left a red glass (0·36 g.) which did not crystallise and was not examined further.

Recrystallisation of the yellow solid from water (25 ml.) gave orange needles II (0·60 g., 22 per cent), m.p. 247–249° (with decomposition), unchanged on further recrystallisation from water. (Found: C, 27·75; H, 3·0. $C_4H_5N_5O_3$ requires C, 28·1; H, 3·1 per cent.) λ_{max} 304–305 mμ (ϵ 4890, in water), unchanged on addition of hydrochloric acid, but changed to λ_{max} 354–355 mμ (ϵ 8630) on addition of aqueous sodium hydroxide. The infra-red spectrum (in Nujol) showed strong bands at 1,660 cm.$^{-1}$ and 3,400 cm.$^{-1}$. (C = O and N–H respectively.) Concentrated aqueous solutions of the product were orange, but more dilute solutions were colourless.

Evaporation of the mother liquors from the above recrystallisation to about 6 ml., followed by rapid cooling gave yellow needles, I (1·8 g., 55 per cent), m.p. 188–190° (with decomposition). (Found: C, 23·6; H, 4·55; N, 47·4. $C_4H_9N_7O_3$ requires C, 23·6; H, 4·4; N, 47·4 per cent.) λ_{max} 345 mμ (ϵ 6310, in water), changed to λ_{max} 302–303 mμ (ϵ 5,690) on addition of hydrochloric acid, and to λ_{max} 353–355 mμ (ϵ 8,900) on addition of aqueous sodium hydroxide. The infra-red spectrum (in Nujol) showed medium bands at 1,690 cm.$^{-1}$ and 3,300 cm.$^{-1}$ (C = O and N–H respectively) and was identical with that of the initial total reaction product. Mixed m.p. of the two compounds showed no depression.

A solution of II (121·5 mg.) in 99–100 per cent hydrazine hydrate (2 ml.) was heated on a steam bath for 1 hr., cooled, and treated with ethanol (100 ml.). The yellow crystalline precipitate, when filtered off and washed with ethanol, was identical with I (m.p., mixed m.p., ultraviolet and infra-red spectra).

I recrystallised from a dilute aqueous solution to give orange needles identical with II (m.p., mixed m.p., ultra-violet and infra-red spectra). Concentration of the mother liquors followed by rapid cooling gave yellow needles, which by m.p., mixed m.p., ultra-violet and infra-red spectral comparisons were shown to be unchanged I.

L-*Histidine hydrazide.* Although this preparation has been briefly described elsewhere (Horii, Murakami, Tamura, Uchida, Yamarnura, Miki and Kato, 1956) full details are recorded below as this paper is in Japanese.

To a solution of L-histidine methyl ester dihydrochloride (Fischer and Cone, 1908) (4·5 g.) in warm, dry methanol (40 ml.) was added a solution of sodium (2·0 g.) in dry methanol (35 ml.). The mixture, protected from atmospheric moisture, was stood at room temperature for 1 hr. with occasional shaking; dry ether (200 ml.) was then added. After a further ½ hr. at room temperature with occasional shaking, the sodium chloride was filtered off and washed with dry ether. Removal of the solvent from the combined filtrate gave a pale-yellow oil (3·2 g.) which did not crystallise, but which was free of chloride ions.

A solution of the above oil in 99–100 per cent hydrazine hydrate (8 ml.), after refluxing for 2½ hr., gave on evaporation a gum (3·5 g.) which did not crystallise. To a solution of this gum in 2N-sulphuric acid (20 ml.) ethanol (25–30 ml.) was gradually added, the oil initially precipitated soon crystallising. The white solid (3·95 g.) was broken up, filtered, and washed with ethanol; after drying *in vacuo* over P_2O_5 it had m.p. 236–239° (with slight decomposition). Repetition of this purification procedure gave histidine hydrazide $1\frac{1}{2}H_2SO_4$ as white prisms 3·8 g., 61 per cent), m.p. 238–240° (with slight decomposition) [Horii, Murakami, Tamura, Uchida, Yamamura, Miki and Kato, 1956, give m.p. 240° (with decomposition)]. (Calc. for $C_6H_{11}N_4O\cdot 1\frac{1}{2}H_2SO_4$: C, 22·4; H, 3·9. Found: C, 22·1; H, 3·75 per cent.)

The following compounds were prepared as described in the literature: imidazole-4(5)-carboxylic acid (Pyman, 1916); imidazole-4(5)-carboxyhydrazide (Balaban, 1930); 4(5)-nitroimidazole-5(4)-carboxylic acid (Windaus and Langenbeck, 1923); DL-α-methylhistidine dihydrochloride (Robinson and Shepherd, 1961a).

Acknowledgements. DL-α-Methyl-5-HTP was kindly supplied by the Upjohn Company, Kalamazoo, Michigan, U.S.A. All other compounds used were obtained commercially.

We are grateful to Dr. P. B. Marshall for helpful advice and discussion. One of us (B.R.) acknowledges the award of a Post-doctoral Research Fellowship by the Wellcome Foundation.

REFERENCES

Balaban, I. E. (1930). *J. chem. Soc.*, 268–273.
Fischer, E. and Cone, L. H. (1908). *Liebigs. Ann.*, **363**, 107–117.
Horii, Z., Murakami, Y., Tamura, Y., Uchida, H., Yamamura, Y., Miki, K. and Kato, M. (1956). *J. pharm. Soc. Japan*, **76**, 1319–1321.
Mackay, D., Riley, J. F. and Shepherd, D. M. (1961). *J. Pharm. Pharmacol.*, **13**, 257–261.

Mackay, D. and Shepherd, D. M. (1960). *Brit. J. Pharmacol.*, **15**, 552–556.
Mackay, D. and Shepherd, D. M. (1962). *Biochim. Biophys. Acta*, in the press.
Pyman, F. L. (1916). *J. chem. Soc.*, **109**, 186–202.
Robinson, B. and Shepherd, D. M. (1961a). *J. chem. Soc.*, 5037–5038.
Robinson, B. and Shepherd, D. M. (1961b). *Biochim. Biophys. Acta*, **53**, 431–433.
Udenfriend, S., Lovenberg, W. M. and Weissbach, H. (1960). *Fed. Proc.*, **19**, 7.
Weissbach, H., Lovenberg, W. and Udenfriend, S. (1960). *Biochem. Biophys. Res. Comms.*, **3**, 225–227.
Windaus, A. and Langenbeck, W. (1923). *Chem. Ber.*, **56**, 683–686.