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HEPARIN, HISTAMINE, AND SEROTONIN IN NORMAL AND MALIGNANT MAST CELLS, AND NANOGRAM DETERMINATION OF SEROTONIN*

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Abstract—The quantities of heparin, histamine, and 5-hydroxytryptamine in the same populations of normal rat mast cells from peritoneal washings, and of malignant (ascites tumor, line P-815) mouse mast cells, were determined per 10^6 cells. Although the normal have about twice the dry weight of the malignant cells, the heparin, histamine, and 5-HT in the former were about 20, 260, and 9 times, respectively, the quantities in the latter. Whereas the number of basic groups of the histamine plus 5-HT exceeds the number of acid groups of the heparin in the normal cells, the reverse is true for the malignant cells. The fluorometric determination of 5-HT was adapted to measurement in the range, 0·005–0·125 μg.

THE CONSIDERABLE interest in heparin, histamine, and 5-hydroxytryptamine in both normal and malignant mast cells, the relative proportions of the substances present, and changes occurring in physiological and pathological states, has led to an extensive literature on the subject which has been thoroughly documented most recently by Selye.[1] However, data are still required of the content of all three constituents in the same sample of normal cells. Such data on malignant cells have been obtained but show wide variation. This study, devoted to accumulation of the data for mast cells from peritoneal washings of normal rats and for ascitic tumor mast cells from mice, continues recent work from this laboratory on ascorbic acid content[2] and biosynthesis of 5-HT in mast cells.[3,4] An adaptation of the fluorometric method developed by Udenfriend and co-workers[5,6] for determination of 5-HT has been elaborated to permit analysis of samples containing 0·005–0·125 μg of 5-HT, compared with 0·2–5 μg in the original method. This brings the sensitivity of the chemical method within range of that of biological assays.[7]

METHODS

Materials. Normal mast cells were obtained from peritoneal washings of the rat as described in detail earlier,[2] but with the addition of human serum albumin (Kabi, Stockholm, Sweden) to a final concentration of 0·1 per cent in the Ficoll solutions and

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Hanks', or balanced salt solutions,[8] used for isolation and washing of the cells. The presence of albumin inhibits cell disruption and reduces contamination from subcellular particulates.[8] Heparin was omitted from the solutions to avoid interference in analysis of the cellular heparin. Malignant mast cells (ascites tumor, line P-815[9]) were obtained from mice, as already described.[2] Cell counting also followed the previous procedure, but without staining, since this is not essential to discern the cell types in the hemocytometer. The normal-cell fraction had 2–10 per cent macrophages, and 0–5 per cent erythrocytes were present with the malignant cells.

Preparation of samples. The following procedure was used for analysis of heparin, histamine, and 5-HT in the same cell suspension. To 30 μl of the suspension, add 90 μl of 0·1 N HCl, freeze and thaw three times to disrupt cells, centrifuge for 3 min (top speed in an International, clinical model CL, head no. 215, carriers no. 369), and use the supernatant fluid for analyses of the three substances. (1) Heparin: neutralize 40 μl supernatant with 30 μl of 0·1 N NaOH and add 30 μl of 0·15 M NaCl. (2) Histamine: to 10 μl supernatant add 0·4 N $HClO_4$ to a final volume of 500 μl. (3) 5-HT: to 50 μl supernatant add 0·1 N HCl to a final volume of 200 μl. In each case mix the solutions, centrifuge briefly, and use the clear solutions for replicate analyses of the respective substances.

Heparin determination. The simple non-metachromatic reaction with azure A was found to be adequate for the analysis of mast cells, and the colorimetric procedure already described[10] was used with a few modifications for convenience: (1) pipette 40-μl portions of the unknown (equivalent to 4 μl cell suspension), standards (0·2–1·0 μg), and blank (0·15 M NaCl) into separate tubes. (2) Add 30 μl of 0·15 M phosphate buffer (pH 7·3) to each tube, mix, and add 30 μl of 0·005% azure A. (3) Mix and centrifuge at 3,000 *g* for 5 min. (4) Transfer the clear solution to micro cells and measure absorption at 600 mμ (Beckman spectrophotometer, model DU, sensitivity and slit settings, 5 and 0·15 mm respectively).

Note: The standard used was heparin sodium, Calbiochem, C grade, 150 units/mg, 4·1% H_2O, 10% S. This was dehydrated over silica gel *in vacuo* and stored at −20°. Stock solution, 1·47 mg/ml ≙ 1 mg/ml of pure free heparin (see below), was stored at −20°. Working solutions and stock solution were diluted with distilled water just before use to 5–25 μg/ml. (Highly purified heparin, e.g. the sample supplied by Jorpes for an earlier study,[10] contains 13% S and has 2·5 SO_3H groups per disaccharide unit. This substance has 1·3 times the sulfur content of the commercial preparation used for this work. To estimate the equivalent in weight to the pure compound of the commercial preparation, and to convert the weight of sodium salt to that of free heparin, the factor, 1·3 × 1·13, or 1·47 was used.)

Histamine determination. The fluorometric *o*-phthaladehyde reaction was employed, without change from the published procedure,[11] on 100-μl volumes of unknown (equivalent to 0·5 μl of cell suspension), standards (2-8 mμg), and blank (0·4 N $HClO_4$).

5-HT Determination. Apparatus: in addition to the more common apparatus used for microchemical work, already described,[12] specific items required are glass-stoppered tubes for extraction (1 ml, volumetric, T 8, Class A, Pyrex, Corning no. 5640, and noncalibrated narrower form, size B, Misco no. S4904, Microchemical Specialties Co., Berkeley, Calif.); glass reaction tubes (about 30 mm long, 3 mm i.d.); shaking device for extraction tubes;[13] mixer (Vortex Jr., Scientific Industries Inc., Queens Village, N.Y.; or Deluxe mixer, Scientific Products, Evanston, Ill.; or home-made devices,

Ref. 12, Vol. I, p. 89; table centrifuge (e.g. same as used for preparation of samples); constriction pipettes (15, 45, 60, and 75 μl), various self-adjusting or automatic pipettes could be substituted (Ref. 12, Vol. 1, pp. 74–85); pipette control or syringe (e.g. no. 0010–1725, Hamilton Co., Whittier, Calif.) attached to the 45-μl constriction pipette with a shoft piece of plastic tubing; syringe (500 μl, 5-μl divisions, Misco no. MNA-500, Microchemical Specialities, or Hamilton no. 750), fitted with a 3-in. stainless-steel hypodermic needle, no. 20, with beveled tip sawed off (for transfer of 200-μl volumes of butanol; correct for needle volume and dead space, usually about 75 μl); automatic pipettes with reservoirs for butanol and heptane (e.g., L/I Repipet, 1 ml, 0·01-ml division, with 125 ml reservoir, Labindustries, Berkeley, Calif.; 1-ml graduated, 0·01-ml division, long tip, conventional serological pipette can be used instead, but less conveniently); microspatula (e.g., Hayman style B, no. 9007, A. H. Thomas, Co., Philadelphia); fluorometer with micro cells (e.g., Aminco Bowman spectrophotofluorometer with slit arrangement no. 3, quartz micro cells and adapter, nos. 4–8114, 4–8119); magnifying lamp (e.g. no. LFM-1, Luxo Lamp Corp., San Francisco, Calif.) to facilitate pipettings. Note: The 45 μl- and 60 μl-constriction pipettes are siliconed, as are the reaction tubes, to facilitate complete transfers of solutions in steps 6 and 7 of the procedure. Siliconing is performed as described (Ref. 12, Vol. 1, pp. 69, 84).

Reagents: *n*-butanol (redistilled); *n*-heptane (redistilled); borate buffer, pH 10 (9.42 g boric acid dissolved in 300 ml distilled water added to 16·5 ml of 10 N NaOH) saturated with butanol and NaCl; 12 N HCl; 0·1 N HCl; NaCl; stock standard solution (4·62 mg 5-HT creatinine sulfate.H_2O, chromatographically pure, per ml of 0·1 N HCl, equivalent to 2 mg 5-HT/ml, stored at −20°); working standard solutions (0·02–0·10 μg/100 μl, freshly prepared for each use); quinine sulfate standard solution (1 μg/ml 0·1 N H_2SO_4).

Procedure: (1) Pipette 75-μl portions of unknown (equivalent to 5 μl of cell suspension), standards, and blank (0·1 N HCl) into separate 1-ml calibrated tubes. (2) To each tube add 75 μl borate buffer, an excess of NaCl (about 80 mg, not weighed each time, but once this amount is placed on the end of a microspatula it can be approximated for subsequent use), and 225 μl butanol. (3) Stopper, shake tubes for 1·5 min, and centrifuge. (4) Transfer 200 μl of the upper butanol layer from each tube to a 1-ml noncalibrated tube, and add 60 μl of 0·1 N HCl and 300 μl heptane. (5) Stopper, shake tubes for 2·5 min, and centrifuge. (6) With a siliconed pipette, transfer 45 μl of the bottom aqueous solution to a siliconed reaction tube, add 15 μl of 12 N HCl and mix. (7) Transfer the solution with the 60-μl pipette to a quartz microcuvette, and after adjusting the spectrophotofluorometer to read 50 at a meter multiplier setting of 0·1 with the quinine standard (350 and 450 mμ exciting and fluorescence wavelengths), measure the fluorescence at 295 and 550 mμ. Correct readings for blank, and obtain values of unknowns from standard curve.

Remarks: In the procedure published earlier[5,6] up to three washings with buffer of the butanol fraction (from step 3 in the procedure given here) are required if appreciable amounts of other 5-hydroxyindoles are present. These washings were found to be unnecessary for the mast cell samples. Thus, an analysis without washing gave 60 $\mu\mu$g of serotonin per 10^6 cells, while a value of 57 was obtained when three washings were included.

Scaling down the analysis to accommodate 100- and 75-μl samples had no sig-

nificant influence. A comparison with 4-ml samples analyzed by the macro procedure showed the same results for the same cell suspension, within experimental error. The latter was found to have a standard deviation of ±4·4 per cent and a standard error of ±1·4 per cent, based on ten replicate analyses.

Recovery experiments revealed that addition of 25-μl portions of a standard 5-HT solution, containing 50 mμg, to 250-μl portions of a rat stomach homogenate, containing an approximately equal amount of native 5-HT, showed that the recovery was within 3 per cent of theoretical.

Experiments were designed to test for possible interference from the presence of histamine and the biological precursors of 5-HT, tryptophan, and 5-hydroxytryptophan (Table 1). When these substances were substituted for 5-HT in the assay, only

TABLE 1. INFLUENCE OF THE PRESENCE OF TRYPTOPHAN, 5-HYDROXYTRYPTOPHAN, AND HISTAMINE ON THE FLUORESCENCE DEVELOPED BY 5-HT

Substance	Quantity (mμmoles)	Fluorescence reading*	
		Without	With (addition of 0·45 mμmole 5-HT)
5-HT	0·45	46	
Tryptophan	0·45	2	46
	4·5	2	48
5-Hydroxytryptophan	0·45	2	54
	4·5	92	118
Histamine	0·45	0	47
	4·5	1	49
	45·0	3	50

* Mean of duplicates, corrected for reagent blank.

5-hydroxytryptophan in a concentration 10 times that of the 5-HT showed appreciable fluorescence. When added to 5-HT in equimolar quantities, 5-hydroxytryptophan alone exerted a significant influence, increasing the fluorescence by about 17 per cent and, at a 10-fold greater concentration, by over 150 per cent. However, it appears that the levels of 5-hydroxytryptophan in animal cells are much lower than those of 5-HT (Ref. 6, p. 112) and, therefore, are not likely to cause interference. Ten- or 100-fold increases in the histamine, which may occur relative to the 5-HT concentration in mast cells and other specimens, had little effect.

RESULTS

From data in Table 2 it follows that the ratios of the content of heparin:histamine:5-HT in the normal rat mast cells were 59:58:1, on a weight basis. When the data were converted from micrograms to millimicromoles, the ratios became 0·8:90:1 (12,000, mol. wt. of heparin). Thus the combined bases exceeded the moles of heparin by about 105 times.

The dry weight of the normal compared to the malignant cells is about twice as great (Diamant and Glick, unpublished). However, the present data show that the amounts of the three substances in the normal were much more than twice those in the malignant cell, the former exceeding the latter by about 20, 260, and 9 times in heparin,

histamine, and 5-HT respectively. The mean ratios of the amounts of the substances in the cells from the gray mice were 28:2:1 and 0·4:3·3:1 on weight and molar bases respectively. Therefore, compared to the normal, the malignant cells had less than 4 per cent of the moles of histamine to each of 5-HT, and together these bases exceeded the moles of heparin by about 11 times, rather than 105 times as in the normal cells.

TABLE 2. CONTENT OF HEPARIN, HISTAMINE, AND 5-HT IN NORMAL AND MALIGNANT PERITONEAL MAST CELLS FROM THE RAT AND MOUSE RESPECTIVELY*

Source	No. of animals	Heparin (μg/10^6 cells)	Histamine (μg/10^6 cells)	5-HT (μg/10^6 cells)
Normal cells				
Male albino rats (Sprague–Dawley)	6	39 ± 8	38 ± 5	0·66 ± 0·10
Malignant cells (P-815)				
Female albino mice (BALB) (1)	3	0·4	0·20	0·03
(2)	3	1·6	0·17	0·05
Female gray mice (DBA/2) (441)	5			0·09
(442)	5			0·07
(443)	5			0·09
(444)	5		0·22	0·10
(464)	5	2·7		
(466)	5	2·9		
(467)	5	2·5		
(476)			0·13	
(477)		1·3	0·12	0·05
(478)		1·3	0·13	0·07
(479)	3	1·7	0·15	0·05
(480)	3	1·7	0·13	0·05
Mean (DBA/2)		2·01 mμmoles/10^6 cells)	0·147 (mμmoles/10^6 cells)	0·071 (mμmoles/10^6 cells)
Normal cells		3·3	342	3·8
Malignant cells, Mean (DBA/2)		0·17	1·3	0·4

* Numbers in parentheses denote the number of the transfer generation. Values are ± standard errors of means.

DISCUSSION

The value, 39 ± 8 μg/10^6 cells, for heparin in normal rat mast cells obtained in this work (Table 2) is comparable to the higher of the values, 39 and 20, in the literature (Table 3). The value found for histamine, 38 ± 5 μg/10^6 cells (Table 2) is higher than the 29 and 31·5 ± 1·9 shown in Table 3, but much greater than the other two values, 13 and 14, reported. In the case of 5-HT, the value, 0·66 ± 0·10 μg/10^6 cells (Table 2) is comparable with certain of the data reported earlier, i.e. 0·78, but appreciably lower than others, 1·34 and ~1 (Table 3). The differences in results may be ascribed to biological variations, differences in environmental conditions, nutrition, etc., and possibly differences in analytical methodology. Data from the other laboratories were not obtained for all three substances on the same samples of cells, and this may add to the variation.

It should be pointed out that the histamine values obtained for the normal rat mast cells by the fluorescence method were higher than the values taken from the literature. The values, 31·5, 13, and 14 (Table 3), were obtained by bioassay and that of 29 by a colorimetric chemical method. It is possible that the normal rat mast cells contain substances other than histamine that give fluorescence with the analytical

reagent used, a problem which, in any case, does not appear to apply to the malignant mouse mast cells whose values are low and not inconsistent with other data. However, further methodological studies would be required to determine whether interfering substances, peculiar to the cell in question, are responsible for the higher values found by the fluorescence method, or whether losses in the bioassay account for the difference.

TABLE 3. MEAN VALUES IN THE LITERATURE OF AMOUNT OF HEPARIN, HISTAMINE, AND 5-HT IN NORMAL RAT AND MALIGNANT MOUSE MAST CELLS
VALUES IN $\mu g/10^6$ CELLS

Source		Heparin	Histamine	5-HT	Reference no.
Normal cells					
Male (Sprague–Dawley)		39*	14*		14
			29*		15
				0·78*	16
Male (Wistar)			31·5 ± 1·9	1·34 ± 0·20	17
R Strain		~20			18
			~13	~1	19
Malignant cells (P-815)					
AKR × DBA/2 F1 Strain	O	0·1	0·19	0·10	20
	T	0·3	0·12	0·10	20
	X-1	0·3	0·08	0·05	20
	X-2	0·9	0·35	0·39	20
	X-1	1·1*†	0·78*†	0·61*†	20

* Calculated from data given in reference.
† Mean of 5 samples collected on the same day.

For malignant mast cells, data in Table 2 seem to reveal a trend toward diminishing values during the 29 generation transfers, but the data are not sufficiently extensive to establish the trend on a statistical basis. By comparison, the P-815 line of cells, cultivated *in vitro* by Schindler *et al.*,[21] showed an increase in 40 generations from 0·12 to 0·54 $\mu g/10^6$ cells for histamine and, correspondingly, from 0·04 to 0·19 for 5-HT. Green and Day[20] also found that heparin, histamine, and 5-HT increased in culture, but decreased when grown in the mouse. Mengel and Trier[22] observed that histamine decreased during 14 generation transfers *in vivo* while 5-HT increased, and both substances gradually returned to stemline levels. Such differences have been ascribed to heterogeneity of the cells *in vivo*.[23] The range of data given by Green and Day[20] encompasses the values obtained in this work.

Hagen *et al.*[24] measured the three constituents in isolated granules from Furth mastocytomas. Losses occurred from the isolation procedure, and ratios of amounts of heparin: histamine: 5-HT were highly variable among preparations.

Data in this paper give a molar ratio of about 105:1 for the two bases to heparin in the normal rat mast cells. An estimate of the number of acid groups per heparin molecule can be made from earlier data[10] that revealed 2·5 acid sulfate and 3·5 total acid groups per disaccharide unit in a sample of purified heparin. The calculated molecular weight per unit was 538, and from a molecular weight of 12,000 for heparin there would be 22 units per mole. The total number of acid groups per mole of heparin would then be 77. These 77 groups are in the cell with the approximately 105 moles of

histamine plus 5-HT present per mole of heparin. In the malignant mouse mast cell, the 77 acid groups have only 11 moles of the combined bases with them. Excess basic groups in the normal cell may be bound to acidic groups in the protein of the cytoplasmic granules that bear all three of the substances, and in the staining of the granules by basic dyes, such as toluidine blue, it is likely that basic groups bound to the heparin are displaced by the dye. This possibility had been mentioned by Smith.[25]

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