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Evidence for a Serotonin Uptake System in Isolated Bovine Pinealocyte Suspensions

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SUMMARY

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Serotonin uptake was investigated in bovine pinealocyte suspensions. Pinealocytes were disaggregated from calf pineal glands by enzymatic treatment with papain followed by mechanical disruption of the tissue fragments. Cells were resuspended in Hank's balanced salt solution which contained 12.5 μM nialamide to inhibit monoamine oxidase activity present in the pinealocytes. The uptake of radiolabeled serotonin as a function of concentration exhibited saturation phenomena when high (10 μM–5 mM) concentrations of serotonin were used. Estimates of the fraction of serotonin bound to the cell membrane were attempted by three methods: (1) lysing cells by exposing them to a hypotonic medium after incorporation of radiolabeled serotonin in standard medium; (2) exposing cells to media of increasing osmolarity in the presence of radiolabeled serotonin; and (3) displacing radiolabeled serotonin from nonspecific binding sites on the cell membrane by cold serotonin. Estimates of the fraction of uptake of serotonin contributed by simple diffusion were made by adding an excess of unlabeled serotonin to 0.76 μM radiolabeled serotonin to saturate specific carrier sites and nonspecific membrane binding sites. Tissue-to-medium ratios exceeded 1 with all concentrations of serotonin used.

INTRODUCTION

Although the pineal has been established as an endocrine gland, not all of its secretory products have been identified. At present, melatonin is considered to be the primary pineal secretory product of the indoleamine series. It has been generally assumed that the main purpose or function of serotonin in pinealocytes is to serve as a precursor for the formation of melatonin. Some evidence from studies in nonmammalian species suggests, however, that serotonin may itself be a secretory product of the pineal gland. In several species it has been demonstrated that pineal granular vesicles contain one or more proteins which could bind serotonin (1, 2, 3) or its precursors and metabolites (2, 4, 5). The presence of serotonin has been demonstrated directly in the secretory granules of the secretory rudimentary photoreceptor (SRP) cells in lizards (6) and parakeets (7). Although it has not been shown that serotonin is stored in secretory vesicles in the mammalian pineal gland, the possibility exists that serotonin may have important functions in the mammalian pineal apart from its role as a precursor of melatonin.

If serotonin is an important product of pineal gland metabolism and is secreted in response to some type of stimulus, it would be important to regulate intrapinealocyte concentrations of this indoleamine. Regulation of the intracellular concentration of serotonin implies that the pinealocyte may have the ability to catabolize, synthesize, and perhaps even transport serotonin across the pinealocyte membrane.

The aspect of intracellular serotonin regulation with which this paper is concerned is that of plasma membrane transport. Investigations of a possible transport system for serotonin in the pineal gland were made with bovine cell suspensions.

MATERIALS AND METHODS

Sterilization procedures. All labware used for tissue collection and preparation procedures was sterilized by exposure to dry heat (350°F) for at least 3 h.

Tissue washing, mincing, and cell dispersion procedures were all done under an ultraviolet hood (Lab Con Co., uv hood).

Source of tissue. Fresh calf pineal glands were obtained at a local slaughterhouse on the day of each experiment. The glands were excised 10 to 40 min postmorten and immediately placed in sterile, ice-cold Hank's balanced salt solution (HBSS; GIBCO) containing 1% (v/v) penicillin–streptomycin (GIBCO, 10,000 units/ml penicillin

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base, 10,000 μg/ml streptomycin base) and 0.5% bovine serum albumin.

Most calves used were between 1 and 4 weeks old and all were less than 3 months old. Almost all of the animals were males, with the greater proportion being Herefords.

Slaughter routinely began at about 6:30 AM, and pineal gland collection was usually completed between 7 and 8 AM. Calves were exposed to a variety of different and nonuniform lighting schedules prior to slaughter. However, all the animals had been exposed to at least 4 h of continuous darkness approximately 15–90 min before the time of death.

Identification of tissue using histochemical techniques. Glands to be used for histological studies to verify the presence of pineal tissue were immediately placed in 10% formalin solution for fixation. Slides of the tissue were subsequently prepared by routine histological methods (courtesy of the Department of Surgical Pathology, University of Rochester).

Preparation of tissue. Pineal glands (usually 22–48 per experiment) were rinsed several times under aseptic conditions in petri dishes with HBSS containing 1% penicillin–streptomycin. After the final rinse, the capsule of each pineal gland was removed, and the stalk of each gland was trimmed free of attendant midbrain elements under a dissecting microscope. The tissue was rinsed thoroughly by successive passage through five to six washes in sterile HBSS. After the final rinse, the tissue was finely minced and again passed through several changes of wash solution, during which small bits of tissue and cells were poured off and discarded. The minced tissue was rinsed until the wash solution appeared clear.

Cell dispersion and isolation procedures. Initially two different enzymatic procedures were tested for the dispersal of pineal cells. Collection, cleaning, and treatment of tissue up to the time of enzyme digestion were identical for both procedures.

(A) Procedure using papain: This method consisted of exposing finely minced pineal tissue to 10 ml of HBSS in a 50-ml Nalgene Erlenmeyer flask containing calcium, magnesium, 1% penicillin–streptomycin, 0.0125% (w/v) papain (Sigma, type 3), and 0.004% (w/v) cysteine hydrochloride. The tissue–enzyme suspension was then placed in a water bath at 37°C for 14 min while the flask was slowly agitated at 60 oscillations per minute. After incubation, 5 ml of ice-cold HBSS was pipetted into the incubation flask, and the clumps of tissue were disrupted by passing them through five or six siliconized pipets with decreasing bore diameters. The resulting tissue suspension was filtered sequentially through a large nylon mesh (Tetko, Inc., Lancaster, N.Y.) (150 μm) and then through a smaller mesh (30 μm) secured by a sterile stainless-steel filter support (Millipore) (25 mm). The cell suspension was forced gently through the filtering apparatus from a disposable 20-ml plastic syringe. The resulting filtrate was layered over two 20-ml portions of a solution containing cold HBSS without calcium and magnesium, 5% bovine serum albumin (BSA), and 1% penicillin–streptomycin. The tubes were then centrifuged at 60*g* for 10 min (International Portable Refrigerated Centrifuge Model PR-2). The resulting supernatants were aspirated, and the loosely packed cells were resuspended in an appropriate amount of HBSS containing calcium and magnesium. Generally, sufficient incubation medium was added to produce a cell concentration of 2×10^6 cells/ml. One pineal reliably produced 2 ml of a final cell suspension containing 2×10^6 cells/ml.

If centrifugation of the final cell filtrate in 5% BSA was carried out above 60*g* or for longer than 10 min, occasional clumps of cells appeared that were difficult to disperse. When clumping occurred, the cell suspension was poured through a 150-μm nylon mesh before use. In each experiment cells samples were routinely set aside, refrigerated, and used for determination of cell viability and yield with a hemocytometer.

(B) Procedure using trypsin: A modified cell dispersion procedure using 0.1% trypsin was employed (8).

The enzyme solution was prepared by dissolving 300 mg of trypsin (Sigma, type 3) in 50 ml of HBSS containing 0.5% BSA and 1% penicillin–streptomycin. After sterilization by filtration, 1-ml aliquots of the solution were placed in capped, sterile polypropylene test tubes and stored at −10°C. When 5 ml of HBSS was added to 1 ml of the stock enzyme solution, the final concentration of trypsin was 0.1%. The enzyme digestion was initiated by adding four to six minced pineal glands to a polypropylene test tube containing 6 ml of the 0.1% trypsin solution. The capped tubes were incubated at 37°C for 30 min with occasional agitation. After 30 min, the trypsin solution was removed and 6 ml of a 2 mM EDTA solution in calcium- and magnesium-free HBSS was immediately poured into the test tube. The minced pineal gland–EDTA mixture was returned to the water bath and reincubated at 37°C for 5 min with occasional agitation. After removal of the samples from the water bath, the EDTA solution was poured off and 3–5 ml of calcium- and magnesium-free HBSS was pipetted into each test tube to wash the tissue. This rinse procedure was repeated three times.

Cells were dispersed in calcium- and magnesium-free HBSS by mechanically forcing the tissue clumps through a Pasteur pipet. The resulting cell suspensions were then layered on calcium- and magnesium-free HBSS containing 4% BSA in 15-ml glass, screw-capped culture tubes and centrifuged at 60–70*g* for 10 min (International Portable Refrigerated Centrifuge Model PR-2). The supernatants were aspirated and the cells were resuspended in an appropriate amount of HBSS containing calcium and magnesium.

Electron microscopy of isolated pinealocytes. It is important to verify that the plasma membranes of the dispersed cells are intact and to evaluate the purity of the final cell suspension. It is also possible that nerve endings may be attached to the pinealocytes. To resolve these issues, transmission electron microscopy was used.

Minced pineal glands were digested and the cells dispersed by the papain method described above.

Tissue was prepared for electron microscopy as follows: Isolated, resuspended cells were fixed with 2% phosphate-buffered (pH 7.5) glutaraldehyde and placed in a 1% osmium tetroxide solution (pH 7.5). The preparation was then rapidly dehydrated in ethanol and infiltrated with and embedded in Dow epoxy resin. The embedded tissue

was sectioned with a Porter–Blum ultramicrotome and the sections were stained with uranyl acetate and lead citrate. Subsequent examination and slides of the tissue sample were made with an RCA EMU-3H electron microscope. No cells observed demonstrated a damaged plasma membrane or exhibited synaptosomal contamination. Some glial contamination was observed. A quantitative estimate of the impurities was not attempted. However, it was suggested (Dr. Michael Sheridan, Department of Anatomy, University of Rochester, personal communication) that the glial contamination in the pinealocyte preparations was insignificant.

Estimation of cellular volume. Cell diameters were estimated from 17 electron micrographs and averaged. The volume of a typical pinealocyte, assuming that it is a perfect sphere, would then be

$$(4/3)\cdot\pi(13\ \mu)^3$$

or 9.2×10^{-9} ml/cell. This calculation was used to estimate tissue-to-medium ratios.

Preparation of ^{3}H-5HT. Hydroxytryptamine binoxalate, 5-[1,2-^{3}H(N)] (serotonin binoxalate, [1,2-^{3}H(N)]), 24.0–29.2 Ci/mmol, and hydroxytryptamine creatinine sulfate, 5-[1,2-^{3}H(N)], 28.2 Ci/mmol, were purchased from New England Nuclear Corp. Prior to use, the purity of the ^{3}H-5HT was tested by unidimensional acending paper chromatography in an *n*-butanol, glacial acetic acid, and water (4/1/5) solvent system. Before each experiment, the ^{3}H-5HT was either diluted with HBSS or mixed with cold serotonin (Sigma) to produce the appropriate concentration of ^{3}H-5HT.

Incubation conditions and procedures. Standard incubation conditions consisted of resuspending isolated cells in HBSS containing 12.5 μM nialamide (Sigma) and 1% (v/v) penicillin–streptomycin. One or 0.9 ml of the resulting cell suspension was pipetted into 15-ml polypropylene beakers at room temperature. The beakers were then preincubated at either 37 or 0°C in an open, shaking water bath for 5 min before the addition of 50 or 100 μl of the appropriate concentration of ^{3}H-5HT.

Incubations were usually terminated after 30 s by pouring the contents of each beaker into individual glass test tubes containing 3 ml of ice-cold HBSS. The test tubes were rapidly transferred to a centrifuge and centrifuged for exactly 3 min at 0°C at 800*g*. The 4 ml of radioactive supernatant was rapidly poured off, and the resulting pellets were resuspended in 2 ml of ice-cold HBSS. After resuspension of the cell pellets in fresh HBSS, centrifugation was repeated. The wash procedure was performed twice. After the final wash, the supernatant was discarded, and the mouths of the test tubes were blotted. One-half milliliter of NCS tissue solubilizer (Amersham Searle Corp.) was added directly to each test tube, the tubes were stoppered, and the pellets were allowed to digest for 2 h at 50°C. The cell digests were then transferred to glass scintillation vials containing 10 ml of Bray's (9) solution, and radioactivity was determined by liquid scintillation spectrometry. Radioactivity from samples incubated at 0°C was routinely subtracted from the total counts obtained at 37°C.

Transport into vesicles or cells reflects uptake into an osmotically reactive space. If uptake decreases with increasing osmolarity of the incubation medium, uptake must be intracellular. On the other hand, binding should not be significantly affected with increases in osmolarity.

Membrane binding was determined in incubation media in which the osmolarity was changed by the addition of various concentrations of sucrose. Osmolarities ranged from 0.285 to 1.00 osmol. Cells were exposed to the varied osmotic environments for at least 40 min before incubation at 37°C. Reactions were terminated by diluting the beaker contents with solutions of the same osmolarity as that in the incubation flasks. All wash solutions during centrifugation maintained the same osmolarity as that in the incubation beaker.

Determination of cell viability. Cell viability was determined routinely after each experiment by trypan blue dye (0.2%) exclusion. Percentage viability was expressed as follows:

$$\%\ \text{viable cells} = \left(1 - \frac{\text{No. of stained cells/0.1 mm}^3}{\text{total cell count/0.1 mm}^3}\right) \times 100.$$

Identity of radioactivity incorporated into cells. The percentage of total radioactivity present as unchanged ^{3}H-5HT was estimated by unidimensional ascending paper chromatography. Incubations were terminated by centrifugation and the resulting pellets were each resuspended in 200 μl of 0.1 N HCl before sonication at 0°C for 10 s (Bronwill Biosonik IV, 4-mm probe, energy setting "Lo-80"). The resulting solutions from various test tubes were combined and centrifuged at 15,000*g* for 15 min. Aliquots of the supernatant were applied to a sheet of Whatman SG81 paper (7 x 7 in.) along with samples of ^{3}H-5HT that had been incubated in cell-free medium. Samples of 0.1% solutions of nonradiolabeled serotonin and 5-hydroxyindole acetic acid (5HIAA; Aldrich Chemical Co., Inc.) were used as standards and were cochromatographed with radiolabeled compound. Chromatograms were developed in an *n*-butanol, glacial acetic acid, and water (4/1/5) solvent system for 4 h in the dark at room temperature.

After air-drying, the chromatograms were cut into strips containing radioactive samples or standards. The strips containing the standards were exposed to I_2 vapor for localization of the indole compounds. The strips containing the radioactivity were cut into 1.0-cm segments. The segments were placed in glass vials and counted in 10 ml of Bray's solution (9) by liquid scintillation spectrometry. Background radioactivity was determined by counting 1.0-cm segments just above the solvent front.

Radioenzymatic assay for serotonin in tissues. Serotonin was measured by the method of Saavedra *et al.* (10). A modification of the radioenzymatic assay procedure was employed as described by Pohl and Gibbs (11). Pineal glands were weighed after cleaning, and the cells were dispersed in the usual manner with papain. After centrifuging in calcium- and magnesium-free HBSS containing 5% BSA, the resulting cell pellets were resuspended in 0.1 N HCl. An aliquot of the suspension was immediately removed to determine cell number. The cell suspension was then homogenized by two successive sonications at 0°C and centrifuged at 6000*g* for 40 min.

Aliquots of four different dilutions of the supernatant were used for the determination of serotonin.

Radioactivity. Radioactivity was determined in a Packard Tri-Carb Model 3385 liquid scintillation spectrometer using glass counting vials containing 10 ml of Bray's solution (9). All samples were counted for 10 min, and counting efficiency was determined by automatic external standardization using a quench curve constructed with tritiated toluene.

Statistical methods. The significance of the difference between experimental groups was calculated using the *U* test (12).

A Hewlett Packard desk-top calculator system (9810 Calculator and 9862A Calculator Plotter) was used to calculate equations of lines and correlation coefficients.

Enzymes. Rat liver *N*-acetyltransferase and bovine pineal hydroxyindole-*O*-methyltransferase were generously donated by Dr. Karl M. Knigge of the Department of Anatomy, University of Rochester.

RESULTS

Comparison of papain and trypsin cell dispersion procedures. The papain and trypsin procedures were compared with regard to (1) the time required for cell dispersion and isolation, (2) the viability of cells as determined by their ability to exclude trypan blue, and (3) the ability of the isolated pinealocytes to take up tritiated serotonin. Table 1 summarizes the results of experiments carried out on 3 separate days with three different preparations.

Viability measurements disclosed no significant differences, although the viability of the cells dispersed with papain was always somewhat higher (Table 1). The trypsin procedure produced more day-to-day variation in cell counts compared to papain treatment; cell yields with trypsin were usually less than 50% of those obtained with papain. The procedure for trypsin dispersal, from initial enzymatic incubation to the final preparation of usable isolated cells, took at least 2 h longer than the papain procedure. The total time required for the papain procedure was about 40 min.

TABLE 1

A comparative study of the papain and trypsin cell dispersion procedures

Expt	Enzyme	Cell viability[a]	Cell count[b]	Average dpm after 60-min incubation with ^{3}H-5HT[c]	
				Per beaker	Per 2 × 10^6 cells
		%	*cells/beaker*		
I	Papain	84	965,000	85,556	177,101
	Trypsin	72	572,500	8,023	27,999
II	Papain	88	1,025,000	91,469	178,365
	Trypsin	85	422,500	13,601	64,331
III	Papain	85	960,000	71,462	148,640
	Trypsin	80	310,000	6,570	42,379

[a] Determined by trypan blue dye exclusion.

[b] Pineal tissue was equally divided before each experiment. Half the tissue was digested with papain and the other half was exposed to trypsin. Isolated cells from both procedures were resuspended in equal amounts of incubation medium and placed in polypropylene beakers. Each beaker contained 1 ml of cell suspension.

[c] Average of triplicates with radioactivity at 0°C subtracted from total counts obtained at 37°C.

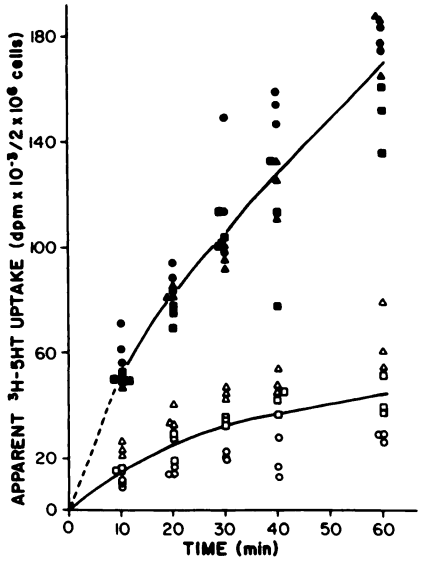

FIG. 1. *The apparent uptake of 0.074 μM tritiated serotonin into pinealocytes after either papain or trypsin treatment*

The papain (closed symbols) and trypsin (open symbols) cell dispersion procedures were compared with regard to the apparent ability of the isolated pinealocytes to take up tritiated serotonin. The apparent uptake of tritiated serotonin with time was significantly ($P < 0.001$) higher with papain-treated cells at all observed time intervals. Experiments were carried out in triplicate on 3 separate days with three different preparations. (○, ●) Experiment I; (△, ▲) experiment II; and (□, ■) experiment III. Each point represents radioactivity associated with a cell suspension obtained at 37°C after counts obtained at 0°C were subtracted.

A significant discrepancy was revealed in the ability of the two different cell groups to take up 0.074 μM tritiated serotonin (Table 1 and Fig. 1). All measurements were done in triplicate on three different preparations. Measurements of uptake at 0°C exhibited no consistent variability whether the cells were obtained by trypsin digestion or by dispersion by papain after adjustment was made for cell number. The apparent uptake of tritiated serotonin after papain was significantly higher than the uptake seen with trypsin-treated cells at 37°C. Consequently, all subsequent experiments were done with the papain dispersion technique.

Characteristics of uptake of ^{3}H-5HT by pinealocytes. The apparent uptake of 10 μM ^{3}H-5HT with time by bovine pinealocytes showed saturation kinetics (Fig. 2). The uptake of radioactivity was linear for approximately 3 min. Therefore, all subsequent experiments, except where noted, employed 30 s incubations of cell suspensions with ^{3}H-5HT to ensure that determinations were made at the initial rate. The apparent uptake of ^{3}H-5HT correlated in a linear fashion with cell number (Fig. 3) and was dose dependent; saturation was obtained with increasing amounts of serotonin (Fig. 4).

Estimation of the fraction of ^{3}H-5HT bound to the cell membrane. Estimates of the fraction of ^{3}H-5HT bound to cell membranes were initially made using two methods: (1) osmotic lysis of cells after exposure to ^{3}H-5HT

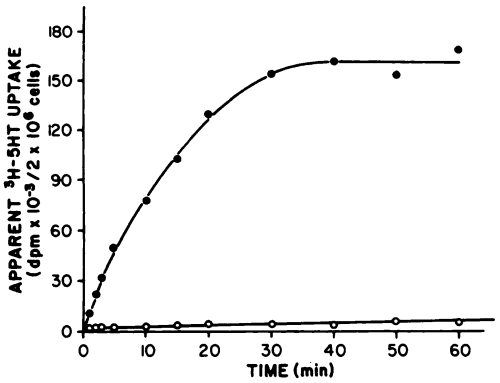

FIG. 2. *The apparent uptake of 10 μM ³H-5HT with time*

The apparent uptake of 10 μM ³H-5HT exhibited saturation kinetics. Linearity was observed for the first 3 min. (●) Radioactivity associated with cell samples obtained at 37°C after subtracting counts obtained at 0°C; (○) radioactivity recovered from cell suspension samples at 0°C. Each point is the average of duplicate determinations.

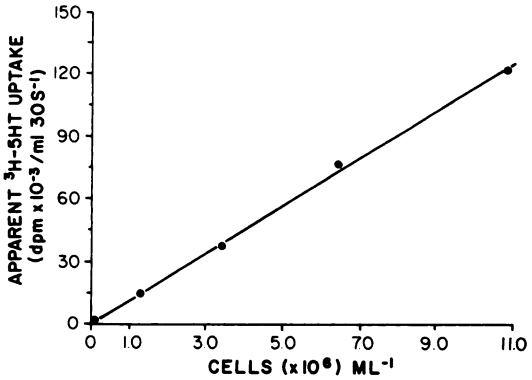

FIG. 3. *Apparent uptake of 10 μM ³H-5HT as a function of cell number*

The apparent uptake of 10 μM ³H-5HT showed a linear relationship with cell number. Points represent means of triplicate or duplicate determinations. The equation for the relationship between apparent ³H-5HT uptake and cell count was computed by the method of least squares.

and (2) modifying the osmolarity of the incubation medium with sucrose. Table 2 and Fig. 5 summarize the results.

Cells were lysed osmotically after incubation with ³H-5HT for various time periods. After the final wash, cells were resuspended in 1 ml of HBSS, 3 ml of deionized distilled water was added to each test tube, and the samples were allowed to incubate at 0°C for about 1 min. The cells were then washed twice in 2 ml of HBSS. Cells exposed to ³H-5HT at 37 or 0°C should show the same amount of radioactivity after rupture if they contain free intracellular ³H-5HT. The cells exposed to 37°C, however, always had a greater amount of radioactivity. This was interpreted to have resulted from some type of intracellular binding that occurred more readily at higher temperatures. Because of this, lysed cell samples that had been exposed to 0°C were used to estimate the amount of ³H-5HT bound to the plasma membrane.

The data in Table 2 indicate that the fraction bound to the plasma membrane after exposing cells to 10 μM ³H-5HT for 30 s is 24%. The relative percentage of ³H-5HT bound decreases with increasing incubation time. After 80 min, when ³H-5HT uptake is at equilibrium, membrane binding is less than 2%. However, a discrepancy was observed between lysed cells incubated at 37°C for 20 and for 80 min. This difference could be due to several factors. For example, nialamide at the concentration used does not inhibit MAO completely (Table 3). Under these conditions, greater amounts of ³H-5HIAA would be expected to be formed after a longer incubation time. The ³H-5HIAA formed might be more loosely bound than ³H-5HT and, therefore, more easily released upon lysis of the cells. Another explanation might be that cells incubated for 80 min become damaged and lose plasma membrane integrity. A greater variability of uptake was always observed with increased incubation times (Fig. 1). After 80 min of incubation, the ability of the cells to bind or retain ³H-5HT might be decreased substantially, especially after osmotic shock.

When cells were exposed to 2 mM ³H-5HT, most of the biogenic amine was membrane bound after a 30 s incubation. The estimated binding is 78%. In the presence of very high concentrations of serotonin (≥2 mM), nonspecific binding becomes the predominant measurable process. The uptake of ³H-5HT (10 μM) after 30 s of incubation decreased when the osmolarity of the incubation medium was increased (Fig. 5). The line intersects the ordinate at 11,021 dpm. When this value was compared with a control value of 44,683 dpm, the fraction bound was estimated to be 24.6%. When 2 mM 5HT was used, only 15% of the amine was bound. This value does not agree with the estimate made with lysed cells, indicating that the amount of bound amine is altered by the increased osmolarity of the incubation medium. Perhaps the resultant folding of the plasma membrane led to the masking of surface binding sites.

Cells from each sucrose-containing medium were counted and their viability was determined after each experiment. This procedure was routinely followed to ensure that decreases in uptake observed with increases in the osmolarity of the incubation medium could not be

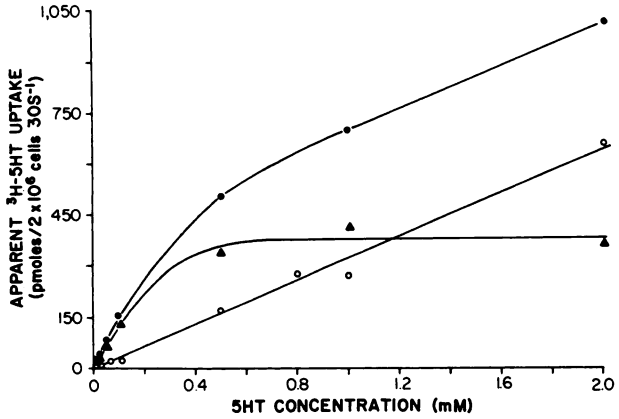

FIG. 4. *The apparent uptake of ³H-5HT as a function of concentration*

The apparent uptake of ³H-5HT exhibited saturation as the concentration of serotonin was increased. Linearity was observed between 10 and 100 μM ³H-5HT. (●) Total radioactivity associated with cell suspensions at 37°C; (○) counts obtained from samples at 0°C; (▲) the difference in counts observed at 37 and 0°C. Each point represents the mean of duplicate determinations.

TABLE 2

Estimation of plasma membrane binding of ^{3}H-5HT as a function of time and concentration[a]

Expt	Incubation time	[^{3}H-5HT]	Treatment	Average dpm/beaker[b]		$C_0/C_{37} \times 100$	$T_{37}/C_{37} \times 100$	$T_0/C_0 \times 100$	$T_0/C_{37} \times 100$
				0°C	37°C				
						%			
I	30 s	10 μM	Osmotic shock	3,131	4,410	43	34	57	24
			Control	5,520	12,755				
II	20 min	10 μM	Osmotic shock	6,846	107,283	5	42	51	3
			Control	13,386	258,355				
III	80 min	10 μM	Osmotic shock	3,587	27,063	6	13	28	2
			Control	12,769	211,175				
IV	30 s	2 mM	Osmotic shock	17,684	26,156	73	115	106	78
			Control	16,654	22,724				

[a] Abbreviations: C_0 = control at 0°C; C_{37} = control at 37°C; T_0 = treated at 0°C; T_{37} = treated at 37°C. The symbol T refers to cells that were exposed to hypotonic medium after incorporation of ^{3}H-5HT.

[b] Average of triplicates.

attributed to either lower cell numbers or decreased viability of cells in the hypertonic medium.

Evaluation of the relative contribution of passive diffusion to ^{3}H-5HT uptake. Evaluation of the relative contributions of passive diffusion and membrane binding to total cellular radioactivity was determined by using the analytical method of Inui and Christensen (13) for defining the fraction of uptake that is subject to competitive inhibition by a given analogue. Cold serotonin was used as a competitive inhibitor of ^{3}H-5HT for specific carrier-mediated transport and nonspecific binding. The fraction of uptake which was not inhibited even in the presence of concentrated solutions of unlabeled 5HT [I] was considered to result from passive diffusion. Solutions of cold 5HT from 0.1 to 5 mM were added to cell suspensions in the presence of 0.76 μM tritiated serotonin. The uptake of radiolabeled 5HT decreased significantly in the presence of unlabeled 5HT (Fig. 6). A smooth curve can be drawn through the points measuring incorporation of radiolabel up to 1 mM cold 5HT. In the presence of 2 mM 5HT, however, a break appears in the curve. This break was interpreted as the displacement of labeled 5HT from nonspecific extracellular binding sites.

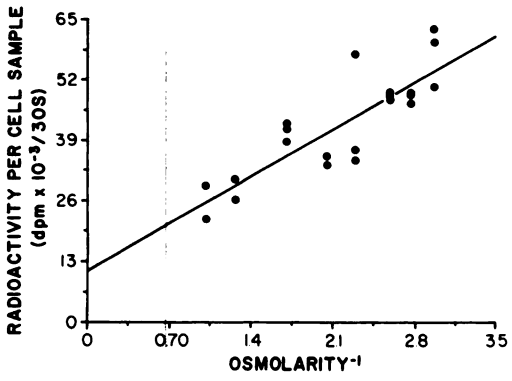

FIG. 5. *Radioactivity associated with cell samples after exposure to 10 μM ^{3}H-5HT as a function of the osmolarity of the incubation medium*

The radioactivity associated with cell suspensions decreased after 30 s of incubation with 10 μM ^{3}H-5HT at 37°C when the osmolarity of the incubation medium was increased. The line intersects the ordinate at 11,021 dpm. When this value was compared to a control value of 44,683 dpm, the fraction bound was estimated to be 24.6%. The equation for the relationship between radioactivity associated with cell samples at 37°C and osmolarity of the incubation medium was computed by the method of least squares. The equation for the line is $y = 10.12 + 15.35x$, with a correlation coefficient of 0.72.

A quantitative estimate of both binding and diffusion was made by plotting (Fig. 7) the reciprocal of the inhibitor concentration ($1/[I]$) against the reciprocal of ^{3}H-5HT uptake inhibition in the presence of inhibitor, $1/(V_0 - V_i)$, where V_0 is the uptake in the absence of inhibitor and V_i is the uptake in the presence of inhibitor. The fraction of total radioactivity contributed by diffusion and binding was calculated from the points at which the extrapolated lines intersect the ordinate.

Percentage diffusion was calculated as follows:

$$0.925 \times 10^{-5} \text{ (Fig. 7)} = 1/(V_0 - V_i).$$

Since V_0 = 121,502 dpm (Fig. 6), then V_i = 13,405 dpm, and the percentage of total radioactivity contributed by diffusion is

$$(13{,}405 \text{ dpm}/121{,}502 \text{ dpm}) \times 100 = 11.0\%.$$

Binding:

$$1.26 \times 10^{-5} \text{ (Fig. 7)} = 1/(V_0 - V_i).$$

Since V_0 = 121,502 dpm, then V_i = 42,480 dpm (binding + diffusion), and the percentage of total radioactivity contributed by membrane binding is

$$(29{,}075 \text{ dpm}/121{,}502 \text{ dpm}) \times 100 = 23.9\%.$$

This estimate of membrane binding using 0.76 μM ^{3}H-5HT correlates with values obtained with 10 μM ^{3}H-5HT when cells were either subjected to osmotic shock after incubation with ^{3}H-5HT (Table 2) or exposed to incu-

TABLE 3

Recovery of radioactivity after incorporationof ^{3}H-5HT by pinealocytes

[^{3}H-5HT]	Time incubated with ^{3}H-5HT	Presence of 12.5 μM nialamide	Percentage recovered as ^{3}H-5HT
2 μM	30 s	No	72
	65 min	No	30
4 μM	70 min	Yes	77

bation media of increasing osmolarities in the presence of labeled serotonin (Fig. 5).

Recovery of radioactivity after incorporation of ^{3}H-5HT by pinealocytes. The total radioactivity remaining in the cell as unchanged ^{3}H-5HT determined by unidimensional ascending paper chromatography is shown in Table 3. The approximate R_f values for 5HT and 5HIAA were 0.46 and 0.88, respectively. In all cases, the decrease in radioactivity associated with the 5HT peaks could be completely accounted for by the increase in the 5HIAA peak.

Estimation of endogenous levels of tissue serotonin in pinealocytes. The assay was performed on four different preparations. Approximately 0.03–0.05 μg of serotonin was recovered per gram of wet weight of whole pineal tissue. Recovery of cells from calf pineal glands was in the range of 5.18–12.6 × 10^7 cells/g of wet weight of tissue. Serotonin content was estimated to be 4–10 × 10^{-7} ng per cell. This suggests that there are 2.3–5.7 × 10^{-8} pmol of serotonin per cell. If it is assumed that the cells are perfect spheres, calculations reveal that each cell has a volume of 9.2 × 10^{-9} ml and that each milliliter of intracellular water contains 250–620 pmol of serotonin.

Estimation of tissue-to-medium ratios of pinealocytes after incorporation of ^{3}H-5HT. Tissue-to-medium ratios were estimated in three separate experiments using three different concentrations of ^{3}H-5HT (Table 4). Data were obtained by counting the radioactivity in 250 μl of supernatant after the initial centrifugation which was used to terminate the reaction. Results are expressed as (dpm/ml of tissue)/(dpm/ml of medium) and (pmol/ml of tissue)/(pmol/ml of medium).

DISCUSSION

When transport phenomena are studied, it is necessary to determine whether the radioactivity associated with the cells reflects binding to the membrane or uptake into the cells. At the start of the present study of 5HT uptake by pinealocytes, methods were not available which would allow an accurate quantitative estimation of membrane binding. Lysing cells after exposure to the radioactive substrate appeared to be the only method available for determining the fraction of radioactivity that is bound to the cell membrane.

Using increasingly hyperosmotic media to measure binding has been used with plasma membrane vesicles (14) isolated from kidney homogenates. As far as could be ascertained, this procedure had not been previously attempted with intact cells. This method was employed with pinealocytes to estimate the amount of membrane binding at initial rate.

Binding measurements were also made at equilibrium with 10 μM ^{3}H-5HT after 80 min of incubation at 37°C. When the radioactivity recovered from cell suspensions was plotted against the reciprocal of the osmolarity of the incubation medium, however, radioactivity fell so rapidly that the line intersected the ordinate at a negative value. These results suggested that exposing cells to hyperosmotic solutions for long periods may damage the cells and cause release of incorporated radioactive substrate. The procedure, however, proved reliable and useful for measuring binding during short incubation periods with the radiolabeled substrate. Data obtained with this method correlated well with those obtained with measurements of binding using lysed cells (Table 2).

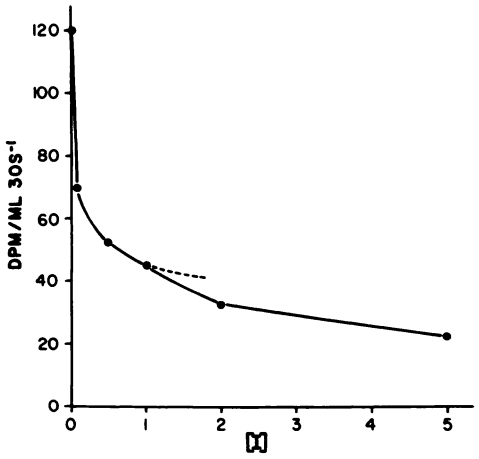

FIG. 6. *Radioactivity associated with cell suspensions in the presence of 0.76 μM ^{3}H-5HT and various concentrations of unlabeled 5HT*

The radioactivity associated with cell suspensions exposed to 0.76 μM ^{3}H-5HT decreased significantly in the presence of increasing doses of unlabeled 5HT. The break (dashed line) which appears in the curve when cell suspensions are incubated in the presence of 2 mM unlabeled 5HT and 0.76 μM ^{3}H-5HT is thought to be due to displacement of radiolabeled 5HT from nonspecific binding sites on the cell membrane. The symbol [*I*] refers to the concentration (mM) of unlabeled 5HT present in the incubation medium.

An unexpected method of determining membrane binding was revealed while using the technique of Inui and Christensen (13) for determining the fraction of

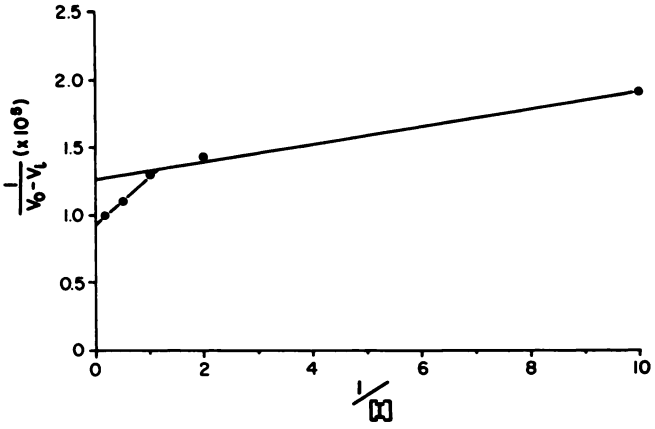

FIG. 7. *A plot of the reciprocal of ^{3}H-5HT uptake inhibition vs the reciprocal of the inhibitor or unlabeled 5HT concentration*

The reciprocal of the inhibitor concentration (1/[*I*]) or unlabled 5HT concentration was plotted against the reciprocal of ^{3}H-5HT uptake inhibition in the presence of various concentrations of inhibitor. $1/(V_0 - V_i)$ represents the reciprocal of ^{3}H-5HT uptake inhibition, where V_0 is the uptake in the absence of inhibitor and V_i is the uptake in the presence of inhibitor. The fraction of total radioactivity contributed by diffusion and binding can be calculated from the points at which the extrapolated lines intersect the ordinate.

TABLE 4

Estimation of tissue-to-medium ratios of pinealocytes after incorporation of ^{3}H-5HT

[5HT]	Time of incubation with ^{3}H-5HT	Tissue/medium ratio[a]	"Actual" tissue/medium ratio[b]
μM	min		
0.5	30	1.7	2.4
	60	2.3	3.0
4.0	70	2.4	3.0
200	30	1.2	1.2
	60	1.5	1.5

[a] Tissue/medium ratios are expressed as (dpm/ml of intracellular water)/(dpm/ml of medium).

[b] Previously estimated average values of endogenous serotonin were included in this calculation. Tissue/medium ratios are expressed as (pmol/ml of intracellular water)/(pmol/ml of medium).

uptake of a substrate that is not inhibited by an analogue. When $1/(V_0 - V_i)$ vs $1/[I]$ is plotted (Fig. 7), a break occurs in the curve, indicating displacement of the radiolabel by the unlabeled serotonin. Using this method, nonspecific membrane binding was 24%, which is in excellent agreement with the values of 24 and 25% obtained with lysis and increasing osmolarity, respectively.

The method of Inui and Christensen allows the use of an analogue of the substrate under investigation or the use of high concentrations of unlabeled substrate. Specific carrier sites become saturated and are blocked by the presence of excess unlabeled serotonin when the uptake of labeled substrate remains constant with the addition of increasing concentrations of unlabeled compound. Although the specific activity of the added serotonin diminishes progressively as the dose of unlabeled substrate increases, uptake of serotonin increases proportionally, producing no observable change in the incorporation of label. In theory, an infinite amount of unlabeled serotonin can be added without a change in uptake of labeled 5HT. This component of uptake, then, is the nonsaturable or diffusional component of serotonin uptake.

Serotonin is found in pinealocytes and in the sympathetic nerves that innervate the pineal gland (15). Neuronal serotonin is thought to be synthesized in the pinealocytes and enters the neurons by way of the norepinephrine reuptake mechanism (16). It has been suggested that only the sympathetic nerves that innervate the pineal contain type A monoamine oxidase (17) and can, therefore, deaminate serotonin. Contrary to these observations, there is evidence to suggest that MAO is important in serotonin metabolism in pinealocytes of nonmammalian species (7, 18).

The results obtained with bovine pinealocytes in this laboratory (Table 3) suggest that serotonin is deaminated to a large extent in the absence of an MAO inhibitor. Nialamide, a nonselective MAO inhibitor, increased the amount of total radioactivity incorporated into pinealocytes by at least twofold (unreported observations). However, a quantitative estimate was not made at this time to determine the fraction of the total radioactivity incorporated into cells as ^{3}H-5HT remaining in the absence of nialamide.

Approximately 0.03–0.05 μg of serotonin was recovered per gram of wet weight of whole pineal tissues when endogenous levels of tissue serotonin were estimated radioenzymatically according to the procedure of Saavedra *et al.* (10). This value is low compared to those of other mammalian species (19) when the time of death was at night; rat pineals contained 10–60 μg and rhesus monkeys contained 10–30 μg of serotonin per gram of tissue. These same species exhibited a much higher serotonin content during the day. Rats were reported to contain 60–120 μg and rhesus monkeys 40–80 μg of the indoleamine per gram of tissue. The only figures available for domestic ungulates were reported without the author's knowledge of the time of death. Goats contained 1.2–7.0 μg/g, and cattle 0.2–0.6 μg/g, when serotonin content was estimated by bioassay and about 2.7 μg/g when a chemical assay was employed; swine and sheep contained, respectively, 20–56 and 1.1–6.6 μg/g of tissue. The reason for the low figures obtained in the present study with calf pineals may be that pineal glands were not directly homogenized and assayed for serotonin content but were first treated with papain. The resulting dispersed cells were assayed for the indoleamine. Recovery of cells by weight from whole glands was low. The number of cells obtained from pineal glands was in the range of $5.18–12.6 \times 10^7$ cells/g of wet weight of tissue. Perhaps cell loss during the dispersion procedure may account for the discrepancy in serotonin concentration.

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