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ADRENALINE INDUCED ACCELERATION OF HISTAMINE FORMATION *IN VITRO*, STUDIED BY TWO ISOTOPIC METHODS

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In a previous study it was observed that administration of adrenaline elevated the histamine forming capacity (HFC) in various tissues including the lung *in vivo* in mice but not in rats (Graham, Kahlson & Rosengren, 1964). Attempts to enhance histamine formation *in vitro* by pharmacological means have so far been unsuccessful (Schayer, 1960; Kahlson, Rosengren & Thunberg, unpublished data quoted by Rosengren, 1966). The present work was undertaken to investigate the effect of adrenaline on the HFC of lung tissue *in vitro*. In this study two different isotopic methods for the determination of tissue HFC have been used; one measures net formation of histamine, and the other determines total formation of $^{14}CO_2$ from ^{14}C-carboxyl labelled histidine. Rationally, the present report consists essentially of two parts, the first dealing with the significance and limitations of the two mehods used, and the second showing elevation of minced mouse lung HFC *in vitro* provoked by adrenaline.

METHODS

Animals

Adult female mice of 25 g (Naval Medical Research Institute strain) and rats of 250 g (Sprague-Dawley strain) were used. The animals were killed by cervical fracture, followed by bleeding from the blood vessels in the neck. The lungs were then excised and pooled samples consisting of tissue from eight mice or three rats were used.

Incubation

The samples were cooled in an ice-bath and minced with scissors. Mincing yielded about 1 mm^3 pieces of the tissue. About 200 mg of minced tissue was transferred to individual incubation vessels and preincubated in 2.7 ml. of sodium phosphate buffer, pH 7.4, containing glucose. The phosphate buffer was isotonic and contained 0.25 mmoles of sodium phosphate and 0.018 mmoles of glucose in 2.7 ml. In experiments designed to show the effect of adrenaline, this compound was added in a final concentration of 5×10^{-4}M. In control experiments 0.9% NaCl was substituted for adrenaline. The samples were preincubated with air as the gas phase for 1 hr at 37° C, unless otherwise stated, in a metabolic shaker before addition of substrate. The addition of substrate increased the final volume to 3.0 ml.

Determination of the formation of 2-ring-^{14}C-labelled histamine (pipsyl method)

After preincubation, aminoguanidine sulphate (10^{-4}M final concentration), pyridoxal-5-phosphate (10^{-5}M final concentration) and 2-ring-^{14}C-labelled-L-histidine (0.8×10^{-4}M final concentration, specific activity 4.9 mc/mmole) were added to the tissue, and incubation continued in nitrogen at 37° C

for 3 hr. Aminoguanidine and nitrogen were used to prevent the catabolism of formed labelled histamine by oxidizing enzymes. The reaction was terminated by adding 66.2 mg of non-radioactive histamine dihydrochloride (equivalent to 40.0 mg of the base) as carrier and perchloric acid in a final concentration of 0.4M. The isotopic dilution technique described by Schayer, as adapted for use in our laboratory (Kahlson, Rosengren & Thunberg, 1963), was used to determine the amount of labelled histamine formed.

Determination of the formation of $^{14}CO_2$ from ^{14}C-carboxyl labelled L-*histidine*

The procedure employed was essentially a combination of the original method described by Kobayashi (1963) and its modification by Levine & Watts (1966). The incubation vessel consisted of a roundbottomed Pyrex test-tube, 125 mm long and with an outer diameter of 25 mm, to which a small side-arm was joined. In principle, this tube resembles a Warburg flask, which, however, was found to be unsuitable for trapping $^{14}CO_2$. One millilitre of 2.0M citric acid was placed in the side-arm. The incubation mixture was added to the main tube and preincubated as described. After preincubation ^{14}C-carboxyl labelled L-histidine (10^{-4}M final concentration, specific activity 1.0 mc/mmole) and pyridoxal-5-phosphate (10^{-5}M final concentration) were added to the tissue. The tubes were closed with a rubber stopper carrying a tiny stainless steel spring to which a 10×25 mm strip of No. 005 Munktells filter paper to which 0.1 mmole of Hyamine 10-X had previously been applied (Rapkin, 1961) was attached. The incubation was carried out in a metabolic shaker for 3 hr at 37° C with air as the gas phase. In the CO_2-method the catabolic fate of the histamine formed is irrelevant, and for this reason the use of nitrogen as gas phase is not required. The incubation was terminated by carefully tipping the citric acid into the incubation mixture. Complete absorption of CO_2 was achieved by continued shaking for 45 min. The filter paper trap was then placed in a counting vessel, 10 ml. of scintillation fluid was added (Bray, 1960), and radioactivity was determined in a liquid scintillation spectrometer.

Blank tests

Carboxyl labelled ^{14}C-histidine as well as 2-ring-labelled ^{14}C-histidine were used in blank tests to measure the non-enzymic formation of $^{14}CO_2$ and ^{14}C-histamine. These blanks were treated in the same way as the samples used for the enzymic assays, but differed in so far as they did not contain any tissue. Values for enzymic formation of histamine were calculated from the difference between readings obtained with samples containing tissue and readings with appropriate blank controls. The effect of adrenaline and sodium ascorbate on the non-enzymic formation of the labelled products was also tested. In samples without any tissue, with adrenaline added, the non-enzymic formation of $^{14}CO_2$ from carboxyl labelled ^{14}C-histidine was considerably greater than the enzymic formation of $^{14}CO_2$ in tissue samples containing adrenaline; this amine is presumably catabolized during 1 hr preincubation in tissue samples. This non-enzymic formation of $^{14}CO_2$ necessitated the omission of adrenaline in control blanks. Thus values for enzymic formation of $^{14}CO_2$ in experiments involving adrenaline are not "fully" corrected because the appropriate blanks did not contain adrenaline.

Chemicals used

L-Adrenaline (Fluka AG); L-ascorbic acid sodium salt (Sigma); hydroxide of Hyamine 10-X, *p*-(diisobutylcresoxyethyl) dimethylbenzylammonium hydroxide (Packard); α-methyl-histidine (Merck, Rahway); α-methyl-DOPA, α-methyl-3,4-dihydroxy-L-phenylalanine (Merck, Rahway); L-histidine-carboxyl-^{14}C (New England Nuclear); L-histidine-ring-2-^{14}C (Radiochemical Centre, Amersham). The commercially obtained ^{14}C-labelled histidines were purified before use by ion exchange chromatography as described by Kahlson, Rosengren & Thunberg (1963).

RESULTS

Part I

Comparison of results obtained by the two methods

Individual samples of minced lungs were investigated concurrently by the pipsyl method and the $^{14}CO_2$ method. The results of these experiments are summarized in Table 1 which

shows that the results obtained by the two methods differ. First, in samples of minced mouse lung, the amount of histamine seemingly formed was larger with the $^{14}CO_2$ method than with the pipsyl method and this discrepancy cannot be due entirely to the difference in substrate concentration. Discrepancies were also found with minced rat lung. Furthermore, adrenaline accelerated the formation of histamine in minced mouse lung as judged by the pipsyl method, whereas with the $^{14}CO_2$ method adrenaline seemed to depress histamine formation in minced mouse and rat lungs. Finally, in blanks containing adrenaline but no tissue, substantial amounts of $^{14}CO_2$ were evolved. This latter phenomenon called for further analysis.

Regarding results presented in Table 1 it should be noted that the ^{14}C-histidine concentration used for the $^{14}CO_2$ method was 20% higher than used for the pipsyl method. This difference is, obviously, irrelevant to the conclusions proper, and is due to the circumstance that determinations with the two methods were begun as rather independent studies.

TABLE 1

SEEMINGLY FORMED HISTAMINE (ng/g) IN MINCED MOUSE AND RAT LUNGS DETERMINED BY TWO METHODS

Histidine concentrations were $0{\cdot}8\times10^{-4}$M (pipsyl method) and $1{\cdot}0\times10^{-4}$M ($^{14}CO_2$-method), and complete conversion to histamine would give 26·7 μg and 33·3 μg of histamine, respectively. In each experiment the figures represent the mean of two determinations on the same pooled tissue sample, corrected for non-enzymic formation of the recorded product except that attributable to adrenaline in the $^{14}CO_2$-method. C, Control; A, adrenaline, 5×10^{-4}M, added.

	Pipsyl method		$^{14}CO_2$ method	
	C	A	C	A
Mouse lung				
Expt. 1	100	160	170	130
2	120	200	210	190
3	70	110	120	120
Rat lung				
Expt. 1	160	160	130	110
2	160	150	140	120
3	150	150	190	180

Formation of $^{14}CO_2$ from ^{14}C-carboxyl labelled histidine in reagent blanks

Under the conditions described, the formation of $^{14}CO_2$ from ^{14}C-carboxyl labelled histidine in reagent blanks (no tissue added) was usually less than 0.02%—that is, equivalent to 70 c.p.m., if they did not contain adrenaline. Addition of adrenaline to blanks, however, increased the amount of $^{14}CO_2$ released. For this reason reagent blanks with adrenaline in final concentrations of 10^{-5}–10^{-3}M were prepared and the yields of $^{14}CO_2$ and ^{14}C-ring labelled histamine formed from the two differently labelled histidines were determined with the $^{14}CO_2$ and pipsyl method, respectively. The formation of $^{14}CO_2$ from carboxyl labelled histidine was found to be directly proportional to the concentration of adrenaline (Table 2 and Fig. 1). Substituting for adrenaline another reducing agent, sodium ascorbate, increased also the $^{14}CO_2$ formation from labelled histidine. To give the same effect, ascorbate was needed in a lower concentration than adrenaline (Fig. 1). On the other hand, none of these compounds was found to cause the formation of significant amounts of ^{14}C-ring labelled histamine from ^{14}C-ring labelled histidine on incubation in identical conditions.

TABLE 2

YIELDS IN TERMS OF % $^{14}CO_2$ AND ^{14}C-RING LABELLED HISTAMINE FORMED FROM THE CORRESPONDING LABELLED HISTIDINES WHEN REDUCING AGENTS ARE PRESENT DURING INCUBATION

Incubations were carried out in Tris-HCl buffer and the gas phase was air in both types of experiment. All figures represent means of duplicates. A 100% yield would have been equal to 33·3 μg histamine base in the $^{14}CO_2$ method and 26·7 μg histamine base in the pipsyl method.

		Yield (%) of labelled reaction product		
		Concentration of agent added (M)		
Agent added	Reaction product	10^{-5}	10^{-4}	10^{-3}
L-Adrenaline	$^{14}CO_2$	0·0223	0·130	0·74
	^{14}C-histamine	0·0085	0·0185	0·0069
Sodium L-ascorbate	$^{14}CO_2$	0·051	0·48	3·03
	^{14}C-histamine	0·0062	0·0108	0·0077

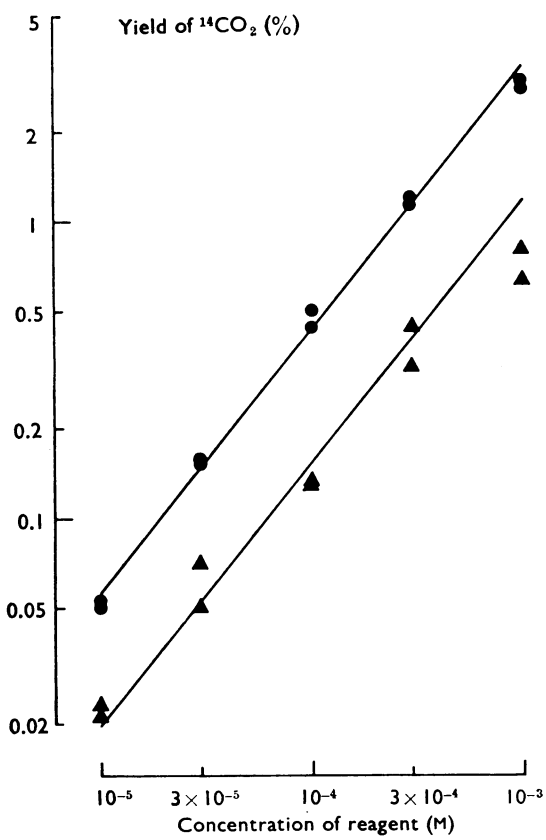

Fig. 1. Effect of increasing concentrations of L-adrenaline (▲——▲) and sodium L-ascorbate (●——●) on the formation of $^{14}CO_2$ from ^{14}C-carboxyl labelled L-histidine. A yield of 100% $^{14}CO_2$ would have been equal to 3.7×10^5 c.p.m. Incubation for 3 hr in Tris-HCl buffer, pH 7.4. Each point represents one individual test.

The optimum pH for the decarboxylation by sodium ascorbate was about 7.5 (Fig. 2) and in phosphate buffer the release of $^{14}CO_2$ by sodium ascorbate was about 8 times higher than in tris-HCl (Tris-[hydroxymethyl]-amino methane) buffer of the same pH and concentration. The recovery of ^{14}C-histamine was unaffected by buffer, pH, or both.

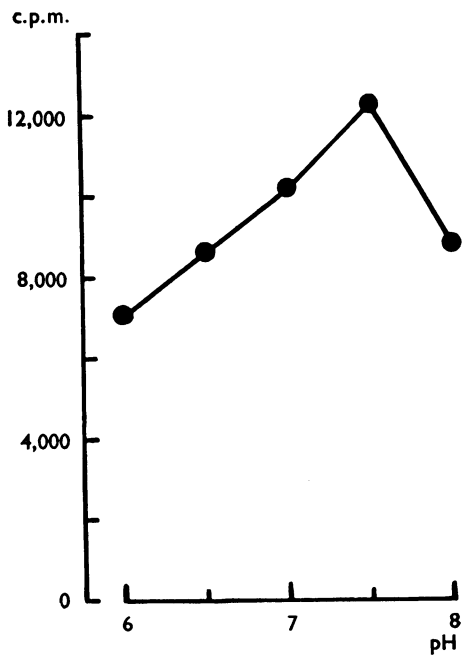

FIG. 2. Relation between pH and formation of $^{14}CO_2$ from ^{14}C-carboxyl labelled L-histidine under the influence of sodium L-ascorbate in a final concentration of 10^{-4}M; phosphate buffer, final concentration 0.08M; 3 hr incubation. Each point represents the mean of two or three determinations.

Part II

All experiments reported in this section were done using the pipsyl method.

Elevation of mouse lung HFC in vitro *by adrenaline*

The HFC of minced mouse and rat lungs after addition of adrenaline in a final concentration of 5×10^{-4}M *in vitro* was studied in nine experiments. The results are given in Table 3. Adrenaline elevated the HFC of minced mouse lungs in all the experiments; the mean increase was 75% but in some experiments the elevation was 100%. In order to see whether histidine decarboxylase activity was lost during the preincubation for 1 hr before adding histidine to the tissue, aliquots were incubated at 4° and 37° C. After adding histidine, incubations were always carried out at 37° C. It was found that activity was not lost during preincubation at 37° C because rather more histamine was formed when the preincubation was done at 37° C than at the lower temperature, irrespective of whether adrenaline was added or not (Table 3).

Samples of rat lungs studied under the same conditions as samples of mouse lungs did not show significant changes in HFC. Adrenaline did not seem to influence HFC in minced rat lungs *in vitro* and the dependence on temperature noted with samples of mouse lungs did not apply to samples of rat lungs.

Obviously, increased amounts of ^{14}C-histamine were formed after addition of adrenaline to minced mouse lungs *in vitro*. The inhibitory actions on this tissue of α-methyl-histidine and α-methyl-DOPA were therefore determined to assess the extent to which histidine decarboxylase was involved. α-Methyl-histidine inhibits histidine decarboxylase competitively while α-methyl-DOPA inhibits a non-specific enzyme, usually referred to as an

aromatic L-amino acid decarboxylase and with a low affinity for histidine. The concentration of the inhibitors in the incubation mixture was 5×10^{-4}M, which in the case of α-methyl-histidine is known to inhibit the formation of histamine in rat gastric mucosa by 50% (Kahlson, Rosengren & Thunberg, 1963). Pertinent results are given in Table 4. It can be seen that α-methyl-histidine inhibited the formation of histamine to almost the same degree whether adrenaline was present or not, whereas the action of α-methyl-DOPA was insignificant.

TABLE 3

HFC OF MINCED MOUSE AND RAT LUNGS AFTER ADDITION OF ADRENALINE, 5×10^{-4}M, *IN VITRO*

Pyridoxal-5-phosphate (final concentration 10^{-5}M) was added only in experiments 7, 8 and 9. In this table all figures of HFC of rat lungs are means of duplicates, as are those of mouse lung except for experiments 1, 7 and 8 which represent one determination only.

Expt.	Temperature at preincubation (°C)	Mouse Histamine formed (ng/g)		Rat Histamine formed (ng/g)	
		Control	Adrenaline	Control	Adrenaline
1	4	80	130	130	130
	37	100	170	120	130
2	4	—	—	170	190
	37	70	90	180	180
3	4	30	40	220	—
	37	40	60	240	220
4	4	40	60	90	80
	37	60	90	90	70
5	4	—	—	160	160
	37	50	140	160	180
6	4	—	—	120	100
	37	50	110	90	100
7	4	130	240	240	—
	37	150	220	210	230
8	4	90	170	—	—
	37	140	200	140	150
9	4	—	—	260	270
	37	—	—	240	240

TABLE 4

EFFECT OF α-METHYL HISTIDINE, 5×10^{-4}M, AND α-METHYL DOPA, 5×10^{-4}M, ON HFC (ng/g) OF MINCED MOUSE LUNG

The figures are means of duplicates (except for α-methyl DOPA in experiment 2, representing one determination only). Adrenaline was used in a concentration of 5×10^{-4}M.

	No inhibitor	α-methyl histidine	α-methyl DOPA
Expt. 1			
Control	140	60	—
Adrenaline	220	100	—
Expt. 2			
Control	110	40	90
Adrenaline	140	50	150
Expt. 3			
Control	130	30	90
Adrenaline	180	60	150

DISCUSSION

The pipsyl method used in this investigation for determining histamine formation from 2-ring-^{14}C-labelled-L-histidine, has a very high specificity and sensitivity (for references, see Kahlson & Rosengren, 1968). It has been used for many years in our laboratory, but is

not ideal because it is laborious and part of the histamine formed during incubation may be metabolized by inactivating enzymes. A simpler procedure for measuring histidine decarboxylase could be based on determining $^{14}CO_2$ formation, as described by Kobayashi (1963). Aures & Clark (1964) used this method in studies of inhibitors of histidine decarboxylase and report, that with some exceptions, there is a correspondence between CO_2 evolved and histamine formed. Smith & Code (1967) feel confident with the $^{14}CO_2$ method, and acclaim it as superior to other methods because it is believed to eliminate a potential error alleged to apply to methods based on the estimating of histamine formed by histidine decarboxylase. Maudsley, Radwan & West (1967) found results obtained with the pipsyl and $^{14}CO_2$ methods comparable, but observed that when histidine decarboxylase activity was low, estimations with the $^{14}CO_2$ method were not possible.

The present finding that in reagent blanks histidine emitted a substantial amount of CO_2 in the presence of adrenaline or sodium ascorbate call for cautiousness in the use of this method for assays of histamine formation. These reactions may also occur when tissue is present. The usefulness of the $^{14}CO_2$ method is gravely limited by our finding that histidine gives off CO_2 from the carboxyl group without concomitant formation of histamine. The reaction mechanism behind this phenomenon is unknown. It seems likely that the first reaction steps produce derivatives of histidine, leaving the carboxyl group unaffected, and subsequent steps may cause the release of CO_2. The phenomenon cannot be explained by the findings of Marasas (1961) who observed that several imidazole acids, as well as histamine, were formed during incubation of histidine with silicate dust possessing oxidizing or hydroxylating properties.

The present study shows that histamine formation can be enhanced *in vitro* by adrenaline in minced mouse lungs, but not in minced rat lungs. This is in agreement with findings *in vivo* (Graham, Kahlson & Rosengren, 1964). The possibility that the enhanced formation does not result from increased histidine decarboxylase activity cannot be excluded because in the presence of adrenaline newly formed histamine may be catabolized to a lesser extent. The evidence, however, points towards the observed changes being the result of a real increase in histamine formation. First, adrenaline added to mouse liver, which is known to be rich in enzymes which methylate and oxidize histamine, did not increase the amount of histamine formed on incubation with histidine *in vitro* (unpublished observations). Second, histamine formation in minced rat lungs was not increased by adrenaline. Third, the relative increase was the same after preincubation at 4° and 37° ; the same applies to the inhibition of HFC by α-methyl histidine in the presence and absence of adrenaline.

The observed temperature dependence of reactions during preincubation remains obscure. Levine & Watts (1966) presented data suggesting the presence of an unidentified soluble inhibitor of histidine decarboxylase in gastric tissue. The question arises whether this supposed inhibitor is destroyed by preincubation at 37° C.

SUMMARY

1. Histidine decarboxylase activity—that is, rate of histamine formation—has been determined in mouse and rat lung *in vitro* with two isotopic methods.

2. Reducing agents, such as adrenaline and sodium ascorbate, accelerate the non-enzymic formation of $^{14}CO_2$ from ^{14}C-carboxyl labelled histidine. This reaction can take place without a concurrent formation of histamine.

3. The radioactive CO_2 method has great limitations and may not be trustworthy for the purpose of measuring the rate of histamine formation, particularly in tissues with low histidine decarboxylase activity.

4. Adrenaline enhances histamine formation *in vitro* in minced mouse lungs but not in minced rat lungs.

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