Biochemical Pharmacology, Vol. 33, No. 23, pp. 3869–3874, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

HISTAMINE UPTAKE IN PIG PLATELETS AND ISOLATED DENSE GRANULES

MIRIAM H. FUKAMI,* HOLM HOLMSEN* and KAMIL UGURBIL†
Thrombosis Research Center, Temple University, Philadelphia, PA 19140, U.S.A.

(*Received* 22 *December* 1983 *accepted* 2 *July* 1984)

Abstract—Histamine, a major constituent of the amine-storage organelles in pig platelets, is taken up by intact platelets in only trace amounts under conditions where 70% of ^{14}C-serotonin is accumulated. Thrombin caused the release of 70–90% of endogenous histamine but only 5–10% of the newly absorbed ^{3}H-amine; however, after 18 hr 30% of the ^{3}H-amine could be specifically released by thrombin. Isolated storage organelles accumulated histamine in a reserpine-sensitive, ATP-dependent manner but at a rate 80–100-fold less than serotonin uptake. Incubation of intact platelets with 1 mM serotonin until amine uptake was saturated caused no changes in platelet histamine content. Similarly, loading of isolated storage organelles with 1 mM histamine or 1 mM serotonin did not affect the levels of the other amine. These results suggested that the storage of each amine is independent of the other. Histidine decarboxylase was not detected in platelet lysates. Since platelets have a short half-life (1–2 weeks) and pig plasma levels of histamine are higher than in other animals, it is concluded that most of the histamine in the storage organelles is probably accumulated in the platelet precursor, the megakaryocyte, either by slow uptake or by synthesis.

Blood platelets have two distinct amine uptake systems, one associated with the plasma membrane and the other with the amine-storage organelles [1]. Serotonin is the principal amine accumulated into platelets by these transport systems and is the major amine constituent of platelets from most species except guinea pig and pig, in which histamine is the more prevalent amine [2]. In a study of the pig platelet dense granules (the amine-storage organelle) and its nucleotide–magnesium complex by ^{1}H-NMR [3], the presence of high levels of histamine was detected in extracts of isolated organelles. NMR studies of intact organelles suggested that histamine is not readily transported into the isolated organelles as is serotonin [3]. The peaks in the spectra of intact organelles were so much broader than those in free solution (e.g. perchlorate extracts of the organelles), that the assignment of peaks to histamine was only tentative. Because of this uncertainty and because it seemed more likely than not that histamine should be transported into and stored in the dense granule by the same mechanisms as serotonin, a study of histamine uptake and storage by conventional methods was undertaken to complement and confirm the NMR studies.

MATERIALS AND METHODS

Methods. Platelets were isolated from pig blood collected in acid-citrate dextrose [4] and washed in 154 mM NaCl, 30 mM Hepes, pH 6.5, with 5 mM EDTA for fractionation preparations and without EDTA for platelet secretion experiments. The latter were resuspended in 154 mM NaCl, 30 mM Hepes, pH 7.4.

Platelet subcellular fractions were prepared essentially as described previously [5] with the following modifications: (1) washed platelets were treated with 20 μM rotenone, 5 mM 2-deoxyglucose and 30 mM δ-gluconolactone for 10 min at 37° prior to nagarse treatment; (2) the isolation medium was changed to 150 mM KCl, 10 mM Hepes and 1 mM EDTA, pH 7.4.

Histamine was assayed enzymatically with histamine methyltransferase and [^{14}C-methyl]-adenosylmethionine as described by Shaff *et al.* [6]. Unlabelled methylhistamine (1-methyl-4-(β-aminoethyl)-imidazole) was added to a neutralized perchloric acid extract and the aqueous phase was extracted with isoamylalcohol–toluene (1:4). ^{14}C in the organic phase was counted and compared to standards treated in the same manner.

Serotonin was assayed as described by Curzon *et al.* [7].

β-*N*-Acetylglucosaminidase was assayed by measuring the hydrolysis of the *p*-nitrophenyl ester [8].

Uptake studies on intact platelets were carried out with platelet-rich plasma. After incubation with histamine or serotonin at room temperature for the specified time periods, a 0.5 ml aliquot of platelet-rich plasma was added to an equal volume of cold medium with 5 mM EDTA and the platelets were sedimented in an Eppendorf centrifuge at 13,000 *g* for 1 min. The platelet pellets were resuspended in ice-cold medium and aliquots were counted or assayed to determine amine uptake. Uptake studies in isolated granules were carried out with millipore filtration exactly as described by Rudnick *et al.* [9].

* Present address: Department of Biochemistry, University of Bergen, N-5000 Bergen, Norway.
† Gray Freshwater Biological Institute, University of Minnesota, College of Biological Sciences, Navarre, MN 55932.

Platelet secretion experiments were done with platelets that had not been exposed to EDTA, because in our hands pig platelets did not secrete well after EDTA treatment. These platelets were washed in a medium of 150 mM NaCl, 10 mM Hepes, pH 6.5, and finally resuspended in the same medium of pH 7.4 [10]. Thrombin and 1 mM Ca^{2+}, final concentration, were added after incubation of platelets at 37° for 5 min; then EDTA was added to give a final concentration of 5 mM before centrifugation to separate the extracellular medium and the platelets. When isotopically labelled platelets were used, the cells were incubated with ^{3}H-histamine or ^{14}C-serotonin in platelet-rich plasma prior to washing.

Per cent secretion was calculated as follows:

$$\frac{\text{conc in thrombin supernatant} - \text{concn in control supernatant}}{\text{conc in total suspension} - \text{conc in control supernatant}} \times 100.$$

Histidine decarboxylase was assayed by the method described for the bacterial enzyme with L-[carboxy-^{14}C]-histidine [11], as well as by a method for the mammalian enzyme using L-[2,5-^{3}H]-histidine [12]. Histidine and histamine were separated by thin-layer chromatography on cellulose with ethanol/ethyl ether/H_2O/ammonium hydroxide (4/5/1/0.1) by volume and detected by ninhydrin staining. The R_f for histidine was 0.04 and 0.4 for histamine.

Materials. ^{14}C-Serotonin (5-[2^{14}C]-hydroxytryptamine binoxalate), 48.6 Ci/mol, ^{3}H(G)-histamine, 5–10 Ci/mmol, *S*-[methyl-^{14}C]-adenosyl-L-methionine, 40 Ci/mol and L-[carboxyl-^{14}C]-histidine, 40 Ci/mol were obtained from New England Nuclear Corp. (Boston, MA). L-[2,5-^{3}H]-Histidine, 57 Ci/mmol was obtained from Amersham International (Buckinghamshire, U.K.). The thrombin used was Bovine Topical Thrombin from Parke-Davis (Detroit, MI). Serotonin, histamine, histidine, *N*-methylhistamine, *o*-phthaladehyde and the metabolic inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). The pig blood was kindly provided by P. Villari and Son, Inc. (Philadelphia).

RESULTS

An NMR spectrum of a neutralized perchloric acid extract of isolated pig platelet dense granules revealed the presence of an amine with two aromatic hydrogens distinct from serotonin hydrogens [3]. Addition of histamine to the extracts resulted in an increase in the peaks of precisely those protons in question. The identification was confirmed by paper chromatography [13] of extracts of control platelets and of supernatants and pellets of centrifuged thrombin-treated platelets, coupling with diazotized anthranilic acid and comparison with serotonin, histamine and tyramine standards (not shown). Serotonin and histamine were the only primary amines present in the platelet extracts (detectable by the sensitivity of this method), and about 90% of both were released by thrombin treatment. Subcellular fractionation of pig platelets showed that the dense granule fraction contained the highest concentration of histamine, 600 nmol per mg of protein (Table 1).

In secretion experiments, the time course and extent of histamine release were similar to those of serotonin and dissimilar to those of β-*N*-acetylglucosaminidase which is released more slowly and to a lesser extent (less than 50%) (Fig. 1).

Uptake studies in intact platelets were carried out as described in Methods. Less than 2% of the histamine label was taken up by platelets after 3 hr (Fig. 2b) and only 5–6% after 18 hr. In the case of serotonin, about 75% of the ^{14}C was associated with the platelets at 1 hr and no more accumulated thereafter (Fig. 2a). Pretreatment of the platelet-rich plasma with 5 μM reserpine or 2 μM imipramine caused significant inhibition of serotonin uptake (Fig.

Table 1. Histamine distribution in pig platelet subcellular fractionations

Fraction	Protein (nmol/mg)	Total	% Distribution
Homogenate	10.2	11,902	
1000 *g* × 10 min pellet	13.8	3888	38.9%
Granules	37.4	4440	44.4%
Membranes	2.35	219	2.19%
Soluble Fraction	1.96	1458	14.6%
Total		10,005	(84.1% recovery)
Granule Subfractions (sucrose gradient)			
A (Top of gradient)	9.3	280	8.94%
B	10.0	132	4.21%
C	15.5	160	5.10%
D	36.2	763	24.4%
E	173	250	7.98%
Pellet (Dense granules)	691	1548	49.4%
Total		3133	(83.3% recovery)

These data represent one of three fractionations preparations carried out as described in Methods, with histamine recoveries of over 80%. The mean histamine content of the purified dense granules for the three preparations was 609 ± 150 (S.D.) nmole/mg of protein.

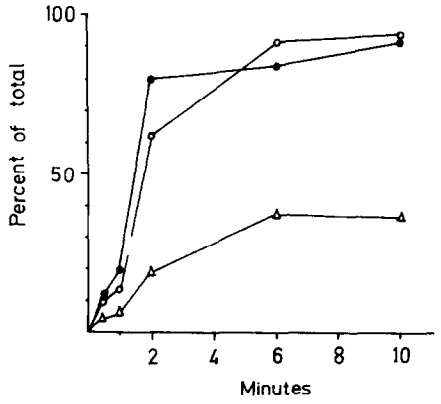

Fig. 1. Time course of thrombin-induced histamine secretion compared to serotonin and β-*N*-acetylglucosaminidase. For experimental details, see Methods. The platelet protein in the reaction mixture was 4.4 mg/m and 5 U/ml of thrombin. The symbols represent: histamine, ●; serotonin, ○; and β-*N*-acetylglucosaminidase, △ (representative of 5 experiments).

2a). These inhibitors appeared to have little effect on histamine uptake (Fig. 2b).

Thrombin treatment (1 U/ml) of platelets preincubated with ^{14}C-serotonin for 3 hr resulted in the secretion of 90% of the ^{14}C label. Less than 10% of the ^{14}C appeared in the extracellular medium in saline-treated control platelets. Thrombin treatment resulted in the release of 52% of the ^{3}H-histamine into the extracellular medium, but 46% of the label also was released by the saline-treated control platelets, resulting in a thrombin-specific secretion of only 6% of the exogenous histamine. Endogenous histamine, however, behaves in the same manner as serotonin and is about 90% secretable (Fig. 1). After 18 hr of incubation of platelet-rich plasma with ^{3}H-histamine, 70% of the ^{3}H label was released by thrombin-treatment, compared to 34% release in saline controls, giving a thrombin-specific secretion of 36%.

Platelets in platelet-rich plasma were incubated with 1 mM serotonin in order to load the storage organelles maximally with the amine to determine if any displacement of histamine would occur. As shown in Fig. 3, the serotonin stores can be increased more than two fold without any decrease in the histamine content. The parallel experiment studying histamine displacement of serotonin could not be done in intact platelets since the rate of histamine uptake in intact platelets is negligible.

Histamine uptake in isolated storage organelles was studied in a mixture of organelles isolated by differential centrifugation at 12,000 *g* for 10 min. The dense storage organelles in this preparation show good integrity and no leakage of storage contents over several hours as indicated by ^{31}P-NMR studies [14]. The organelle mixture was incubated in the uptake medium described by Rudnick [9] with either ^{3}H-histamine or ^{14}C-serotonin. At low concentrations, <2 μM, ^{3}H-histamine uptake was found to be dependent on ATP and was sensitive to inhibition by reserpine (Fig. 4b). Net uptake of serotonin or histamine was also studied in isolated granules with 1 mM concentrations of amines. The net histamine

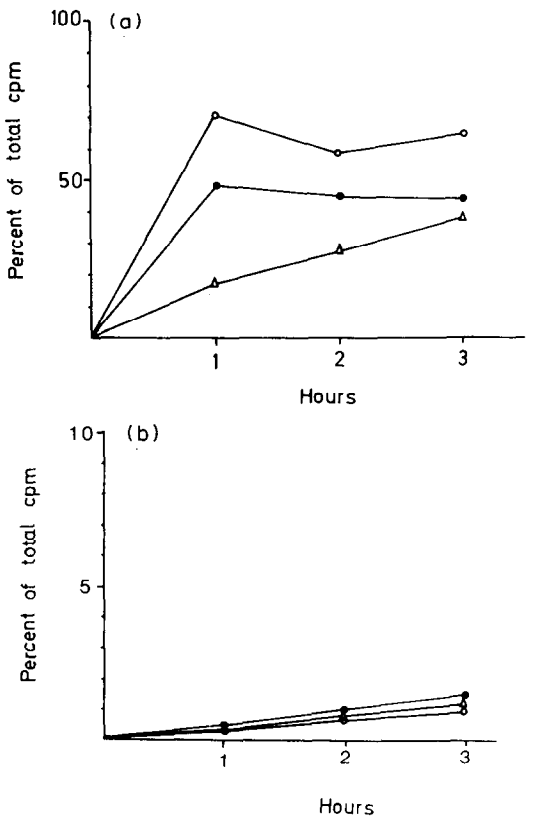

Fig. 2. Uptake of histamine by intact platelets compared to serotonin. Platelet-rich plasma (20 ml) was incubated with (a) ^{14}C-serotonin 0.2 μCi, 0.4 μM, or (b) ^{3}H-histamine, 1.0 μCi, 0.5 μM. At the times indicated, aliquots were removed and processed as described in Methods. The symbols are: serotonin or histamine alone, ○; with reserpine 5 μM, ●; with imipramine 1 μM, △ (representative of 3 experiments).

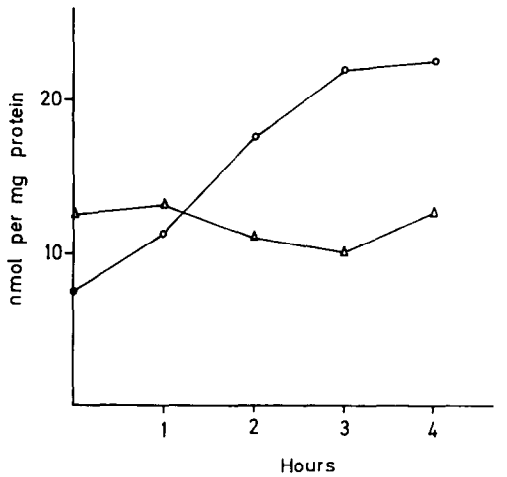

Fig. 3. Effect of serotonin loading on histamine stores in intact platelets. Platelet-rich plasma with 5 mM EDTA was incubated with 1 mM serotonin and sampled hourly for serotonin, ○, and histamine, △, assays as described in Methods. The mean increase in serotonin was 2.4-fold ±0.4 in 3 experiments while histamine remained the same (1.01 ± 0.11).

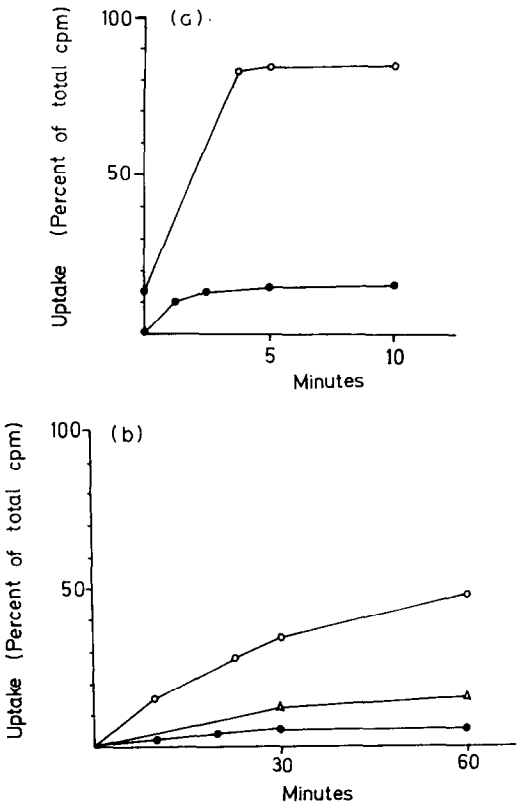

Fig. 4. Uptake of ^{3}H-histamine by isolated granules compared to uptake of ^{14}C-serotonin. Granules were incubated in a medium of 300 mM sucrose, 10 mM Hepes, 2.5 mM $MgSO_4$, 5 mM KCl, pH 8.5, with 5 mM ATP and (a) ^{14}C-serotonin 0.2 μCi, 0.4 μM or (b) ^{3}H-histamine 2.5 μCi, 1.0 μM. Aliquots of 0.2 ml were taken at the indicated times from a total reaction volume of 2 ml containing 5.5 mg of granule protein and processed as described in reference 8. The symbols represent: serotonin or histamine alone, ○; with reserpine 5 μM, ●; without ATP, △ (representative of 4 experiments).

content increased by approximately 15% in 30–60 min with ATP supplementation of the medium every 30 min (Fig. 5). At this high histamine concentration of 1 mM, the uptake was about 50% inhibited by reserpine at 30 min. The serotonin content of the storage organelles increased 100% under similar conditions (Fig. 5). Incubation of the isolated granules with serotonin and histamine simultaneously, or preincubation with one amine followed by a wash and incubation with the other amine did not alter the net uptake of either (not shown).

Histidine decarboxylase activity was not detected in freshly prepared lysates of pig platelets using conditions described by Gale [11], with L-carboxy-^{14}C-histidine, 0.1 μCi and 0.4 mM substrate concentration, with a sodium hydroxide $^{14}CO_2$ trap. The assay for the mammalian enzyme with 20 μM pyridoxal-5'-phosphate, dithiothreitol, nitrogen atmosphere and 0.5 mM histidine with 7 μCi of ^{3}H-label showed no histidine decarboxylase activity. In both assays, the product measured after 30 and 60 min incubations was not more than the zero time incubations, in which perchloric acid was added to the platelet lysate before the labelled substrate and the incubation at 37°; neither the $^{14}CO_2$ nor the ^{3}H-histamine formation exceeded 3% of the label in the added substrate.

DISCUSSION

The presence of high concentrations of histamine in pig platelets reported by others [2, 15, 16] has been confirmed by these studies. The secretion experiments with endogenous histamine and subcellular fractionations showed that the histamine is contained in the same compartment as serotonin, in the dense storage organelles. The uptake studies with intact platelets show that histamine is not readily transported into the cell. The histamine which is still associated with platelets after they are washed to remove excess histamine in the medium does not involve the plasma membrane serotonin carrier since this association is not inhibited by imipramine. Unlike serotonin, the histamine absorbed by intact platelets is not immediately sequestered in the dense storage organelles, as shown by the secretion experiments. After a 3 hr labelling period followed by wash and resuspension, 40–50% of the ^{3}H-histamine becomes dissociated from the platelets during incubation at 37°. Treatment with thrombin causes the release of only 5–10% more. Only the increment released by thrombin can be considered to be released from the secretory organelles [17]. These results indicate that about half of the ^{3}H-histamine which becomes associated with platelets after 3 hr is bound in a manner that withstands washing with cold medium, but is readily released at 37°. Another 40% or so of the histamine seems to be within the platelet in a compartment other than the secretory organelle, probably the cytoplasm. Over time, more of the amine moves from this compartment into the secretory organelle, so that after 18 hr, 30–40% of the accumulated label was specifically released by thrombin. In contrast to these findings, it has been reported that *in vivo* administration of labelled histamine to rabbits 3 hr before isolation of platelets

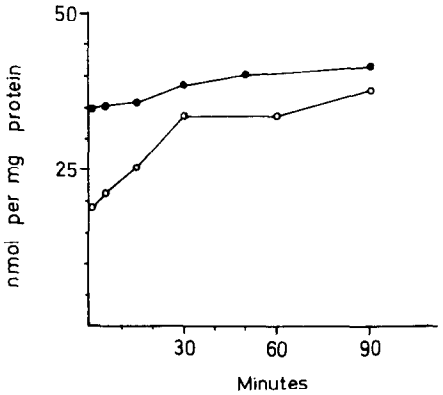

Fig. 5. Histamine and serotonin loading in isolated granules. The granule mixture was incubated with 1 mM histamine, ●, or 1 mM serotonin, ○, in the medium described in Fig. 4. Aliquots of 0.1 ml were taken at the indicated times from a 1 ml reaction volume containing 1.6 mg of granule protein and processed as in Fig. 4 (mean of 2 experiments).

leads to a labelled histamine distribution practically identical to that of endogenous serotonin in the dense storage organelles [18]. However, these workers did not monitor the overall recovery and distribution of histamine in their fractionation procedure. Others have shown that under certain experimental conditions even serotonin can accumulate in a non-secretable compartment, the cytosol [19, 20]. Studies in rabbit platelets, which contain a lesser proportion of histamine to serotonin compared to pig platelets, 0.42 [21], have shown that histamine uptake in this species is also much slower than serotonin uptake and is reserpine-sensitive [22].

At the level of the storage organelle, the transport of histamine apparently occurs via the dense granule serotonin carrier [1, 23], since histamine uptake is both ATP-dependent and reserpine-sensitive. Dense storage organelles isolated from platelets of reserpinized pig are depleted in both histamine and serotonin [3], indicating that the same transport mechanisms are involved in amine accumulation, as in rabbit platelets [24]. However, the rate of histamine uptake is 80–100-fold less than the rate of serotonin uptake; similar rates have been found for histamine uptake in isolated rabbit platelet organelles [25]. This difference in uptake rates at the dense granule membrane correlates well with the results from the uptake-secretion experiments which show that the small amount of histamine transported across the plasma membrane does not become available for secretion for some time.

One approach to the study of storage mechanisms that we have attempted is to determine what effects, if any, the uptake of large amounts of one amine has on the other (e.g. displacement). 5,6-Dihydroxytryptamine has been reported to displace serotonin so that substantial accumulation of 5,6-dihydroxytryptamine was associated with a decrease in the serotonin content of the dense granule [26]. These results imply that the same storage sites are involved for 5,6-dihydroxytryptamine and serotonin. In uptake studies with isolated organelles in the presence of 1 mM amine, the serotonin content was increased by 100% without having any effect on the endogenous histamine content. Similarly histamine levels were increased 20% in isolated organelles with no changes in serotonin levels. In intact platelets, the serotonin content was increased three fold without any decrease in platelet histamine. These results indicate that the storage sites for histamine and serotonin are independent of each other.

The findings reported in this paper are in agreement with the ^{1}H-NMR results [3]. Serotonin was shown to associate with ATP–Mg^{2+} complexes in aqueous solution and exhibited similar chemical shifts in the intact storage organelles [3]. Addition of serotonin to isolated organelles resulted in increases of the peaks assigned to the amine. Histamine, on the other hand, had little affinity for ATP–Mg^{2+} complexes in aqueous solution at pH 5.5 which is the pH of dense granule interior [27]. DaPrada and co-workers have described interactions between ATP and histamine by two methods, but their studies were carried out at pH 6.5 [28, 29]. Given that the imidazole ring of histamine has a pK of about 6.5, there would be a large difference in the average charge of histamine between pH 5.5 and 6.5 Also, the nucleotide-divalent cation–amine ratio used in the NMR studies were approximately the same as that found in dense granules from pig platelets [3] and different from those used by DaPrada *et al.* Both the difference in pH and the presence of 2 Mg^{2+} per nucleotide would affect profoundly the electrostatic interactions in these systems and explain the discrepancies between the NMR studies [3] and those of DaPrada *et al.* [28, 29]. Addition of histamine to isolated organelles resulted in no changes in the ^{1}H-NMR spectrum within the time observed. As reported in this paper, the rate of uptake of histamine is 80–100-fold slower than that of serotonin, which would explain why uptake was not seen during ^{1}H-NMR scanning. Intermolecular nuclear Overhauser effects between the aromatic protons of serotonin and adenine nucleotides confirm that high molecular weight aggregates of adenine nucleotides and Mg^{2+} are associated with serotonin [3]. It is postulated that these high molecular weight storage complexes which reduce the effective osmotic pressures contributed by the high concentrations of adenine nucleotides and divalent cations also serve as a storage matrix for serotonin. Since histamine does not show similar interactions with ATP–Mg^{2+}, either in aqueous solutions or in intact storage organelles, based on tentative assignment of peaks [3], it is probably stored by a different mechanism than serotonin. This conclusion is compatible with the loading experiments described here in which accumulation of saturating levels of each amine had no displacement effect on the other.

No hemostatic function can be attributed to platelet histamine and its release. It is noteworthy that histamine levels in all organs and tissues of the pig are higher than in any other species examined [30]. The platelet-free plasma concentrations of histamine in pig are reported to be 37–50 ng/ml or about 0.3–0.4 μM compared to 0.6 ng/ml in human platelet-free plasma [16, 30]. From the rate of histamine uptake in the isolated pig organelle mixture found here, 5 pmole/min/mg of protein, it can be calculated that it would take about 5 days or 120 hr for the 37 nmole of histamine/mg of protein in the granule mixture (Table 1) to be accumulated. Since the platelet life span is only 1–2 weeks, it seems unlikely that all of the histamine is accumulated by the circulating platelet. Histamine synthesis in the circulating platelet also seems to be an unlikely source since histidine decarboxylase levels are negligible. However, megakaryocytes, the stem cell of platelets, have been shown to be capable of storing exogenous serotonin at specific subcellular sites [31]. Therefore, the possibility exists that most of the pig platelet histamine is accumulated at the megakaryocyte stage from the plasma or possibly by synthesis.

Acknowledgements—This research was supported by USPHS Grant no. HL 14217 and HL 26089. The skilled technical assistance of Gail L. Pakstis and Carol A. Dangelmaier is gratefully acknowledged.

REFERENCES

1. M. DaPrada and A. Pletscher, *Br. J. Pharmac.* **34**, 591 (1968).

2. M. DaPrada, J. G. Richards and K. Kettler, in *Platelets in Biology and Pathology 2* (Ed. J. L. Gordon), pp. 107–147, Elsevier North-Holland, Amsterdam (1981).
3. K. Ugurbil, M. H. Fukami and H. Holmsen, *Biochemistry* **23**, 416 (1984).
4. R. H. Aster and J. H. Jandl, *J. clin. Invest.* **43**, 843 (1964).
5. L. Salganicoff, P. A. Hebda, J. A. Yandrasitz and M. H. Fukami, *Biochim. biophys. Acta* **385**, 394 (1975).
6. R. E. Shaff and M. Beaven, *Analyt. Biochem.* **94**, 425 (1979).
7. G. Curzon, B. D. Kantamaneni and M. D. Tricklebank, *Br. J. Pharmac.* **73**, 555 (1981).
8. H. J. Day, H. Holmsen and T. Hovig, *Scand J. Haematol. Suppl.* **7**, 3 (1981).
9. G. Rudnick, H. Fishkes, P. J. Nelson and S. Schuldiner, *J. biol. Chem.* **255**, 3638 (1980).
10. R. L. Kinlough-Rathbone, A. Chahil and J. F. Mustard, *Am. J. Physiol.* **224**, 941 (1973).
11. E. F. Gale, in *Methods of Enzymatic Analysis*, Vol. 4, (Ed. H. U. Bergmeyer), pp. 1662–1668. Verlag Chemie Weinheim, Academic Press, New York (1974).
12. D. Aures and R. Haakonson, in *Methods in Enzymology*, Vol 17, part B (Eds. H. Tabor and C. W. Tabor), pp. 667–677. Academic Press, New York (1971).
13. T. Wood, *J. Chromatogr.* **35**, 352 (1968).
14. K. Ugurbil, M. H. Fukami and H. Holmsen, *Biochemistry* **23**, 409 (1984).
15. S. K. Ainsworth, R. E. Newman and R. A. Harley, *Br. J. ind. Med.* **36**, 35 (1979).
16. A. P. Almeida, W. Flye, D. Deveraux, Z. Horakova and M. A. Beaven, *Comp. Biochem. Physiol.* **67C**, 187 (1980).
17. H. Holmsen, in *Mechanisms of Hemostais and Thrombosis*, (Eds. C. H. Mielke, Jr. and R. Rodvien), pp. 73–109. Symposia Specialists, Miami, FL (1978).
18. M. DaPrada and A. Pletscher, *Eur. J. Pharmac.* **7**, 45 (1969).
19. H.-J. Reimers, D. J. Allen, J.-P. Cazenave, I. A. Feuerstein and J. F. Mustard, *Biochem. Pharmac.* **26**, 1645 (1977).
20. J. L. Costa, D. L. Murphy and M. S. Kafka, *Biochem. Pharmac.* **26**, 517 (1977).
21. M. DaPrada, A. Pletscher, J. P. Tranzer and H. Knuchel, *Nature, Lond.* **216**, 315 (1967).
22. J. Tuomisto, *Ann. Med. exp. Fenn.* **46**, 330 (1968).
23. H. Fishkes and G. Rudnick, *J. biol. Chem.* **257**, 5671 (1982).
24. M. DaPrada, A. Pletscher, J. P. Tranzer and H. Knuchel, *Life Sci.* **7**, 477 (1968).
25. M. DaPrada and A. Pletscher, *Life Sci.* **8**, 65 (1969).
26. M. DaPrada, R. A. O'Brien, J. P. Tranzer and A. Pletscher, *J. Pharmac. exp. Ther.* **186**, 213 (1973).
27. R. G. Johnson, A. Scarpa and L. Salganicoff, *J. biol. Chem.* **253**, 7061 (1978).
28. M. DaPrada, R. Obrist and A. Pletscher, *J. Pharm. Pharmac.* **27**, 63 (1975).
29. K. H. Berneis, A. Pletscher and M. DaPrada, *Nature, Lond.* **224**, 281 (1969).
30. W. Lorenz, H. Barth, J. Kusche, H. J. Reimann, A. Schmal, E. Matejka, Ch. Mathias, M. Hutzel and E. Werle, *Eur. J. Pharmac.* **14**, 155 (1971).
31. J. P. Tranzer, M. DaPrada and A. Pletscher, *J. Cell Biol.* **52**, 191 (1972).