Studies of Platelet 5-Hydroxytryptamine (Serotonin) in Storage Pool Disease and Albinism

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ABSTRACT Platelets in patients with storage pool disease are markedly deficient in a nonmetabolic (storage) pool of ADP that is important in platelet aggregation. They are also deficient in ATP, although to a lesser degree. In seven patients with this disorder, including one with albinism, platelet 5-hydroxytryptamine (5-HT) levels were reduced in proportion to the reduction in ATP ($r = 0.94$). Their platelets show diminished capacity to absorb [^{14}C]5-HT, and the type of defect was similar to that produced in normal platelets by reserpine, a drug known to inhibit the uptake of 5-HT by the platelet dense granules. Storage pool-deficient platelets also converted more [^{3}H]5-HT to [^{3}H]5-hydroxyindoleacetic acid than did normal platelets, and the platelets in one of two patients studied contained increased amounts of 5-HT metabolites. The above findings, together with those reported previously, support the conclusion that the capacity of the dense granules (which may be either diminished or functionally abnormal) for storing 5-HT is decreased in storage pool disease; as a result, the 5-HT that enters the platelet may be more exposed to monoamine oxidases present on mitochondrial membranes. This diminished storage capacity (for 5-HT) may also explain why preincubating platelet-rich plasma with 5-HT for 45 min without stirring inhibits subsequent platelet aggregation by 5-HT to a greater degree in patients with storage pool disease than in normal subjects. The latter finding is also consistent with the theory that the aggregation of platelets by 5-HT is mediated by the same receptors on the plasma membrane that are involved in its uptake. The diminished release of platelet-bound [^{14}C]5-HT by collagen that we found in these patients, as well as findings in previous studies, suggests that the release reaction may also be abnormal in storage pool disease.

INTRODUCTION

The 5-hydroxytryptamine (5-HT, serotonin)[1] present in blood is found in the platelets (1) and originates from enterochromaffin cells, particularly those in the gastrointestinal tract (2). The transfer of 5-HT from plasma to platelets can occur at a considerable concentration gradient (3), and at least two subcellular sites have been implicated in this transport mechanism. There is evidence that active transport of 5-HT through the platelet membrane (4) may be mediated by specific receptors that appear to be of the classical D-type (5). Within the platelets, 5-HT is stored in granules of high electron density (dense bodies) (6–9), and it has been suggested that the localization of 5-HT within these granules may protect it from the action of metabolizing enzymes (10). In particular, platelets contain monoamine oxidase (11), (presumably located on the external membrane of mitochondria), and the formation of the principal metabolites of 5-HT, 5-hydroxytryptophol and 5-hydroxyindoleacetic acid (5-HIAA) (10, 11), is considerably enhanced if the platelets are treated with reserpine, a drug that liberates 5-HT from its storage organelles (10–13). Platelets are also aggregated by 5-HT (14), and recent studies by Baumgartner and Born suggest that 5-HT-induced platelet aggregation is mediated by the same receptors responsible for its uptake at the plasma membrane (15–17).

The platelet dense granules that store 5-HT also contain appreciable amounts of bivalent cations (18), as well as a special metabolically inactive pool of adenine nucleotides (the storage pool) that is important in platelet aggregation (19–20). We have previously reported a group of patients with a congenital disorder of hemostasis in whom the contents of the platelet dense granules

are diminished (21–23). As a result of the diminished amounts of ADP in the storage pool, collagen-induced platelet aggregation is impaired, and the second phase of epinephrine and ADP-induced aggregation is either absent or diminished (24–27). In addition, the release mechanism may also be abnormal in these patients (28). The type of congenital defect present in the platelets of these patients with "storage pool disease" makes them uniquely suitable for studying certain aspects of 5-HT uptake, metabolism, and release, and 5-HT-induced aggregation.

METHODS

Patients

The study included six patients with storage pool disease. Three patients (E. P., L. G., and S. N.) are unrelated, while three others (D. C., S. C., and R. C.) are members of a previously described family (family C) (25). In previous studies, all patients showed diminished collagen-induced platelet aggregation and absent or diminished second-phase aggregation with epinephrine or ADP (24–27). In addition, the amounts of storage pool ADP and ATP in their platelets were diminished (21, 22). Platelet 5-HT levels were decreased in three patients (L. G., D. C., and S. N.), and normal in one patient (E. P.) (22). The levels were not measured in patients R. C. and S. C. In the latter study, the normal levels of platelet 5-HT were 126 ±31.6 nmol/10^{11} platelets. A unique phospholipid abnormality, consisting of an increased lecithin: phosphatidyl ethanolamine ratio, was also found in family C but not in the other unrelated patients (29). Through the courtesy of Dr. James A. Wolff, we also studied a 10-yr-old girl (S. M.) of Puerto Rican descent with oculocutaneous albinism (30), on whom some of the findings at age 2 have been previously reported (31). She has light blonde hair, and the color of her skin is considerably lighter than that of her parents or siblings. Her irises are blue-grey, and continuous coarse nystagmus was prominent. She has continued to show easy bruising, frequent epistaxis, and excess bleeding after tooth extractions. Our studies showed a platelet count of 233,000/mm^3, absent second wave of aggregation with 5 μM epinephrine, markedly diminished collagen-induced aggregation (4%, compared with normal [±2 SD] values of 73±16% obtained with a dilute suspension of collagen [25, 26]), and disaggregation, after normal initial aggregation, with 4 μM ADP.

Control subjects were normal hospital employees. Both control subjects and patients took no drugs for at least 1 wk before the study.

Blood

Venous blood was mixed with 3.2% (0.108 M) sodium citrate (9:1) and centrifuged at 1,500 g and 20°C for 3 min to obtain platelet-rich plasma (PRP). To obtain platelet-poor plasma (PPP), the blood was centrifuged at 2,400 g for 30 min at 4°C. All glassware was silicone-coated (SC-87 Dri-Film, General Electric, Schenectady, N. Y. 15% in toluene).

Platelet aggregation

2 ml of PRP were stirred at 37°C in the cuvette of a Payton dual-channel aggregation module (Payton Asso-

ciates, Buffalo, N. Y.), and platelet aggregation was recorded continuously as the increase in light transmittance (relative to a PPP blank) on a Riken-Denshi (Tokyo, Japan) recorder with a chart speed of 30 mm/min. We determined the maximum slope of the aggregation curve by drawing a tangent to the curve at the point of inflection, and the velocity of aggregation in mm/min was calculated as the (slope) × (chart speed).

Determination of ATP and ADP in platelets

PRP was mixed in an ice bath for 10 min with an equal volume of 96% ethanol containing 10 mM EDTA. After centrifugation at 4°C and 14,000 g for 20 min, the ethanol extracts were stored at −60°C. Determination of ADP and ATP in the extracts was by the method of firefly luminescence (22, 32) with dessicated firefly lantern extracts (Fle-50, Sigma Chemical Co., St. Louis, Mo.) as the source of the luciferase. The reaction was carried out in a dark cell, and the light flash was measured with a photometer coupled to an Aminco-Bowman photomultiplier microphotometer (American Instrument Co., Inc., Silver Spring, Md.) and recorded on a 10-in strip chart potentiometric recorder (Beckman Instruments, Inc., Fullerton, Calif.).

Measurement of 5-HT in platelets

The method used was a modification of the method of Crosti and Luchelli (33, 34) as previously described, (22), except that in the present study fluorescence was measured in an Aminco-Bowman spectrofluorometer with excitation at 295 nm and emission at 540 nm, whereas in the previous study the instrument was a Farrand Ratio Fluorometer with a 295 nm interference filter for excitation light (Farrand Optical Co., Inc., Valhalla, N. Y.) and a Corning filter (3-69, Corning Glass Works, Science Products Div., Corning, N. Y.) for the fluoresced light. Since the fluorescence properties of all 5-hydroxyindoles are similer at pH values below 2 (2), the values obtained by this method are not specific for 5-HT.

5-HT uptake

[side chain-2-^{14}C]5-HT creatinine sulfate (57 mCi/mmol, Amersham/Searle Corp., Arlington Heights, Ill.), dissolved in 70% ethanol to a concentration of 2 mM, was stored at −20°C. 10 μl of this solution of radioactive 5-HT were added to 10 ml of PRP (adjusted to a platelet count of 200,000/mm^3 with autologous PPP), to give a final 5-HT concentration of 2 μM. The mixture was incubated in a plastic tube at 37°C, and at intervals of 1, 3, 5, 10, 30, and 60 min, 1-ml samples were removed and immediately placed in melting ice. At the end of 60 min, all specimens were centrifuged together at 10,000 g and 4°C for 30 min in a Sorvall RC2-B centrifuge (Ivan Sorvall, Newtown, Conn.). The supernate was removed, and the radioactivity in 100 μl was determined in a liquid scintillation vial containing 15 ml of Bray's solution, with a Tri-Carb dual-channel liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The amount of radioactivity remaining in the supernatant plasma was compared with the total radioactivity added (the latter determined from a sample of the incubation mixture in which the platelets had been lysed by the addition of 1/20 vol of 20% Triton X-100, Rohm and Haas Co., Philadelphia), and results were expressed as the percent activity taken up by the platelets. In most studies, we also measured the radioactivity in the

platelet pellet directly. This was done by resuspending the pellet in 1 ml of PPP containing 1% Triton X-100 and counting the radioactivity as described above. When measured in this way, the calculated percent uptake of radioactive 5-HT into platelets was generally 1–5% lower than when calculated by the first method, in which it was assumed that all of the loss of radioactivity from the plasma could be accounted for by uptake into the platelets. This was observed in both normal subjects and in patients.

Collagen-induced release of platelet-bound [^{14}C]5-HT

Studies by Holmsen, Østvold, and Day have shown that the release of platelet-bound [^{14}C]5-HT by aggregating agents parallels that of endogenous 5-HT and therefore can be used to assess the platelet release reaction (35). In this study, we used a suspension of human connective tissue, undiluted or diluted 1:2 in saline and prepared as previously described (36), as a source of collagen. Platelets were labeled with radioactive 5-HT by incubating PRP with 2 μM [^{14}C]5-HT for 30 min, and the percent of the radioactivity incorporated into platelets was determined on a sample as described above. Two other 2-ml samples were stirred with 0.1 ml of either undiluted or diluted collagen in the aggregation module for 6 min and then centrifuged immediately for 10 min in a Clay-Adams serofuge (Clay-Adams, Inc., Parsipanny, N. J.). The supernate was removed, and the percent of the platelet-bound [^{14}C]5-HT released by collagen was determined.

Metabolism of platelet-bound radioactive 5-HT

Incubation of PRP with radioactive 5-HT and preparation of extracts for chromatography. Citrated PRP, adjusted to a platelet count of 200,000/mm^3 with autologous PPP, and containing less than 500 red blood cells/mm^3, was incubated at 37°C for 5 h with 5 μM [^{3}H]5-HT. Studies on patient E. P. and a control subject studied on the same day were performed with [G-^{3}H]5-HT creatinine sulfate (6.8 Ci/mol, Amersham/Searle Corp.) and on all other patients (and controls run the same day) [side chain-1,2-^{3}H-(N)]5-HT binoxalate (1.3 Ci/mmol, New England Nuclear, Boston, Mass.). (These two chemical forms of [^{3}H]-5-HT could be used interchangeably, as indicated by the virtually identical results obtained in parallel studies on control subjects). The pH at the end of the incubation period was 8.18±0.04 for all specimens. After incubation, 4 ml PRP was centrifuged at 12,000 g and 4°C for 10 min. The platelet pellet and 2 ml of the supernatant plasma were extracted with 2 ml and 1 ml of 1 M HClO$_4$, respectively, for 10 min at 4°C, and the protein precipitates were sedimented by centrifugation at 20,200 g for 5 min. 1.5 ml of each supernate was then treated with either 1.5 ml (pellet supernate) or 0.5 ml (plasma supernate) of 0.6 M K$_2$CO$_3$ to bring the pH to 7.4 and to remove ClO$_4$⁻. The KClO$_4$ was sedimented by high speed centrifugation for 5 min, and the neutralized extracts were mixed (1:1) with a standard solution (37) that contained 5-HT (Sigma), 5-HIAA (Sigma), and 5-hydroxytryptophol, (Regis Chemical Co., Morton Grove, Ill.) in concentrations of 1 mM. The extract-standard mixtures were stored at − 60°C.

Paper chromatography. The above extracts were thawed, 20-μl aliquots were spotted under nitrogen on Whatman no. 1 chromatography paper (1 × 10-in strips) and the indoles were separated by ascending chromatography for 6 h in the dark with isopropanol: H$_2$O: concentrated am-
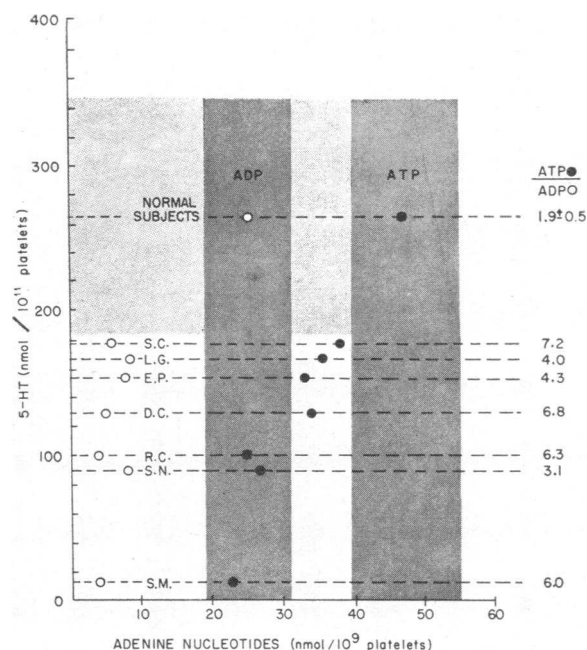
monia (20:2:1) solvent (38). The strips were dried and sprayed with HCl/methanol for visualization of the spots under ultraviolet light. They were then cut into six zones, and the radioactivity was eluted with 4 ml of methanol, twice, followed by 4 ml of acetone, twice. The combined elutants from each zone were evaporated overnight at 70°C in counting vials, and radioactivity was determined in the Tri-Carb scintillation counter after addition of 11 ml of Bray's solution to each vial. Counting efficiency was 8–15%. Recovery of radioactivity in the neutralized extracts was 60–80% and from the extracts after chromatography and elution was 95–100%. Results, expressed as picomoles of radioactive 5-HT or metabolites per milliliter of PRP, were calculated from the counts per minute, corrected for counting efficiency, extraction recovery, and dilutions. The radioactivity in the zone that contained the 5-HIAA (zone 2) were doubled to account for the 50% loss of specific activity that occurs in the conversion of [^{3}H]5-HT binoxalate to [^{3}H]5-HIAA.

Detection of endogenous 5-HT metabolites in platelets

Extraction of platelets. Citrated PRP was centrifuged at 12,000 g and 4°C for 10 min, and the platelet pellet was resuspended in 2.5 ml of 0.04 M Tris, pH 7.3, containing 27 mM EDTA. After addition of 2 ml of 1 M HClO$_4$, the protein precipitate was removed by centrifugation, and the

5-HT metabolites (5-HIAA and 5-hydroxytryptophol) in the acidic supernate were extracted with 4 ml of ethyl acetate. Under these conditions, Bartholini, Pletscher, and Bruderer (12) showed, using thin layer chromatography (TLC), that 5-HT remains in the aqueous phase while 5-HIAA and 5-hydroxytryptophol are found in the organic phase, and we confirmed this observation. After evaporation of the ethyl acetate at 37°C under a flow of N_2, the 5-HT metabolites were dissolved in 100 μl of methanol.

TLC. 50 μl of the methanol extracts were spotted under N_2 on a silica gel TLC plate. The extracts were chromatographed, together with similar volumes containing 5-HIAA and 5-hydroxytryptophol standards, for 2½ h with isopropanol: methyl acetate: H_2O: concentrated NH_3 (35:45: 2:18) as solvent. The plates were sprayed with HCl/ methanol, and fluorescence of the spots was developed by exposure to ultraviolet light. This method could detect 2–3 nmol of 5-HIAA or 5-hydroxytryptophol.

Quantitative determination of 5-hydroxyindoles in extracts. 5-hydroxyindoles in the aqueous phase, containing 5-HT, and organic phase, containing 5-HIAA and 5-hydroxytryptophol, obtained after extraction of the acidic supernates into ethyl acetate (see above) were determined in 3 N HCl by the spectrofluorometric method described in a previous section. The amount of metabolite was expressed as the percent of the total 5-hydroxyindoles.

Drugs

Reserpine phosphate and imipramine HCl were obtained from the CIBA Pharmaceutical Company, Div. of the Ciba-Geigy Corporation (Summit, N. J.) and were dissolved in water for in vitro studies. 5-HT creatinine sulfate was purchased from the Sigma Chemical Co., and was dissolved in 0.9% NaCl. Epinephrine HCl (Parke, Davis & Company, Detroit, Mich.) was also dissolved in saline.

Statistical methods

SD, SE, and equations for the regression line between two variables were determined by standard methods (39).

RESULTS

5-HT and adenine nucleotide content of platelets. Results are shown in Fig. 1. In normal subjects, the values obtained for 5-HT were 265±40 (SD) nmol/10^{11} platelets. The values obtained in this study, in which a spectrofluorometer was used to measure fluorescence, were approximately twice those obtained in a previous study in which fluorescence was determined by a filter fluorometer. The reason for this discrepancy is not known, but
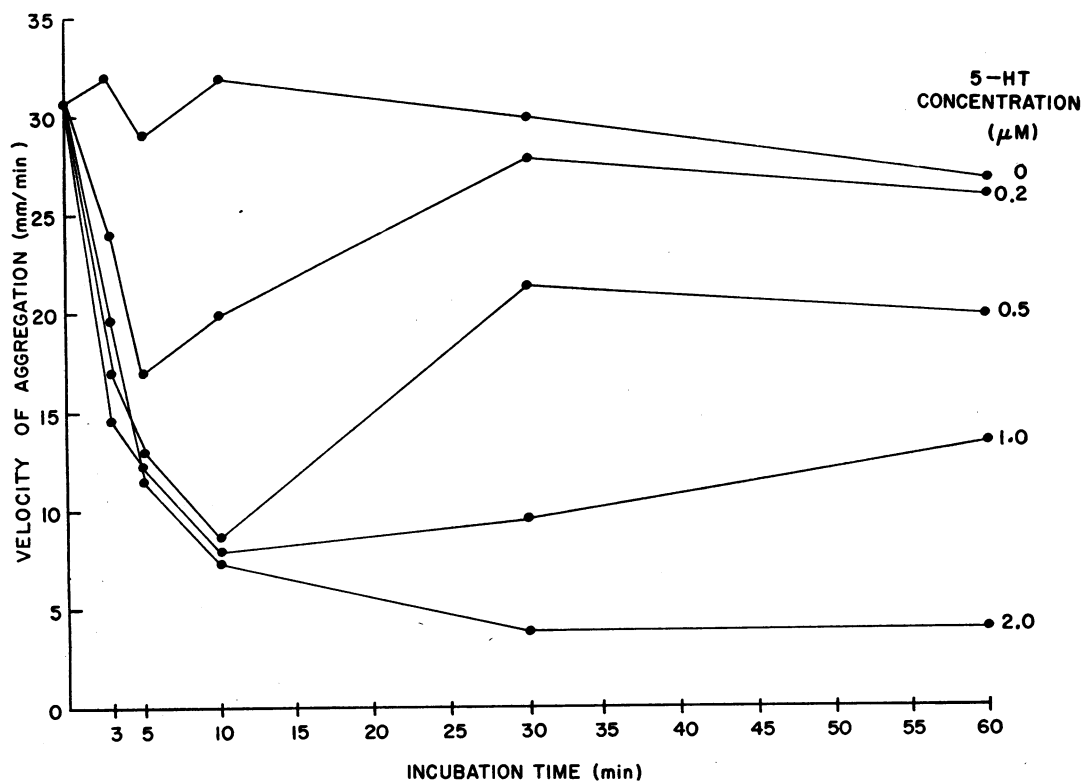

FIGURE 2 Effect of preincubating normal PRP with different concentrations of 5-HT for varying periods of time on the subsequent platelet aggregation by 5-HT (epinephrine). Citrated PRP from a normal subject was incubated at 37°C (without stirring) with 1/20 vol of 5-HT in the final concentrations indicated. Samples were transferred to the aggregometer cuvettes at the time indicated and 1/20 vol of a solution containing 5-HT and epinephrine (final concentrations of 5 μM and 1 μM, respectively) was added to the stirred PRP. The velocity of aggregation was determined as described in Methods.
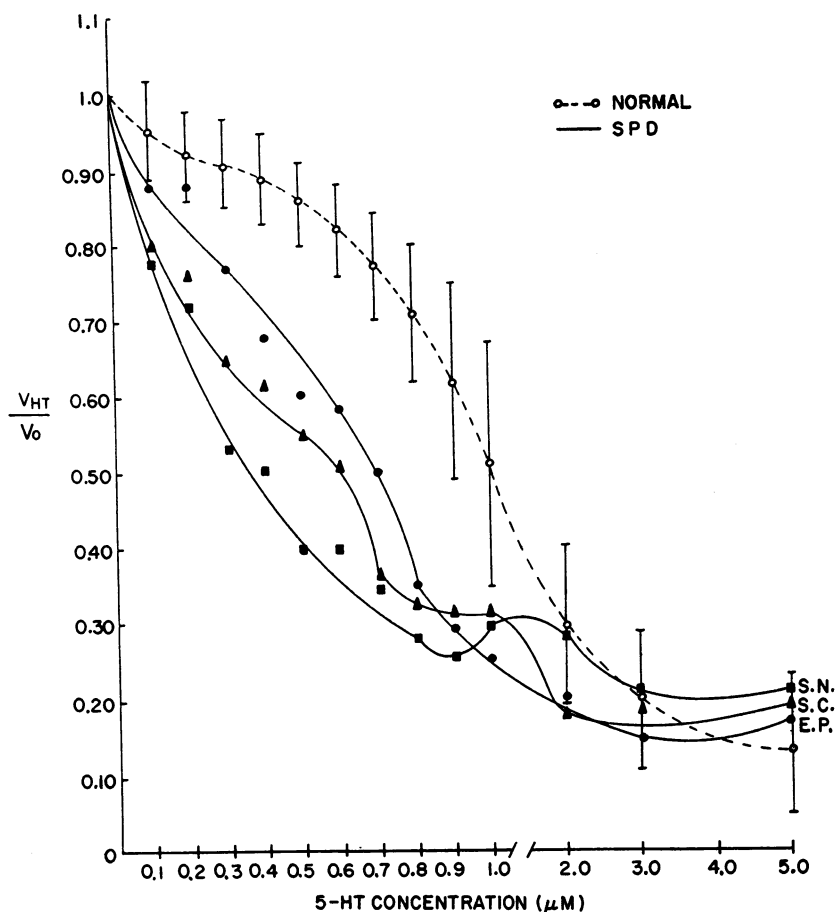
FIGURE 3 Effect of preincubating PRP for 45 min with varying concentrations of 5-HT on subsequent platelet aggregation by 5-HT (epinephrine). Conditions were similar to those in Fig. 2. Results are expressed as the ratio of the velocity of aggregation obtained after preincubation with 5-HT (V_{HT}) to the velocity obtained on the same PRP incubated with saline ($V_0 = 0$ concentration of 5-HT). The inhibitory effects of preincubating the PRP with 5-HT are more pronounced in patients S. N., S. C., and E. P. with storage pool disease (SPD) than in normal subjects, whose mean±SD values are connected by the dashed line.

exactly the same findings have been reported recently by Holmsen et al. (35). The amount of 5-HT in the platelets of patients with storage-pool disease was decreased in all cases. The most striking defect was obtained in the patient (S. M.) with albinism, whose platelets contained only 13 $nmol/10^{11}$ cells (5% of the normal value). In patients S. N., R. C., and D. C., the values of 90, 100, and 125 nmol were 34–47% of the mean value obtained in normal subjects. The values of 180, 167, and 155 nmol, obtained in patients S. C., L. G., and E. P., represented only modest reductions in the 5-HT content of their platelets. The decreased content of platelet ADP (and to a somewhat lesser extent of ATP) and the increase in the ATP/ADP ratio previously described for patients with storage pool disease (21, 22) were again obtained. Similar findings were also obtained in the patient (S. M.) with albinism.

There was a good correlation ($r = 0.94$) between the amounts of 5-HT and ATP in the platelets (Fig. 1).

Aggregation of platelets by 5-HT and by a mixture of 5-HT and epinephrine. As reported by others (14, 15), we found that 5-HT produces weak aggregation of human platelets, consisting of a single, reversible wave. However, the aggregation of platelets by 5-HT is markedly increased by the simultaneous addition of epinephrine in concentrations that, by themselves, produce only a small first wave (15, 16). We therefore used a mixture of 5-HT (final concentration 5 μM) and epinephrine (final concentration 1 μM) to aggregate platelets in this study. The velocity (V) of the first wave of aggregation produced by this mixture of 5-HT and epinephrine will henceforth be referred to as 5-HT (epinephrine)-induced platelet aggregation. We confirmed the findings of Baumgartner and Born that 5-HT (epineph-

rine)-induced platelet aggregation was diminished by prior incubation without stirring with 5-HT (15, 16). This inhibitory effect of 5-HT was dose-dependent and progressive during the first 5-10 min of incubation (Fig. 2). With continued incubation (except for the highest concentration of 5-HT), the platelets gradually regained their ability to aggregate with 5-HT (epinephrine).

We next studied whether the behavior of platelets in storage pool disease was similar to that described above in normal subjects. We incubated PRP with increasing concentrations of 5-HT (0.1–5.0 μM) for 45 min at 37°C and then studied 5-HT (epinephrine)-induced platelet aggregation. The results are shown in Fig. 3 and demonstrate that for each concentration of 5-HT used during the 45-min incubation period, the subsequent aggregation of storage pool platelets by 5-HT (epinephrine) (V_{HT}) was less, relative to the control value, V_o, than that obtained with normal platelets. The results of these studies indicate that the inhibition of 5-HT (epinephrine)-induced aggregation by prior incubation

of the platelets with 5-HT, first described by Baumgartner and Born (15, 16), is more apparent in patients with storage pool disease than in normal subjects.

Uptake of radioactive 5-HT into platelets. PRP was incubated with [^{14}C]5-HT, as described in Methods. The uptake of radioactivity into the platelets of normal subjects was rapid during the first 5-10 min, and saturation levels of 95±2 (SD)% uptake were obtained after 30 min (Fig. 4). In five patients with storage pool disease, the saturation levels (but not the initial rates of uptake) were decreased (Fig. 4). The most pronounced abnormalities were observed with the platelets of patient S. N. and in the patient (S. M.) with albinism.

The shape of the 5-HT uptake curves in patients with storage pool disease were similar to those produced by incubating normal PRP with varying concentrations of reserpine, a drug that inhibits the uptake of 5-HT into the platelet granules (40–42). Reserpine had no effect on the initial rate of uptake, but decreased the saturation levels of [^{14}C]5-HT (Fig. 5). By contrast, imipramine, a membrane-active drug (40, 43), inhibited the initial
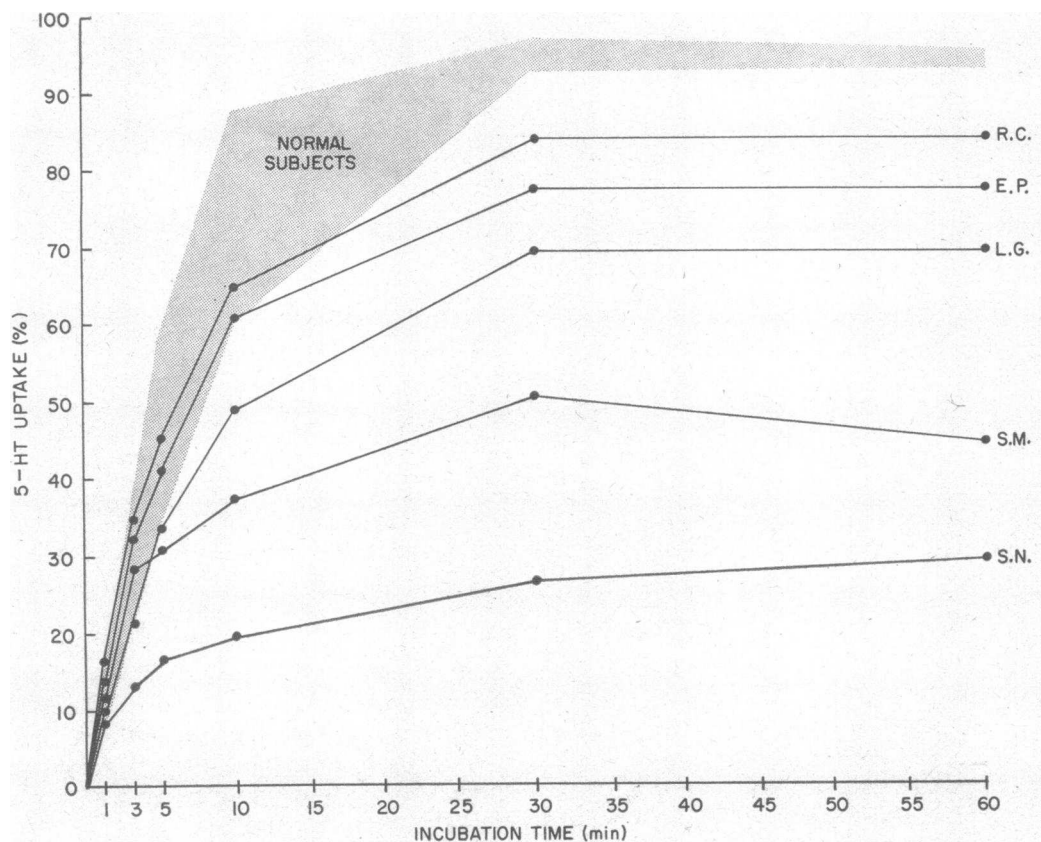
FIGURE 4 Uptake of radioactivity into platelets obtained during incubation of PRP with [^{14}C]5-HT. PRP was incubated at 37°C with a 2 μM concentration of [^{14}C]5-HT and the percent of the total radioactivity that was incorporated into the platelets was determined. The stippled area encloses the mean±SD obtained in normal subjects. Closed circles are values obtained in patients with storage pool disease.

426   H. J. Weiss, T. B. Tschopp, J. Rogers, and H. Brand

FIGURE 5 Effect of reserpine and imipramine on the uptake of [¹⁴C]5-HT into normal platelets. Experimental conditions were similar to those in Fig. 4 except that PRP contained 1/20 vol of reserpine (above) or imipramine (below) in the concentration indicated.
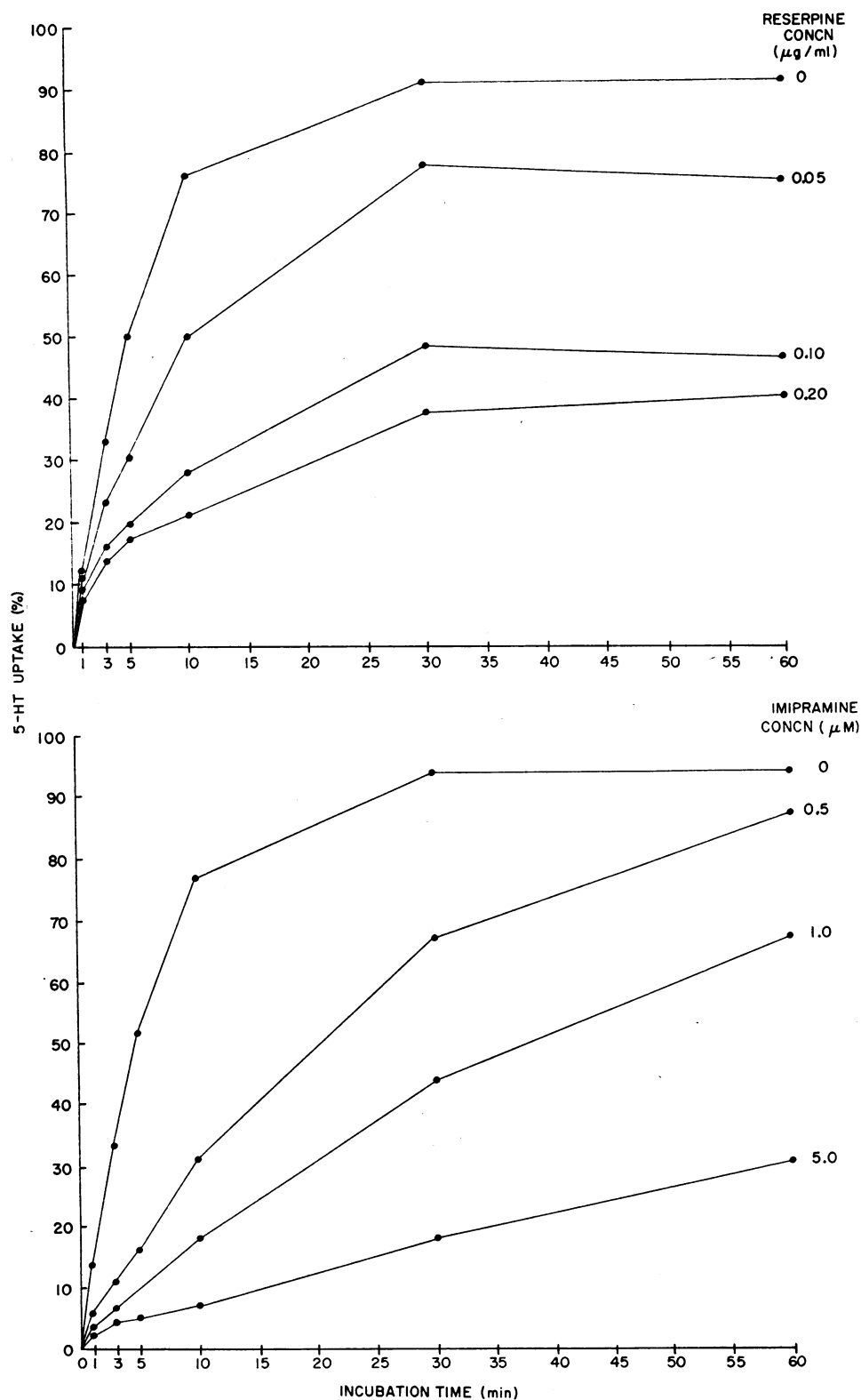
Platelet 5-Hydroxytryptamine (Serotonin) in Storage Pool Disease   **427**

rate of uptake, but the platelets continued to accumulate 5-HT during the incubation period (Fig. 5).

Release of platelet-bound [^{14}C]*5-HT.* In addition to the impairment of the 5-HT uptake described above, the release of platelet-bound [^{14}C]5-HT by collagen was also impaired in patients with storage pool disease. This is demonstrated by the findings shown in Fig. 6. When stirred with PRP from normal subjects, undiluted or diluted collagen released 77±12 (2 SD)% or 63±12 (2 SD)% of the platelet-bound [^{14}C]5-HT into the plasma. Impaired release of 5-HT was obtained in all four patients with storage pool disease studied. The figure also shows, for comparison, the well-known inhibitory effects of aspirin on the platelet release reaction (36, 44).

Metabolism of radioactive 5-HT by platelets. PRP was incubated for 5 h with [^{3}H]5-HT, and the platelets and plasma were separated and analyzed by paper chromatography for 5-HT and its two principal metabolites, 5-HIAA and 5-hydroxytryptophol (see Methods and Fig. 7). Previous studies have shown that radioactive metabolites of 5-HT that are formed in the platelets can pass into the plasma (12). In addition, 5-HT is not metabolized by human plasma (45, 46). Therefore, the
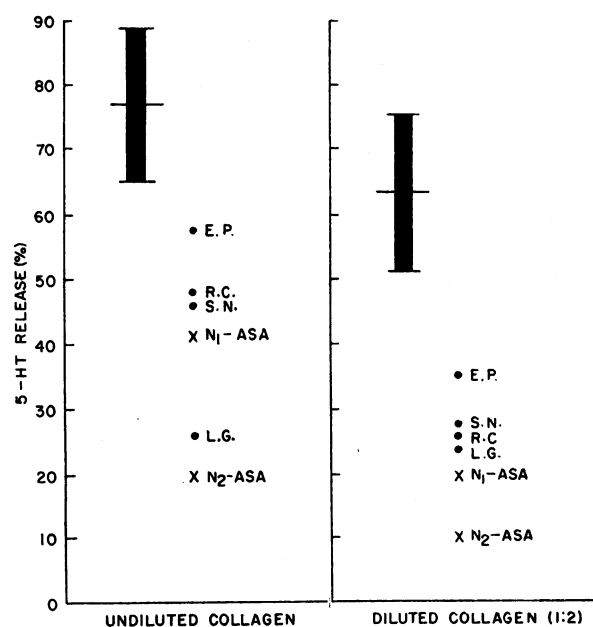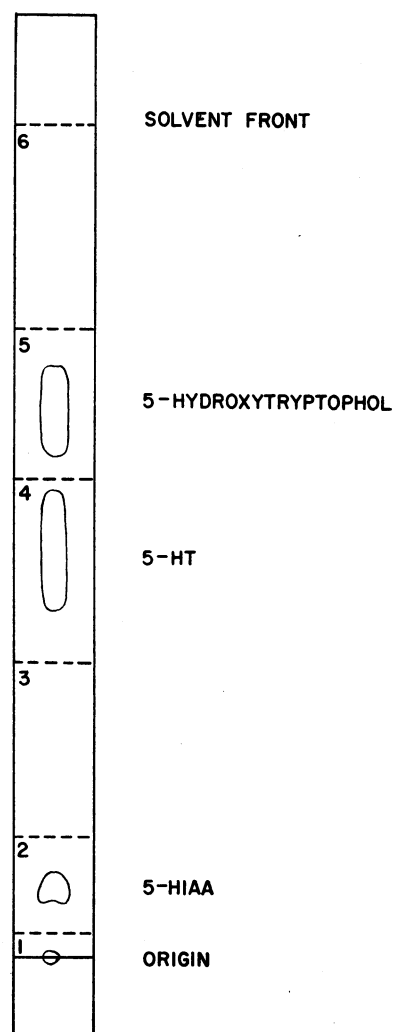

FIGURE 7 Separation of radioactive hydroxyindoles by paper chromatography. Paper chromatography was performed on neutralized platelet and plasma extracts (see section in Methods on radioactive metabolites) obtained after incubation of PRP for 5 h with [^{3}H]5-HT. The diagram depicts the relative positions of 5-HT, 5-HIAA, and 5-hydroxytryptophol. The strips were cut into six sections along the dashed lines, and the radioactivity in the strips was eluted and counted (see Table I). Radioactivity in zone 3 could be either an unidentified metabolite or trailing from the adjacent 5-HT zone.

FIGURE 6 Release of platelet-bound [^{14}C]5-HT. PRP was incubated first for 30 min at 37°C with 2 μM [^{14}C]5-HT. A 2-ml sample was then stirred in the aggregation module for 6 min with 0.1 ml of either undiluted or diluted (1:2) collagen and the amount of released radioactivity (expressed as a percent of the amount taken up by the platelets) was determined. The solid bars depict the mean±SD values obtained in normal subjects. Individual values are those obtained in patients with storage pool disease (●) and in two normal subjects (N_1 and N_2) who had ingested 1.2 g of aspirin 2 h before their blood was obtained (X).

ratio of the total amount of ^{3}H metabolites (in platelets and plasma) to that of [^{3}H]5-HT in the platelets is an indication of the extent to which the [^{3}H]5-HT in the platelets has been metabolized. The results are shown in Table I. The ratio of ^{3}H metabolites to [^{3}H]5-HT in normal subjects was 0.44±0.06 (SD). Strikingly increased values were obtained in patients E. P. and L. G., and the albino patient S. M. (2.08, 0.78, and 7.56, respectively). In all cases, this increase was primarily due to an increased amount of [^{3}H]5-HIAA. By con-

TABLE I

Amount of Radioactive Hydroxyindoles after Incubation of PRP with [³H]5-HT

| | Platelets | | | | | | Plasma | | | | | | [³H]metabolites‡ | |
	Zone 1*	5-HIAA	Zone 3	5-HT	Tpol	Zone 6	Zone 1	5-HIAA	Zone 3	5-HT	Tpol	Zone 6	Total	Platelet [³H]5-HT
							pmol [³H]hydroxyindoles/ml of PRP							
Controls (n = 7)														
Mean	54	285	167	2,710	58	1	161	670	406	507	171	2	5,196	0.44
SD	8	71	59	148	28	1	51	141	48	115	46	3	22	0.06
SPD patients														
E. P.	60	636	119	1,183	25	2	379	1,614	388	523	182	22	5,133	2.08
L. G.	48	541	121	2,253	53	0	155	926	386	492	232	2	5,209	0.78
S. M.§	86	1,170	64	436	7	0	266	1,857	413	650	262	4	5,215	7.56
D. C.‖	55	399	138	2,737	44	3	146	582	369	451	187	5	5,116	0.44
R. C.	36	227	136	2,615	22	1	186	816	347	540	182	2	5,110	0.48
S. C.	60	238	144	2,696	15	1	177	715	293	561	208	6	5,114	0.44

* PRP was incubated with [³H]5-HT (5,000 pmol/ml) for 5 h and 5-hydroxyindoles were separated by paper chromatography (Fig. 7). The origin is enclosed in chromatogram zone 1. After chromatography of [³H]5-HT alone, 95% of the radioactivity was recovered in the 5-HT zone.
‡ Metabolites are the total amounts of [³H]5-HIAA and [³H]5-hydroxytryptophol (Tpol) in platelets and plasma.
§ S. M. is a patient with oculocutaneous albinism.
‖ D. C., R. C., and S. C. are members of family C.

trast, radioactive metabolites were not increased in any of the three members of family C.

Content of endogenous 5-HT metabolites in platelets. The above studies indicated that the platelets of some patients with storage pool disease could metabolize [³H]-5-HT more rapidly than normal platelets. This suggested

TABLE II

Endogenous Metabolites (5-HIAA + 5-Hydroxytryptophol) in Platelets

| | PRP | No. of platelets extracted (× 10⁻¹¹) | 5-Hydroxyindole content* | | % Metabolites |
			5-HT	5-HIAA + Tpol	
	ml		nmol/10¹¹ platelets		% total indoles
Control subjects					
D. Y.	66	0.24	225	12	5.2
S. S.	26	0.12	178	13	6.8
Patients					
L. G.	105	0.24	163	10	5.8
E. P.	25	0.09	126	20	13.7

* Platelets were extracted as described in Methods. The values shown for 5-HT are the amounts of indoles present in the aqueous phase and those for the metabolites 5-HIAA and 5-hydroxytryptophol (Tpol) are the amounts in the organic phase after extraction of the acidic supernate with ethyl acetate. TLC on the organic phase was also performed for patient L. G. and control D. Y.

that their platelets might contain more metabolites than normal subjects. To examine this possibility, we extracted the platelets of patients L. G. and E. P and two normal subjects, starting with the amount of PRP indicated in Table II, by the procedure outlined in Methods. By TLC, no metabolites were detectable (by fluorescence) in either patient L. G. or control D. Y. Since 2–3 nmol of either metabolite could have been detected by this method, the findings indicated that neither was present in an amount greater than 10% of the total hydroxyindoles in the platelets of these two subjects. Actual measurement of the hydroxyindole content in the extracts indicated that the total content of metabolites (5-HIAA + 5-hydroxytryptophol) in the two normal subjects and in patient L. G. was 5–7% of the total platelet hydroxyindoles. In patient E. P., however, the percentage of metabolites was 14%. (This patient also metabolized [³H]5-HT more extensively than patient E. G.; see Table I). These studies were not done in patient S. M.

DISCUSSION

The diagnostic features of storage pool disease are the markedly diminished content of platelet ADP and the relatively lesser diminution of ATP, resulting in an increased ATP/ADP ratio (Fig. 1). As discussed in detail previously (22, 47), these findings are due to the deficiency of a special, nonmetabolic pool of adenine nucleotides (the storage pool) that in normal platelets

has a particularly high content of ADP. We found a decreased content of platelet 5-HT in all seven patients with storage pool disease studied, and the levels showed a good correlation with those obtained for ATP. The latter finding is consistent with previous suggestions that 5-HT and ATP in platelets are complexed, with bivalent cations, as high molecular-weight aggregates (18, 48, 49). In contrast to the striking decrease in ADP obtained in all patients, the extent to which 5-HT and ATP were decreased was more variable (Fig. 1). The most striking decreases were obtained in the patient S. M. with albinism. Decreased 5-HT (31, 50, 51) and adenine nucleotide (50) values have also been reported in other patients who have both albinism and a bleeding disorder, and the findings in the present study suggest that the platelet defects (31, 50–52) in these patients may differ only in degree from those in patients with "idiopathic" storage pool disease.

In addition to the diminished levels of 5-HT and adenine nucleotides, the platelets of patients with storage pool disease and albinism are also deficient in Ca^{+2} (23) and show diminished numbers of the electron-dense granules (51, 53) in which these substances are stored (6–9). As also described in patients with albinism (31, 50), their platelets showed an impaired capacity for absorbing radioactive 5-HT, and the type of defect appeared to be similar to that observed in normal platelets treated with reserpine, a drug that specifically blocks the incorporation of 5-HT into the platelet granules (40–42). These findings suggest that the basic defect in storage pool disease may be either a decreased number of the dense granules or a functional defect in their storage capacities. An impaired capacity of the dense granules for storing 5-HT would appear to provide the most satisfactory explanation for the diminished 5-HT content of their platelets.

The findings in the present study further suggest that the platelet 5-HT in patients with storage pool disease (and the albinism variant of this disorder) may be metabolized more rapidly than that in normal platelets. For example, incubation of PRP from patients E. P., L. G., and S. M. with [^{3}H]5-HT for 5 h resulted in the appearance of more [^{3}H]5-HIAA (S. M. $\gg$ E. P. $>$ L. G.), particularly in the plasma, than in similar studies performed with the PRP of normal subjects. Using somewhat different experimental conditions, Hardisty, Mills, and Ketsa-Ard also found an increased amount of an unidentified radioactive metabolite of 5-HT after incubating a suspension of washed platelets obtained from their albino patient for 1 h with [^{14}C]5-HT (50). It is not clear why increased amounts of radioactive metabolites of 5-HT were not found in the three members of family C who were studied. Other differences between the platelet defects in this family and those in

other, unrelated patients with storage pool disease have been previously noted (26, 27). In addition to an increased metabolism of [^{3}H]5-HT, we also found increased amounts of endogenous 5-HT metabolites in the platelets of patient E. P. (S. M. was not studied in this regard). Future studies to detect a possible increase in the urinary excretion of 5-HT metabolites in patients with storage pool disease are anticipated. The increased capacity for metabolizing platelet 5-HT in storage pool disease is best explained at present by the diminished capacity of their platelets for accumulating and sequestering 5-HT in storage granules. As a result of this latter defect, a relatively larger percentage of the 5-HT transported through the plasma membrane may remain unprotected from monoamine oxidase present on mitochondrial membranes.

The impaired release of platelet-bound [^{14}C]5-HT by collagen that we found in storage pool disease could be due to a relatively smaller percentage of the absorbed 5-HT entering the storage granules, if the latter are the source of the 5-HT extruded from the cell during the release reaction (54). However, in previous studies we found that the release of heparin-neutralizing activity (platelet factor 4, PF-4) by collagen or epinephrine was also impaired in storage pool disease (28), although the total content of PF-4 in their platelets was normal, and Hardisty et al. reported that the thrombin-induced release of acid hydrolases was decreased in the albinism variant of this syndrome (50). In addition, synthesis of prostaglandins E_2 and $F_{2\alpha}$, which is closely associated with the release reaction (55), is also impaired in storage pool disease (56). These findings suggest that the release mechanism may be defective in this disorder, and this would explain the impaired release of platelet-bound [^{14}C]5-HT that we found.

We confirmed the observations of Baumgartner and Born that the aggregation of platelets by 5-HT is inhibited by their prior incubation with 5-HT (15–17). These authors suggested that during the preincubation period, 5-HT is transported through the membrane by receptors from which the amine then dissociates and is taken up in the storage granules. With increasing concentrations of 5-HT (see Fig. 2) or where the storage capacity of the platelet granules is inhibited by reserpine (16, 17), the receptors become increasingly saturated with 5-HT and, hence, are unable to react with the second dose of this amine. Therefore, 5-HT-induced aggregation is inhibited, and this effect was more pronounced in patients with storage pool disease. The latter finding is consistent both with the previous conclusions that the platelets of these patients have a limited capacity for storing 5-HT and with the hypothesis of Baumgartner and Born that the aggregation of platelets by 5-HT is mediated by the same receptors on the

plasma membrane that are involved in its uptake (15–17).

As the role of 5-HT in hemostasis is not clear at present, the clinical significance of the abnormalities in 5-HT content, metabolism, release, and aggregation that we found in patients with storage pool disease remains to be determined. Recently, a type of platelet defect similar to that in storage pool disease has been described in an inbred strain of rat (57). As in storage pool disease, the platelets of these rats (the fawn-hooded strain) are deficient in both adenine nucleotides and 5-HT (57). In addition, the behavior of these rats appears to be markedly different than that of other strains of this animal,[2] and significant behavioral differences, evaluated by a variety of tests, have recently been documented.[3] Since some, but by no means all, of the patients with storage pool disease have had psychiatric problems of varying severity, it is entirely possible that biochemical abnormalities involving 5-HT in platelets may sometimes reflect abnormalities of a more general nature in both man (58–60) and experimental animals.

ACKNOWLEDGMENTS

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