Biochemical Pharmacology, Vol. 18, pp. 2639–2654. Pergamon Press, 1969. Printed in Great Britain

PRODUCTION OF $^{14}CO_2$ FROM HISTIDINE-^{14}COOH BY *N*-(2′-CARBOMETHOXY)CYCLOPENTYLIDENE, CYANOACETHYDRAZIDE AND RAT PYLORIC HISTIDINE DECARBOXYLASE

W. LIPPMANN

Biogenic Amines Laboratory, Ayerst Laboratories, Montreal, Quebec, Canada

(*Received* 14 *January* 1969; *accepted* 11 *April* 1969)

Abstract—Production of $^{14}CO_2$ from DL-histidine-^{14}COOH was observed with both *N*-(2′-carbomethoxy) cyclopentylidene, cyanoacethydrazide (AY-17,224) and a female rat pyloric stomach preparation. Each exhibited an optimum pH of 7·0. The activities of AY-17,224 and the pyloric enzyme were found to be dependent upon the type and concentration of buffer employed. Protein and pyridoxal phosphate caused a decrease in activity of AY-17,224. AY-17,224 neither inhibited nor potentiated the pyloric enzyme. AY-17,224 was partially inhibited whereas the pyloric enzyme was stimulated maximally by pyridoxamine phosphate. α-Methyl-histidine inhibited AY-17,224 but had no effect on the pyloric enzyme, whereas the reverse was observed with KCN. Phenylhydrazine inhibited whereas phenylethylhydrazine stimulated and NSD-1055 had no effect on the activity of AY-17,224; each of these compounds inhibited the pyloric enzyme. The K_m of AY-17,224 with respect to histidine-^{14}COOH was $1{\cdot}35 \times 10^{-4}$M while that of the pyloric enzyme was $1{\cdot}25 \times 10^{-3}$ M.

THE DECARBOXYLATION of L-histidine has been extensively studied both *in vitro* and *in vivo*. It appears that there are at least two different mammalian histidine decarboxylases. One is a nonspecific L-aromatic amino acid decarboxylase (EC 4.1.1.26) (a.a.d.) which acts on histidine as well as on other aromatic amino acids, such as dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (5-HTP).[1,2] This nonspecific enzyme (a.a.d.) is found in large amounts in the kidney of the guinea pig[1–6] and rabbit.[7–9] The other enzyme, the specific histidine decarboxylase (EC 4.1.1.22), is stereospecific for L-histidine and exhibits properties different from those of the a.a.d.[3,5,10] This specific enzyme is found to be concentrated in the fetal rat[9–16] (particularly in the liver[11]), in the rat hepatoma[4,5,17,18] and in the mouse mast cell tumor.[3,9,12] These enzymes from the latter sources appear to be identical.[9,12]

A histidine-decarboxylating system has been shown to be present in the stomach of many species, such as the human, rat, mouse, cat, dog, guinea pig and frog.[19] The nature of this rat gastric enzyme system is not clear, since it has been variously identified as the specific enzyme[20] or as a mixture of both enzymes.[21,22] The localization of this system within the stomach has not yet been conclusively determined; it has been reported to be present exclusively in the pyloric (thick glandular) portion,[21] the specific enzyme to occur in the fundic and the a.a.d. in the pyloric segment,[23] or both enzymes in different types of cells in the pylorus.[21]

In an extensive study, it has been shown that a specific histidine decarboxylase

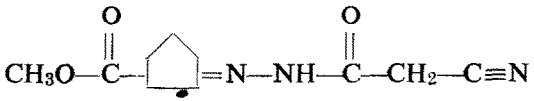
occurs almost exclusively in the enterochromaffin-like cells of the glandular region, with very little activity in the rest of the stomach. The a.a.d. is concentrated in a different type of cell.[24]

A bacterial histidine decarboxylase has been found and extensively studied;[25] it differs from both of the mammalian enzymes in various properties.[26]

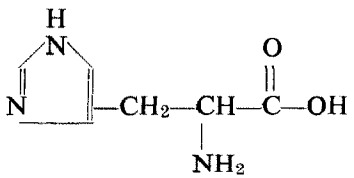
Most mammalian decarboxylases require pyridoxal phosphate (Pyr. P) for activity. The Pyr. P is apparently attached to the protein molecule through the phosphate ester, while the 4-aldehyde is involved in the formation of a Schiff base with the substrate.[27] The specific fetal histidine decarboxylase has been shown to be absolutely dependent on Pyr. P[13,16] and observed inhibitions of the enzyme in some cases might have been due to the combination of the inhibitor with the coenzyme.[15] The effect of Pyr. P on the pyloric enzyme is not clear, since there have been reports of dependence[28] and nondependence[29] on Pyr. P.

This report concerns the finding that AY-17,224 [*N*-(2′-carbomethoxy) cyclopentylidene, cyanoacethydrazide] causes production of $^{14}CO_2$ from histidine-^{14}COOH. The nature of this activity has been investigated in relation to the decarboxylation of histidine by rat pyloric histidine decarboxylase and the results are reported here.

AY-17,224



HISTIDINE



MATERIALS AND METHODS

DL-Histidine-^{14}COOH (10·0 mc/m-mole) was purchased from the California Corp. for Biochemical Research (Calbiochem) as were L-histidine (free base), pyridoxal phosphate and analogues, and streptomycin sulfate. α-Methyl-histidine was from General Biochemicals. NSD-1055 (4-bromo-3-hydroxybenzyloxyamine) was supplied by Smith & Nephew Research Ltd. and phenylethylhydrazine (Nardil) by Warner–Lambert Research Laboratories. Bacterial histidine decarboxylase (*Cl. welchii* NCTC6785) was obtained from Schwartz Bioresearch. AY-17,224 [*N*-(2′-carbomethoxy) cyclopentylidene, cyanoacethydrazide] was synthesized by Dr. R. Laliberté of Ayerst Laboratories.

The animals used were female, Charles River, albino rats, 250–300 g, obtained from Canadian Breeding Laboratories (Laprairie, Que.).

Enzyme preparations. At least six animals were sacrificed by decapitation and the stomachs immediately removed. The stomachs were cut along the lesser curvature and the pyloric (thick glandular) portion was separated from the rest of the stomach,

rinsed in cold water and then finely minced. The tissue was homogenized in 3 vol. of cold buffer [0·2 M sodium–potassium phosphate (Na–K phosphate), pH 7·0] in a Sorvall Omnimixer (15 sec on, 15 sec off, repeated four times). In experiments where more than one buffer was used, the tissues were minced into a homogeneous mixture and then equal aliquots were removed for homogenization in the different buffers. After homogenization, the preparations were centrifuged in a Sorvall centrifuge at 2000 *g* for 30 min. The supernatant fluids were decanted through a piece of gauze, placed in a refrigerator and used within 1 hr.

Histidine decarboxylase assay. Rat pyloric histidine decarboxylase activity was estimated by the measurement of $^{14}CO_2$ evolved from DL-histidine-^{14}COOH. This measurement was originally described by Kobayashi[30] and subsequently modified by others.[14,20] The estimation of the $^{14}CO_2$ evolved was performed according to the method of Lippmann, as described by Buyske.[31] The reaction vessel for incubation studies consisted of a Pyrex glass tube (25 × 150 mm) with a side arm attached 75 mm from the top. A small glass hook (40 mm) was suspended from a rubber stopper. Trichloroacetic acid (0·3 ml of a 50% solution) was placed in the side arm. A cylinder of Whatman No. 3 filter paper (made from a 22 mm-disc) with 0·1 ml of 10 N NaOH absorbed was hung on the glass rod to collect the evolved $^{14}CO_2$ in the closed vessel. The usual incubation mixture consisted of 0·05 ml of 1×10^{-3} M Pyr. P; 1·25 ml of 2×10^{-1} M Na–K phosphate buffer, pH 7·0; and 0·6 ml of enzyme preparation in 2×10^{-1} M Na–K phosphate buffer or 1·70 ml of 2×10^{-1} M Na–K phosphate buffer; and 0·2 ml of AY-17,224 in a total volume of 1·9 ml. This was incubated in a Dubnoff metabolic shaker for 15 min, at which time 0·1 ml of substrate containing 0·4 μc DL-histidine-^{14}COOH and 5×10^{-7} moles of unlabeled L-histidine (in 1×10^{-4} N HCl) was added and the stopper placed firmly in the tube. The incubations were carried out at 37°. Precautions were taken to avoid allowing the filter paper to touch the wall of the vessel during the assay. The usual incubations were of 2-hr duration. The tube was tipped in order to terminate the reaction and the mixture was shaken for an additional 45 min. The filter paper was removed, placed in a scintillation vial containing 10 ml of 0·4% 2,5-diphenyloxazole and 0·005% 1,4-*bis*-(5-phenyloxazol-2-yl)-benzene in toluene, and the activity was determined in a liquid scintillation spectrometer. When denatured enzyme preparations were used, the enzyme was heated 5 min in boiling water previous to addition to the reaction vessel.

In regard to the nature of the volatile compound from the reaction mixture as being $^{14}CO_2$, filter paper cylinders obtained from a typical experiment were eluted with distilled water, and unlabeled sodium carbonate (as carrier) and barium chloride were added to the eluates in order to precipitate the highly insoluble barium carbonate. The precipitates were then transferred to the main compartment of another incubation tube, hydrochloric acid was added and the evolved gas was collected on filter paper cylinders impregnated with sodium hydroxide. The radioactive content of the cylinders was then determined. Quantitative recovery of the original radioactivity was achieved both with known quantities of $Na_2{}^{14}CO_3$ and the radioactive contents of the cylinders from the assay reactions.

All compounds insoluble in distilled water were dissolved in 10% dimethylsulfoxide (DMSO); the final test concentration of DMSO (1%) did not affect the enzyme or AY-17,224 activity. All experiments were performed at least twice.

Bacterial histidine decarboxylase activity was assayed as described by Gale.[32]

The $^{14}CO_2$ measuring system was tested by utilizing the bacterial histidine decarboxylase preparation and was found to be linear up to at least 24,000 cpm (85 μmoles histidine decarboxylated). This activity was much higher (at least four times) than any of the subsequent assays.

RESULTS

The effects of varying the pH on the production of $^{14}CO_2$ from histidine by AY-17,224 and pyloric histidine decarboxylase are shown in Fig. 1. Both AY-17,224 ($2{\cdot}5 \times 10^{-4}$ M) and pyloric histidine decarboxylase exhibited a pH optimum of 7·0 with a secondary peak at pH 7·4, with added 0·05 M sodium–potassium phosphate buffer.

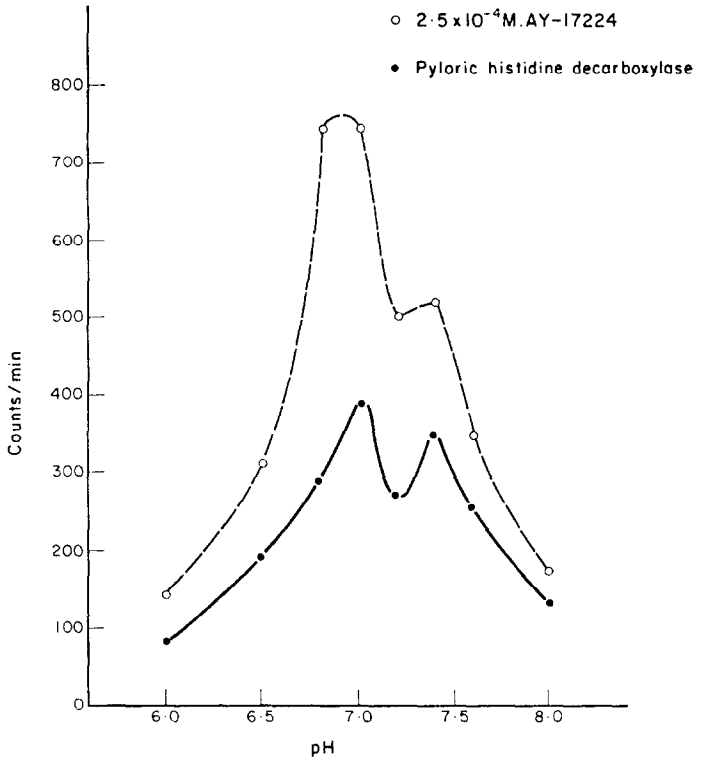


FIG. 1. Effect of pH on the production of $^{14}CO_2$ from histidine-^{14}COOH by AY-17,224 and rat pyloric enzyme. All experiments were carried out with 0·05 M Na–K buffer and performed twice, in duplicate.

The activity of both AY-17,224 and rat pyloric histidine decarboxylase varied with different types of buffers (pH 7·0) (Table 1). AY-17,224 exhibited similar activities with 0·05 M Na–K phosphate buffer and sodium phosphate buffer (Na–Na phosphate), and 0·5 times these activities with Tris (hydroxymethyl)-aminomethane buffer (Tris). Increasing the ionic strength of the added phosphate buffer to 0·2 M caused an increase in activity of 2·8-fold. The maximum increase of 5-fold occurred with potassium arsenate buffer (K-arsenate). With sodium acetate (Na-acetate) and sulfate

(Na-sulfate) buffers, there was reduced activity. No activity was observed with either potassium arsenite (K-arsenite) buffer or sucrose.

The rat pyloric histidine decarboxylase showed activity similar to that observed with AY-17,224 with the 0·05 M Na–K phosphate, Na–Na phosphate, Tris and 0·2 M phosphate buffers. No stimulation was observed with the 0·2 M arsenate buffer and the level with 0·2 M sulfate was similar to that of the 0·05 M phosphate buffers. Activity was observed with the sucrose and this was somewhat higher (1·5 times) than that with the 0·05 M phosphates. No appreciable activity was observed with 0·2 M arsenite.

TABLE 1. EFFECTS OF DIFFERENT BUFFER SYSTEMS ON THE PRODUCTION OF $^{14}CO_2$ FROM HISTIDINE-^{14}COOH

Buffer system*	Activity†	
	AY-17,224‡ (% of control)	Pyloric histidine decarboxylase§ (% of control)
0·05 M Na–K phosphate	100	100
0·05 M Na–Na phosphate	119	100
0·05 M Tris	51	57
0·2 M Na–K phosphate	280	235
0·2 M Na-acetate	85	0
0·2 M K-arsenate	514	92
0·2 M K-arsenite	2	20
0·2 M Na-sulfate	62	114
0·25 M Sucrose	5	144

* All buffers were adjusted to pH 7·0, except sucrose, which was pH 6·8. All experiments were performed twice, in duplicate.

† All results are corrected for background activity in the absence of AY-17,224 (or histidine decarboxylase) utilizing the appropriate buffer. All activities are compared to the 0·05 M Na–K phosphate as 100 per cent, which was assayed simultaneously with each of the other buffers.

‡ AY-17,224 = 5×10^{-4} M; control activity = 700 cpm.

§ Control activity of histidine decarboxylase = 200 cpm.

The observation that a higher buffer concentration yielded increased production of $^{14}CO_2$ with both AY-17,224 and the pyloric histidine decarboxylase was examined further. With each, a maximum activity occurred with 0·2 M, with higher concentrations of the added buffer causing a reduction in activity (Fig. 2). At each of the various buffer concentrations employed, the pH remained constant throughout the experiment (pH 7·0). When the final ionic strength of the solution was increased from 0·05 to 0·2 M (with NaCl) there was no change in activity, whereas above 0·2 M there was a decrease in activity.

At the higher buffer concentrations, increasing the amount of trichloroacetic acid (TCA) added to the incubation mixture did not increase the amount of $^{14}CO_2$ released, thus demonstrating that sufficient TCA was being utilized under these conditions.

Under conditions with the 0·2 M Na–K phosphate, pH 7·0 and 37°, the rates of production of $^{14}CO_2$ from histidine-^{14}COOH by both AY-17,224 and the rat pyloric preparation were linear over a period of 2·5 hr (Fig. 3). Thus, subsequent incubations were carried out for 2 hr.

The linearity of the system with increasing concentrations of AY-17,224 (at least

1–5 $\times$ 10^{-4} M) is demonstrated in Fig. 4. The system was also linear with increasing concentrations (at least 4–20 mg protein) of pyloric enzyme. The $^{14}CO_2$ production from histidine-^{14}COOH by AY-17,224 (5 $\times$ 10^{-4} M) did not occur when the pyloric enzyme was present in the incubation mixture; with increasing levels of pyloric enzyme (4–20 mg) the activities observed in the presence and absence of AY-17,224 were similar. Furthermore, 4 mg of heat-denatured enzyme also inhibited the activity

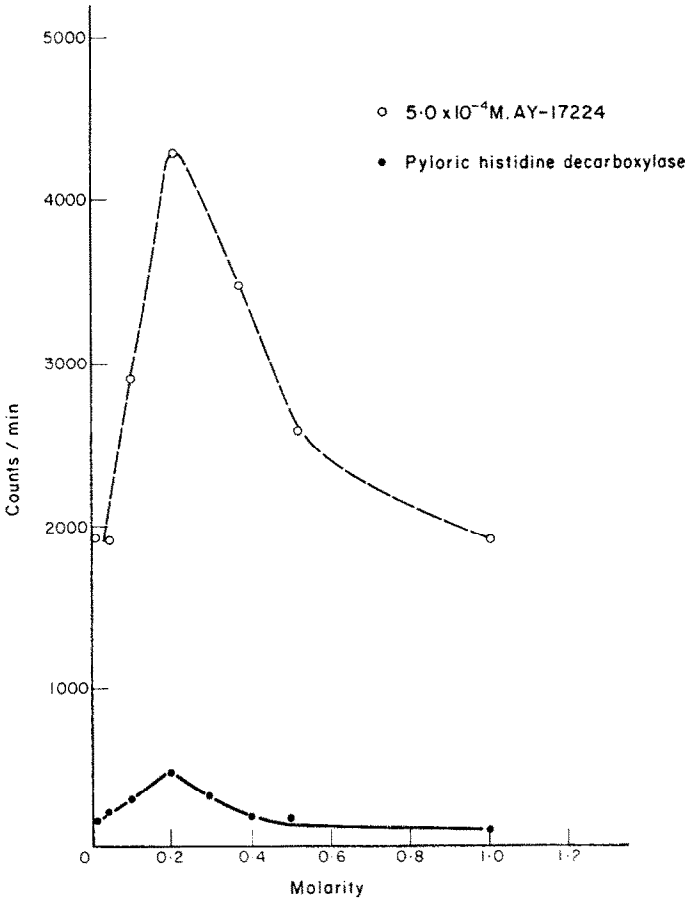


FIG. 2. Effect of phosphate buffer concentration on the production of $^{14}CO_2$ from histidine-^{14}COOH. The molarity indicated is the concentration of the buffer added to the incubation mixture and the molarity was increased by increasing the strength of the Na–K phosphate buffer. All points were determined at least twice and in duplicate.

of AY-17,224. Another protein, bovine serum albumin (BSA), also caused inhibition of the production of $^{14}CO_2$ from histidine-^{14}COOH by AY-17,224 (Fig. 5). There was a rapid decline in the level of $^{14}CO_2$ produced in the presence of increasing amounts of BSA and at 1 mg there was a decrease of one-half. The level of activity continued to decrease with higher levels of BSA, although the rate of decrease was not as great.

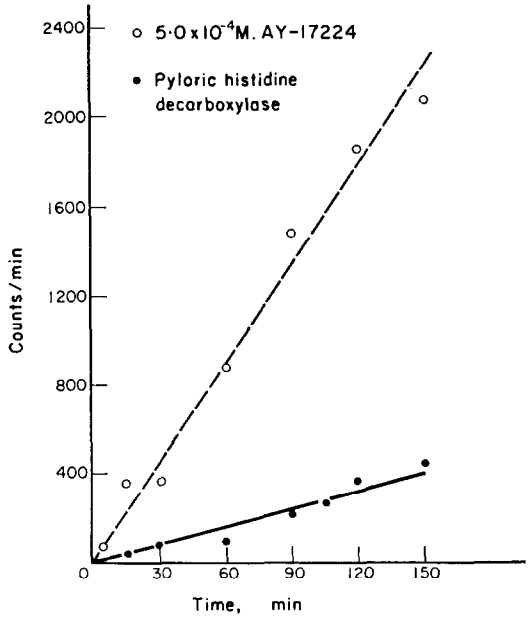


FIG. 3. Effect of incubation time on AY-17,224 and pyloric histidine decarboxylase activity. All experiments were performed with 0·2 M Na–K buffer (pH 7·0) and each point is the average of two duplicates.

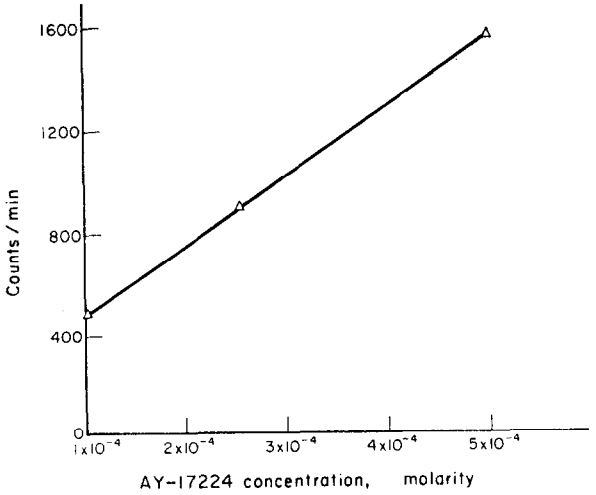


FIG. 4. Effect of AY-17,224 concentrations on the production of $^{14}CO_2$ from histidine-^{14}COOH. All points were determined (at least twice) with 0·2 M Na–K buffer (pH 7·0).

At 5 mg, the remaining AY-17,224 activity had declined to about one-fourth the original.

Various agents and assay conditions were examined for their effects on the production of $^{14}CO_2$ from histidine-^{14}COOH by AY-17,224 (5×10^{-4} M) and the rat pyloric stomach preparation (Table 2). No histidine decarboxylase activity was found in the fundic (rumen) portion of the stomach of fed animals. The pyloric enzyme preparations from rats fasted for 24 hr before sacrifice were greatly decreased in

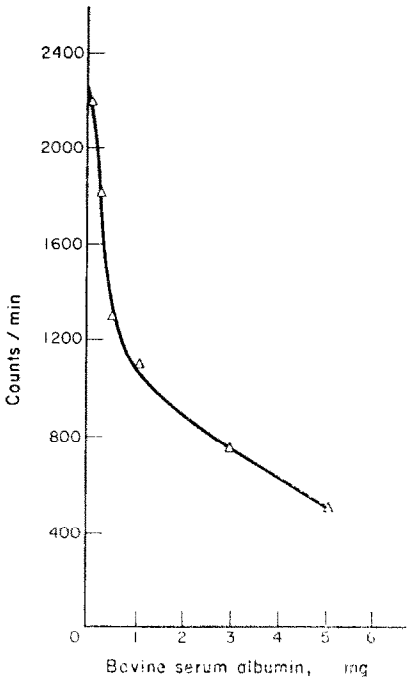


FIG. 5. Inhibition of AY-17,224 $^{14}CO_2$ production from histidine-^{14}COOH by bovine serum albumin. The concentration of AY-17,224 was 5×10^{-4} M. Each point was performed twice, in duplicate.

activity. Organic solvents such as DMSO (1%) or benzene (20 mg) had no effect on AY-17,224 activity; benzene only slightly inhibited (25 per cent) the enzyme activity. Streptomycin sulfate (1×10^{-4} M), an inhibitor of possible bacterial contamination,[14,30] had no effect on either preparation. Lowering the reaction to 20° caused large decreases in both AY-17,224 (67 per cent) and rat pyloric enzyme (79 per cent) activities. α-Methyl-histidine ($1{\cdot}25 \times 10^{-3}$ M) diminished the AY-17,224 activity greatly (73 per cent), but did not cause any appreciable change in the enzyme activity. With $2{\cdot}5 \times 10^{-4}$ M α-methyl-histidine the activity with AY-17,224 declined 44 per cent. Histamine (5×10^{-4} M) also caused an inhibition of AY-17,224 activity (46 per cent). Potassium cyanide ($2{\cdot}5 \times 10^{-3}$ M) exhibited only a slight inhibition (14 per cent) of AY-17,224, whereas it inhibited the rat pyloric enzyme completely. NSD-1055 at 1×10^{-4} M inhibited the AY-17,224 activity (35 per cent), whereas at 1×10^{-5} M no inhibition was observed; the rat pyloric enzyme was completely inhibited at both concentrations. Phenylhydrazine (1×10^{-4} M) decreased the

AY-17,224 activity (55 per cent) and caused complete loss of enzymatic activity. Phenylethylhydrazine stimulated AY-17,224 production of $^{14}CO_2$ from histidine-^{14}COOH to 173 per cent but inhibited (85 per cent) the enzyme activity. Pyr. P (1×10^{-5} M) caused a slight inhibition (15 per cent) of AY-17,224 activity (5×10^{-4} M). (At the lower concentrations of AY-17,224 of $2{\cdot}5 \times 10^{-4}$ M and 5×10^{-5} M the levels of inhibition by Pyr. P were increased to 37 and 57 per cent respectively.) The activity of the enzyme was increased only slightly (15 per cent) by the addition

TABLE 2. EFFECT OF VARIOUS AGENTS AND ASSAY CONDITIONS ON THE PRODUCTION OF $^{14}CO_2$ FROM HISTIDINE-^{14}COOH BY AY-17,224 AND PYLORIC HISTIDINE DECARBOXYLASE

Conditions*	AY-17,224† (% of control)	Pyloric histidine decarboxylase‡ (% of control)
0·2 M Na–K phosphate, pH 7·0		
+Pyr.P, 10^{-5} M	85	100
−Pyr.P, 10^{-5} M	100	85
Fundic enzyme preparation		0
24 hr fasting		38
1% DMSO	100	100
20 mg Benzene	100	75
0·25 M Sucrose		
+Pyr.P, 10^{-5} M	2	61
−Pyr.P, 10^{-5} M	0	24
20°	33	21
α-Me-histidine,		
$1{\cdot}25 \times 10^{-3}$ M	27	90
$2{\cdot}5 \times 10^{-4}$ M	56	92
Histamine, 5×10^{-4} M	54	116
KCN, $2{\cdot}5 \times 10^{-3}$ M	86	10
NSD-1055,		
10^{-4} M	65	10
10^{-5} M	100	10
Phenylhydrazine, 10^{-4} M	45	0
Phenylethylhydrazine, 10^{-4}M	173	15

* All experiments were performed at least twice with 0·2 M phosphate buffer, pH 7·0, except for the experiment performed in 0·25 M sucrose.
† AY-17,224 = 5×10^{-4} M; control activity = 1900 cpm.
‡ Control activity = 500 cpm.

of Pyr. P (1×10^{-5}M). In sucrose, the addition of Pyr. P caused a stimulation of the enzyme preparation activity. AY-17,224 was inactive in sucrose medium and the addition of Pyr. P had no effect.

Increasing the concentration of Pyr. P in the presence of AY-17,224 (5×10^{-4} M) caused a decline in the production of $^{14}CO_2$ (Fig. 6). At 2×10^{-4} M Pyr. P, the level of activity decreased 58 per cent and was completely lost at 5×10^{-4} M. By utilizing the method of Dixon,[33] the K_i of Pyr. P for AY-17,224 with respect to histidine was found to be $2{\cdot}75 \times 10^{-5}$ M.

The effects of various analogues of Pyr. P at 5×10^{-4} M on the production of $^{14}CO_2$ from histidine-^{14}COOH by AY-17,224 (5×10^{-4} M) and the pyloric enzyme were determined and are shown in Table 3. Pyr. P was the only compound which greatly inhibited (100 per cent) the activity of AY-17,224. Only slight inhibitions in activity were observed with pyridoxal (28 per cent), pyridoxine phosphate (16 per

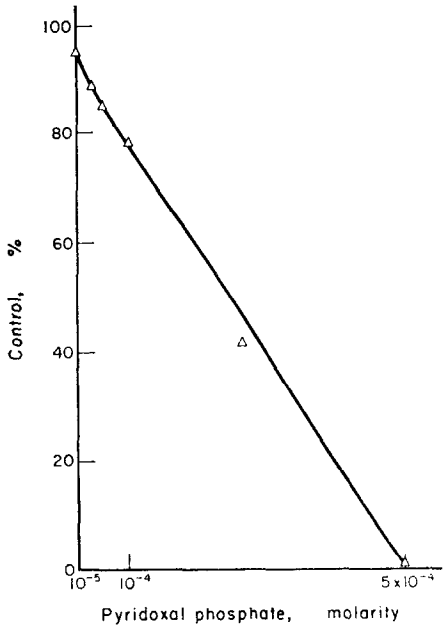


FIG. 6. Inhibition of AY-17,224 production of $^{14}CO_2$ from histidine-^{14}COOH by pyridoxal phosphate. The concentration of AY-17,224 was 5×10^{-4} M. Each point was repeated twice, in duplicate.

TABLE 3. EFFECT OF PYRIDOXAL PHOSPHATE ANALOGUES ON AY-17,224 AND PYLORIC HISTIDINE DECARBOXYLASE ACTIVITIES

Compound added*	AY-17,224† (% of control)	Pyloric histidine decarboxylase‡ (% of control)
Control (no Pyr.P)	100	100
Pyridoxal phosphate, 5×10^{-5} M	85	118
5×10^{-4} M	0	69
Pyridoxal, 5×10^{-4} M	72	59
Pyridoxine phosphate, 5×10^{-4} M	84	72
Pyridoxine, 5×10^{-4} M	78	107
Pyridoxamine phosphate, 5×10^{-4} M	65	168
Pyridoxamine, 5×10^{-4} M	70	94

* All experiments were performed twice, in duplicate.
† AY-17,224 = 5×10^{-4} M; control activity = 2650 cpm.
‡ Control activity = 525 cpm.

cent), pyridoxine (22 per cent), pyridoxamine phosphate (35 per cent) or pyridoxamine (30 per cent). At the same concentration, the activity of the pyloric enzyme was inhibited by pyridoxal phosphate (31 per cent), pyridoxal (41 per cent) and pyridoxine phosphate (28 per cent). Neither pyridoxamine nor pyridoxine caused any appreciable change. Pyridoxamine phosphate stimulated (68 per cent) the enzymatic activity.

The K_m values for the production of $^{14}CO_2$ were obtained from Lineweaver–Burk plots[33] (Figs. 7 and 8). The K_m for AY-17,224 was found to be $1{\cdot}35 \times 10^{-4}$ M and that for the pyloric enzyme was $1{\cdot}25 \times 10^{-3}$ M.

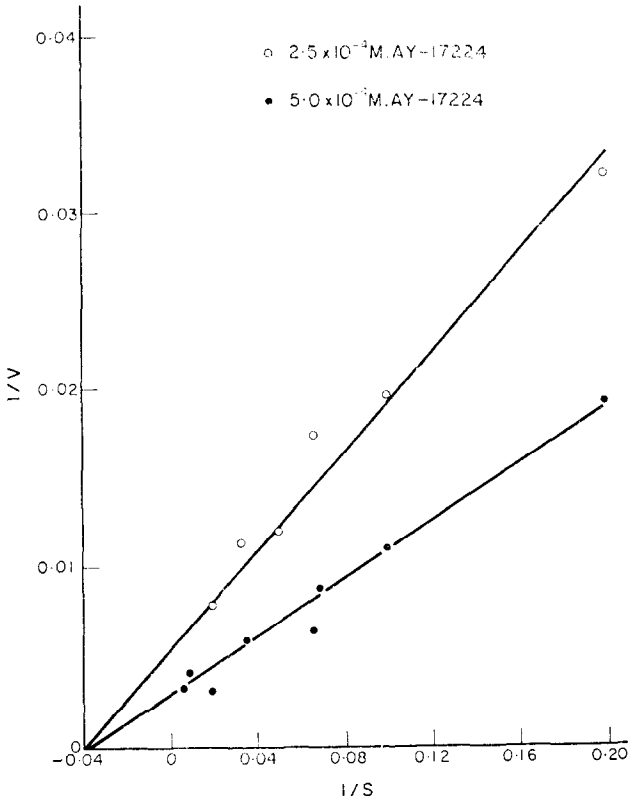


FIG. 7. Lineweaver–Burk plot for K_m AY-17,224 with respect to histidine-^{14}COOH. Each point was determined twice, in duplicate.

DISCUSSION

AY-17,224 has been shown to cause the production of $^{14}CO_2$ from histidine-^{14}COOH *in vitro* and the nature of the activity was determined in relation to the decarboxylation of histidine by the histidine decarboxylase in the female rat pyloric stomach.

It is of interest that from the present studies there appear to be two pH peaks (7·0

and 7·4) for the production of $^{14}CO_2$ from histidine-^{14}COOH with AY-17,224 or the pyloric histidine decarboxylase. With respect to the enzymatic activity, the observation that there is a pH optimum of 7·0 is in accord with the findings of others using similar enzyme preparations.[8,14,29,34] A pH optimum of 7·5 has been reported for an enzymatic preparation consisting of the pyloric region of the rat stomach, with another larger peak occurring at a lower pH in the fundic section.[23,35] The significance of the secondary pH peak with AY-17,224 is unclear, but may be associated with the

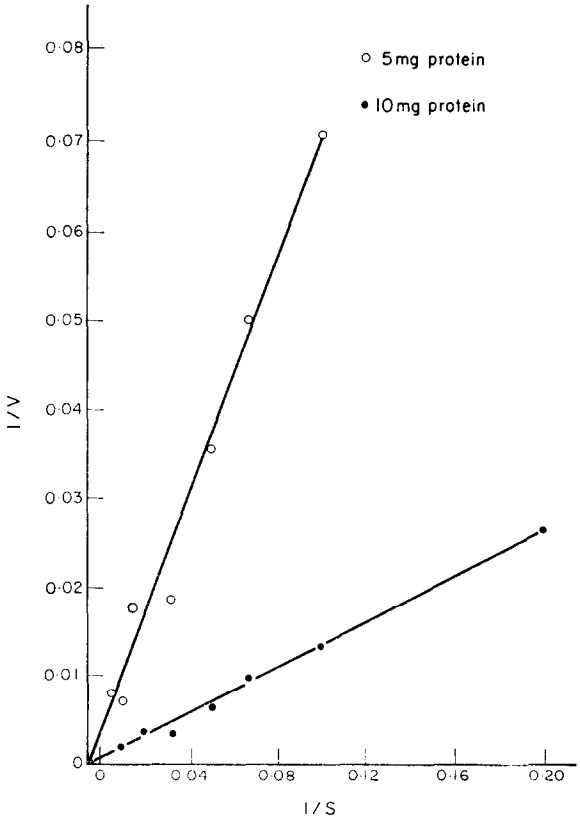


FIG. 8. Lineweaver–Burk plot for K_m rat pyloric enzyme with respect to histidine-^{14}COOH. Each point was performed twice, in duplicate.

fact that histidine undergoes changes in its ionic form as the pH is altered.[13] In relation to this, it has been reported that the pH optimum of the fetal enzyme is dependent upon the substrate concentration;[13,21] however, others did not observe such a relationship when the pH optimum was determined for the gastric histidine decarboxylase.[34]

The type of buffer employed in the activity measurements is more critical with respect to AY-17,224 than the enzyme. AY-17,224 appears to require a trivalent anion for maximum activity, with arsenate being the most effective examined. In another trivalent ion buffer, phosphate, the activity is about one-half that of arsenate

but about four times that of the monovalent acetate and the divalent sulfate. In the divalent arsenite and in sucrose, which is not highly ionized, no activity is observed. In contrast, the enzyme exhibits the high activity in phosphate, sucrose, sulfate and arsenate, with the difference being a maximum of 2-fold between the phosphate and the arsenate; slight activity in arsenite and no activity in acetate is observed. With respect to the ionic strength, there is a maximum concentration which is of importance since, as with phosphate, both AY-17,224 and the rat pyloric enzyme show a 2·5-fold increase in activity when the ionic strength of the added buffer is increased from 0·05 to 0·2 M; higher concentrations cause a decline in activity from the maximum. Also, at high levels of ionic strength, increased by the addition of sodium chloride, the level of AY-17,224 activity is decreased.

Production of $^{14}CO_2$ from histidine-^{14}COOH by AY-17,224 is inhibited by the presence of protein and this inhibition is of a nonspecific nature in relation to the type of protein. This is indicated by the finding that addition of the pyloric enzyme, serum albumin or even heat-denatured pyloric enzyme preparation prevented the activity of AY-17,224. There are multiple active sites in the AY-17,224 molecule available for potential combination with a protein, and attachment to the protein could cause a loss in the activity of AY-17,224. With respect to the pyloric enzyme, the $^{14}CO_2$-forming activity is unchanged by the addition of AY-17,224, suggesting that the latter does not attach to the active site of the enzyme or in any manner that alters the protein molecule in such a way as to cause an inhibition of activity.

In the comparative studies with the pyloric enzyme preparation, it was observed that the fundic (thin-walled) portion of the stomach of the female Charles River rat does not exhibit any appreciable histidine decarboxylase activity. Other investigators have reported similar findings, i.e. the histidine decarboxylase activity of the rat stomach is concentrated in the pyloric (thick glandular) portion,[21,24,29,36–41] although the terminology used by others may differ.[24] However, it has also been reported that the histidine decarboxylase activity is concentrated in different regions of the stomach, such as the fundus,[35] rumen,[34] the acid-secreting cells in the glandular area[19] or the enterochromaffin-like cells[24] (the latter two regions are contained within the preparation presently used). Some of these discrepancies may be due to differences in sex (e.g. males used instead of females[34,35]), differences in enzyme preparation (pH, buffer, mucosa) or differences in designation as to the different parts of the stomach.[19,24]

In the present studies the preparations from rats which had been fasted for 24 hr were of low activity; this has also been reported by others.[19,23,34,37] A second histidine-decarboxylating system has been reported to be present in the rat stomach and is unaffected by starvation.[19,23]

Neither the activity of AY-17,224 nor that of the pyloric enzyme was affected by 1% DMSO. Also, benzene did not have any effect on the activity of AY-17,224. Benzene and other organic solvents have been reported to stimulate some histidine decarboxylase preparations [rabbit kidney[7,8] (a.a.d.), rat kidney and duodenum[29] (a.a.d.), adult rat liver[29,38] or rat pyloric enzyme[23]]; to have no effect (pyloric stomach,[29] fetal liver[37] or fundic stomach[23]); or to inhibit strongly (glandular rat stomach[8]). In the present studies benzene slightly inhibited the rat pyloric enzyme (25 per cent). Streptomycin sulfate[39] and other antibacterial agents[40] have been shown to prevent activity from bacterial contamination of the histidine decarboxylase *in vitro*. Under the conditions used in the present studies, streptomycin sulfate (10^{-4} M)

had no effect on the production of $^{14}CO_2$ from histidine-^{14}COOH by either AY-17,224 or the female rat pyloric histidine decarboxylase.

AY-17,224 and the pyloric enzyme were similarly affected by a lowering of the assay temperature, since the activities of both were decreased.

α-Methyl-histidine caused inhibition of the activity of AY-17,224; in contrast, even at a higher concentration α-methyl-histidine did not inhibit the pyloric histidine decarboxylase. α-Methyl-histidine has been shown to inhibit various types of mammalian histidine decarboxylase *in vitro* (female rat lung,[42] female rat gastric mucosa,[36] male rat pyloric enzyme[23]) and *in vivo*,[43,44] and not to affect others (rat fundic enzyme[23]). A low concentration of histamine (with respect to substrate concentration) caused an inhibition of AY-17,224 activity. In contrast, the pyloric enzyme was not inhibited by histamine; this is in agreement with the findings *in vitro* of other investigators with both the pyloric enzyme[15] and the fetal enzyme.[14] It has been reported that in large doses histamine will inhibit the rat pyloric enzyme; the gastric mucosal histidine decarboxylase can apparently be inhibited by histamine *in vivo*.[19,24]

At the lower concentration employed (10^{-5} M), NSD-1055 was not inhibitory and exhibited only partial inhibition of the activity of AY-17,224 at the higher concentration (10^{-4} M). A complete inhibition of the pyloric enzyme by NSD-1055 (10^{-5} M) was observed in the present studies and this is in accord with the results of others [pyloric rat stomach,[14] rat mast cell enzyme[45] (specific) and fetal enzyme[14,46] (specific)]. KCN did not show any appreciable inhibition of AY-17,224 activity even though it was utilized at a higher concentration ($2{\cdot}5 \times 10^{-3}$ M) than the other compounds; however, it caused inhibition of the pyloric enzyme. KCN has also been reported to inhibit the specific rat mast cell histidine decarboxylase.[45] Phenylhydrazine partially inhibited, whereas phenylethylhydrazine caused a stimulation of AY-17,224 activity; both compounds, however, inhibited the rat pyloric histidine decarboxylase. Hydrazines have also been shown to cause inhibition of other histidine decarboxylases [rabbit kidney[7] (a.a.d.), guinea pig kidney (a.a.d.) and rat hepatoma[5] (specific)]. The nature of the differences in the effects of the hydrazines on the AY-17,224 activity is being further investigated.

Although well established for the specific rat fetal histidine decarboxylase,[16,45] the requirement of Pyr. P for the activity of the gastric histidine decarboxylase is still in question.[14,15,29] In the present studies, at a level of 10^{-5} M Pyr. P, only a very slight stimulation of gastric histidine decarboxylase was observed. Using a female rat pyloric stomach preparation, Telford and West[29] also observed that there was little or no stimulation of activity upon addition of Pyr. P. In contrast to the effect on the pyloric enzyme, Pyr. P caused a slight inhibition of AY-17,224 activity. At the high concentration of 5×10^{-4} M, both AY-17,224 and the rat pyloric enzyme are inhibited. With respect to this inhibition, since it has been shown that Pyr. P can interact with histidine[27] in addition to interaction with the enzyme,[16] it is possible that the histidine becomes unavailable for decarboxylation activity. In the case of AY-17,224 this inhibitory action by Pyr. P appears to be rather specific, since various other pyridoxal analogues did not exhibit a large inhibition. However, with the rat pyloric enzyme this specificity did not exist, as similar decreases were caused by other analogues. The activities of AY-17,224 and the pyloric enzyme differ with respect to pyridoxamine phosphate in that this analogue caused a slight inhibition of AY-17,224 but caused a stimulation of the pyloric enzyme.

In the present studies the K_m of AY-17,224 for histidine was found to be $1{\cdot}35 \times 10^{-4}$ M, whereas that of the pyloric histidine decarboxylase was found to be $1{\cdot}25 \times 10^{-3}$ M, thus indicating that the AY-17,224 has a higher affinity for histidine.

Utilizing the observation that AY-17,224 causes a production of $^{14}CO_2$ from histidine-^{14}COOH, it has been found that quantitative determination of graded amounts of unlabeled histidine can be accomplished because the amount of $^{14}CO_2$ produced is inversely proportional to the unlabeled histidine in the test mixture.[47]

Acknowledgements—The author acknowledges the technical assistance of Mr. K. G. Lloyd, Mrs. I. Marotta and Mrs. S. Schaal.

REFERENCES

1. C. T. Clark, H. Weissbach and S. Udenfriend, *J. biol. Chem.* **210**, 139 (1954).¶
2. W. Lovenberg, H. Weissbach and S. Udenfriend, *J. biol. Chem.* **237**, 89 (1962).
3. H. Weissbach, W. Lovenberg and S. Udenfriend, *Biochim. biophys. Acta* **50**, 177 (1961).
4. D. Mackay, J. F. Riley and D. M. Shepherd, *J. Pharm. Pharmac.* **13**, 257 (1961).
5. B. Robinson and D. M. Shepherd, *J. Pharm. Pharmac.* **14**, 9 (1962).
6. W. Dawson, D. V. Maudsley and G. B. West, *J. Physiol., Lond.* **181**, 801 (1965).
7. N. G. Waton, *Br. J. Pharmac. Chemother.* **11**, 119 (1956).
8. R. W. Schayer, *Am. J. Physiol.* **189**, 533 (1957).
9. P. O. Ganrot, A. M. Rosengren and E. Rosengren, *Experientia* **17**, 263 (1961).
10. R. J. Levine, T. L. Sato and A. Sjoerdsma, *Biochem. Pharmac.* **14**, 139 (1965).
11. G. Kahlson, E. Rosengren and T. White, *J. Physiol., Lond.* **151**, 131 (1960).
12. A. Burkhalter, *Biochem. Pharmac.* **11**, 315 (1962).
13. R. Hakanson, *Biochem. Pharmac.* **12**, 1289 (1963).
14. R. J. Levine and D. E. Watts, *Biochem. Pharmac.* **15**, 841 (1966).
15. R. J. Levine and D. E. Watts, *Biochem. Pharmac.* **16**, 993 (1967).
16. R. Hakanson, *Eur. J. Pharmac.* **1**, 383 (1967).
17. G. Kahlson, E. Rosengren and T. White, *J. Physiol., Lond.* **145**, 30P (1959).
18. D. Mackay, P. B. Marshall and J. F. Riley, *J. Physiol., Lond.* **153**, 31P (1960).
19. G. Kahlson, E. Rosengren, D. Svahn and R. T. Thunberg, *J. Physiol., Lond.* **174**, 400 (1964).
20. S. Snyder and P. Epps, *Molec. Pharmac.* **4**, 187 (1968).
21. R. Hakanson and C. Owman, *Biochem. Pharmac.* **15**, 489 (1966).
22. R. Hakanson, *Nature, Lond.* **208**, 793 (1965).
23. A. G. Radwan and G. B. West, *J. Pharm. Pharmac.* **19**, 305 (1967).
24. D. Aures, R. Hakanson and A. Schauer, *Eur. J. Pharmac.* **3**, 217 (1968).
25. J. Rosenthaler, B. M. Guirard, G. W. Chang and E. E. Snell, *Proc. natn. Acad. Sci. U.S.A.* **54**, 152 (1965).
26. A. M. Rothschild and R. W. Schayer, *Biochim. biophys. Acta* **34**, 392 (1959).
27. D. E. Metzler, M. Ikawa and E. E. Snell, *J. Am. chem. Soc.* **76**, 748 (1954).
28. I. E. Skidmore and M. W. Whitehouse, *Biochem. Pharmac.* **15**, 1965 (1966).
29. I. M. Telford and G. B. West, *J. Pharm. Pharmac.* **13**, 175 (1961).
30. Y. Kobayashi, *Analyt. Biochem.* **5**, 284 (1963).
31. D. A. Buyske, in *Isotopes and Experimental Pharmacology* (Ed. J. L. Roth), p. 275. Univ. Chicago Press, Chicago (1965).
32. E. F. Gale, in *Methods of Enzyme Analysis* (Ed. H. U. Bergmeyer), p. 373. Academic Press, New York (1965).
33. M. Dixon, *Biochem. J.* **55**, 170 (1953).
34. P. E. Powell and M. A. Kumar, *Analyt. Biochem.* **22**, 485 (1968).
35. D. V. Maudsley, A. G. Radwan and G. B. West, *Br. J. Pharmac. Chemother.* **31**, 313 (1967).
36. G. Kahlson, E. Rosengren and R. Thunberg, *Am. J. Physiol.* **169**, 467 (1963).
37. A. Castellucci, *Experientia* **21**, 219 (1965).
38. I. M. Telford and G. B. West, *J. Physiol., Lond.* **157**, 306 (1961).

39. B. A. CALLINGHAM, Y. KOBAYASHI, D. V. MAUDSLEY and G. B. WEST, *J. Physiol., Lond.* **179**, 44P (1965).
40. A. G. RADWAN and G. B. WEST, *Br. J. Pharmac. Chemother.* **33**, 177 (1968).
41. R. W. SCHAYER, *Am. J. Physiol.* **187**, 63 (1956).
42. B. GRAHN and E. ROSENGREN, *Br. J. Pharmac. Chemother.* **33**, 472 (1968).
43. G. KAHLSON, E. ROSENGREN and S. E. SVENSSON, *Nature, Lond.* **194**, 876 (1962).
44. A. G. RADWAN and G. B. WEST, *Br. J. Pharmac. Chemother.* **30**, 392 (1967).
45. M. CABUT and D. HAEGERMARK, *Acta physiol. scand.* **73**, 62 (1968).
46. F. J. LEINWIEBER, *Molec. Pharmac.* **4**, 337 (1968).
47. W. LIPPMANN and K. LLOYD, in preparation.