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EVIDENCE FOR RAPID HISTAMINE TURNOVER AND LOSS OF HISTAMINE FROM IMMATURE RAT MAST CELLS

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Abstract—Histamine synthetic activity which is high in young mast cells decreases as the cells mature [Beaven *et al., J. Pharmac. exp. Ther.* **224**, 620 (1983)]. In this study we show that a substantial proportion of newly formed histamine in young mast cells leaked to the extracellular environment. The cells acquired the full ability to sequester newly formed histamine once the numbers of intracellular granules and the supply of sulfated mucopolysaccharide material within them had increased. Rat peritoneal mast cells were separated into successive fractions of increasing size and maturity by counter current elutriation. Loss of histamine from fractions of immature cells was demonstrated by a progressive accumulation of histamine in the medium without any decrease in intracellular histamine content. The estimated turnover time of histamine was less than 10 hr. In fractions of more mature cells, the proportion of cellular histamine released into the medium was substantially lower, giving estimated turnover times of 20 hr or longer. Studies with radiolabeled histidine also indicated that little, if any, newly formed histamine was lost from fractions of mature cells. Both release of endogenous histamine and formation of radiolabeled histamine from labeled histidine were inhibited by the histidine decarboxylase inhibitor α-fluoromethylhistidine (10 μM). Histamine turnover times were similar in the presence or absence of external histidine, a possible indication that the supply of intracellular histidine was sufficient to maintain normal histamine synthetic activity.

Over twenty-five years ago, Schayer first reported that intact rat peritoneal mast cells synthesized histamine, although he noted that histamine synthetic activity was substantially reduced upon cell disruption [1]. The soluble cell extract was found to contain a pyridoxal phosphate-dependent histidine decarboxylase activity [2]. Others identified a histidine decarboxylase activity (EC 4.1.1.22) that was highly specific for histidine (K_m 0.2 to 0.4 mM) in a mouse transplantable mast cell tumor [3] and mouse mastocytoma [4]. Intact mast cells [5–9], blood basophils [10, 11], and cultured rat leukemic basophils [12] have since been shown to possess the ability to take up and decarboxylate radiolabeled histidine and to incorporate the newly synthesized histamine into intracellular granules [5, 7, 10, 12]. Recently we have shown that histamine synthetic activity is high in immature mast cells and that this activity declines as the mast cells mature [13]. Histamine content, on the other hand, is low in immature cells and increases as the cells mature. We now report that a substantial portion of the histamine is not retained by immature mast cells but is lost to the extracellular space.

MATERIALS AND METHODS

Materials. Compound 48/80 (Lot No. 46664) was a gift from Burroughs Wellcome Research Laboratories, Tuckahoe, NY. α-Fluoromethylhistidine was supplied to us by Dr. Kollonitsch, Merck Sharp & Dohme Laboratories, Rahway, NJ. A stock solution of 10^{-2} M was prepared in 0.01 N HCl and was diluted to required concentration with the incubation medium. L-[*carboxyl*-^{14}C]Histidine was purchased from the New England Nuclear Corp., Boston, MA, and L-[*ring*-2-^{14}C]histidine from Amersham-Searle, Arlington Heights, IL. Other reagents were obtained from sources noted previously [13].

Collection and histochemical examination of cells. Peritoneal cells (~150 × 10^6 cells) were collected from male Sprague–Dawley (6–8 weeks of age) rats and were separated into twelve fractions of 100 ml by counter current separation in a Beckman elutriator as described earlier [13]. With this procedure mast cells were separated into successive fractions of increasing cell size and maturity (in Fractions 5 through 12). Most monocytes were recovered in Fractions 3 and 4, although they do elutriate along with immature mast cells in Fractions 5 and 6 [13].

Cell numbers and size were determined by the use of a Celloscope counter (Particle Data Inc., Elkhart, IN). Ethidium bromide/fluorescein diacetate dyes were used to estimate cell viability [14], and Wright's stain was used to differentiate mast cells from other cell types [13]. The degree of sulfation of mucopolysaccharide in mast cell granules was assessed by Alcian blue/safranin dyes [15–17]. Destruction of granule structure, which was a problem in our earlier studies [13], was avoided by changing the protocol for fixation of cells. Slides were prepared from each of the elutriated fractions by cytocentrifuge and air dried as described previously [13]. The slides were fixed in Carnoy's fixative for 30 min and then rehydrated by quick immersion in 100, 95 and then 70% ethanol. They were stained by immersion in 0.5% solution of Alcian blue in 0.25 mM sodium phosphate buffer, pH 1.0. They were rinsed in the same buffer (without Alcian blue) and then stained for 1 min with

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a 0.5% Safranin solution in 70% ethanol. The slides were dehydrated by successive immersion in 70, 95 and 100% ethanol. The slides were cleared with xylene and then mounted in Permount.

Measurement of formation of $^{14}CO_2$ and radiolabeled histamine from [^{14}C]carboxyl and ring labeled histidine. Cells were recovered from the elutriated fractions by centrifugation (200 *g* for 10 min) and were washed once with a modified Hanks' medium which contained *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, 10 mM) and bovine serum albumin (0.1%). The cells were resuspended in the same medium to give a suspension of 1.25×10^6 cells in each ml. Aliquots of 20 μl (to measure $^{14}CO_2$ release) or 60 μl (to measure intracellular [^{14}C]histidine or [^{14}C]histamine) were dispensed into 1.5-ml polypropylene tubes. To each of these tubes was added an equal volume (i.e. 20 or 60 μl) of reagent that consisted of L-[*carboxyl*-^{14}C] histidine and L-[*ring*-2-^{14}C] histidine (0.5 μCi of each/1 ml; sp. act. 55 mCi/mmole). This reagent was prepared in modified Hanks' medium containing 10 μM pyridoxal phosphate and/or α-fluoromethylhistidine where needed. For control (blank) determinations medium was substituted for the cell suspension. In all experiments, assays were performed in triplicate.

For measurement of $^{14}CO_2$ release, the tubes were placed in glass counting vials that contained 20 μl of hyamine hydroxide to trap $^{14}CO_2$. The vials were capped and then incubated in a water bath (37°) for 60 min. Trapped $^{14}CO_2$ was assayed as described previously [9].

For assays of intracellular radioactive compounds, 60 μl of the cell/reagent suspension was layered on top of 250 μl of a mixture of dioctyl- and dibutylphthalate (47.5:52.5, v/v) in small (400 μl) polypropylene tubes. The cells were deposited through the organic phase by centrifuging the tubes for 90 sec in a Beckman Microfuge. The liquid phase was removed by aspiration, and the cell pellets were recovered by cutting off the centrifuge tube tips with a razor blade. The tips were placed in either scintillation mixture to assay total radioactivity or 100 μl of 0.01 N HCl to extract soluble radioactive compounds. The HCl extracts were lyophilized and reconstituted in 35 μl of 0.01 N HCl before application to thin-layer cellulose powder plates. Radioactive histidine and histamine were separated by chromatography in a butanol–ethanol–ammonia solvent system (80:10:30, by vol.) and assayed as reported previously [18].

The above procedures were used for the study of histamine synthesis in mature mast cells. Such studies were not performed with immature mast cells in Fractions 5 and 6 because of the predominance (up to 90%) of monocytes in these fractions [13].

Preliminary experiments in which fraction 9 cells were incubated (60 min) with L-[*carboxyl*-^{14}C]histidine indicated that the percentages of label recovered as $^{14}CO_2$, intracellular histidine and histamine were, respectively, 0.37 ± 0.06, 0.14 ± 0.05 and 0 (mean value ± S.E.M., five experiments). Based on these results the assumption was made that, in the presence of both *ring-* and *carboxyl*-labeled histidines the intracellular [^{14}C]-labeled histamine was derived exclusively from the L-[*ring*-2-^{14}C]histidine. [^{3}H]-Labeled histidine was not employed because of problems with [^{3}H]-exchange reactions. We found that up to 30% of the [^{3}H]-label was lost from the available commercial preparations of [^{3}H]-labeled histidine when these were used in the procedures described above.

Histamine release in different fractions of elutriated cells. Spontaneous histamine release was determined by incubating (37°) suspensions of cells (0.2×10^6 cells/0.5 ml) in modified Hanks' medium (with 10 mM HEPES and 0.1% bovine serum albumin). Where noted, the histidine decarboxylase inhibitor, α-fluoromethylhistidine, or L-histidine was added. At 0, 1, 2 and 3 hr, 100 μl of cell suspension was removed from each tube for centrifugation (200 *g* for 10 min). The supernatant and pellet fractions were collected. The pellets were resuspended in 100 μl of the Hanks' medium and were disrupted by sonification. Histamine in both fractions was assayed in 10-μl aliquots by the single step radioenzymatic assay [13] or in samples with low histamine content by the more sensitive assay described in Ref. 13. Cell viability was assayed by ethidium bromide/fluorescein diacetate stains described above.

RESULTS

Morphology of mast cells in various elutriated fractions. Rat peritoneal mast cells consist of subpopulations of cells at different stages of maturity. These subpopulations can be categorized into four stages, viz. I, II, III and IV by their reaction to different histochemical dyes [15–17] (Fig. 1).

The distribution of the different subpopulations of cells in the various elutriated fractions was similar to that reported previously [13], i.e. immature Stage I cells were predominant (55–75% of mast cells) in Fractions 5 and 6, partially differentiated Stage II and III cells constituted 50–60% of the cells in Fractions 7–9 and mature Stage IV cells were predominant (45–55%) in Fractions 10–11. In the latter fractions, greater than 80% of the cells were Stage III and IV cells. The presence of sulfated constituents in the intracellular granules was generally apparent by Stage II of growth, and the extent of sulfation increased as cells matured through Stage III and IV of growth (Fig. 1). Although it was impossible to count the number of intracellular granules, visual inspection of cell photomicrographs indicated that the number of granules increased markedly upon cell maturation (Fig. 1).

Spontaneous histamine release and turnover upon incubation of different fractions of elutriated cells. With all fractions tested (5–11), the amount of histamine present in the medium steadily increased over the course of 3 hr whereas the amount of intracellular histamine remained unchanged (representative experiments are shown in Fig. 2). The rates of histamine release in all fractions varied from 0.17 to 0.42 pg/mast cell/hr with the highest values for fractions of immature cells (5 and 6) and fractions of large cells (10 and 11) which also contain degenerating mast cells [13]. The proportion of histamine released, however, was greater from immature cells (Fractions 4–7) than from mature cells (Fractions

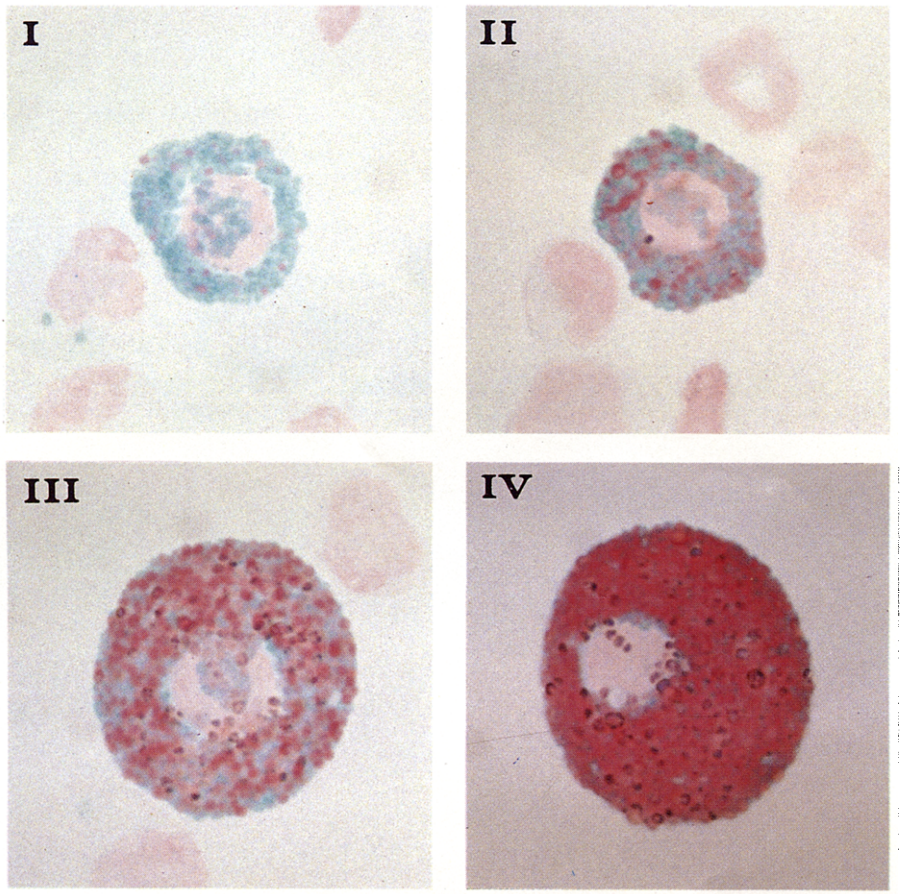

Fig. 1. Photomicrographs of rat peritoneal mast cells that were stained with Alcian blue and safranin [15]. Cells from different elutriated fractions were selected to indicate typical examples of cells at Stages I, II, III and IV of growth. Unsulfated mucopolysaccharides stain blue, and sulfated mucopolysaccharides stain red. Note the large nucleus in Stage I cells and the increase in granule numbers and increasing degree of sulfation of granular constituents as cells mature. Stage I cells do not exhibit the characteristic metachromatic stain, with toluidine blue or Wrights' stain [13]. Stage I cells were most prevalent in Fractions 5 and 6 of elutriated cells; Stage II cells in Fraction 7; Stage III in Fraction 9 and Stage IV cells in Fractions 10 and 11 (see text for further details). Magnification 500×.

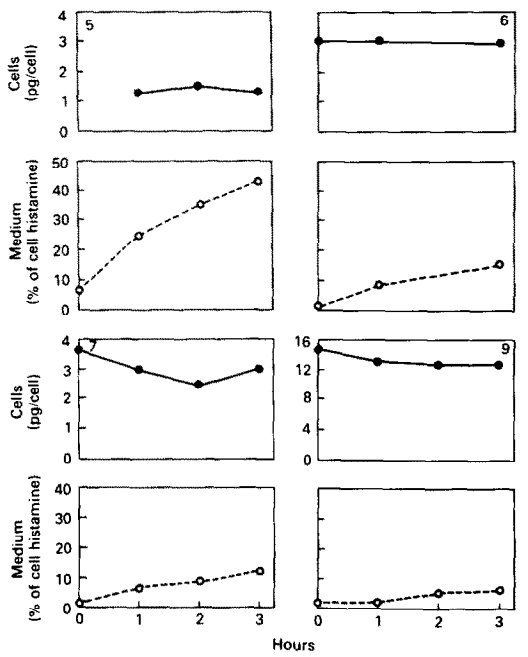

Fig. 2. Spontaneous release of histamine into the incubation medium with different fractions of elutriated rat peritoneal cells. The data were from representative experiments with Fractions 5, 6, 7 and 9 as indicated by the number of each panel. Note the constancy of intracellular histamine levels (see also data in Fig. 4). Cells were recovered from the elutriated fractions by centrifugation. They were resuspended in modified Hanks' medium and then incubated at 37°. Aliquots (100 μl) were removed at the indicated times to separate cells from medium as described in Materials and Methods. Points are mean of two samples prepared from each fraction. Viability, as determined by vital stains, was >90% for all fractions at 3 hr.

8–11) (Fig. 3). The calculated fractional turnover rate (i.e. proportion of cellular histamine pool released into the medium over the first hour) ranged from 0.12 ± 0.02/hr for Fraction 5, which has a high proportion of immature stage I cells, to 0.04/hr or lower for Fractions 8–11, which have high proportions of stage III and IV cells (see Fig. 3). It should be noted that the calculated values may underestimate actual turnover rates because of nonlinearity of histamine loss from fractions of immature cells (Fig. 3).

Effects of histidine and α-fluoromethylhistidine on spontaneous leakage. The presence of L-histidine (0.25 mM) resulted in no significant change in histamine levels or rates of endogenous histamine release in all cell fractions tested. In Fraction 5 that contained small cells, both cellular histamine levels and rates of release were reduced by the histidine decarboxylase inhibitor α-fluoromethylhistidine (10 μM) (Fig. 4, left panel). In fractions that contained large cells (Fig. 4, right panel), only the rates of release were reduced markedly (about 66%), even though isotopic studies indicated that this concentration of inhibitor suppressed by 90% both the formation of radiolabeled histamine and the release of $^{14}CO_2$ from L-[*carboxyl*-^{14}C]histidine in fractions of mature cells (Table 1). The stoichiometric relationship between $^{14}CO_2$ release and intracellular accumulation of [^{14}C]-labeled histamine at 60 min indicated that most of the newly formed histamine was retained within the mature mast cells (Table 1).

DISCUSSION

Rat peritoneal mast cells can be separated on the basis of size into subpopulations that differ markedly in their histamine contents, histamine synthetic activities [13], and abilities to retain newly formed histamine (this study). These subpopulations can also be distinguished on the basis of their cytochemical characteristics (Fig. 1). Fractions of small cells that consist primarily of post-mitotic (Stage I) cells contain few granules, have low histamine content and leak substantial amounts of histamine. Upon cell maturation, there is a progressive increase in the numbers of granules, degree of sulfation of granule mucopolysaccharides, and ability of cells to sequester newly formed histamine. Conversely, histamine synthetic activity, which is high in small immature mast cells, declines as cells mature [13].

Changes in rates of histamine synthesis have also been observed during the growth cycle of transformed rat basophil (2H3) cells. Synthetic activity was highest as cells approached "S" phase of growth. It then declined as cells approached division and increased again as daughter cells increased in size [12]. Turnover of histamine in 2H3 cells was also rapid, and changes in histamine content closely followed changes in synthetic activity [12]. The small total pool of histamine and high rates of histamine turnover in 2H3 cells were attributed [12] to the low glycosoaminoglycan content. In these respects, small mast cells resemble 2H3 cells. Both have low sulfated mucopolysaccharide content and both exhibit high rates of histamine turnover. The turnover time for histamine in immature cells was probably of the order of 10 hr (Fraction 5, Fig. 2) compared to an estimated time of 12 hr in 2H3 cells [12]. Mature mast cells, in contrast, retained most if not all of the newly synthesized histamine over a 60-min period (data in Table 1). The finding that α-fluoromethylhistidine caused a more rapid decline in intracellular histamine levels in fractions containing small mast cells than in those containing large mast cells (Fig. 4) provided further evidence that histamine turnover is more rapid in the small cells.

The critical steps in histamine synthesis and storage in intact cells include histidine uptake, histidine decarboxylation and translocation of newly formed histamine into the storage granules. An uptake system with high affinity for histidine (K_m, 20–50 μM) has been identified both in rat mast cells [7] and 2H3 cells [12, 19]. This system, which cotransports α-fluoromethylhistidine [19], is inhibited by histidine analogs, glutamine and aromatic amino acids [7, 9, 19–21] and concentrates both histidine and α-fluoromethylhistidine within the cell [12, 19]. The histamine synthetic activity of mast cells [9] or 2H3 cells [12] is largely destroyed upon cell lysis but it is retained upon degranulation of mast cells [13]. From immunohistochemical studies the enzyme appears to be present in discrete patches on the inner surface of the mast cell plasma membrane [22].

Little is known about the process of translocation

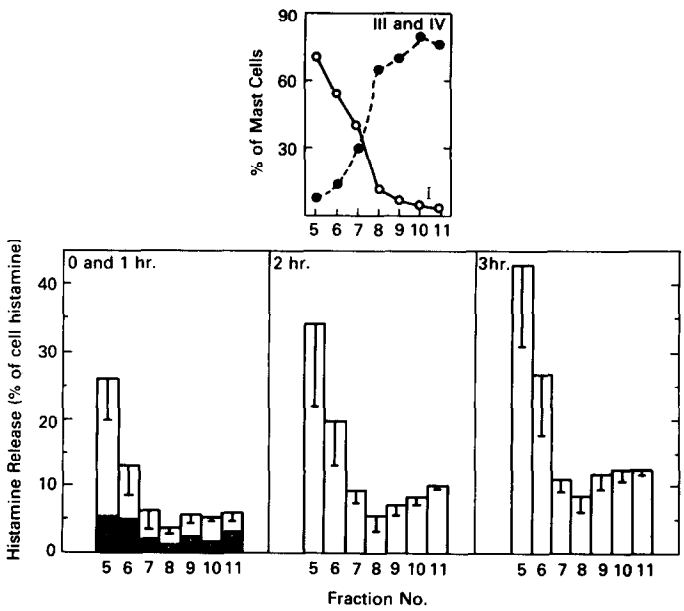

Fig. 3. Extent of spontaneous histamine release from different fractions of elutriated rat peritoneal cells at 0 (solid bars), 1, 2 and 3 (open bars) hr. The experimental protocols were as described in the legend of Fig. 2. Histamine levels in medium are expressed as percentage of that recovered in cell pellets at 0 hr (1.7, 3.8, 5.8, 8.7, 12.4, 12.6 and 8.7 pg/mast cell for Fractions 5–11 respectively). No significant decrease in intracellular histamine levels was observed in any fraction during the course of the experiment. Values are mean ± S.E.M. of four separate experiments. The upper panel shows the distribution of stage I (○——○) and stage III and IV (●– – –●) mast cells in each fraction.

of newly formed histamine into granules. Whether or not the system is fully functional in young mast cells or 2H3 cells remains to be determined. The process, which is relatively rapid in mast cells [7] and slow in 2H3 cells [12], is suppressed when cellular ATP levels are reduced (by 87%) by treatment with antimycin-A [21]. In rat peritoneal mast cells most (>90%) of the radiolabeled histamine formed from radiolabeled histidine is incorporated into the granular pool within 5 min [7], whereas in 2H3 cells only 66% of the newly formed labeled histamine is incorporated into this pool by 60 min [12].

The maintenance of constant intracellular histamine levels in immature mast cells in the absence of histidine (Figs. 2 and 4) suggests that either intracellular histidine levels or the supply of amino

Table 1. Stoichiometric relationship between $^{14}CO_2$ release and intracellular accumulation of [^{14}C]-labeled histamine in fractions of mature rat peritoneal mast cells

	% Label recovered as	
Experiment/Substrates	$^{14}CO_2$	[^{14}C]Histamine
Control		
L-[*carboxyl*-^{14}C]Histidine	0.42 ± 0.06	
L-[*ring*-2-^{14}C]Histidine		0.46 ± 0.06
α-Fluoromethylhistidine, 10 μM		
L-[*carboxyl*-^{14}C]Histidine	0.05 ± 0.02	
L-[*ring*-2-^{14}C]Histidine		0.045 ± 0.01

Values are mean ± S.E.M. for three experiments and have been corrected for assay blanks (medium only). The two labeled histidines were added to suspensions (samples were in triplicate) of elutriated rat peritoneal cells (Fraction 9, 85–95% mast cells) to give an equal number of cpm for each compound. Samples of this mixture were incubated (37°, 60 min), in the absence or presence of α-fluoromethylhistidine, and assayed for $^{14}CO_2$ release or accumulation of intracellular labeled histamine as described in Materials and Methods. To calculate percent recovery, the assumption was made (see Materials and Methods) that $^{14}CO_2$ was derived exclusively from L-[*carboxyl*-^{14}C]histidine and intracellular labeled histamine was derived from L-[*ring*-2-^{14}C]histidine. The percentages of label recovered as $^{14}CO_2$ and [^{14}C]histamine in the assay blanks were 0.09 ± 0.01 and <0.02 respectively.

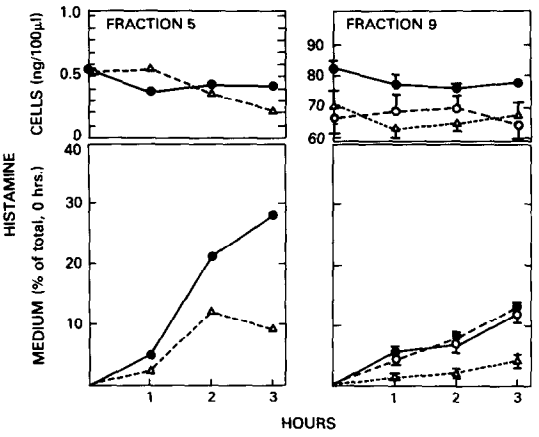

Fig. 4. Appearance of histamine in medium in the absence (●——●) or presence of 2×10^{-4} M L-histidine (○- - -○) or 10 μM α-fluoromethylhistidine (△- - -△). Cells were recovered from elutriated Fractions 5 and 9. The experimental protocol was identical to that in Fig. 2. Values are mean of two experiments (Fraction 5) or, for Fraction 9, mean ± S.E.M. from three experiments. Values for histamine in the medium have been corrected for histamine content of medium at 0 hr (2–4% of total histamine).

acid from endogenous sources is sufficient to maintain steady rates of histamine synthesis. This suggestion, however, cannot be confirmed directly because fractions that contain small cells contain monocytes in addition to immature mast cells and thus the intracellular histidine concentrations measured in our experiments reflect the concentrations in both kinds of cells. Studies with 2H3 cells, however, have shown that intracellular histidine concentrations decline from 1.8 to 0.4 mM with no decline in intracellular histamine levels (0.1 mM) when cells are incubated in histidine-free medium for 3 hr.

Several conclusions can be drawn from the present work. Although small immature mast cells do not release histamine in response to compound 48/80 [13] or to the Ca^+ ionophore A23187 (E. WoldeMussie, unpublished work), they do release histamine spontaneously at a steady rate. If histamine is also released from small mast cells *in vivo*, it seems plausible that inflammatory lesions associated with developing colonies of young or malignant mast cells, such as urticaria pigmentosa, mastocytomas, keloids and hypertrophic scars [23], might be due to high rates of spontaneous histamine release rather than to mast cell degranulation. If so, α-fluoromethylhistidine could be of value as a suppressant of spontaneous release (Fig. 4).

The present findings may also help to clarify the concepts of "nascent" histamine or inducible histidine decarboxylase, promoted principally by Schayer [24] and Kahlson and Rosengren [25]. These concepts were introduced to explain the findings by these authors that increases in tissue histidine decarboxylase activity or rates of histamine formation were not accompanied by increases in tissue histamine levels in developing fetus, regenerating tissues, tissues subjected to pathological stimuli, and in animals that were treated chronically with histamine liberators [24]. It is possible that some of these experimental situations were associated with growth of new (stage I) mast cells which do not stain metachromatically with toluidine blue [13]. In the case of fetal development, liver regeneration and wound healing, mature mast cells (as recognized by toluidine blue stain) become apparent at later stages of these processes [26, 27].

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