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Serotonin Deficiency and Prolonged Bleeding in Beige Mice (39137)[1]

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Beige is a pleotropic autosomal recessive mutation of the mouse (1). Principal phenotypic manifestations of the trait include coat color dilution, brought about through aggregation of melanin granules (2), enlarged lysosomes in cells of the granulocytic series (3, 4), decreased resistance to bacterial infections (5), impaired *in vitro* chemotaxis and microbial killing by neutrophil leukocytes (6), ultrastructural abnormalities of the golgi–endoplasmic–reticular system of the liver (7), and the vacuolar system of the kidney (8, 9). On the basis of these characteristics the beige mouse is accepted as an animal model of the Chediak–Higashi syndrome (CHS) of man (10, 11).

The present report describes another manifestation of the beige mouse syndrome, the impaired uptake and storage of serotonin (5HT) by blood platelets, which is associated with a parallel but quantitatively dissimilar adenine nucleotide deficiency. In addition, beige mice are observed to bleed excessively either spontaneously, due to fighting trauma, or from experimental wounds. A causal relationship between the observed biochemical abnormalities and the bleeding defect is suggested by our observation that prolonged bleeding does not occur in mice that have been pretreated with small parenteral doses of exogenous serotonin.

Materials and methods. Inbred beige (bg/bg) mice were obtained from the original line maintained by Dr. Liane B. Russell (Oak Ridge National Laboratory) on a C57BL background. F_1 hybrids (+/bg) were produced by mating female inbred C57BL/6 mice to beige males. The resultant progeny were phenotypically normal. Controls (+/+) were C57BL/6J mice of the same inbred line used to produce the hybrids. Because larger blood volumes would be obtained, only healthy 4–8 month old males were used in these experiments. Mice were given pasteurized Purina 5010C and given, to inhibit bacterial growth, hyperchlorinated-acidified water *ad lib.*

Collection of whole blood and separation of platelets. Mice were anesthetized by a brief exposure to CO_2. The thorax was reflected and heart blood was collected through a 25-gauge needle into a 1-cc syringe containing 0.1 ml of 3% (w/v) sodium citrate. The blood was mixed well with anticoagulant and after removing the needle, transferred to precooled siliconized glass centrifuge tubes. The volume of blood collected was recorded and all samples were diluted to 3 ml with cold saline. After mixing a sample was removed and platelets were counted by phase microscopy. The tubes were spun for 10 min at 150*g*. The platelet rich plasma (PRP) layer was removed using a siliconized pasteur pipette, the volume was recorded, and all was adjusted to 3 ml with cold saline. A sample was removed from the PRP tube for the determination of percentage recovery of platelets after centrifugation of the saline diluted blood. Platelets were spun out of the plasma by centrifugation for 10 min at 1000*g*. The plasma supernate was discarded and the platelet pellet resuspended in 3 ml of distilled water. The distilled water platelet lysate was used in subsequent assays for ATP, ADP, 5HT, and protein.

Bleeding time and reversal by 5HT. Beige mice were anesthetized with sodium phenobarbital and the terminal 2-mm portion of the tail was severed using a sharp razor blade. Bleeding time was measured by stopwatch to the nearest second using a filter paper disc intermittently applied to the adherent drop of blood.

The systemic effects of exogenous 5HT

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were evaluated by injecting anesthetized beige mice via tail vein with various dilutions of serotonin creatinine sulfate complex (Sigma, Lot 72C-1650) in a total volume of 0.2 ml of saline. The serotonin was >98% pure by thin-layer chromatography and was freshly prepared immediately before use. The concentration of serotonin injected was calculated as the free base assuming the anhydrous molecular weight of serotonin and its creatinine sulfate complex were 176 and 387 g/mole, respectively. On this basis 2.2 mg of the serotonin complex was equivalent to 1.0 mg of serotonin. One minute after injection, the tail was severed and bleeding time was determined.

Platelet 5HT assay. The method of Ashcroft *et al.* (12) was used. Sets of standards, a reagent blank and 1-ml aliquots of the distilled water platelet lysate were deproteinized and made 3 *M* with HCl. Samples were assayed using a Turner 110 filter fluorimeter equipped with a 7-54 primary and 4-97 and 2A-12 secondary filters.

Platelet adenine nucleotide assay. Adenine nucleotides were determined using a modification of the firefly photoluminescence method of Holmsen *et al.* (13). One millimeter of the distilled water platelet lysate was heated to 100° for 8 min. Of this heat inactivated extract, 0.1 ml was mixed with 2.0 ml phosphoenolpyruvate–pyruvate kinase (Sigma) for the assay. Pure standards of ATP and ADP (Sigma) were run concurrently. Luminescence intensity was measured using an Aminco Photomultiplier Microphotometer (Model 10-280) equipped with a Chem-Glow reaction chamber (Model J4-7441).

Platelet protein assay. The method of Lowry *et al.* (14) was used on 0.1 ml of the distilled water platelet lysate against a reference standard of crystalline bovine albumin.

Measurement of 5HT uptake by platelets in vivo. Groups of nine C57BL/6 and nine beige mice were injected via tail vein with 1 μCi of tritiated 5HT (Amersham/Searle, Lot TRK223), specific activity 29 mCi/mmole, in 0.1 ml saline. At 20, 40, 80, 160, 240, and 320 min after injection, groups of three mice from both mutant and control groups were anesthetized and heart bled. The PRP obtained as described previously was divided into 1-ml replicates and the platelets were pelleted by centrifugation for 10 min at 1000*g*. Only those preparations that yielded 50% or greater platelet recovery were assayed in order to exclude the rare sample that had begun to clot. There were no significant differences between mutant and control % recovery. The supernatant plasma was retained for scintillation counting. The platelet pellet was resuspended in 1 ml of saline and recentrifuged. The second supernatant was also counted and represents the wash fraction. The final platelet pellet was resuspended in 1 ml of saline. The original supernatant, wash, and platelet fractions were each mixed with 10 ml of a cocktail containing 1 part Triton X-100 and 2 parts toluene with Liquaflor as a scintillator.

Electron microscopy of platelets. Platelet pellets from beige and control mice were overlayed with cold phosphate buffered (pH 7.3, 0.1 *M*) 3% glutaraldehyde. After fixation for 3 hr, the pellets were washed with phosphate buffer and postfixed in 1% osmium tetroxide in the same buffer. After dehydration in graded alcohols and propylene oxide, the pellets were infiltrated overnight and subsequently embedded in Epon 812. Thin sections were placed on Formvar-coated grids, stained with uranyl acetate and lead citrate, and examined in a Hitachi 11-E electron microscope operated at 75 kV.

Statistics. All values are expressed as means ± the standard error unless otherwise specified. Student's *t* test was used to compare mutant and hybrid values against control. Only those comparisons that gave a $P < .05$ were considered significant.

Results. Bleeding time, platelet counts, and percentage recovery. Average platelet counts, corrected for anticoagulant and saline dilution, were the same for all three genotypes (Table I). The percentage recovery of platelets from dilute whole blood was also very similar, suggesting that platelet size or density did not differ appreciably among mice of various genotypes. Phase microscopy of the platelets did not reveal obvious differences in size or shape among the three genotypes. Bleeding time in mu-

tant (bg/bg) was significantly longer than either hybrid or normal. Beige bleeding time is reported in excess of 10 min because seldom did hemostasis occur spontaneously in this period of time. Bleeding could be arrested by firm pressure or silver nitrate cautery.

Bleeding time reversal. Prolonged bleeding in beige mice was prevented by prior treatment with exogenous 5 HT (Table II). A crude estimate of bleeding rate was obtained by inspection of the spot diameter on the filter disc at each successive 30-sec interval. Doses in excess of 6 μg/10 g were equally effective in reversing the bleeding tendency, while a dose of 3 μg/10 g the effect was either transient or absent.

TABLE I. AVERAGE WHOLE BLOOD PLATELET COUNT, BLEEDING TIME, AND PERCENTAGE PLATELET RECOVERY IN BEIGE, HYBRID, AND CONTROL MICE.[a]

Genotype	Number of observations	Platelet count/mm³ × 10³	Recovery (%)	Bleeding time (sec)
bg/bg	15	1204 (58)	67 (2)	>600
+/bg	15	1152 (47)	69 (3)	300 (45)
+/+	15	1204 (50)	66 (2)	255 (22)

[a] Numbers in parenthesis are standard errors.

TABLE II. HEMOSTASIS IN BEIGE MICE AFTER INTRAVENOUS INJECTION OF SEROTONIN CREATININE SULFATE.

Amount of 5HT (mg/10 g body wt)	Number of mice	Average bleeding time (sec)	Comment
0.1	4	270 (24)[a]	Rate reduced
0.05	4	270 (35)	Rate reduced
0.025	4	232 (29)	Rate reduced
0.0125	4	210 (0)	Rate reduced
0.006	4	232 (15)	Rate reduced
0.003	4	255 (30)	Rebleeding observed
0.0015	4	600	No effective hemostasis

[a] ±1 SD.

Adenine nucleotide, 5 HT, and protein content of platelets. Beige platelets contain significantly less ATP, ADP, and 5 HT than either hybrid or normal platelets (Table III). While protein values follow the general trend, the differences were not significant at the 5% level. F_1 hybrid (+/bg) levels were also less than normal but the differences were not significant. The ratio of ATP:ADP was 3.4, 2.8, and 2.9 in beige, hybrid, and normal, respectively. The relative deficiencies of ATP, ADP, and 5 HT were not proportional; both ATP and ADP were reduced approximately 3-fold while 5 HT was reduced 35-fold.

Uptake and binding of exogenous 5 HT by platelets. Twenty minutes after *in vivo* injection of tritiated 5 HT, beige mice had only 63% as much radioactivity in platelet-rich plasma as controls (Table IV). The label that associated with beige platelets was not stable, as evidenced by the proportionately greater amount that either remained in the plasma or eluted from the platelets in the wash fraction. Normal platelets not only took up the label rapidly but the levels remained essentially constant over the time of the assay (320 min). Since the activity decreased sharply in plasma of beige mice, if it is assumed that catabolism and excretion of label were similar in mutant and control, this would suggest that beige platelets were not competent to bind and protect the administered 5 HT (Fig. 1).

Platelet electron microscopy. Structures typical of catecholamine specific storage organelles were readily demonstrated in normal platelets (Fig. 2). Similar structures were not demonstrated in multiple sections

TABLE III. AVERAGE ADENINE NUCLEOTIDE, 5HT, AND PROTEIN CONTENT OF ISOLATED PLATELETS FROM BEIGE, HYBRID, AND CONTROL MICE.[a]

Genotype	Number of observations	ATP (μg)	ADP (μg)	5HT (μg)	Protein (mg)
bg/bg	15	0.89 (0.06)	0.26 (0.04)	0.011 (0.003)	0.22 (0.01)
+/bg	15	2.07 (0.18)	0.74 (0.09)	0.327 (0.015)	0.24 (0.01)
+/+	15	2.61 (0.22)	0.90 (0.12)	0.388 (0.030)	0.25 (0.01)

[a] All values are expressed per 100 million platelets. Numbers in parenthesis are standard errors.

TABLE IV. THE ABSOLUTE AND RELATIVE BINDING OF [^{3}H]5HT BEIGE AND CONTROL MICE PLATELETS *IN VIVO*.

Animal	Time after injection (min)	Number of animals	Total activity (cpm/ml × 10^3)	(SE)	Percentage distribution		
					Platelet	Plasma	Wash
C57BL/6 control							
	20	5	41	(8)	91.2 (0.4)	7.6 (0.4)	1.2 (0.2)
	40	6	38	(4)	92.5 (0.7)	6.0 (0.7)	1.5 (0.2)
	80	6	34	(4)	93.8 (0.6)	4.3 (0.3)	1.8 (0.4)
	160	6	31	(5)	95.0 (0.5)	3.8 (0.3)	1.3 (0.2)
	240	6	29	(4)	95.6 (0.2)	3.2 (0.2)	1.2 (0.2)
	320	5	32	(3)	95.6 (0.2)	3.2 (0.2)	1.2 (0.2)
C57BL/S beige							
	20	6	26	(2)	74.6 (1.5)	21.3 (1.2)	4.0 (0.4)
	40	6	19	(2)	76.3 (1.3)	19.8 (1.2)	3.8 (0.2)
	80	6	10	(1)	75.5 (1.0)	20.8 (0.9)	3.6 (0.3)
	160	6	8	(0.6)	76.2 (1.4)	20.3 (1.1)	3.5 (0.4)
	240	4	5	(0.2)	72.5 (0.9)	24.0 (1.0)	3.8 (0.2)
	320	6	4	(0.2)	70.6 (1.6)	25.5 (1.5)	3.8 (0.6)

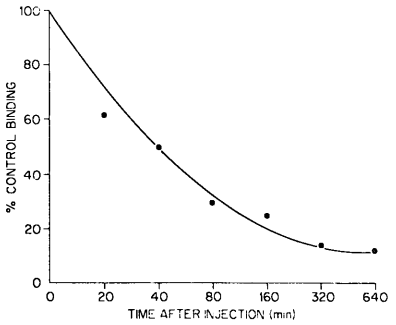

FIG. 1. Relative amount of labeled 5HT bound to beige mouse platelets at intervals after injection.

of beige mouse platelet pellets; however, electron-lucent cores surrounded by a clear halo were occasionally seen (Fig. 3). It is possible that these structures represent modified storage organelles but are deficient in 5HT. A second type of cytoplasmic organelle, the alpha granule, was not visibly affected in the beige platelet.

Discussion. Beige mice are subject to a mild bleeding disorder with a prolonged bleeding time that is reversed by small doses of exogenous serotonin. Platelet counts are normal and differences in clotting time or clot retraction that would account for the observed bleeding tendency were not observed (15). Analysis of a distilled water lysate of isolated platelets revealed a 3-fold reduction in adenine nucleotides, a 35-fold reduction in serotonin, and a slight but significant reduction in platelet protein. The *in vivo* uptake and binding of exogenous serotonin by beige mouse platelets was also impaired. Visible evidence of the combined adenine nucleotide and 5HT deficiency with reduced uptake and storage was obtained by the observation that beige mouse platelets lack structures typical of storage organelles or dense bodies (16).

Chediak–Higashi syndrome. This syndrome of beige mice is similar to the (CHS) in man and to models of CHS in other mammalian species (10, 11, 17). Observations in the various species, therefore, may be relevant and useful in constructing a working hypothesis to account for the observed bleeding tendency in beige mice.

Page *et al.* (18), in studies of two CHS children, found an absolute deficiency of blood serotonin coupled with normal platelet counts and normal excretion of urinary tryptophan catabolites both before and after metabolic loading with l-tryptophan. He concluded that the defect responsible for reduced whole blood 5HT levels was the result of an inability of blood platelets to take up or store serotonin. The observations of Page were extended to the mink, cow, and mouse models, where it was observed that whole blood serotonin levels were greatly depressed and *in vitro* uptake of serotonin by platelets was reduced (15,

20). It has been reported recently that in addition to decreased whole blood serotonin levels, adenine nucleotide levels were less than 18% of normal in CHS cattle and mink while *in vitro* aggregation of CHS platelet rich plasma by 1×10^{-6} *M* adenosine diphosphate was normal (21). Response to collagen or thrombin was markedly altered (21). Phillips *et al.* (19) could find no difference in prothrombin time, partial thromboplastin, thrombin time, Factor II, Factor V, Factor VIII, fibrinogen, platelet count, platelet adhesiveness, or fibrinolytic factors that would explain bleeding

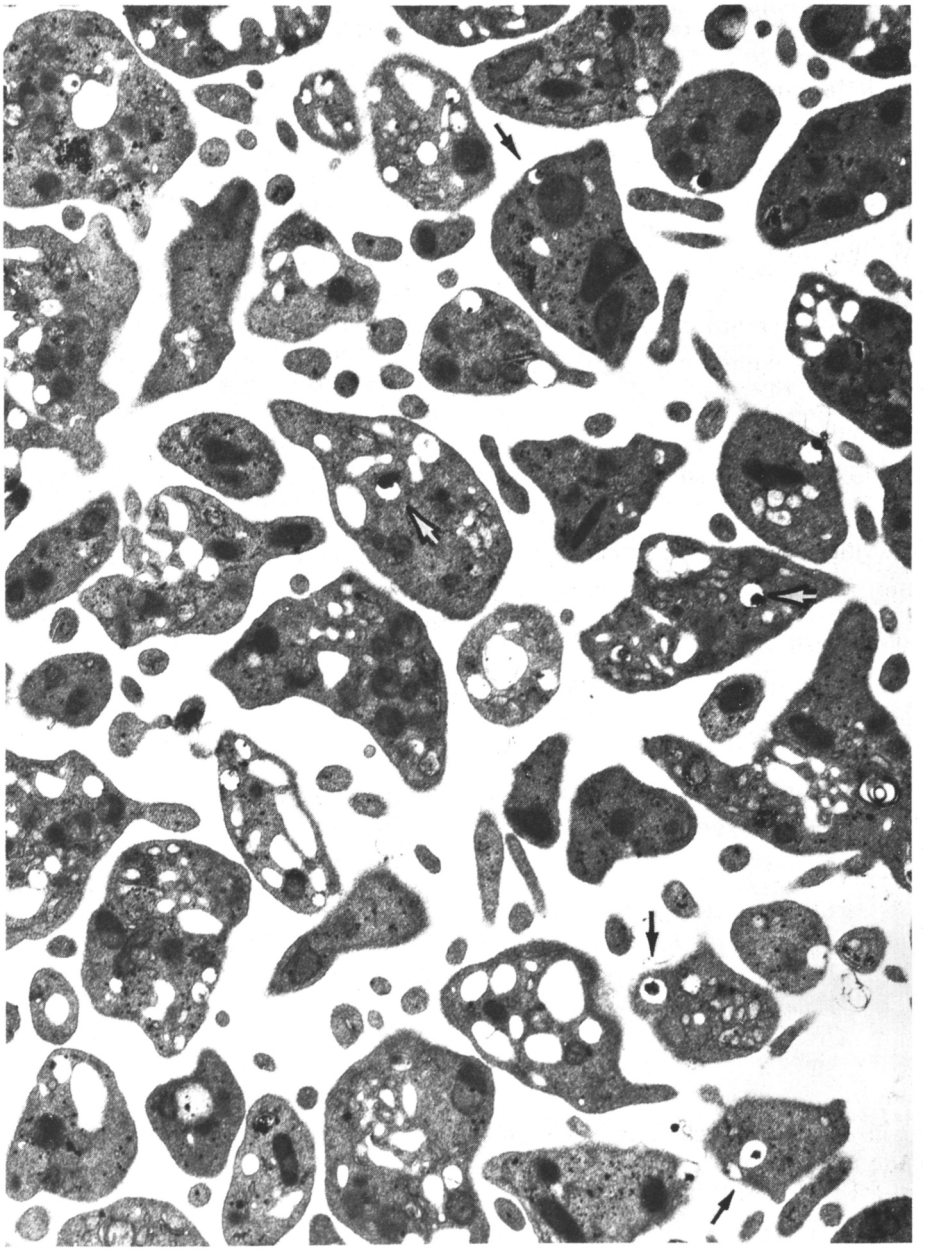

FIG. 2. Electron photomicrograph of representative control platelets illustrating numerous osmiophilic dense bodies (arrows). × 14,500.

tendencies in Aleutian mink or partial albino hereford cattle, animal models of the CHS.

These observations suggest to us that the beige mouse and possibly the other animal models of the CHS manifest a heritable, autosomal recessive trait that shares many features with the platelet storage pool diseases of man (22, 23), especially the variant that includes oculocutaneous albinism (24, 25). Features which the CHS shares with storage pool disease are a decrease in plate-

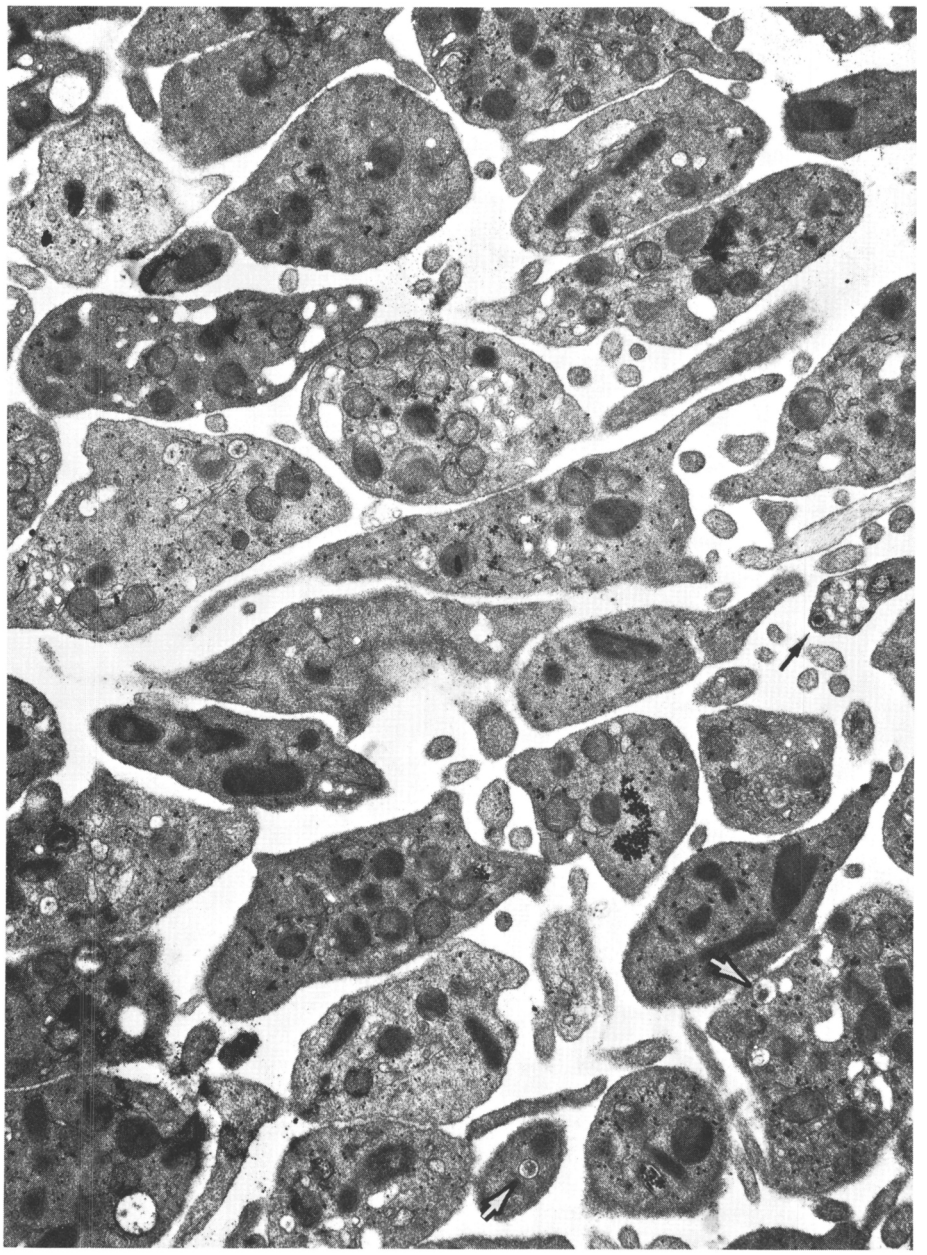

FIG. 3. Electron photomicrograph of a representative field of beige mouse platelets. Abnormalities include a relative deficiency of osmiophilic dense bodies and the occasional presence of electron lucent structures (arrows) which may represent modified dense bodies. × 14,5000.

let adenine nucleotides, increased ATP-ADP ratio, and parallel decrease in serotonin associated with prolonged bleeding time.

Other principal manifestations of the CHS in man and animal models are not reported to accompany storage pool disease. They include peroxidase-positive, iron-negative, enlarged azurophil granules in neutrophil leukocytes associated with pigmentary abnormalities brought about through aggregation of melanin granules (2–4). Decreased resistance to bacterial infections is also regularly observed (10, 11). Thus, the storage pool diseases and the CHS and its models are not identical.

The primary mechanism responsible for defective hemostasis in the beige mouse or for impaired storage of adenine nucleotides and serotonin by platelets is unknown, but the observation of defective collagen and thrombin induced aggregation (21) suggest that the platelet release reaction may be impaired. This would result in defective hemostatic plug formation (26), as has been proposed to explain bleeding tendencies in storage pool disease of man.

Alternate interpretations are also possible. The observation of reversal of the bleeding tendency by prior treatment with small doses of serotonin suggests that, in beige mice, platelet serotonin deficiency may account for prolonged bleeding time either independently or synergistically with impaired adenine nucleotide release. It is possible that serotonin, bound to receptors on platelet surface membrane (27), is transported via an energy dependent mechanism against considerable concentration gradients (28), taken up, and stored by specific platelet organelles in a complex with adenine nucleotides and divalent cations (29). Serotonin is also a potent vasoconstrictor and a product of the release reaction and capable of causing irreversible platelet aggregation (30). In addition, serotonin will arrest bleeding from wounds in normal rats and guinea pigs (31), as well as in beige mice. These associations with platelets and ADP, the physiologic action on the vasculature, and reversal of a bleeding tendency all suggest that serotonin may be important in hemostasis. Principal evidence against accepting a physiologic role for platelet bound serotonin in primary hemostasis stems from observations that serotonin levels in blood and platelets of reserpinized humans, rabbits, rats and guinea pigs are depressed to a degree comparable to the levels detected in beige mice without a demonstrable increase in bleeding time or a predisposition to a bleeding tendency (32–34). These observations have been accepted as evidence that platelet serotonin is not an essential component of the hemostatic process. We wish to offer an alternative interpretation of the reserpine data that would explain the apparent paradox.

Reserpine is a powerful and long-lasting releaser of serotonin from storage sites throughout the body including the platelet (30). The drug accomplishes release of serotonin without interference with either continued synthesis by gut enterochromaffin cells or catabolism via the amine oxidase pathway (35, 36). If reserpine mobilized depot serotonin, while inhibiting storage (37), without affecting continued synthesis or accelerating catabolism of serotonin, it follows that small but physiologically active amounts of serotonin may continue to circulate in plasma. Since it has been shown (31) that parenteral administration of small amounts of serotonin will effect hemostasis, we infer that circulating serotonin can participate in the hemostatic process. In summary, a reserpinized individual might have a small amount of serotonin continuously available for participation in hemostasis.

A full understanding of the relationships that exist, if any, among any of the genetic disorders of platelet function must await the accumulation of additional data. However, we anticipate that the availability of a convenient animal model, the beige mouse, will contribute substantially to an increased understanding of heritable defects in mammalian platelet function as well as afford an opprotunity to evaluate the efficacy of potential therapies.

Summary. Beige mice have been observed to bleed excessively from small wounds. Platelets obtained from these mice were deficient in adenine nucleotides and serotonin and *in vivo* uptake of labeled sero-

tonin was impaired. Heterozygotes of beige and C57BL/6 mice have adenine nucleotide and serotonin levels comparable to control and do not manifest a bleeding tendency, consistent with the recessive mode of inheritance of the beige mouse syndrome. Morphologic confirmation of the observed biochemical defects was obtained by the demonstration that serotonin storage organelles were not observed in electron photomicrographs of beige mouse platelets. The demonstration that small doses of serotonin were effective in reversing the bleeding tendency in beige mice suggested a causal role for defective storage and release of endogenous serotonin as the basis for the bleeding tendency, acting either independently or synergistically with impaired release of adenine nucleotides.

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