Serotonin Storage Pools in Basophil Leukemia and Mast Cells: Characterization of Two Types of Serotonin Binding Protein and Radioautographic Analysis of the Intracellular Distribution of [^{3}H]Serotonin

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ABSTRACT We studied binding of serotonin to protein(s) derived from rat basophil leukemia (RBL) cells and mast cells. We found two types of serotonin binding protein in RBL cells. These proteins differed from one another in molecular weight and eluted in separate peaks from sephadex G-200 columns. Peak I protein ($K_D = 1.9 \times 10^{-6}$ M) was a glycoprotein that bound to concanavalin A (Con A); Peak II protein ($K_{D1} = 4.5 \times 10^{-8}$ M; $K_{D2} = 3.9 \times 10^{-6}$ M) did not bind to Con A. Moreover, binding of [^{3}H]serotonin to protein of peak I was sensitive to inhibition by reserpine, while binding of [^{3}H]serotonin to protein of peak II resisted inhibition by that drug. Other differences between the two types of binding protein were found, the most significant of which was the far more vigorous conditions of homogenization required to extract peak I than peak II protein. Neither peak I nor peak II protein resembled the serotonin binding protein (SBP) that is found in serotonergic neurons of the brain and gut. Electron microscope radioautographic analysis of the intracellular distribution of [^{3}H]serotonin taken up in vitro by RBL cells or in vivo by murine mast cells indicated that essentially all of the labeled amine was located in cytoplasmic granules. No evidence for a pool in the cytosol was found and all granules were capable of becoming labeled. The presence of two types of intracellular serotonin binding proteins in these cells may indicate that there are two intracellular storage compartments for the amine. Both may be intragranular, but peak I protein may be associated with the granular membrane while peak II protein may be more free within the granular core. Different storage proteins may help to explain the differential release of amines from mast cell granules.

The cellular biology of serotonergic neurons is difficult to study in the central nervous system (CNS) because the tissue is complex and because glial cells take up and bind serotonin (10, 18). As a result, the mechanisms involved in the storage and release of serotonin by serotonergic neurons are still rather poorly understood (11). To circumvent some of the problems inherent in studying the CNS, serotonin uptake and release have been examined in several simpler model systems. The myenteric plexus of the gut, for instance, contains serotonergic neurons that share many of the characteristics of their CNS counterparts (12): they both take up serotonin by a similar mechanism and both contain a highly specific serotonin binding protein (SBP). Since SBP has also been found to be moved proximodistally in serotonergic axons by fast transport and to be concentrated in synaptic vesicles (29), it seems likely that SBP is a component of serotonergic synaptic vesicles and is, at least in part, released together with the transmitter by exocytosis (21).

Non-neuronal cells have also been studied in the hope that they will manifest serotonergic mechanisms identical to those of neurons and thus serve as proxies for neurons. Such cells include platelets, thyroid parafollicular cells, and mast cells.

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Platelets are perhaps the best known of the non-neuronal putative models (6), although they do not contain an SBP that is similar to that found in neurons (27). Parafollicular cells are of neural crest origin, synthesize and store serotonin (8, 14, 15, 20), and also contain the neuronal-type SBP (4, 5).

Mast cells of rodents take up and store serotonin (3, 17, 34). These cells are thought to store serotonin and histamine together, probably within their large secretory granules, and both amines are known to be secreted simultaneously by stimuli that cause exocytosis (23, 25); however, mast cells have recently been shown to be capable of releasing serotonin independently of histamine (2). This has led to the suggestion that serotonin might either be stored in the granules and in another site as well, or be released separately, perhaps as a complex with a binding protein. Here we identify proteins that bind serotonin in rat peritoneal mast cells. Because mast cells cannot be obtained in great abundance, we studied in detail rat basophil leukemia (RBL) cells that, like mast cells, take up, store, and release serotonin (9). RBL cell-derived proteins that bind serotonin were characterized and compared to those of neurons. In addition, we obtained radioautographic evidence that all of the serotonin taken up by RBL cells or mast cells is indeed stored in their secretory granules.

MATERIALS AND METHODS

RBL Cell Culture

Rat basophil leukemia cells (RBL, HR+, subline 2H3) were provided by Dr. Henry Metzger (National Institutes of Health) and grown in stationary cultures at 37°C as described previously (19). For cell disruption, the medium and cells in suspension were discarded and 3 ml of a trypsin-EDTA mixture (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) (10 times) was added and incubated at 37°C for 5 min. The flasks were then shaken vigorously to dislodge the cells and 10 ml of culture medium were added at room temperature. The cells were pooled and sedimented (180 *g* for 10 min). The supernatant was discarded and the cells were resuspended in 3.0 ml cold HEPES buffer (10 mM, pH 7.0). They were then broken by 20 strokes in a Teflon-glass mechanical homogenizer at 1,200 rpm. Additional HEPES (2 ml) was added to wash off any remaining material and the final 5 ml were stored at −120°C.

Mast Cells

Peritoneal mast cells were collected by peritoneal lavage of male Sprague Dawley rats (~450 g, Charles River Breeding Laboratories, Wilmington, MA) and purified on a bovine serum albumin (BSA) gradient as previously described (33). The cells were then suspended in HEPES buffer (10 mM, pH 7.4) and homogenized as described above.

Preparation of Proteins that Bind Serotonin from Mast Cells or from RBL Cells

Disrupted cell suspensions were thawed and further disrupted either by homogenization with a Polytron (Brinkmann Instruments, Inc., Westbury, NY) for 3 min, position 5 at 4°C, or with a motor-driven homogenizer using a serrated tight pestle (10 strokes at 4°C). Homogenates were centrifuged (100,000 *g* for 60 min), and the supernatant fluid was fractionated with ammonium sulfate (15–60% saturation). The precipitate containing the proteins that bind serotonin was collected by centrifugation (15,000 *g* for 10 min), dissolved in potassium phosphate buffer (0.02 M, pH 7.5), and was dialysed against the same buffer for 90 min.

Molecular Sieve Chromatography for Separating the Two Proteins that Bind Serotonin

The 15–60% ammonium sulfate fraction was applied to 1.2 × 26.5 cm columns of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with potassium phosphate buffer (0.02 M, pH 7.5). The columns were eluted with the same buffer at 4°C, and 1-ml fractions were collected at a flow rate of 5 ml/h.

Binding Assay

The assay for serotonin binding proteins has been described previously (30). It involves incubation of samples for 15 min with 0.2 μM [^{3}H]serotonin (11.0 Ci/mmol, Amersham/Searle, Arlington Heights, IL) in the presence of Fe^{+2} (0.1 mM). The resulting protein-Fe^{+2}-[^{3}H]serotonin complex is then separated from free serotonin on Sephadex G-50. Nonspecific binding, measured in the presence of a 1,000-fold excess of nonradioactive serotonin, was subtracted from the total binding to obtain the specific binding. To determine the dependence of serotonin binding on the serotonin concentration, incubation with [^{3}H]serotonin was prolonged to 60 min to ensure that equilibrium had been achieved. Dissociation constants were calculated by plotting the data according to Scatchard. When the effect of inhibitors or drugs on serotonin binding was tested, the test agent was added together with [^{3}H]serotonin. To investigate the effect of gangliosides, the protein containing sample was preincubated (10 min) with G_{Ds} (disialosyllactosyl ceramide isolated from chicken brain) in the presence of a sixfold (wt/wt) excess of egg lecithin (Sylvana Chemical Corp., Grand Island, NY) as described previously (28). The binding of [^{3}H]serotonin was determined by adding Fe^{+2} and the radioactive amine to the protein-ganglioside suspension and then incubating for an additional 15 min. The bound [^{3}H]serotonin was separated from the free amine using a Sephadex G-50 column.

Affinity Chromatography Employing a Column of Anti-BSA IgG Covalently Linked to Sepharose

CN-Br activated Sepharose 4B (3 g; Pharmacia Fine Chemicals) was added (after washing) to a solution of the IgG fraction of a rabbit antiserum to BSA (anti-BSA [N. L. Cappel Laboratories, Inc., Cochranville, PA] 50 mg in 5 ml 0.1 M sodium bicarbonate buffer [pH 8.3] containing 0.5 M NaCl). The suspension was rotated at 170–175 rpm for 15 min at 24°C and then for 16 h at 4°C. The coupled Sepharose was washed with 50 ml of alternating sodium acetate buffer (0.1 M, pH 4.0, containing 0.5 M NaCl and Tris hydrochloride (0.1 M, pH 8.0, containing 0.5 M NaCl) and was poured into a column (1.5 × 3.5 cm), that was equilibrated with sodium bicarbonate (0.1 M, pH 8.3) and run at the rate of 10 drops/min. The BSA bound to the column was eluted with 30 ml glycine hydrochloride buffer (0.2 M, pH 2.4).

Removal of Heparin and of DNA

Heparin and DNA were removed from homogenates by the method of Yurt and Austen (36). The cells were disrupted with a Polytron, as described above, and were incubated with DNase (10 μg/ml) and $MgCl_2$ (5×10^{-4} M) for 5 min at 25°C. Solid NaCl was then added to a final concentration of 1 M and the incubation was continued for an additional 20 min. Undisrupted cells and debris were removed by centrifugation (1,000 *g* for 10 min). The resultant supernatant was applied to a 1 × 15 cm column of Dowex-1-Cl-x2 chloride (50–100 mesh; Bio-Rad Laboratories, Richmond, CA). The column was equilibrated and washed with 1 M NaCl in 0.02 M potassium phosphate buffer, pH 7.5. Proteins that bind serotonin did not bind to this column. Heparin was eluted with 3 M NaCl. The 3 M eluate was pooled, dialyzed overnight against H_2O, and lyophilized. The dry powder was taken up in potassium phosphate buffer (0.02 M, pH 7.5). Proteins in the effluent and eluent were assayed for serotonin binding capacity.

Chromatography on a Con A-Sepharose Column

A column (1.5 ml) of Con A-Sepharose (Pharmacia Fine Chemicals) was constructed in a pipette (0.9 × 8 cm) and equilibrated with potassium phosphate buffer (0.02 M, pH 7.5). Proteins obtained from the excluded fractions (peak I) or peak II of a Sephadex G-200 column were fractionated subsequently on this column by applying a sample of 2 ml (2 mg protein or less at 4°C) and washing with 6 ml of the above buffer. Glycoproteins were then eluted with alpha-methyl-D-mannoside (0.5 M) in potassium phosphate buffer (0.02 M, pH 7.5), after allowing the column to equilibrate with the monosaccharide for 1 h.

PAGE

Electrophoresis on 7.5% polyacrylamide tube gels was carried out, using a modification of the SDS buffer system of Laemmli (22, 24). For electrophoretic analysis, the protein or protein-iron-[^{3}H]serotonin complex was dissolved in a buffer containing SDS and mercaptoethanol (27). Gels were stained with Coomassie Blue and destained as described by Fairbanks (7) or they were stained with the periodic acid-Schiff (PAS) technique (27). To locate radioactivity, the gels were sliced, dissolved in tissue solubilizer (TS-1; Research Products, Elk Grove Village, IL), and counted.

Radioautography

To prevent catabolism of injected [^{3}H]serotonin, mice were injected intraperitoneally with the monoamine oxidase inhibitor, pargyline (100 mg/kg, Abbott Diagnostics, Diagnostic Products, North Chicago, IL). Animals received [^{3}H]-serotonin (1.2 mCi/mouse, 16 Ci/mmol; New England Nuclear, Boston, MA) intraperitoneally and then were killed 4 h later. The dorsal foot skin was fixed and prepared for electron microscope radioautography as described previously (1). RBL cells (4×10^7 in 2.5 ml) were incubated with 200 μCi [^{3}H]serotonin for 60 min at 37°C. Labeled cells were then washed twice in 10 ml growth medium, twice in 10 ml isotonic saline, and then were resuspended in 3 ml saline. Immediately thereafter, 3×1 ml aliquots of cells were added to double strength fixative containing 3% sucrose (1). The cells were fixed for 2 h at 24°C, centrifuged at 5,500 *g* for 15 min at 4°C, and the pellet was resuspended in 0.2 M cacodylate buffer and placed in 0.5 ml Eppendorf tubes that were centrifuged at 15,000 *g* for 3 min before postfixation, dehydration, and embedding. After embedding, silver gray thin sections were cut and placed on collodion-coated glass slides. A thin carbon layer was then shadowed over the slides. Ilford L4 emulsion was layered on the carbon-coated slides in a film that produced a purple interference color. Slides were exposed for 3 wk in an atmosphere of dry CO_2, and developed with Kodak Microdol-X developer for 3 min. After fixation, washing, and drying, the collodion layer carrying the sections and emulsion was floated off the glass slides. The sections were then picked up on bare copper grids and treated with alcohol for 5 minutes to remove the collodion. The grids were then rehydrated through a descending series of ethanols and stained with uranyl acetate and lead citrate. Grids were examined with a JEM 100C electron microscope. Pictures were taken of every silver grain on each grid examined.

Analysis of Radioautographs

Radioautographs were analyzed by the method of Williams (35). Essentially, probability circles with a radius of (1.5 times the half distance (HD) (the distance from a radioactive line within which 50% of the developed silver grains fall) (26) were drawn around each silver grain and the potential radioactive sources within these circles were scored. A regular grid of similar circles placed over the tissue and scored similarly served to evaluate the distribution that would have been expected if the radioactive material were disposed randomly in the tissue. Grain distribution was compared with the circle distribution (expectation for a random distribution) by means of a chi-square. Cellular elements were defined as labeled if significantly more grains fell over them than would have been expected for a random distribution ($P < 0.001$).

RESULTS

Preparation of Proteins that Bind Serotonin from RBL Cells

After ammonium sulfate precipitation and dialysis of protein in the high speed (100,000 *g*) supernatant obtained from ~10^8 homogenized RBL cells (20 mg total protein), serotonin binding activity was found in the fraction of 15–60% saturation that had ~30% of the total protein of the high-speed supernatant and a serotonin binding capacity of 146,200 cpm/mg protein. Ammonium sulfate precipitation caused almost no denaturation of serotonin binding protein and thus resulted in a two- to threefold purification. When the RBL cells were disrupted using a Polytron rather than by a glass/Teflon homogenizer, a greater amount of protein was extracted from the cells (0.8 mg/ml instead of 0.5 mg/ml) and it also had a threefold higher serotonin binding capacity (483,200 cpm/mg protein) than protein extracted by glass/Teflon homogenization. Nonspecific binding observed in the presence of a 1,000-fold excess of cold serotonin was 25% of total binding.

Properties of Crude Proteins that Bind Serotonin

Binding of serotonin to the proteins was 50% inactivated by heating at 80°C for 20 min; binding was dependent on protein integrity, since incubation with trypsin (0.1 mg for 10 min at 37°C) decreased the specific binding by 90%. Other enzymes such as hyaluronidase or ribonuclease had no effect on serotonin binding. Linear binding of [^{3}H]serotonin was observed up to 140 μg protein/ml. The binding was enhanced as much as sixfold by the presence of Fe^{+2}, and this effect was specific since other divalent cations, such as Ca^{+2}, Mg^{+2}, or Co^{+2}, as well as Fe^{+3}, had much less effect (Table I). There was some stimulation by Ca^{+2}. This represents another difference from neuronal SBP; serotonin-binding to neuronal SBP is inhibited by Ca^{+2}. The concentration of salts normally found in extracellular fluids (Krebs-Ringer solution) strongly interfered with [^{3}H]serotonin binding. Histamine and heparin, components of RBL granules, when present at 10^{-5} M did not inhibit the binding of [^{3}H]serotonin and no proteins able to bind [^{3}H]-histamine were detected. Binding of serotonin was saturable and only one dissociation constant ($K_D = 3.9 \times 10^{-7}$ M) was observed when the data was analyzed by a Scatchard plot. At this stage of purification, the serotonin binding proteins were not stable and purification had to be continued immediately without storage of the material.

Separation and Isolation of Two Binding Proteins from Polytronized RBL Cells

When the 15–60% ammonium sulfate fraction was applied to a Sephadex G-200 column, two separate protein peaks (detected by measuring optical density at 280 nm) contained serotonin binding capacity (Fig. 1). Peak I was in the void volume (eluted at ~10 ml), contained 16% of the protein applied to the column and had a binding capacity of 529,600 cpm/mg protein. Peak II (a broader protein fraction that eluted at ~14 ml), contained 35% of the proteins applied on the column, and had a binding capacity of 312,500 cpm/mg protein. The activity of this second fraction varied considerably from preparation to preparation. A similar pattern of fractionation was observed when a Sepharose 4B column was used. Since the Sepharose 4B exclusion volume contains proteins with a molecular weight $>5 \times 10^6$, peak I proteins that bind serotonin are probably aggregates, whereas peak II proteins are likely to have a molecular weight of close to that of the BSA marker (Fig. 1).

The possibility that a heparin complex with proteins was emerging from the Sephadex column as peak I was investigated. The supernatant of the disrupted cells was treated with high salt concentrations to minimize macromolecular interactions and the heparin was removed by passing the mixture through a column of Dowex-1-Cl-×2. Heparin was bound to the column while proteins were recovered in the effluent and fractionated on Sephadex G-200. The profile and binding

TABLE I

Effect of Various Metals on the Binding of Serotonin to Proteins from RBL Cells *

Metal	SBP	Relative binding
10^{-4} M	cpm/mg	
None	14,130 ± 1,500	1.0
Fe^{+2}	82,610 ± 7,700	5.9
Ca^{+2}	42,390 ± 3,700	3.0
Mg^{+2}	30,980 ± 3,100	2.2
Mn^{+2}	22,280 ± 1,800	1.6
Cu^{+2}	21,210 ± 3,500	1.5
Fe^{+3}	10,330 ± 1,500	0.73

* Precipitated at 15–60% saturated $(NH_4)_2SO_4$; 60 μg protein/ml. Each value is the mean ± SE of three experiments.

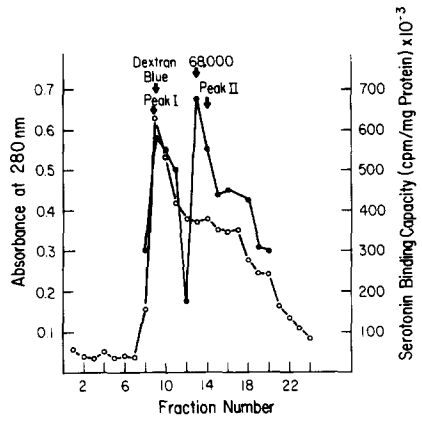

FIGURE 1 Preparation of SBPs from RBL cells. Protein (5–10 mg of $[NH_4]_2SO_4$ fraction 15–60%) was applied to a Sephadex G-200 column. Fractions (1 ml) were collected and assayed for protein concentration and serotonin binding capacity. The column was calibrated with Dextran Blue (>1,000 kdaltons) and BSA (68 kdaltons). (○) Absorbance at 280 nm. (●) Serotonin binding capacity.

capacity of the eluted proteins were very similar to those obtained when ammonium sulfate fraction was chromatographed (Fig. 1). Isolated heparin did not bind [^{3}H]serotonin tightly enough to be detectable by molecular sieve chromatography. Moreover, peak I did not consist of proteins complexed with DNA because addition of DNase had no effect on the subsequent fractionation of [^{3}H]serotonin binding proteins.

Con-A-Sepharose Column

To further characterize the SBPs that were eluted in peaks I and II, protein mixtures (up to 4 mg protein) were passed through Con-A-Sepharose columns. Glycoproteins fraction were looked for that bound to the column, were not eluted with 0.5 M NaCl, but were eluted with alpha-methyl-D-mannoside. When peak I was used as the starting material, the protein of peak I that bound [^{3}H]serotonin did bind to the Con-A-Sepharose column and was eluted with alpha-methyl-D-mannoside; however, when peak II protein (3.5 ml) was used 85 ± 5% of the serotonin binding activity passed through the column. The serotonin binding protein of peak I was therefore considered to be a glycoprotein that binds to Con A, while the SBP of peak II was not. Results of electrophoresis (see below) were consistent with this conclusion.

Determination of Whether BSA in Peak II Could Account for Serotonin Binding

The apparent similarity in molecular weight between peak II proteins that bind serotonin and BSA prompted us to test whether the binding activity of peak II is due to the presence of BSA. Since RBL cells were grown in a medium that contained 10% fetal calf serum, it is conceivable that RBL cells had taken up BSA by endocytosis, or some BSA could have adhered to the plasma membrane of the RBL cells despite the intensive washing that followed harvesting. For example, we recently demonstrated that washed rat platelets contain an albuminlike protein that binds serotonin (27).

Proteins eluted in peak II (0.6 mg total protein with a binding capacity of 468,000 cpm/mg protein) were applied to a Sepharose column to which the IgG fraction of rabbit antiserum to BSA had been coupled covalently. In the first experiment, ~25% of the total protein applied did not bind to the column and contained ~70% of the serotonin binding capacity of the proteins in peak II. When the column was eluted with glycine hydrochloride buffer (0.2 M, pH 2.4) to remove any proteins bound to the anti-BSA column, an additional 45% of the original protein applied to the column was recovered (serotonin binding capacity could not be measured because low pH denatures the protein irreversibly). Thus, 30% of the total protein could not be eluted. The eluent was subjected to electrophoresis on 7.5% SDS gels. No BSA (<1 μg) could be detected in the protein eluted from the column. When ^{125}I-BSA (600 μg containing 68,000 cpm) was applied to the column, 94.5% of the radioactivity was bound. This experiment showed that the antibodies attached to Sepharose retained their ability to recognize BSA and that the bulk of the 600 μg BSA applied to the column could be bound.

In a second experiment, when 900 μg of proteins that eluted in peak II were applied to the same column, 80% of the total protein did not bind to the anti-BSA Sepharose and passed through. The specific binding capacity of this protein was calculated to be 15% higher than that originally applied to the column. The recovery of those proteins able to bind serotonin was therefore 95%. It was concluded that the protein present in peak II that binds serotonin is not BSA. The higher recovery in the second experiment was most probably due to saturation of nonspecific protein binding sites on the Sepharose achieved during the first experiment.

Binding Properties of RBL Cell-derived Peak I and Peak II Proteins

Serotonin binding was concentration dependent and saturated as the concentration of serotonin in the incubation mixture was increased. A Scatchard plot of the data (Fig. 2) revealed one dissociation constant for the protein in peak I (K_D = 1.9 = 10^{-6} M) and two dissociation constants for the protein(s) in peak II ($K_{D1} = 4.5 \times 10^{-8}$ M and $K_{D2} = 3.9 \times 10^{-6}$ M). The binding of serotonin to both proteins was Fe^{+2} dependent. Both preparations were very labile and lost 75% of

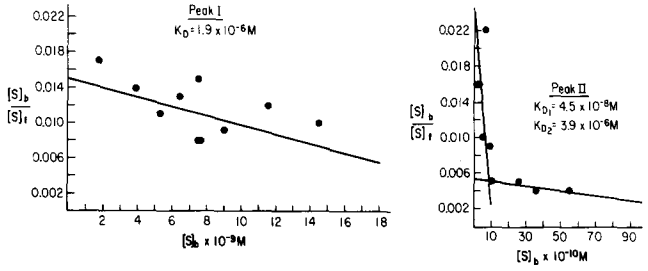

FIGURE 2 Scatchard plot analysis of the binding of serotonin to the glycoprotein in peak I (left) and protein eluted in peak II (right). The respective proteins eluted from Sephadex G-200 column were incubated in the presence of Fe $(NH_4)_2(SO_4)_2$ (0.1 mM) with indicated amounts of G-^{3}H-serotonin for 1 h at 30°C. Binding capacity was assayed as described in Materials and Methods. The ratio of concentrations of bound to free serotonin $[S]_b/[S]_f$ was plotted against the concentration of bound serotonin $[S]_b$; mol/l. The K_D's were calculated from the slopes of the lines (slope = $-K_D^{-1}$). The line represents the results of the linear least square analysis of the experimental data. One dissociation constant was determined for the peak I glycoprotein and two dissociation constants were determined for the binding protein eluted in peak II. The experiment was carried out four times for each protein.

their serotonin binding capacity even when kept overnight at −20° in 10% glycerol.

Effect of Reserpine on the Binding of Serotonin

Gentle homogenization of RBL cells with a motor driven Teflon homogenizer released serotonin binding protein(s) that had different sensitivity to reserpine than the protein(s) released by vigorous homogenization (Polytron). Most preparations obtained by gentle homogenization showed a binding capacity that was not antagonized by reserpine (up to 10^{-5} M), while a few showed no more than 40% inhibition. Polytronization of the cells increased the recovery of SBPs and the binding of serotonin by these additional proteins was now sensitive (80 ± 10% inhibition) to reserpine (10^{-5} M) in ten out of ten experiments. Proteins released by Polytron homogenization were separated on a Sephadex G-200 column into peak I and peak II fractions as described earlier, which differed in their sensitivity to reserpine. The binding of serotonin to peak I proteins was inhibited by reserpine (78.5 ± 8.2% in 8 out of 8 experiments), while the binding of serotonin to peak II proteins was largely unaffected by reserpine, although in a few experiments there was an inhibition of ~30% (Table II). This result is consistent with the existence of two sites of cellular compartmentation of SBPs that differ in their resistance to disruption and their sensitivity to reserpine. The variation among experiments may be caused by the inability to consistently disrupt the different storage compartments to the same extent.

Effect of Inhibitors on Serotonin Binding to Proteins Separated on Sephadex G-200

Several psychoactive drugs have been found recently to inhibit the release of histamine, but not that of serotonin from mast cells (2, 32). Some of these compounds were tested for their ability to affect RBL cell-derived proteins that bind serotonin (Table II). Chloropromazine, imipramine, and pargyline (5×10^{-6} M) inhibited binding of serotonin to proteins in peak I by 85%, 82%, and 65%, respectively. Peak II proteins were affected to a lesser degree by chlorpromazine and imipramine (37% and 30% inhibition, respectively), whereas pargyline had no inhibitory effect. Fluoxetine (5×10^{-6} M), a specific serotonin uptake inhibitor, had no effect on the binding of serotonin to either protein.

TABLE II

Properties of SBPs Extracted from Rat Basophil Leukemia Cells

	% Inhibition	
Inhibition of serotonin binding	Peak I	Peak II
Reserpine*	79 ± 8 SE‡	20 ± 10 SE‡
Imipramine*	82	30
Chlorpromazine*	85	37
Pargyline*	65	0
Fluoxetine*	0	0
Krebs-Ringer	90	90
ATP§	50	50
	% Increase	
G_{D3} (disyalosyllactosy ceramide) plus lecithin	200	300–500

‡ The difference is significant $P < 0.01$.
* 5×10^{-6} M.
§ 5×10^{-4} M.

Histamine, a major component of mast cell and RBL cell granules, did not affect the binding of serotonin to proteins in the 15–60% ammonium sulfate fraction. After dialysis and fractionation on Sephadex G-200, however, serotonin binding capacity of the proteins in peak II was inhibited (80%) by high concentrations of histamine (10^{-4} M), while that of the proteins in peak I was hardly affected. Some variability observed between different preparations may be due to incomplete removal of endogenous histamine, which is generally ~10 times more concentrated than serotonin.

We also tested the effect of ATP on the binding of serotonin to proteins derived from RBL cells because ATP has been found to inhibit the binding of serotonin to proteins of neuronal (brain and myenteric plexus) and non-neuronal (parafollicular cells of sheep thyroid and blood platelets) origin (5, 16, 27). This nucleotide (500 μM) caused 50% inhibition of serotonin binding to both of the Sephadex G-200 protein peaks. Inhibition by ATP is therefore not specific to neuronal proteins and is most probably due to the interaction of the nucleotide with the ligand (31).

Effect of Gangliosides

Since serotonin binding to SBP isolated from neuronal tissue or from thyroid (28) was shown to be enhanced (three- to fivefold) in the presence of G_{D3} (disyalosyllactosyl ceramide) and lecithin, we tested the effect of these lipids on the RBL cell proteins that bind serotonin. The ganglioside-lecithin mixture was found to enhance the binding of serotonin by protein in peak I by a factor of 2 and to produce a three- to fivefold increment in serotonin binding by proteins in peak II (Table II). The RBC cell proteins thus resemble neuronal SBP with repect to potentiation of serotonin binding by gangliosides.

Electrophoresis

Proteins eluted from Sephadex peak I or from Con-A-Sepharose (50 μg) were subjected to electrophoresis on SDS-mercaptoethanol acrylamide gels and stained for proteins and glycoproteins. Both preparations were quite heterogeneous as judged by Coomassie Blue staining; however, no material was found in the gels corresponding to a molecular weight between 10,000–40,000. (Mast cell chymase has a molecular weight of 23,000) (36). In both preparations, specific staining for glycoprotein revealed the presence of only two glycoproteins; a doublet with a molecular weight of ~150,000. In addition, a very fast migrating band that stained with Schiff's reagent but not with Coomassie Blue was present only in material from Sephadex peak I. This band was not found in the purified glycoprotein eluted from Con A-Sepharose. When proteins eluted in peak II were subjected to electrophoresis and stained similarly, no glycoprotein could be detected; however, since this stain is relatively insensitive, peak II could possibly contain low concentrations of glycoprotein(s) that do not bind to Con A.

To isolate the SBPs on gels, the complex of protein-Fe^{+2}-[^{3}H]serotonin was prepared by incubating protein (50 μg) with Fe^{+2} (0.1 mM) and [^{3}H]serotonin (10^{-5} M). The complex (9,500 cpm), separated from free serotonin on a Sephadex G-50 column, was applied to 5% acrylamide gels. Most of the recovered radioactivity comigrated with the marker dye (where free serotonin migrates). A small amount of radioactivity was detected at the origin of the gel. These results indicate that either the protein that binds serotonin is positively charged and

therefore migrates away from the anode, or that the complex was in an aggregated form and did not penetrate the gel. The first possibility could not be tested because the protein-[^{3}H]-serotonin-Fe^{+2} complex dissociated at the low pH required for electrophoresis of basic proteins. We tried to eliminate the effect of aggregation and the charge of the proteins on their mobility in gels by performing electrophoresis of the complex in the presence of a low concentration of SDS and mercaptoethanol; however, the complex was not stable under these conditions and we were unable to determine its mobility. This result contrasts with our previous observations (27) that the neuronal SBP-Fe^{+2}-^{3}H-serotonin complex is stable in the presence of SDS. The difference is stability between the complexes prepared from RBL cells and neurons is most probably due to the difference in the affinity of the respective binding proteins for serotonin. This result further demonstrates that different proteins are responsible for serotonin binding in neurons and RBL cells.

Preparation of Proteins that Bind Serotonin from Rat Mast Cells

A frozen-thawed homogenate of 5×10^6 purified (90%) rat mast cells (1.6 mg total protein) was further disrupted with a Polytron for 3.5 min and then centrifuged at 100,000 *g* for 60 min. The serotonin binding capacity of the 15–60% ammonium sulfate fraction obtained from this fraction was 33,400 cpm/mg protein. Binding of [^{3}H]serotonin was linear with respect to protein concentration and, as with protein from RBL cells, required the presence of Fe^{+2}. Thus, proteins that bind serotonin were also present in mast cells and seemed similar to those of RBL cells; however, since RBL cells could be obtained in greater abundance, the properties of binding proteins from RBL cells were studied more extensively.

Radioautography

[^{3}H]Serotonin was localized to RBL cells and mast cells by electron microscope radioautography (Table III and IV; Figs. 3, 4 and 5). In vivo, none of the extracellular elements surrounding mast cells concentrated the labeled amine. Neighboring fibroblasts also failed to concentrate [^{3}H]serotonin. Within mast cells, a statistical analysis of the distribution of radioautographic silver grains revealed labeling only of mast cell granules (Table III). In heavily labeled cells (Fig. 4), all granules appeared to be labeled. The pattern of labeling of RBL cells in vitro was essentially similar to that of mast cells (Fig. 5). Although these cells have vacuolated-appearing granules, labeling was confined to the granules (Table IV) and all granules seemed to be capable of becoming labeled.

DISCUSSION

We undertook this study to determine whether RBL cells contain serotonin binding proteins and whether such proteins are similar to those of neurons. We also sought to derive information about the mechanisms of serotonin storage in basophils, RBL, and mast cells, and to gain insight into how differential release of mediators thought to be stored in mast cell granules can be effectuated. We found at least two proteins that specifically bind [^{3}H]serotonin in RBL cells. These proteins differ in molecular weight and thus elute in separate peaks from Sephadex G-200. Peak I protein has only a single dissociation constant when complexed with [^{3}H]serotonin ($K_D = 1.9 \times 10^{-6}$ M) while peak II protein has two ($K_{D1} = 4.5 \times 10^{-8}$ M; $K_{D2} = 3.9 \times 10^{-6}$ M); moreover, peak I protein appears to be a PAS-demonstrable glycoprotein that binds to Con A-Sepharose while peak II proteins are not. If the proteins eluted in peak II, therefore, are glycoproteins, they must have binding specificity for lectins other than Con A and must be present in a form or amount not detected by PAS. The proteins of the two peaks also differ with respect to reserpine sensitivity and ease of extraction from the cells. Compared to peak II protein, binding of serotonin to protein of peak I is more inhibitable by reserpine, and peak I protein requires more vigorous cell homogenization to be extracted. It can be concluded, therefore, that RBL cells contain at least two specific serotonin binding proteins. Rat mast cells also contain serotonin binding proteins that are present in the same ammonium sulfate fraction as RBL proteins and also require Fe^{+2} for binding.

Yurt and Austen (36) have shown that high concentrations of serotonin inhibit mast cell granule chymase activity presumably by binding to that enzyme. Chymase in turn binds to heparin, and these investigators envision an intragranular complex of serotonin bound to chymase bound to heparin. Based on molecular weight, neither of the two types of serotonin binding protein we have found corresponds to chymase or heparin; nor is heparin involved in the binding of serotonin by peak I or peak II proteins. Chymase is smaller and apparently has a much lower affinity for serotonin than the proteins of peaks I and II (36); however, if chymase aggregates to form multimers that have higher affinity for serotonin (the affinity

TABLE III

[^{3}H]Serotonin Grain Distribution in Rodent Mast Cells

Item	% Grains	% Circles	RSA (% Grains/% Circles)	Observed grains	Expected grains	X^2
Extracellular connective tissue elements (collagen + matrix)	2.2	42.6	0.05	24	467	420
Fibroblasts	0	1.0	0	0	10	10.0
Mast cell						
Granules	73.0	25.0	2.9	801	274	1014*
Cytosol + endoplasmic reticulum	21.2	20.6	1.0	233	225	0.3
Nucleus	2.6	9.9	0.3	29	108	58
Mitochondria	0	0.1	0	0	2	2
Golgi apparatus	0.9	0.7	1.2	10	8	0.5
				$\epsilon = 1097$		$\epsilon = 1505$‡

* $P < 0.001$. Observed greater than expected (labeled).
‡ $P < 0.001$. The distribution differs from random.

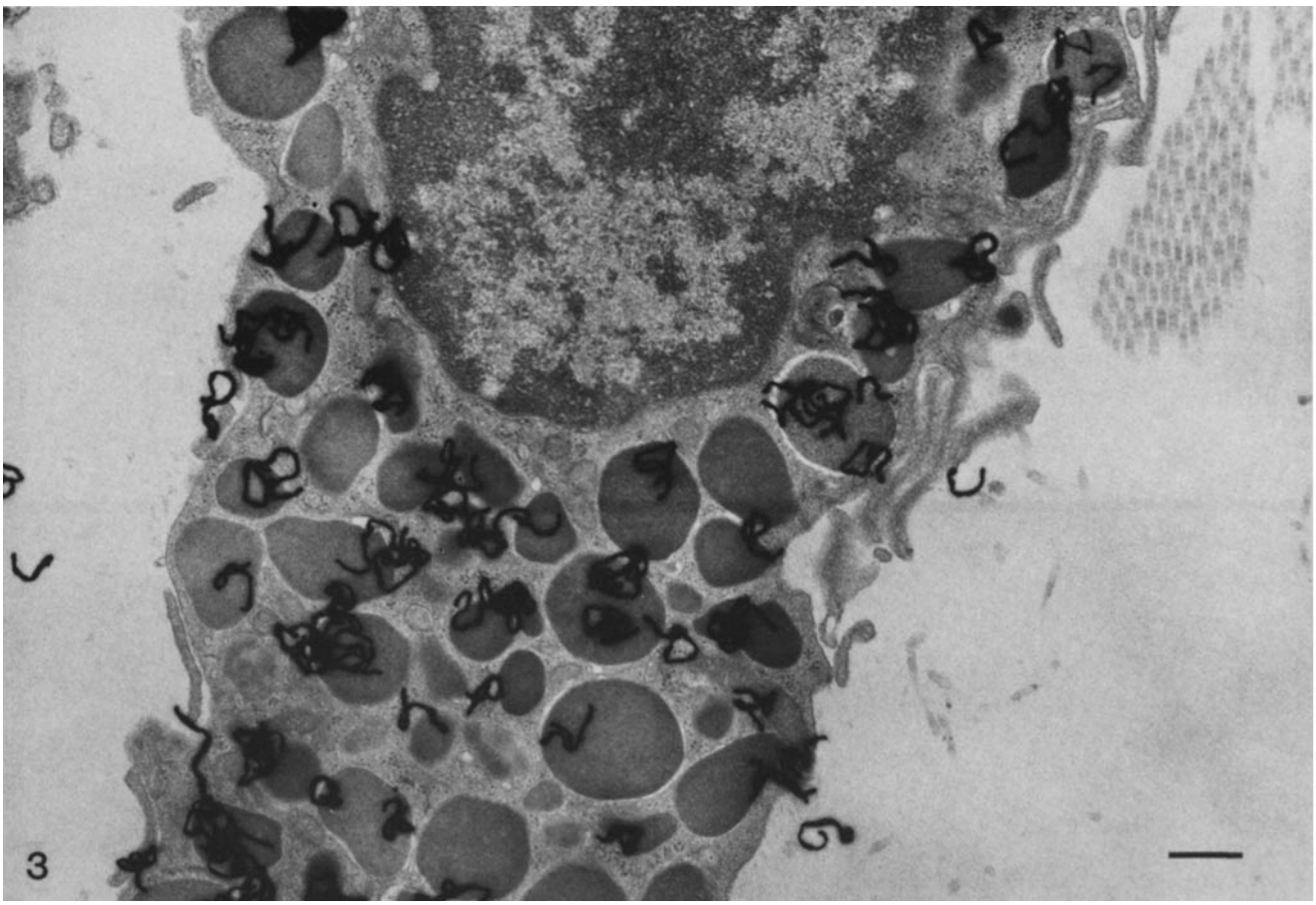

FIGURE 3 An electron microscope radioautograph of a portion of the cytoplasm of a mast cell labeled with [^{3}H]serotonin. Note the apparent association of silver grains with the amine storage granules and the paucity of grains over the nucleus or other cytoplasmic structures; however, because of the uncertainty of identification of the radioactive source for each silver grain, a statistical analysis of grain distribution (Tables III and IV; 35) was done to determine which mast cell components were labeled in these experiments. Only granules are labeled. Bar, 0.5 μm. × 20,000.

would have to increase 100-fold), then chymase could contribute to serotonin binding by peak I proteins. Since no material was found in SDS gels of material derived from peak I that migrates at 23,000 daltons (chymase), it seems unlikely that this material contains a chymase like that of mast cells.

The serotonin binding proteins of RBL cells do not resemble the SBP of neurons because the neuronal protein is not a glycoprotein, has a higher affinity for serotonin and differs in molecular weight (5, 16, 21). Although the neuronal serotonin binding protein is probably a component of synaptic vesicles (21, 29), the intracellular localization of the RBL serotonin binding proteins is unclear; however, the vigorous homogenization necessary to release peak I protein suggests it is sequestered within a cellular organelle. The sensitivity of peak I protein to reserpine suggests that it may be involved in serotonin storage. In fact, the observation that the proteins of both peaks can be liberated into solution without adding a detergent indicates that these proteins are not integral components of membranes.

The presence of two intracellular proteins that bind serotonin and that require different conditions for extraction, suggests that the two proteins are derived from different intracellular compartments. If both proteins are involved in serotonin binding within the cells, this would, in turn, imply that there are two different storage compartments for serotonin. These compartments might correspond to an intragranular pool and a cytosol pool of serotonin. The difficult-to-extract, reserpine-sensitive peak I protein would be a prime candidate for the intragranular serotonin storage protein. The more readily liberated peak II protein might be located in the cell cytosol. Other possibilities, however, may be more likely. Both compartments might be intragranular. One type of granule containing peak II protein, would then be more fragile than that containing protein of peak I. Another alternative would be the localization of both types of proteins uniformly in all granules, with peak I protein preferentially associated with the granular membrane (although not an integral protein) while peak II protein might be free in the granular core. Such a difference could account for the greater difficulty encountered in liberating peak I than peak II proteins by homogenization. Radioautographic studies of the intracellular localization of [^{3}H]-serotonin are most consistent with the last possibility. Essentially all of the labeled amine was found to be stored in granules of both RBL cells and mast cells; moreover, all of the granules appeared to store [^{3}H]serotonin (Table III and IV; Figs. 3, 4, and 5). No evidence was obtained to favor storage in the cytosol, despite inhibition of monoamine oxidase, and no differences between populations of granules could be discerned.

The reserpine insensitive peak II protein could help explain observations concerning the effect of reserpine on delayed type hypersensitivity (DTH) in mice. When mice are treated with reserpine, their mast cells are depleted of serotonin, and DTH

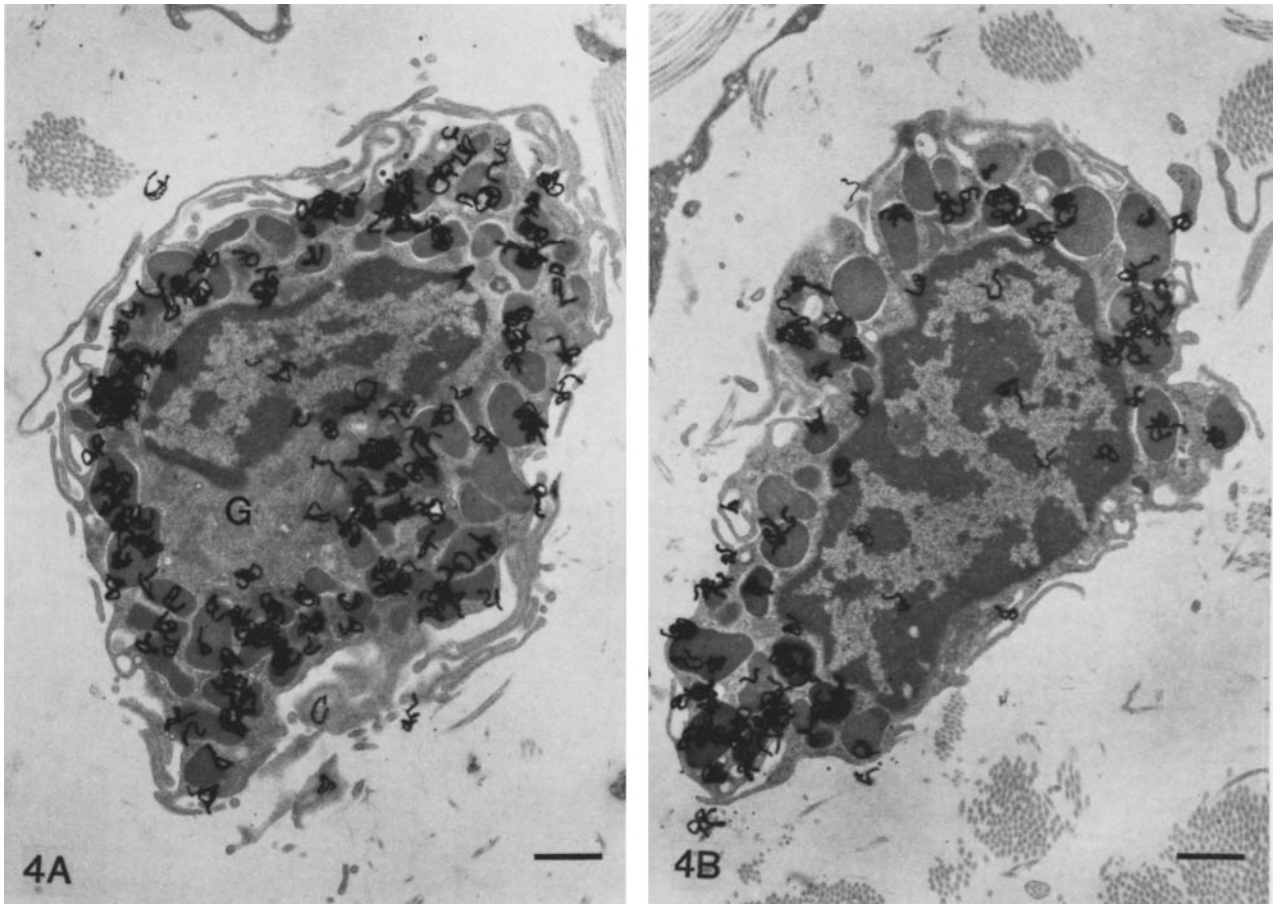

FIGURE 4 Electron microscope radioautographs of two mast cells labeled with [^{3}H]serotonin. Despite the heavy labeling of these cells, note the restriction of silver grains to the cells themselves. Note also that virtually every granule of each cell is labeled. Silver grains are scarce over other cellular structures such as the Golgi apparatus (G). Bar, 1.0 μm. × 8,500.

TABLE IV

[^{3}H]Serotonin Grain Distribution in RBL Cells

Item	% Grains	% Circles	RSA (% Grains/% Circles)	Observed grains	Expected grains	X^2
Extracellular space	1	15	0.07	2	33	29
RBL cell						
Granules	58	10	5.8	130	22	530*
Cytosol + endoplasmic reticulum	16	36	0.4	36	80	24
Nucleus	12	21	0.6	26	47	9
Mitochondria	0	2	0	0	4	4
Golgi apparatus	6	4	1.5	14	9	3
Microvilli	7	11	0.6	15	25	4
				$\epsilon = 223$		$\epsilon = 604$‡

* $P < 0.001$. Observed greater than expected (labeled).
‡ $P < 0.001$. The distribution differs from random.

is abolished (1, 13). This is because release of serotonin by mast cells is required to increase vascular permeability to aid in delivery of leukocytes to sites of DTH. Monoamine oxidase inhibition eliminates this action of reserpine (13). Thus, it seems that reserpine releases serotonin intracellularly from a protected compartment to one where serotonin is vulnerable to catabolism by monoamine oxidase. It is possible that reserpine releases serotonin from peak I protein but does not affect serotonin bound to protein of peak II. The peak II reserpine-insensitive SBP thus could account for mast cell's retaining sufficient serotonin to mediate the vascular changes of DTH when reserpine is given to a monoamine oxidase inhibited animal. If so, it would follow that serotonin bound to peak II protein may be susceptible to attack by monoamine oxidase. The intracellular localization of both proteins remains to be determined.

No matter what the final localization of peak I and peak II proteins ultimately turns out to be, the presence of two types

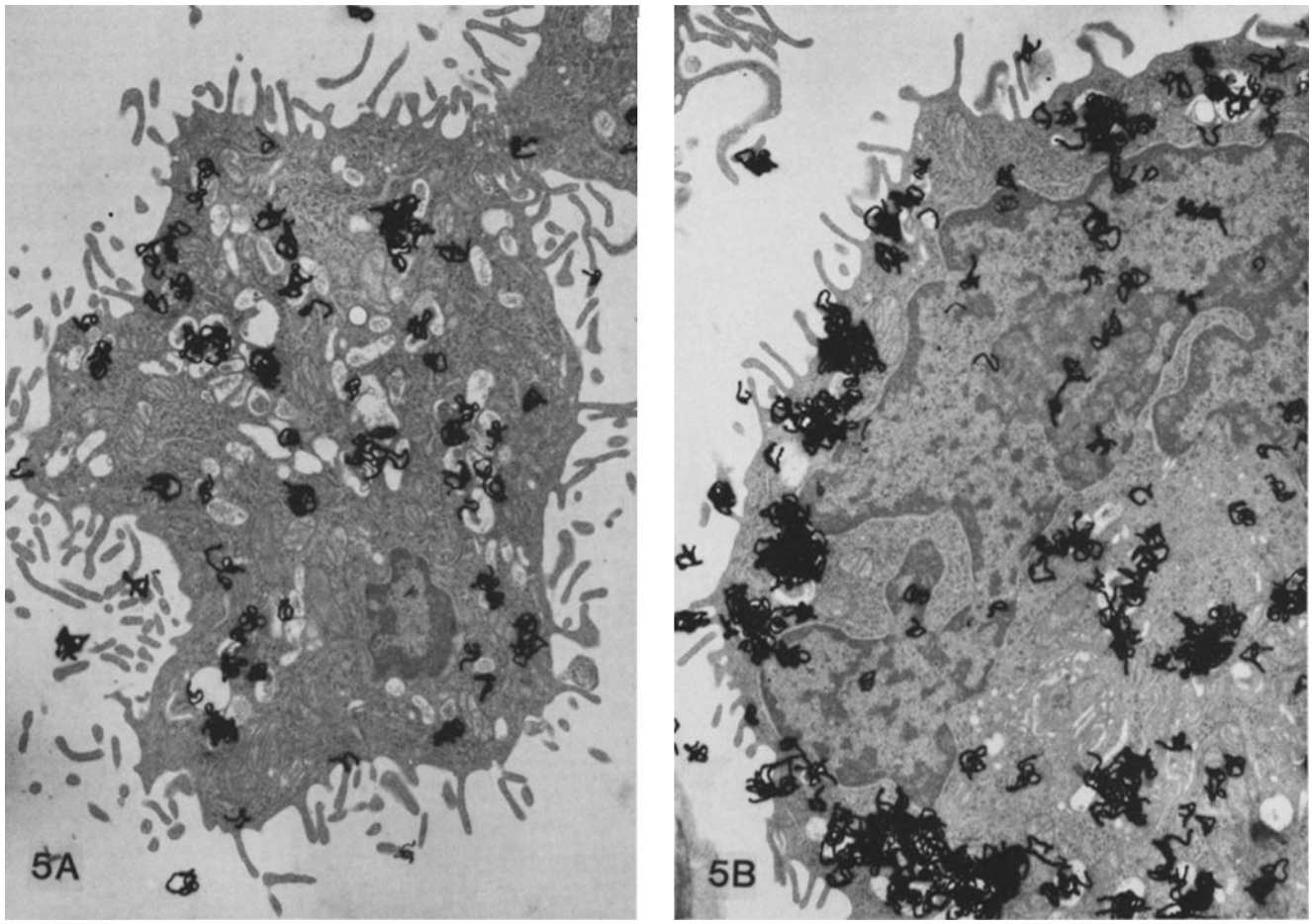

FIGURE 5 Electron microscope radioautographs of two RBL cells labeled with [^{3}H]serotonin. The granules of these cells appear quite vacuolar and are incompletely filled with wispy granular material. The restriction of label to these granules and their structure can be seen in A. The cell in B has been overexposed. Essentially all of the granules are labeled. Bar, 1.0 μm. × 10,000.

of SBPs and the possibility of the different storage pools for this amine could explain how serotonin and not histamine might be differentially released from mast cells.

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REFERENCES

1. Askenase, P. W., S. Bursztajn, M. D. Gershon, and R. K. Gershon. 1980. T cell-dependent mast cell degranulation and release of serotonin in murine delayed-type hypersensitivity. *J. Exp. Med.* 152:1358–1374.
2. Askenase, P. W., and T. C. Theoharides. 1980. Differential release of serotonin and histamine in the mast cell. *Fed. Proc.* 39:905.
3. Benditt, E. P., L. R. Wong, M. Arase, and E. Roeper. 1955. 5-Hydroxytryptamine in mast cells. *Proc. Soc. Exp. Biol. Med.* 90:303–304.
4. Bernd, P., M. D. Gershon, E. A. Nunez, and H. Tamir. 1981. Separation of dissociated thyroid follicular and parafollicular cells: association of serotonin binding protein with parafollicular cells. *J. Cell Biol.* 88:499–508.
5. Bernd, P., E. A. Nunez, M. D. Gershon, and H. Tamir. 1979. Serotonin binding protein: characterization and localization in the parafollicular cells of the sheep thyroid. *In* Molecular Endocrinology. I. McIntyre and M. Szelke, editors. Elsevier/North Holland Biomedical Press, New York. 123–132.
6. DaPrada, M., and A. Pletscher. 1968. Isolation of 5-hydroxytryptamine organelles of rabbit blood platelets: physiological properties and drug induced changes. *Br. J. Pharmacol.* 34:591–597.
7. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analyses of the major polypeptides of the human erythrocytes membrane. *Biochemistry.* 10:2607–2617.
8. Falck, B., and C. Owman. 1968. 5-Hydroxytryptamine and related amines in endocrine cell systems. *Adv. Pharmacol.* 6A:211–231.
9. Fewtrell, C., A. Kessler, and H. Metzger. 1979. Comparative aspects of secretion from tumor and normal mast cells. *Adv. Inflammation Res.* 1:205–221.
10. Fillion, G., D. Beaudoin, J. C. Rouselle, and J. Jacob. 1980. [^{3}H]-5HT binding sites and serotonin sensitive adenylate cyclase in glial cell membrane fractions. *Brain Res.* 198:361–374.
11. Gershon, M. D. 1977. Biochemistry and physiology of serotonergic transmission. *In* Handbook of Physiology. J. M. Brookhart, V. B. Mountcastle, E. R. Kandel, and S. R. Geiger, editors. American Physiology Society, Bethesda, MD.
12. Gershon, M. D. 1981. The enteric nervous system. *Annu. Rev. Neurosci.* 4:227–272.
13. Gershon, R. K., P. W. Askenase, and M. D. Gershon. 1975. Requirement for vasoactive amines for production of delayed-type hypersensitivity skin reactions. *J. Exp. Med.* 142:732–747.
14. Gershon, M. D., and E. A. Nunez. 1970. Histochemical and radioautographical studies of serotonin and parafollicular cells in the thyroid gland of prehibernating bat. *Endocrinology.* 86:160–166.
15. Gershon, M. D., and E. A. Nunez. 1976. Uptake of biogenic amines by thyroid gland. *Mol. Cell. Endocr.* 5:169–180.
16. Gershon, M. D., and H. Tamir. 1981. Serotonin binding protein: role in transmitter storage in central and peripheral neurons. *In* Serotonin: Current Aspects of Neurochemistry and Function. B. Haber, editor. Plenum Press, New York. 37–49.
17. Green, J. P. 1966. Synthesis, uptake and binding of histamine and 5-hydroxytryptamine in mast cells. *In* Mechanisms of Release of Biogenic Amines, U.S. Von Guler, S. Rosell, B. Uvnas, editors. Pergamon Press, New York. 125–145.
18. Hertz, L., F. Baldwin, and A. Schousboe. 1979. Serotonin receptors on astrocytes in primary culture: effect of methysergide and fluoxetine. *Can. J. Physiol. Pharmacol.* 57:223–226.
19. Isersky, C., H. Metzger, and D. M. Buell. 1975. Cell cycle-associated changes in receptors for IgE during growth and differentiation of a rat basophilic leukemia cell line. *J. Exp. Med.* 141:1147–1162.
20. Jaim-Etcheverry, G. J., and L. M. Zieher. 1968. Cytochemical localization of monoamine stores in sheep thyroid gland at the electron microscope level. *Experientia (Basel).* 24:593–595.
21. Jonakait, G. M., H. Tamir, A. R. Gintzler, and M. D. Gershon. 1979. Release of serotonin and its binding protein from enteric neurons. *Brain Res.* 174:55–69.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T_4. *Nature (Lond.).* 227:680–685.
23. Lagunoff, D., and G. Y. Chi. 1980. Cell biology of mast cells and basophils. *In* Cell

Biology of Inflammation: Handbook of Inflammation. G. Weissmann, editor. Elsevier, Amsterdam. 2:217–265.
24. Mahadik, S. P., A. Korenovsky, and M. M. Rapport. 1976. Slab gel analysis of polypeptide components of rat brain subcellular organelles. *Anal. Biochem.* 76:615–633.
25. Morrison, D. C., J. F. Roser, P. M. Henson, and C. G. Cochrane. 1974. Activation of rat mast cells by low molecular weight stimuli. *J. Immunol.* 112:573–582.
26. Salpeter, M. M., L. L. Bachmann, and E. E. Salpeter. 1969. Resolution in electron microscope autoradiography. *J. Cell Biol.* 41:1–20.
27. Tamir, H., R. Bebirian, F. Muller, and D. Casper. 1980. Differences between intracellular platelets and brain protein that bind serotonin. *J. Neurochem.* 35:1033–1044.
28. Tamir, H., W. Brunner, D. Casper, and M. M. Rapport. 1980. Enhancement of the binding of serotonin to serotonin binding protein (SBP) by gangliosides. *J. Neurochem.* 34:1719–1724.
29. Tamir H., and M. D. Gershon. 1979. Storage of serotonin and serotonin binding protein in synaptic vesicles. *J. Neurochem.* 33:35–44.
30. Tamir, H., A. Klein, and M. M. Rapport. 1976. Serotonin binding protein: enhancement of binding by Fe^{+2} and inhibition of binding by drugs. *J. Neurochem.* 26:871–878.
31. Tamir, H., and K. P. Liu. 1981. On the nature of the interaction between serotonin and serotonin binding protein: effect of nucleotides, ions and sulfhydryl reagents. *J. Neurochem.* 38:135–141.
32. Theoharides, T. C., and P. W. Askenase. 1980. Amitriptyline permits release of serotonin but not histamine from stimulated mast cells. *Eur. J. Cell. Biol.* 22:181.
33. Theoharides, T. C., and W. W. Douglas. 1978. Somatostatin induces histamine secretion from rat peritoneal mast cells. *Endocrinology.* 102:1637–1640.
34. Uvnas, B. 1978. Chemistry and storage function of mast cell granules. *Dermatology* 71:76–80.
35. Williams, M. W. 1969. The assessment of electron microscopic autoradiographs. *Adv. Opt. Electron Microsc.* 3:219–272.
36. Yurt, R., and K. F. Austen. 1977. Preparative purification of the rat mast cell chymase: characterization and interaction with granules components. *J. Exp. Med.* 146:1405–1419.