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Characterization of Serotonin Uptake in Isolated Bovine Pinealocyte Suspensions

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SUMMARY

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The uptake of radiolabeled serotonin as a function of concentration exhibited saturation when low (0.005–2 μM) concentrations of serotonin were used. Kinetics of the initial rate (30 s) of entry of radiolabeled serotonin into pinealocytes were examined using various concentrations of the substrate. The effects of temperature, changes in external ion concentrations, drugs, metabolic inhibitors, and other compounds on the uptake of serotonin were investigated. High concentrations (10 μM–5 mM) of serotonin appear to use amino acid transport systems, e.g., L-lysine, L-leucine, and L-alanine. Uptake is partially sodium dependent, temperature dependent, sensitive to metabolic inhibitors, and inhibited by various amino acids. Low concentrations (0.005–1 μM) of serotonin appear to use a highly specific, allosteric carrier for transport. Uptake is sodium dependent, temperature dependent, and sensitive to a variety of metabolic inhibitors.

INTRODUCTION

It has been established that the mammalain pineal is an endocrine gland, although neither all of its active principles nor their target organs have been identified. The subcellular localization of organelles that may act as storage sites for potential secretory products has yet to be elucidated. However, evidence exists which implies that there may be at least two secretory processes in the mammalian pineal. One of the processes suggests that serotonin (5-HT) may be an important secretory product of the pineal gland. This process is characterized by the production of granular vesicles by the saccules of the Golgi apparatus (1). The presence of granular vesicles has been described in the pineals of practically all vertebrates studied. Several investigators (2–4) have suggested that these vesicles contain melatonin or serotonin. Juillard *et al.* (5) have suggested that the indoleamine (presumably serotonin) functions in the metabolism, storage, and/or release of a proteinaceous compound(s) in the granules.

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.

The aspect of intracellular serotonin regulation that concerns this paper is that of plasma membrane transport. Previous observations made in this laboratory on bovine cell suspensions suggested the existence of a transport system for serotonin in the pineal gland (6).

In the work described in this paper the various procedures used to characterize the mechanism of serotonin uptake into bovine pinealocytes were the following: the kinetics of uptake, ionic requirements, temperature and energy dependence, and effects of certain drugs and compounds on the uptake of serotonin into pinealocytes.

MATERIALS AND METHODS

Cell dispersion and isolation procedures. A description of cell dispersion and isolation, preparation of the tissue, and sterilization procedures is given in the preceding paper (6).

Preparation of ^{3}H-5HT. 5-Hydroxy[G-^{3}H]tryptamine creatinine sulfate, 10.8 Ci/mmol, was purchased from Amersham Searle Corp. Hydroxytryptamine creatinine sulfate, 5-[1,2-^{3}H(N)], 28.2 Ci/mmol, was purchased from New England Nuclear Corp. Preparation of ^{3}H-5HT was as previously described (6).

Incubation conditions and procedures. Standard incubation conditions were as previously described (6).

Temperature effects were determined by incubating samples at 22, 30, 37, and 42°C. Radioactivity at 0°C was always subtracted from the total counts observed at each of the temperatures.

Fifty microliters of an appropriate amount of a metabolic inhibitor or drug was added to a beaker containing 0.9 ml of the cell suspension; the beaker was then immediately placed in a water bath at 37°C. After either a

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10-min (metabolic inhibitors) or a 20-min (drugs) preincubation, control samples were transferred to a 0°C water bath. Incubation continued for another 5 min (metabolic inhibitors) or 10 min (drugs), resulting in a total preincubation time of 15 min with metabolic inhibitors and 30 min with drugs. Fifty microliters of ^{3}H-5HT was then added to each beaker; incubations were terminated after 30 s.

Amino acids (Sigma) and biogenic amines (Sigma) were added simultaneously with ^{3}H-5HT. Appropriate concentrations of these compounds were premixed and 100 μl of this mixture was added to a 0.9-ml cell suspension that had been preincubated for 5 min at either 0 or 37°C.

When effects of preloading with various compounds on the uptake of ^{3}H-5HT were examined, cell suspensions were incubated with the appropriate concentration of substrate at 37°C for 30–40 min. Uptake was terminated by layering the cell suspensions on ice-cold calcium- and magnesium-free HBSS containing 5% BSA and 1% penicillin–streptomycin and centrifuging at 0°C for 10 min at 60*g*. The cells were resuspended in standard incubation medium. Fifty microliters of ^{3}H-5HT was added to the beakers containing 1 ml of the cell suspension after a 5-min preincubation at either 37 or 0°C.

Effects of external ion concentrations and replacement of sodium chloride with equiosmolar amounts of lithium chloride, cesium chloride, potassium chloride, choline chloride, or sucrose were also determined. Osmolarities of these solutions were determined routinely by freezing point osmometry (Advanced Instruments Model 31). When magnesium or calcium was removed, no replacements were made. After isolation, individual cell pellets were resuspended in the appropriate medium. Uptake of ^{3}H-5HT was observed for 30 s after preincubation at 37 and 0°C.

Determination of cell viability. Cell viability was determined after each experiment by trypan blue (0.2%) dye exclusion in a hemocytometer.

Expression and analysis of results. Standardization of cell suspensions consisted of expressing the results as ^{3}H-5HT uptake per 2×10^6 cells. Most of the uptake studies were carried out for 30 s and, therefore, results are expressed as dpm or pmol ^{3}H-5HT/2×10^6 cells/30 s.

The K_i of serotonin uptake inhibitors was determined by the method of Dixon (7).

Kinetic studies were evaluated graphically according to Lineweaver and Burk (8). V_{max} and apparent K_m values were determined by the method of least squares using a Hewlett Packard desk-top calculator system (9810 Calculator and 9862A Calculator Plotter).

Radioactivity. Radioactivity was determined as previously described (6).

Sources of drugs and metabolic inhibitors. *d*,l-Amphetamine sulfate (Arenol Chemical Corp.); chlorpromazine (Smith, Kline and French); cocaine hydrochloride (University of Rochester Department of Pharmacy); 1-(3′,4′-dichlorophenyl)-2-isopropylaminoethanol hydrochloride (DCI; dichloroisoproterenol; Aldrich Chemical Company, Inc.); imipramine (U.S.V. Pharmaceuticals); (±)-metaraminol bitartrate (Merck, Sharp & Dohme); reserpine phosphate (Ciba Pharmaceutical Company); 2,4-dinitrophenol (Sigma); *N*-ethylmaleimide (Calbiochem); *p*-hydroxymercuribenzoate, sodium salt (Sigma); iodoacetamide, crystalline (Sigma); iodoacetic acid (Sigma); mercuric chloride (J. T. Baker Chemical Company); ouabain octahydrate (Sigma); potassium cyanide (Fisher Scientific); sodium azide (Fisher Scientific); and sodium fluoride (Fisher Scientific).

RESULTS

Characteristics of ^{3}H-5HT uptake by pinealocytes. The apparent uptake of 0.07 μM ^{3}H-5HT by pinealocytes was linear for at least 2 min (Fig. 1); the initial uptake of 0.01 μM ^{3}H-5HT produced a sigmoid curve with cell number. Linearity was observed between 1 and 3.5×10^6 cells/ml. Uptake at the initial rate over a range of 0.025 to 3.25 μM ^{3}H-5HT is shown in Fig. 2. The uptake of ^{3}H-5HT was dose dependent and exhibited saturation as the concentration of serotonin was increased. Figure 3 shows the characteristics of uptake at the lower range of the ^{3}H-5HT concentrations represented in Fig. 2 on an expanded scale. The rate of uptake between 0.025 and 0.25 μM ^{3}H-5HT appeared to represent a different type of saturation process from that seen at higher concentrations. This observation was made regularly. The initial rate of uptake of very low concentrations of ^{3}H-5HT was linear over a small range of concentrations (up to 0.1 μM) and time (5–10 min); thereafter, rates changed abruptly. Linearity was again observed between about 0.2 and 1.0 μM ^{3}H-5HT before the uptake process approached equilibrium. The recovery of radioactivity from cells incubated at 0°C was linear throughout the range of 0.025–2.0 μM. This suggests that binding and diffusion are not decreased proportionally in the concentration range between 0.025 and 0.25 μM ^{3}H-5HT and that the uptake relative to higher concentrations of ^{3}H-5HT is greater.

The initial rate of ^{3}H-5HT uptake was determined at

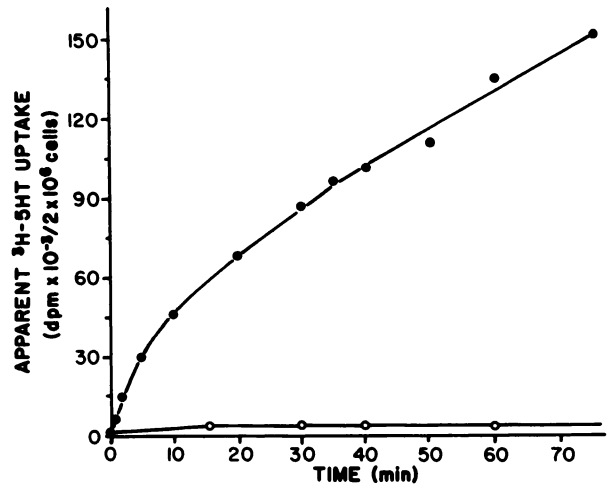

FIG. 1. *The apparent uptake of 0.07 μM ^{3}H-5HT as a function of incubation time*

Uptake did not exhibit saturation kinetics even after 75 min. Linearity was observed for at least the first 2 min. (●) Mean values obtained at 37°C after subtracting determined or actual mean values observed at 0°C; (○) mean values of the radioactivity recovered from cell suspension samples at 0°C. Each point is the average of triplicate determinations.

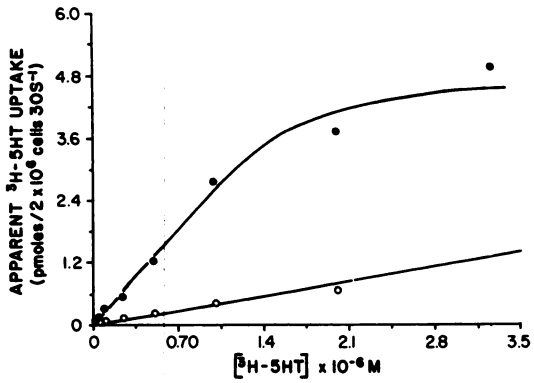

FIG. 2. *The apparent uptake of ³H-5HT as a function of concentration*

Uptake exhibited saturation as the concentration of serotonin was increased. (●) Radioactivity associated with cell suspensions at 37°C after subtracting counts obtained from samples incubated at 0°C; (○) counts obtained from samples incubated at 0°C. Each point represents the mean of duplicate determinations.

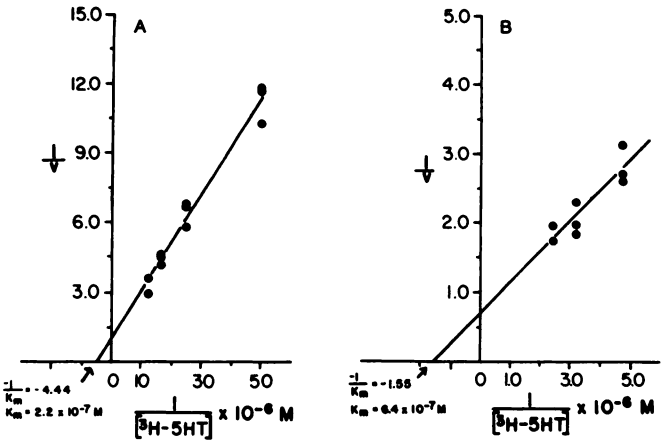

FIG. 4. *Lineweaver-Burk plots of the initial uptake of ³H-5HT into pinealocytes*

(A) 0.01–0.08 μM ³H-5HT; (B) 0.2–0.8 μM ³H-5HT. Velocity (v) is expressed as picomoles of serotonin accumulated per 2 × 10^6 cells per 30 s. An equation for each line was determined by the method of least squares.

various concentrations between 0.01 and 0.08 μM ³H-5HT and between 0.2 and 0.8 μM ³H-5HT. Figure 4A is a typical Lineweaver-Burk plot from a single experiment using concentrations of between 0.01 and 0.08 μM ³H-5HT. Statistical treatment of three different experiments revealed an apparent K_m of 2.1 ± 0.4 × 10^{-7} M and V_{max} of 0.79 ± 0.25 pmol ³H-5HT/2 × 10^6 cells/30 s. Figure 4B is a typical Lineweaver-Burk plot from a single experiment using concentrations of between 0.2 and 0.8 μM ³H-5HT. Statistical treatment of three different experiments revealed an apparent K_m of 6.5 ± 0.7 × 10^{-7} M and V_{max} of 1.86 ± 0.41 pmol ³H-5HT/2 × 10^6 cells/30 s. These values are characteristic of a high-affinity uptake process for biogenic amines (9, 10).

The initial rate of ³H-5HT uptake by pinealocytes was determined at various serotonin concentrations between 10 and 100 μM which had been shown previously to exhibit linearity. Statistical treatment of three different experiments revealed the following kinetic constants: apparent K_m = 191 ± 41.6 μM; V_{max} = 230.7 ± 113.2 pmol ³H-5HT/2 × 10^6 cells/30 s. These values are characteristic of a low-affinity process (11).

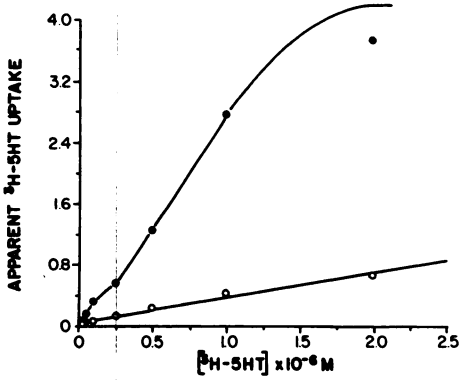

FIG. 3. *The apparent uptake of ³H-5HT as a function of concentration*

(●) Radioactivity associated with cell suspensions at 37°C after subtracting counts observed at 0°C; (○) counts obtained from samples incubated at 0°C. The radioactivity recovered from cell samples at 0°C showed linearity throughout the range 0.025–2.0 μM.

Effects of temperature and metabolic inhibitors on ³H-5HT uptake. Uptake was markedly temperature dependent for various concentrations of ³H-5HT: 0.05, 0.5, 5, 10 and 200 μM. Uptake increased exponentially between 22 and 42°C and most sharply between 37 and 42°C. Q_{10} values were always above 3.

Various metabolic inhibitors or the absence of glucose (Table 1) inhibited the uptake of ³H-5HT. The most effective inhibitors of initial uptake appeared to be sulfhydryl reagents, i.e., mercuric chloride, *N*-ethylmaleimide, and *p*-hydroxymercuribenzoate. Sulfhydryl inhibitors, such as *p*-chloromercuribenzoate, *N*-ethylmaleimide, 2,4-dinitrofluorobenzene, and ethacrynic acid, have been reported to inhibit the (Na^+ + K^+)-ATPase transport system, suggesting that intact sulfhydryl groups are required for this system (12).

Effects of the absence of or substitution for NaCl on the initial uptake of ³H-5HT. Uptake of 0.16 μM ³H-5HT was partially sodium dependent when either choline chloride or sucrose was substituted for sodium chloride in the incubation medium. No significant difference was observed between sucrose and choline chloride when they were used to maintain the osmolarity of media where sodium was omitted. For example, when the concentration of Na was 5 mM and the concentration of either sucrose or choline chloride was 138 mM, ³H-5HT uptake was depressed by about 50%. Consequently, choline chloride was used exclusively to replace sodium chloride in all subsequent experiments.

Figures 5A–C summarize the effects of various concentrations of NaCl and its substitution by choline chloride, LiCl, and CsCl on 0.05, 0.5, 5, and 200 μM ³H-5HT uptake. The initial rate of ³H-5HT uptake was determined at various concentrations between 0.04 and 0.08 μM ³H-5HT and 0.2 and 0.8 μM ³H-5HT in the presence (143 mM Na^+) and absence (5 mM Na^+) of NaCl. Inspection of the Lineweaver-Burk plots revealed the following kinetic parameters for uptake employing concentrations of between 0.04 and 0.08 μM ³H-5HT in the presence of 143 mM sodium ion. An apparent K_m of 2.4 × 10^{-7} M and V_{max} of 0.65 pmol ³H-5HT/2 × 10^6 cells/30 s were obtained. In

TABLE 1

Effect of metabolic inhibitors on the initial uptake of ^{3}H-5HT into pinealocytes[a]

^{3}H-5HT	Metabolic inhibitor	Percentage uptake
μM		
0.1	None	100
	Iodoacetamine, 10 mM	41
	Iodoacetate, 1 mM	64
	Ouabain, 1 mM	50
	Potassium cyanide, 1 mM	104
	Sodium azide, 10 mM	88
	2,4-Dinitrophenol, 0.5 mM	3
	Sodium fluoride, 20 mM	78
	Mercuric chloride, 1 mM	18
0.5	None	100
	Absence of glucose	79
	Iodoacetate, 1 mM	76
	Ouabain, 0.1 mM	55
	Sodium azide, 10 mM	84
	N-Ethylmaleimide, 1 mM	20
	p-Hydroxymercuribenzoate, 0.1 mM	35
200	None	100
	Absence of glucose	80
	Iodoacetate, 1 mM	54
	Ouabain, 0.1 mM	79
	Sodium azide, 10 mM	62
	2,4-Dinitrophenol, 0.1 mM	54
	N-Ethylmaleimide, 1 mM	28
	p-Hydroxymercuribenzoate, 1 mM	20

[a] Results are averages of triplicate determinations from single cell preparations.

the presence of 5 mM sodium ion, an apparent K_m of 5.4 $\times 10^{-7}$ M and V_{max} of 0.61 pmol ^{3}H-5HT/2 $\times 10^6$ cells/30 s were obtained.

Double reciprocal plots using concentrations of between 0.2 and 0.8 μM ^{3}H-5HT revealed an apparent K_m of 7.2×10^{-7} and V_{max} of 2.27 pmol ^{3}H-5HT/2 $\times 10^6$ cells/30 s in the presence of 143 mM sodium ion. The kinetic parameters obtained in the presence of 5 mM sodium ion were as follows: apparent K_m of 5.3×10^{-7} M and V_{max} of 1.02 pmol ^{3}H-5HT/2 $\times 10^6$ cells/30 s.

Li^+ seemed to partially substitute for Na^+ in the uptake of 0.05, 0.5, and 5 μM ^{3}H-5HT. Li^+ appeared to be most ineffective in substituting for Na^+ in the uptake of 0.05 μM ^{3}H-5HT. Partial replacement of NaCl by CsCl resulted in a marked stimulation of ^{3}H-5HT uptake in all cases (Figs. 5A–C). However, as the Cs^+ concentration was increased, and the Na^+ concentration was decreased in proportion to the increase in the Cs^+ concentration, ^{3}H-5HT uptake was inhibited.

When the Na^+ concentration was decreased to 5 mM and the Cs^+ concentration was 138 mM, the uptake of ^{3}H-5HT decreased or increased, depending upon its concentration.

Cesium concentration was 46 mM and Na^+ concentration was 97 mM when a marked stimulation of ^{3}H-5HT uptake was seen (Figs. 5A–C). However, no such stimulation was apparent with Li^+ at this concentration.

If K^+ is totally removed and replaced by choline chloride, the uptake of 0.05 and 0.5 μM ^{3}H-5HT is inhibited by about 28% (Table 2). The uptake of 200 μM ^{3}H-5HT appears to be only slightly inhibited. If the effects of

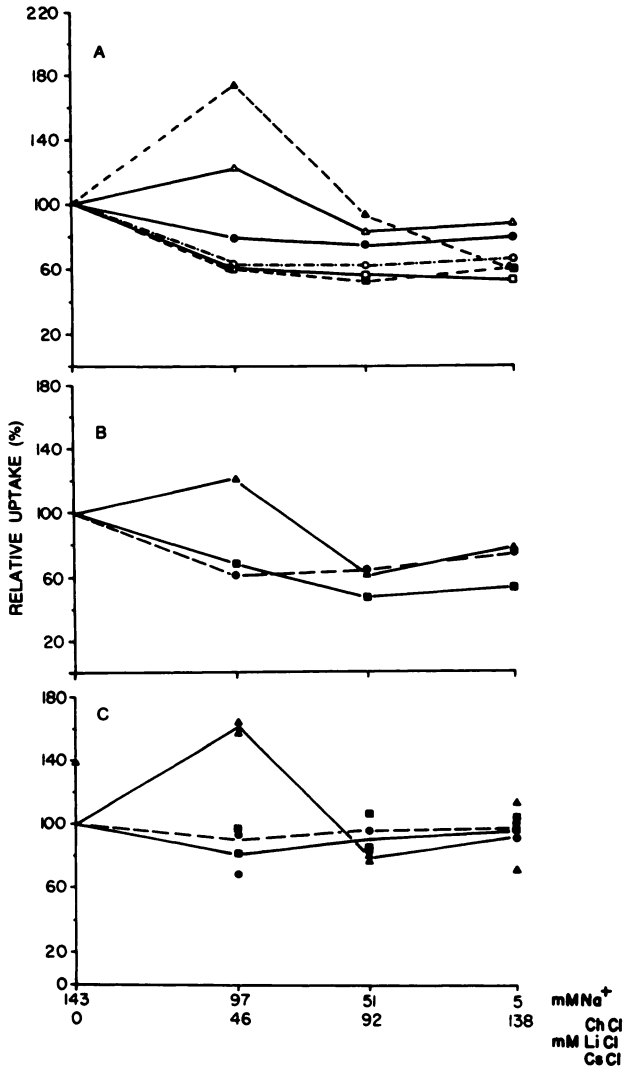

FIG. 5. *The uptake of 0.05 and 0.5 μM (A), 5 μM (B), and 200 μM (C) ^{3}H-5HT as a function of various concentrations of cations*

In A open symbols represent 0.05 μM and closed symbols represent 0.5 μM ^{3}H-5HT. The cations substituted for NaCl in the medium are indicated in all three parts as follows: (●) LiCl; (▲) CsCl; (■) choline chloride.

increasing the K^+ concentration above control values are examined, the uptake of 0.05 μM ^{3}H-5HT appears to be higher in the presence of a greater concentration of K^+ when compared to a medium where NaCl was replaced with choline chloride. In the case of 0.5 μM ^{3}H-5HT, if the effects of increasing the K^+ concentration above control values are examined, the uptake of ^{3}H-5HT is lower in the presence of higher concentrations of K^+ when compared to a medium where NaCl was replaced with choline chloride. These observations suggest that K^+ can partially substitute for Na^+ in the uptake of 0.05 μM ^{3}H-5HT. Potassium ion, however, is inhibitory and competes with Na^+ when the uptake of 0.5 μM ^{3}H-5HT is considered.

The absence of magnesium ion in the incubation medium reduced the specific uptake of 0.05, 0.5, and 5 μM ^{3}H-5HT. When cell suspensions incubated in magnesium-free HBSS were compared to cells incubated in standard

medium, the specific uptake observed in magnesium-free medium was about 60% of control. The absence of magnesium ion in the incubation medium did not appear to have any significant effect on the specific uptake of 200 μM ^{3}H-5HT.

No significant effects on specific ^{3}H-5HT uptake at 37°C were observed in the absence of calcium after the radioactivity recovered from cell suspensions incubated at 0°C was subtracted.

To determine if amino acids or biogenic amines affected ^{3}H-5HT uptake, several different compounds were tested. Table 3 shows the effects of various amino acids and biogenic amines on the initial uptake of ^{3}H-5HT. No amino acid was an effective inhibitor of 0.20, 0.25, or 0.5 μM ^{3}H-5HT uptake. L-Lysine, L-leucine, and L-tryptophan were the most effective inhibitors of 200 μM ^{3}H-5HT uptake. Histamine (free base) failed to produce consistent effects on the uptake of 0.2 and 0.5 μM ^{3}H-5HT at low and high concentrations but markedly stimulated the uptake of 100 and 200 μM ^{3}H-5HT. The uptake of 200 μM ^{3}H-5HT was increased by approximately 300% over the control in the presence of 1 mM histamine.

The potency of L-tryptophan as an inhibitor of ^{3}H-5HT uptake was determined graphically (7); the reciprocal of ^{3}H-5HT uptake ($1/v$) was plotted against the concentration of the amino acid. K_i was determined directly from a Dixon plot. Tryptophan is a weak competitive inhibitor of both 0.25 and 0.5 μM ^{3}H-5HT and 100 and 200 μM ^{3}H-5HT uptake. The K_i of tryptophan in the presence of 0.25 and 0.5 μM ^{3}H-5HT was 3.4×10^{-3} M. The K_i of tryptophan in the presence of 100 and 200 μM ^{3}H-5HT was 2.8×10^{-3} M.

Loading cells with high concentrations of certain substrates and then exposing them to low concentrations of the same or different compounds can produce a transitory stimulation of uptake of the latter. This phenomenon has been termed *accelerative exchange diffusion* (13), *trans stimulation*, or *countertransport* (12). ^{3}H-Leucine and ^{3}H-tryptophan exhibited *trans* stimulation when incubated with pinealocytes (data not shown). Serotonin (200 μM) exhibited this phenomenon (Table 4) only when cells had been preloaded with L-lysine. When cells were loaded with either 0.5 or 1 mM cold serotonin and subsequently exposed to 0.037 μM ^{3}H-5HT, uptake of the latter was markedly suppressed (Fig. 6).

The effects of a variety of drugs on 0.5 μM ^{3}H-5HT uptake were investigated. In initial experiments, a comparison of the effects of various drugs on the initial uptake of 0.5 μM ^{3}H-5HT was made in the presence and absence of 12.5 μM nialamide. In most cases a large discrepancy existed between the results obtained in the presence and absence of nialamide. The inhibition of serotonin uptake observed with some drugs may be modified by monoamine oxidase (MAO). For example, reserpine, which supposedly excludes serotonin from storage sites, may make serotonin more available to the action of MAO. Consequently, the MAO inhibitor, nialamide, was not used in subsequent experiments.

Uptake of 200 μM ^{3}H-5HT was inhibited by chloro-

TABLE 2

Effect of varying the extracellular concentration of potassium ion on the initial uptake of ^{3}H-5HT[a]

^{3}H-5HT	Extracellular concentration (mM)			Initial uptake
	Na^+	K^+	Choline chloride	
μM				% of control
0.05	143	0	5	72
	105	5	38	37
	105	43	0	42
	80	5	63	50
	80	68	0	80
0.5	143	0	5	72
	105	5	38	61
	105	43	0	32
200	143	0	5	97

[a] Each value represents the average of triplicate determinations.

TABLE 3

Effect of biogenic amines and amino acids on the initial uptake of ^{3}H-5HT into pinealocytes[a]

Inhibitor	Initial uptake (% of control)				
	^{3}H-5HT				
	0.2 μM	0.25 μM	0.5 μM	100 μM	200 μM
Dopamine (μM)					
6	82	—	68	—	—
12	81	—	50	—	—
Histamine (μM)					
0.5	117	—	109	—	—
1.0	100	—	123	—	—
2.0	86	—	97	—	—
250	—	—	—	112	140
500	—	—	—	155	180
1 mM	—	—	—	227	291
L-Norepinephrine (μM)					
4	88	—	69	—	—
8	82	—	61	—	—
L-Alanine (mM)					
0.1	—	—	99	—	—
1.0	—	—	94	—	—
2.0	—	—	96	—	—
4.0	—	—	89	—	—
10.0	—	—	—	—	59
L-Leucine (mM)					
0.1	—	—	100	—	—
10.0	—	—	—	—	43
L-Lysine (mM)					
0.1	—	—	92	—	—
10.0	—	—	—	—	32
α-(Methylamino) isobutyric acid (mM)					
0.1	—	—	100	—	—
0.5	—	—	100	—	—
1.0	—	—	100	—	—
2.0	—	—	100	—	—
4.0	—	—	100	—	—
10.0	—	—	—	—	62
L-Tryptophan (mM)					
1.0	—	90	91	—	—
2.0	—	64	75	65	68
3.5	—	—	—	51	57
5.0	—	49	56	—	46

[a] Each value represents the average of triplicate determinations.

TABLE 4

Effect of preloading cells with various compounds on subsequent ^{3}H-5HT uptake[a]

Preloading compound[b]	Uptake (% of control)	
	^{3}H-5HT	
	0.5 μM	200 μM
L-Alanine	75	89
L-Histidine	72	88
L-Leucine	72	61
L-Lsine	69	136
Serotonin	28	32

[a] Each value is the average of triplicate determinations.

[b] Isolated cells were exposed to various amino acids or serotonin at a concentration of 10 mM at 37°C for 30 min. Uptake was terminated by centrifuging cells through calcium- and magnesium-free HBSS containing 5% BSA at 0°C before subsequent incubation with ^{3}H-5HT.

promazine and imipramine, inhibited slightly and not at all by reserpine and dichloroisoproterenol, respectively, and unexpectedly stimulated by amphetamine, cocaine, and metaraminol (Table 5).

Table 6 lists the type of inhibition by selected drugs and the K_i values obtained when 0.25 and 0.5 μM ^{3}H-5HT uptakes were plotted by the method of Dixon (7).

DISCUSSION

The uptake of all concentrations of ^{3}H-5HT investigated showed a marked temperature and energy dependence.

Partial sodium dependence was observed with 0.16, 0.05, and 0.5 μM ^{3}H-5HT (Fig. 5A) and 5 μM ^{3}H-5HT uptake (Fig. 5B). Maximum uptake in the presence of 5 mM Na^+ was about 50% of control with all concentrations of ^{3}H-5HT. Only a slight sodium dependence was noted for uptake at 200 μM ^{3}H-5HT.

Two effects are probably being observed with Cs^+ when CsCl is substituted for NaCl: some type of direct or indirect stimulation of ^{3}H-5HT uptake and its ability to substitute for Na^+. Only one effect is seen with Li^+: its

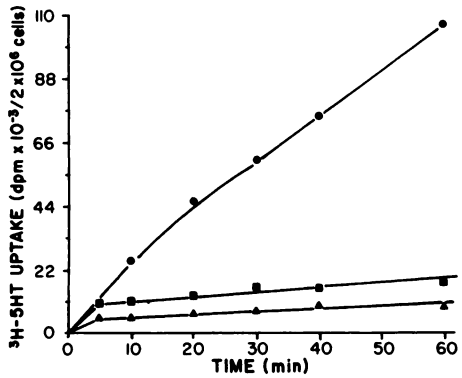

FIG. 6. *Uptake of 0.037 μM ^{3}H-5HT after preloading pinealocytes with nonradiolabeled serotonin*

Uptake of 0.037 μM ^{3}H-5HT did not exhibit *trans stimulation* but was instead markedly diminished in cells previously exposed for 40 min to either 0.5 mM (■) or 1 mM (▲) serotonin. A curve representing uptake of 0.037 μM ^{3}H-5HT by nonpreloaded cells (●) is shown for comparison. Each point represents the mean of triplicate determinations.

TABLE 5

Effect of various drugs on the initial uptake of 200 μM ^{3}H-5HT[a]

Drug	Concentration	Uptake
	μM	% of control
Amphetamine	4000	136
Chlorpromazine	40	28
Cocaine	40	111
Dichloroisoproterenol	40	100
Imipramine	40	61
Metaraminol	400	120
Reserpine	0.61 μg/ml	95

[a] Each value is the average of triplicate determinations.

ability to substitute for Na^+. When the concentration of Na^+ is decreased below 97 mM, Cs^+ no longer has a stimulatory effect. The uptake of ^{3}H-5HT as Cs^+ concentrations of 92 and 138 mM reflects its ability to substitute for Na^+. Cesium substitutes most effectively for Na^+ when the ^{3}H-5HT concentration is 0.05 μM (Fig. 5A). Cesium appears to substitute equally with Li^+ when the ^{3}H-5HT concentration is 5 or 200 μM (Figs. 5B and 5C). Cesium does not substitute for Na^+ when the concentration of ^{3}H-5HT is 0.5 μM (Fig. 5A). These results correlate with those obtained with K^+. Potassium (Table 2) could partially substitute for Na^+ when 0.05 μM ^{3}H-5HT uptake was observed but not with 0.5 μM ^{3}H-5HT uptake.

Uptake of all concentrations of ^{3}H-5HT, except 200 μM, was decreased in the absence of Mg^{2+}. This effect may be due to inhibition of a Mg^{2+}-dependent (Na^+ + K^+)-ATPase.

Although subcellular localization of serotonin storage sites or compartmentalization was not attempted with bovine pinealocytes, the observation that reserpine inhibited ^{3}H-5HT uptake suggests that storage vesicles containing serotonin may exist in the bovine pineal. Reserpine presumably impairs serotonin accumulation specifically by its effects on amine storage vesicles (14, 15).

The uptake mechanism(s) of higher concentrations of serotonin (5–200 μM) is probably an amino acid transport system. Physiologically, these mechanisms are probably insignificant but, nevertheless, will be discussed here briefly in terms of Christensen's (12) classification of the transport systems for the neutral and basic amino acids in Ehrlich ascites tumor cells. The low-affinity uptake mechanism (K_m, approximately 200 μM) may be a combination of two or three amino acid transport mecha-

TABLE 6

Estimation of the type of inhibition and K_i values of various drugs reducing the uptake of ^{3}H-5HT[a] *into pinealocytes*

Inhibition	Drug	K_i
		M
Competitive	Chlorpromazine	1.6×10^{-6}
	Cocaine	7.5×10^{-7}
	Dichloroisoproterenol	2.6×10^{-6}
	Imipramine	5.2×10^{-7}
	Metaraminol	400×10^{-6}
Noncompetitive	Ouabain	2.9×10^{-6}
	Reserpine	3.6×10^{-7}

[a] The concentrations of ^{3}H-5HT used were 0.25 and 0.50 μM.

nisms. It was observed (Table 3) that 200 μM ^{3}H-5HT uptake was inhibited by the following amino acids (10 mM) in decreasing order of potency: L-lysine > L-leucine > L-alanine > α-(methylamino)isobutyric acid. High concentrations (i.e., 200 μM) of serotonin can probably use all of these transport systems. It appears, however, that high concentrations of serotonin use mainly the sodium-independent lysine transport system. This was the only amino acid that participated in *trans stimulation* with serotonin (Table 4). This result was unexpected. It was expected that high concentrations of serotonin would use mainly the Na-independent L system used by tryptophan and leucine. The slight sodium sensitivity (Fig. 5C) of 200 μM ^{3}H-5HT uptake suggests that a mechanism of transport utilized by this concentration of serotonin is sodium dependent. This is more apparent when the uptake of 5 μM ^{3}H-5HT is observed (Fig. 5B). This transport system may be System A, as designated by Christensen (12) and used by alanine. The stimulation of uptake of 100 and 200 μM ^{3}H-5HT by histamine (Table 3) and the stimulation of uptake of 200 μM ^{3}H-5HT observed with some drugs (Table 5) may represent types of *trans* stimulation. Histamine may be taken up very rapidly by pinealocytes by one mechanism and then may exchange for serotonin across the membrane using another transport system. Metaraminol and amphetamine may displace endogenous substrates, causing an exchange with exogenous radiolabeled serotonin. The possibility cannot be excluded, however, that these substrates may increase the uptake of ^{3}H-5HT directly (*cis* stimulation) by modifying the carrier in some manner.

The uptake mechanism observed at lower concentrations of serotonin (less than 0.8 μM) is highly specific (Table 3; Figs. 4A and B). When concentrations between 0.04 and 0.08 μM were plotted according to Lineweaver and Burk in the presence of sodium (143 mM Na^+) and in the absence of sodium (5 mM Na^+), the V_{max} was the same but the apparent K_m of serotonin more than doubled in the absence of sodium. The capacity of the carrier, however, remained the same. This agrees with observations by other investigators. Biogenic amine transport is visualized as being of high affinity but low capacity (10). Its transport is carrier mediated (16), and the affinity of the carrier for the amine is regulated by electrolytes (17).

The capacity (V_{max}) for uptake of ^{3}H-5HT between 0.2 and 0.8 μM in the absence of sodium (5 mM) was diminished by approximately 55%, but the apparent K_m was the same.

To explain the data obtained, an allosteric carrier model for low concentrations of serotonin is proposed (Fig. 7). It should explain the following points.

(1) Dopamine and L-norepinephrine inhibit 0.5 μM ^{3}H-5HT uptake more strongly than the uptake of 0.2 μM ^{3}H-5HT.

(2) Cs^+ and K^+ can partially substitute for Na^+ in the uptake of 0.05 μM ^{3}H-5HT but not for the uptake of 0.5 μM ^{3}H-5HT.

(3) Li^+ can partially substitute for Na^+ in the uptake of 0.5 μM ^{3}H-5HT but not for 0.05 μM ^{3}H-5HT uptake.

(4) When Na^+ is removed from the medium, the V_{max} of uptake for concentrations of between 0.02 and 0.08 μM ^{3}H-5HT does not change, but the K_m more than doubles.

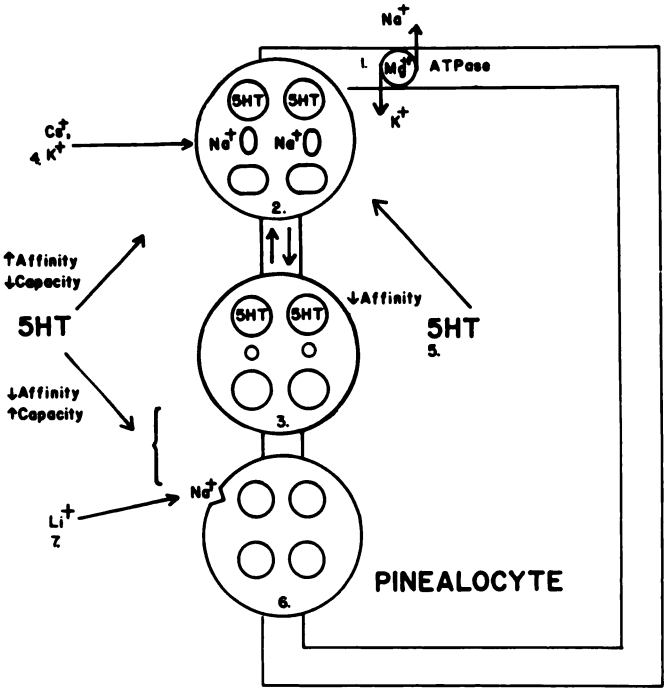

FIG. 7. *A proposed model for the allosteric carrier of ^{3}H-5HT in bovine pinealocytes*

The numbers represent the following: (1) a magnesium-dependent ($Na^+ + K^+$)-ATPase in the pinealocyte membrane; (2) the high-affinity, low-capacity configuration of the allosteric carrier; (3) the low-affinity configuration of the allosteric carrier; (4) the site at which Cs^+ and K^+ can partially substitute for Na^+ on carrier 2; (5) the intracellular accumulation of exogenous 5HT causes carrier 2 to change its configuration to 3; (6) the low-affinity carrier of 5HT (this carrier is visualized as being identical to carrier 3 except that it is sodium dependent); (7) the Na^+ ion on carrier 6, which can be partially replaced by Li^+.

(5) When Na^+ is removed from the medium, the K_m of uptake for concentrations of between 0.2 and 0.8 μM ^{3}H-5HT does not change, but the V_{max} is diminished by approximately 55%.

(6) The uptake between 0.02 and 0.08 μM ^{3}H-5HT and the uptake between 0.2 and 0.8 μM ^{3}H-5HT are energy dependent.

(7) Both ranges of concentrations are inhibited maximally by approximately 55% when ouabain is present or Na^+ is absent.

(8) When low (i.e., 0.037 μM ^{3}H-5HT (Fig. 6) and 0.07 μM ^{3}H-5HT (Fig. 1)) concentrations of serotonin are taken into cells as a function of time, the rate of uptake decreases after about 5 or 10 min.

(9) When the uptake of low concentrations of ^{3}H-5HT is observed as a function of concentration, the rate of uptake slows between approximately 0.1 and 0.2 μM ^{3}H-5HT but then increases with concentrations greater than 0.2 μM.

The transport of ^{3}H-5HT into bovine pinealocytes is visualized as follows (Fig. 7).

Two independent ^{3}H-5HT carriers exist in the pinealocyte membrane, 2 and 6. The movement of carrier 6 is sodium dependent, while the movement of carrier 2 is

sodium independent. Carrier 2, however, provides a limited number of high-affinity sites for serotonin. The affinity of serotonin at these sites is sodium dependent. The sodium ion associated with carrier 2 can be partially replaced by K^+ or Cs^+, but not by Li^+. Carrier 2 is allosteric and can change its configuration to 3 under the following conditions: (a) the absence of Na^+ in the incubation medium; (b) the presence of extracellular concentrations of serotonin in excess of 0.2 μM; and (c) the presence of appropriate intracellular concentrations of serotonin.

These three conditions cause the removal of Na^+ from its sites on the carrier with either subsequent or concomitant configurational changes of the carrier. As the concentration of serotonin is increased intra- or extracellularly, serotonin displaces or "squeezes" out Na^+ ions by causing conformational changes on the carrier. The sites available on the carrier are now of a lower affinity. Therefore, when low concentrations of serotonin are exposed to Na^+-free (5 mM Na^+) medium, the uptake of ^{3}H-5HT decreases by approximately 50% (Fig. 5A) because the apparent K_m has more than doubled. V_{max} remains the same because carrier 3 is the only mechanism available for 5HT transport in sodium-free medium. The combination of carrier 3 and 6 provides a low-affinity and high-capacity "carrier." In the presence of ouabain or the absence of sodium, carrier 6 would become nonfunctional. There would be an approximately 50% decline in the uptake of ^{3}H-5HT. This was observed as a change in V_{max} when 5HT concentrations of between 0.2 and 0.8 μM were used. The sodium ion associated with carrier 6 can be partially replaced by Li^+ (Fig. 5A) but not by Cs^+ or K^+ (Table 2).

Since the affinity of serotonin for specific carrier sites decreases in the presence of serotonin concentrations exceeding 0.2 μM, the relative inhibitory capacity of dopamine and L-norepinephrine can be increased (Table 3) in the presence of 0.5 μM serotonin if the affinity of these compounds for the specific carrier sites on carriers 3 and 6 is increased or decreased less than the decrease in affinity of serotonin for these same sites.

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