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SUBCELLULAR DISTRIBUTION OF 5 HYDROXYTRYPTAMINE IN PIG PLATELETS

B. F. MINTER* and N. CRAWFORD

Department of Biochemistry, University of Birmingham, Edgbaston, Birmingham 15

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Abstract—The subcellular distribution of 5 hydroxytryptamine (5HT) in pig platelets before and after incubation of the cells with the amine has been investigated by density gradient fractionation. Conditions of homogenization and fractionation were designed to separate the platelet surface and intracellular membrane structures from the granular components. The major fractions have been partially characterized by electron microscopy and by certain marker enzymes. While a low density membrane zone separates well with minimal contamination the higher density granule fraction is a heterogeneous mixture of mitochondria and α-granules, small osmiophilic dense bodies and numerous unidentified organelles. Evidence has been presented for the existence of two separate storage compartments for 5HT. One site in the granule zone accounts for about 10 per cent of the total cell 5HT content stored as a relatively strongly bound complex. The other major storage compartment is within the extragranular cytoplasmic pool and this accounts for about 80 per cent of the cell 5HT. In this compartment the amine is either weakly associated or freely diffusible. The major proportion of the 5HT absorbed by the platelet during incubation in the presence of 5HT can be recovered from the low density fraction of the cell as a soluble component. Platelet granules exhibit a low capacity but have a high affinity for the absorbed amine.

THE MAJOR fraction of the circulating blood 5 hydroxytryptamine (5HT) is associated with the blood platelets in a physiologically inactive form. Normally the platelets are not fully saturated, and when exposed either *in vivo* or *in vitro* to 5HT they are capable of increasing their content many times. This property of 5HT absorption has been investigated by a number of workers[1–8] and although at low external levels of 5HT (1–2 μg/ml) the absorption mechanism displays many of the features of an active transport process, at higher external levels (> 5 μg/ml) a diffusion process appears to predominate.[4,9] A wide variety of tissue amine releasing agents are now known and many of these have been specifically tested against whole platelets for the way in which they affect the 5HT content either by promoting its release or by inhibiting the transfer of 5HT unidirectionally across the membrane and into the cell. In contrast however, there have been few investigations of the intracellular localization of endogenous and absorbed 5HT and little attention directed towards identifying the drug-sensitive storage compartments.

In 1958 Sano *et al.*[10] working with rabbit platelets (their 5HT content is many times higher than other laboratory animals) failed to demonstrate a bound form of the amine. Hughes and Brodie[11] using both lysed rabbit platelets and platelets homogenized in sucrose, also deduced that 5HT was not associated with any identifiable organelle but later Schultz *et al.*[12] found that in human platelets 95 per cent of the 5HT was associated with a non-granular hyalomere fraction. Davis and Kay[13] in an autoradio

* Present address: The Biochemistry Department, Messrs. Pfizer Limited, Sandwich, Kent.

graphic study using high activity tritiated 5HT were able to demonstrate on electron-microscopy a significant localization of 5HT within the cell but a specific organelle association could not be firmly ascertained. Buckingham and Maynert[14] by differential centrifugation of platelet homogenates found that much of the homogenate 5HT was associated with a sedimentable granule fraction. Unfortunately electron micrographs were not presented with this study and the morphological nature of their amine-binding subcellular component is not known. In 1965 Wurzel *et al.*[15] established by sucrose density gradient centrifugation of rabbit platelet homogenates that a granular fraction contained tightly bound 5HT. They too however were unable to identify with certainty the type of structure involved. Later Marcus *et al.*[16] in a study of the intracellular localization of certain enzymes in human platelets fractionated by sucrose gradient centrifugation, showed that their platelet granule fraction contained α-granules, mitochondria and large membrane fragments. Day *et al.*[17] have shown particulate association of 5HT in their gradient studies but were not able to definitively identify the organelles involved. Evidence for the association of 5HT in platelets with small osmiophilic dense bodies smaller than the α-granules has been provided by Pletscher *et al.*[8,18] in studies using a combined chemical and electron micrographic approach. These workers found that the number of these small dense bodies within the platelet cytoplasm increased with an increase in cell 5HT content and decreased following exposure of the platelet to the 5HT releasing alkaloid reserpine. Maynert and Isaac[19] however have since questioned the separate identity of these 5HT organelles and suggest that they may in fact be α-granules that differ from their usual appearance due to sectioning in oblique planes.

In the present investigation isolated pig platelets have been homogenized and subfractionated on sucrose density gradients. The distribution of 5HT between the various subcellular fractions has been studied, before and after exposure of the platelets to 5HT. In some experiments [^{14}C] 5HT has been used to aid the localization of both the endogenous and absorbed amine. The major fractions which have been separated with the present scheme included a low density membrane fraction and a higher density granule fraction. The fractions are separated by an intermediate non-particulate zone. Evidence is presented for the existence of at least two morphologically distinct storage compartments for 5HT within the platelet. One storage site is associated with the granular components and has a high affinity but relatively low storage capacity for the amine. The other, and the major site of location when the platelet is saturated with 5HT, is extragranular and here the 5HT appears to be present in the form of a soluble, freely exchangeable storage pool. A preliminary account of this work has been presented.[20]

Reagents and apparatus

Unless otherwise stated all chemicals were obtained from British Drug Houses Ltd., and were of Analytical Grade wherever possible. 5 hydroxytryptamine (May and Baker Ltd., Dagenham, Essex, or the Regis Chemical Company, Chicago) was used as the creatinine sulphate salt but concentrations are given as the free base. Batch samples from the commercial sources were checked for purity by chromatography and fluorimetric spectral characterization. [^{14}C] 5HT-creatinine sulphate with a specific activity of 39·7 mc/mM was obtained from the Radiochemical Centre, Amersham. Reagents were prepared with glass distilled water and all pipettes and vessels used

in the handling of platelet rich plasma and platelet suspensions were of plastic or well-siliconized glass. The siliconized surfaces were renewed at frequent intervals using a 20% solution of Repelcote (Hopkins & Williams) in acetone. For the fluorimetric measurements both an Aminco–Bowman Spectrophotofluorimeter and a Farrand Recording Spectrofluorimeter have been used. These two instruments gave exactly the same activation and emission spectral plots with pure solutions of 5 hydroxytryptamine in HCl. Sucrose density gradients were prepared in 9 ml capacity MSE polypropylene tubes and centrifuged in a MSE "Superspeed 40" refrigerated centrifuge using a 3 × 10 ml swing out rotor.

Collection of blood and preparation of platelet suspensions

Pig blood was collected from the slaughter house. Immediately after the animal had been killed the throat was slit and the blood allowed to flow freely into 5 l. polythene vessels containing 500 ml of 1·5% disodium EDTA (ethylene diamine tetra-acetic acid) in 0·9% saline buffered to pH 7·4 with 0·154 M tris–HCl buffer. During collection the blood was mixed with anticoagulant by gentle swirling. Blood samples were processed within 0·5 hr of collection.

On arrival at the laboratory, the pig blood was transferred to 1-l. plastic containers and centrifuged at 300 *g* for 20 min. The supernatant platelet-rich plasma was removed by gently siphoning through wide polyethylene tubing into further plastic vessels. The platelet rich plasma samples were again centrifuged at 300 *g* for 20 min to remove any remaining red cells. The upper two-thirds supernatant of platelet-rich plasma was transferred by siphoning into plastic tubes.

Variations in the viscosity of the pig blood, presumably due to haemoconcentration, occasionally resulted in the need for a further low speed centrifugation step to ensure red cell-free platelets. When platelet suspensions were required or platelet pellets for homogenization, the cells were deposited from the plasma by a higher speed centrifugation (2000 *g* for 20 min) and the pellet suspended in a wash solution consisting of 0·154 M sodium chloride, 0·077 M tris–HCl buffer pH 7·4 and 0·077 M disodium EDTA in the ratio 90:8:2 v/v. Washed platelets were deposited by centrifugation and resuspended in a solution containing 0·154 M NaCl 0·077 M tris–HCl buffer pH 7·4 (90:10; v/v). Resuspension volumes were usually arranged to approximately one hundredth of the volumes of platelet-rich plasma from which the cells were derived.

Incubation conditions

Fresh platelet-rich plasma with platelet counts between 200,000 and 500,000/mm^3 were shaken at 37° in siliconized flasks or plastic vessels at 60 excursions/min. After incubation the samples were transferred to an ice bath to arrest absorption and the platelets separated by centrifugation without delay. Although small changes in pH may occur during incubation with this procedure, this was considered preferable to the possible artefacts which may result from resuspension of platelets in synthetic buffer media, especially since the effect of pH on the uptake of 5-hydroxytryptamine is small.

Homogenization of platelets

Washed platelet pellets were suspended in sucrose solutions of the following composition: for homogenization using the Teflon plunger and glass tube, 0·3 M sucrose

solution containing 0·001 M disodium EDTA; using the MSE blendor procedure, 0·25 M sucrose in 0·3 M imidazole buffer pH 7·4 containing 0·1 % disodium EDTA. In the earlier investigations, the commercial Potter–Elvehjem type homogenizer consisted of a smooth walled glass tube with a Teflon plunger which gave a side-wall clearance of approximately 0·005 in. at 4°. This gave poor homogenate yields even with homogenization times of 5–10 min. To overcome this difficulty a Teflon pestle was constructed to give "nil clearance" at room temperature and a radial clearance of 0·002 in. after contraction at 4°. In constructing these plungers it is necessary to ensure that the steel drive rod penetrates the Teflon cylinder as far as possible to avoid uneven contraction of the plunger during cooling. With this homogenizer five complete excursions of the plunger though the homogenate, performed over a period of 2–3 min, with intervals for cooling provided a satisfactory homogenate yield. The tube was immersed in ice during the procedure and the speed of rotation was approximately 3000 rev./min. In some of the later experiments a MSE top drive blade blendor was used with homogenization times of between 2 and 4 min at a speed of rotation of approximately 2000 rev./min. After the homogenization the unbroken cells and larger cell debris were removed by centrifugation (3000 *g* for 20 min) before proceeding with the experiments. The relative merits of these two homogenizers particularly with respect to homogenate yield and organelle preservation will be commented upon in the Results section of this paper.

Preparation of sucrose gradient and centrifugation

Sucrose density gradients were prepared by successive layering in 9 ml. MSE polythene tubes of 1 ml volumes of sucrose solutions containing 0·001 M EDTA of molarity 2·0 – 1·0 M decreasing in 0·2 M steps. The gradients were usually prepared on the day before use and allowed to stand at 4° overnight. A linearity check of these gradients after overnight diffusion and centrifugation, using the methyl orange procedure of Luck[21] and 0·5 ml increments, showed a good linearity throughout most of the gradient but with some slight distortion at the low and high density ends. Volumes of homogenate between 0·5 and 1·0 ml were applied to the upper surface of the gradients and the tubes centrifuged at 100,000 *g* for 90 min at 4°. Fractions from the gradients were removed either successively from the upper surface by a "J" type siliconized pipette, or when a larger number of equal subfractions were taken, by aspirating from the bottom of the tube using a 1 ml plastic syringe and polythene cannula. For further subdivision of the major fractions into particulate and supernate, the fractions were diluted to a sucrose concentration of approximately 0·3 M and centrifuged at 100,000 *g* for 60 min in a MSE 8 × 10 ml angle head rotor. Aliquots of the supernatant were removed directly for analysis and the deposited particles first washed and then resuspended for analysis in a buffered sucrose solution containing 0·154 M NaCl 0·077 M EDTA 0·077 M tris–HCl pH 7·4 and 0·3 M sucrose.

Protein. The procedure used was essentially that of Lowry *et al.*[22] but with the minor modification to the reagents recommended by Price.[23] This modification avoids exceeding the solubility product of the copper tartrate in the stock solutions. Commercial Folin and Ciocalteau reagent was routinely adjusted to 1 N by titration and addition of distilled water. Standard curves were prepared from crystalline bovine plasma albumin (Armour & Co. Ltd.).

Marker enzymes. The following enzymes were measured in the subcellular fractions

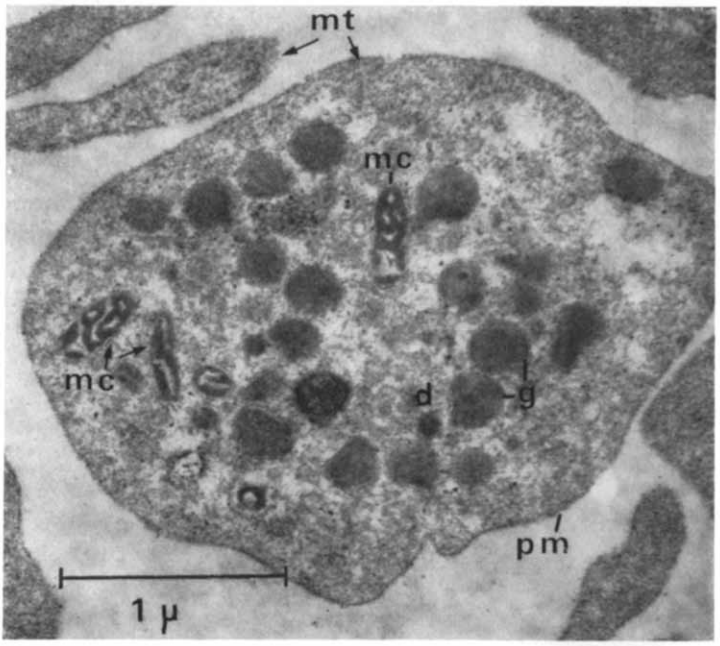

FIG. 1. Electron micrograph of pig platelet showing following intracellular features: g, granules; mc, mitochondria; pm, plasma membrane; mt, microtubular structures; d, dense bodies.

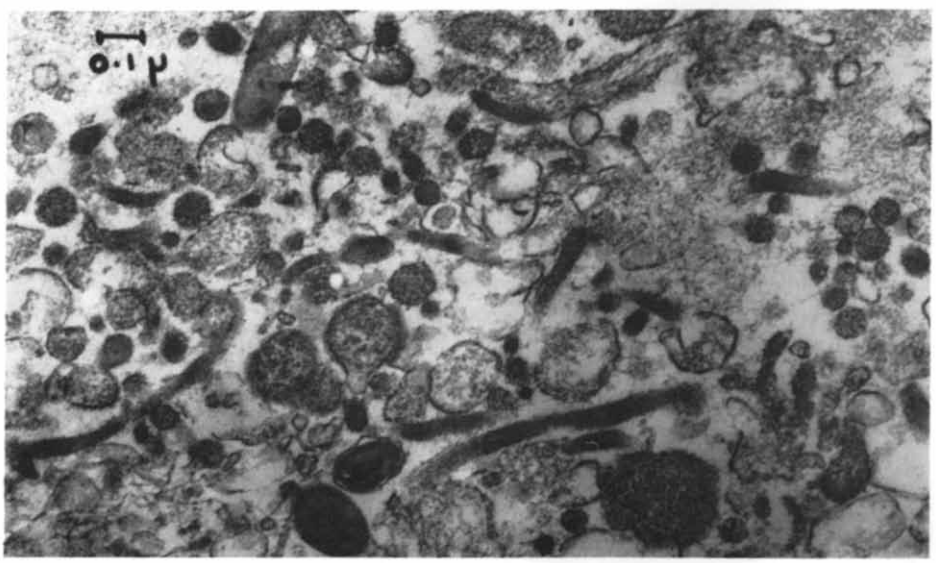

FIG. 2. Electron micrograph of homogenate prepared with the blendor homogenizer.

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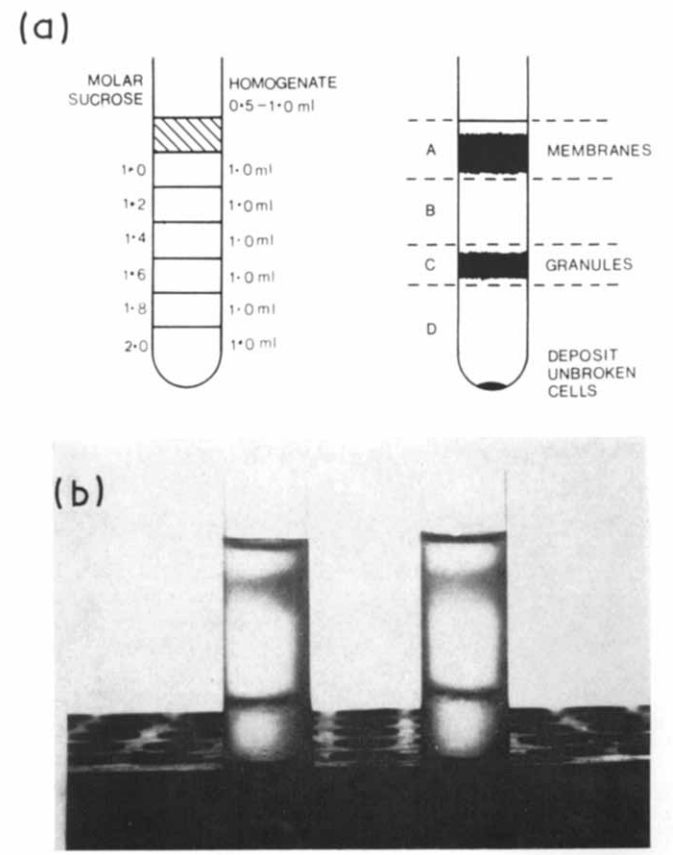

FIG. 3. Preparation of sucrose gradient and location of major zones. (a) diagrams of gradients before and after centrifugation (100,000 *g* 90 min) (b) photograph of two gradient separations of platelet homogenates.

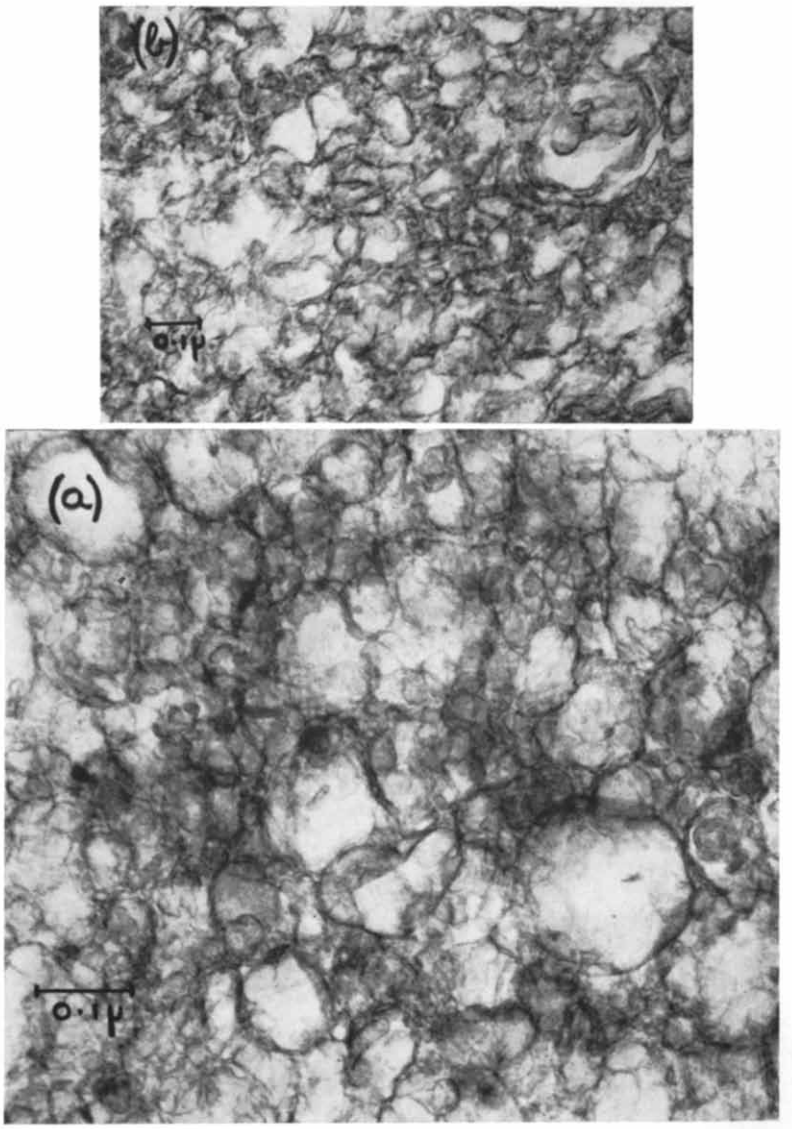

FIG. 4. Electron micrographs of membrane fractions from sucrose density gradients. (1) homogenate prepared by blendor (b) homogenate prepared by nil-clearance Teflon pestle.

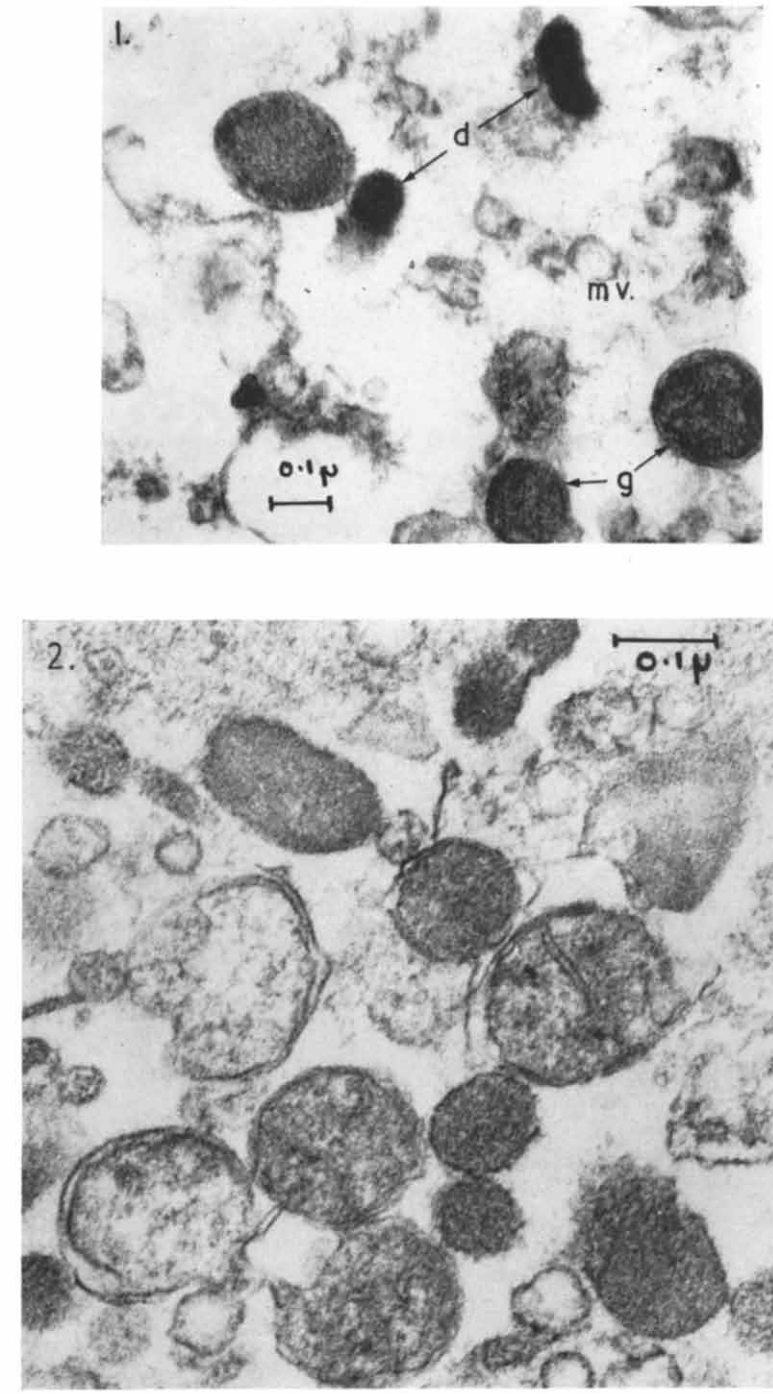

FIG. 5. Electron micrographs of granule fractions (fraction C) showing (1) dense bodies (d), α-granules (g) and membrane vesicles and fragments (mv) (2) whole and distorted α-granules.

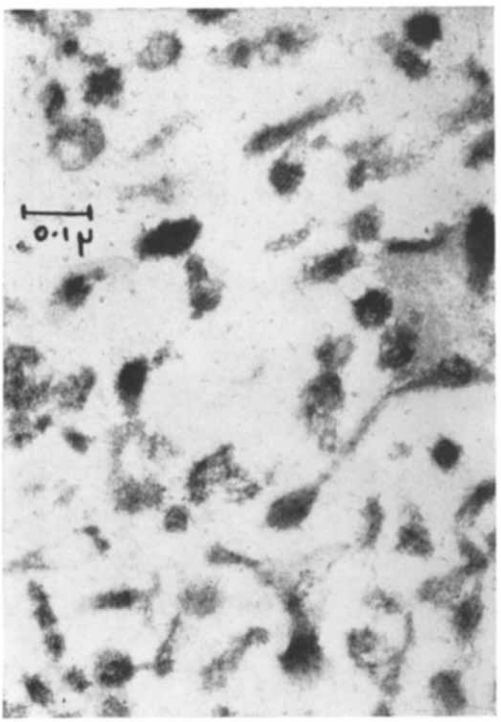

FIG. 10. Electron micrograph of high density subfraction C_3 of the major granule zone showing high concentration of dense bodies.

to assist in the localization of membranes and other cytoplasmic constituents: glucose 6 phosphatase (EC 3.1.3.9) for endoplasmic reticulum,[24] succinate dehydrogenase (EC 1.3.99.1) as succinate-2-(*p*-iodophenyl) 3-(*p*-nitrophenyl)-5-phenyl tetrazolium reductase, for mitochondria,[25] and 5'nucleotidase (3.1.3.5)[26] for plasma membrane.

5-Hydroxytryptamine. In earlier experiments 5-hydroxytryptamine was measured by the fluorescence procedure of Crawford and Rudd[27] after precipitation of the protein with 10% trichloroacetic acid. To avoid occasional interference by sucrose and to increase the sensitivity with subfractions, the ninhydrin fluorescence reaction of Vanable[28] was applied to the final extraction stage. Instead of the extraction with 3 N HCl as in the Crawford–Rudd procedure this extraction was carried out with 4 ml 0·05 M phosphate buffer pH 7·0. The fluorescence was developed by the addition of 0·6 ml of 0·24% ninhydrin solution to the buffer extract after removal of the upper organic phase. The mixture was heated for 30 min at 70°, allowed to cool and read in the spectrophotofluorimeter after standing at room temperature for 1 hr. An activating wavelength of 384 nm was used and fluorescent emission measured at 500 nm. Standards in the range 200–900 ng of serotonin were taken through the full procedure with every batch of analysis. For the measurement of [^{14}C] 5 hydroxytryptamine, 0·2 ml of solution was added to 10 ml of scintillation liquid in a counting vial. The phosphor mixture was prepared by adding 2 vol. of Triton X-100 to 1 vol. of a solution containing 6·0 g 2-5-diphenyloxazole and 0·12 g 2-*p*-phenylene bis (5-phenyl oxazole) in 1000 ml xylene. The samples were counted in a Packard liquid scintillation spectrometer (Model No. 527). Counts were corrected to 100 per cent efficiency by the channel ratio method.

Electron microscopy. The homogenates and fractions from the sucrose density gradients were fixed in buffered glutaraldehyde (6·25% in 0·2 M cacodylate buffer pH 7·4). Periods of fixation varied from a few minutes to 1 hr and depended upon the appearance and quantity of material. After washing several times with buffer the fixed material was treated with 1·0% osmium tetroxide for 30 min and the preparation dehydrated through a range of ethanol–water mixtures, followed by propylene oxide, and embedded in Araldite. Ultrathin sections were cut using a Huxley ultramicrotome, stained with uranyl acetate or tungstoborate, and examined with a Siemens Elmiskop 1 microscope.

RESULTS

Homogenization—General considerations

The blood platelets from human subjects and most animal species measure between 2–4 μ in diameter and are bounded by a surface membrane which will apparently withstand a considerable degree of both mechanical and osmotic shock.[28] Pig platelets show a similar appearance to human platelets under the electron microscope (Fig. 1) and most of the intracellular organelles seen in the human platelet are readily identifiable in the pig cells. In choosing the optimal conditions for homogenization a number of difficulties have been encountered in the present study. Mild conditions, using a loose-fitting Teflon pestle and short homogenization times, left the majority of the cells intact and a homogenate yield that was quite inadequate for analytical purposes. The low-clearance homogenizer (radial clearance 0·002 in. at 4°) used in the earlier experiments with homogenization times of 3–5 min, considerably increased

the yield of material, but the degree of organelle preservation was not ideal. The small osmiophilic 5HT storage bodies survived this procedure well, but some of the larger α-granules had lost their contents and only rarely were mitochondria seen in electron micrographic preparations. In the later experiments using the MSE blade homogenizer with homogenization times of 2–5 min the homogenate yield was much higher and the electron micrographic studies suggested an improvement in organelle preservation although this was difficult to evaluate in quantitative terms. Results from both homogenization schemes however have been presented in the present investigations since they gave essentially the same biochemical profiles. During homogenisation the platelets were suspended in 0·3 M sucrose containing 0·001 M EDTA. The inclusion of EDTA in the medium followed the recommendations of Roodyn *et al.*[29] for the preservation of mitochondria with other tissues. Figure 2 shows the appearance of the blendor homogenate immediately before applying to the gradients. It is comprised of many granule structures interspersed with membrane vesicles and fragments. Both large and small membrane bound granules are present of varying electron densities and a number of elongated structures of a less well defined nature. A particular problem with the blood platelet is that it has a very short life span (7–10 days) and consequently displays a wider spectrum of maturation and senescence changes than many other cell types. This is reflected in differences in the appearance and integrity of the intracellular organelles in whole cell sections and produces difficulties in ascertaining the optimum conditions for their preservation in subcellular fractionation studies. In retrospect, from the present study, since platelet mitochondria are few (2–3/cell section) show coarse cristae and are possibly more robust than the α-granules (10–15/cell section with a very thin boundary membrane) it is now considered that future experimental procedures should be directed towards a better preservation of these latter structures as a more sensitive index of organelle survival.

Sucrose density gradient fractionation and electron microscopy of major fractions

Density gradient centrifugation on sucrose gradients has been applied to homogenates prepared from control platelets and platelets which have been incubated for varying periods with 5 hydroxytryptamine at different levels. All these gradients consistently produced two major particulate fractions A and C. These two fractions have been tentatively designated as the "membrane fraction" (A) and the "granule fraction" (C). A diagram and a photograph of typical gradients are shown in Fig. 3. The upper low density membrane fraction (A) generally locates between densities 1·120 and 1·154 and the higher density granule band (C) between densities 1·205 and 1·230. In some gradients a narrow and slightly turbid pink zone is present just above the membrane fraction and in most analytical studies this zone has been included in fraction A. Electron micrographs of the membrane fraction A (Figs. 4a and 4b) show that it is comprised almost entirely of small vesicles with occasional larger sheet membrane structures. The proportion of sheet membrane structures to vesiculated forms varied from preparation to preparation in an as yet unexplained way. Investigation with marker enzymes (5′-nucleotidase, glucose-6-phosphatase) have failed to reveal any significant differences between these two structural forms to account for this. The granule fraction C (Figs. 5a and 5b) is a more heterogeneous fraction with whole and distorted α granules, occasional intact mitochondria, and small dense bodies similar in structure to the

5HT storage organelles described by Pletscher *et al.*[30,31] Membrane fragments were also seen in fraction C. In some of the gradient separations fraction C showed two and occasionally three distinct particulate layers. These fractions were difficult to isolate without contamination from adjacent zones but when analysed separately have been referred to as C1, C2 etc., where C1 represents the lowest and C3 the highest density granule subfraction. A visible sub-division of the major fractions was not however a consistent feature of the gradients. The intermediate zone between fractions A and C was usually slightly opaque but contained no sedimentable material and the layer between fraction C and the bottom of the gradient was always crystal clear. In some of the earlier separations a small deposit located at the bottom of the gradient tube was included with fraction D. Electron microscopy of this sediment revealed that it consisted largely of unbroken cells and large cytoplasmic fragments. This deposit was subsequently excluded from the gradients by a second centrifugation stage (3000 *g* for 20 min), immediately before applying the homogenate to the top of the gradients.

Protein and enzyme distribution in the major fractions and in the soluble and particulate components of the membrane and granule fractions A and C

Table 1 shows the distribution of total protein and the enzymes 5′nucleotidase, glucose-6-phosphatase and succinic dehydrogenase in the gradients from 6 different platelet homogenates prepared with the "nil clearance" Teflon homogenizer. Approximately 87 per cent of the total protein was recovered in the membrane fraction A and 10 per cent in fraction C, containing the granules. It was also found that whereas approximately 96 per cent of the 5′nucleotidase activity and 87 per cent of the glucose-6 phosphatase activity was recovered in fraction A only 33 per cent of the total succinic dehydrogenase activity was present in this fraction with 66 per cent of the activity located in fraction C.

TABLE 1. DISTRIBUTION OF PROTEIN, 5′ NUCLEOTIDASE, GLUCOSE-6-PHOSPHATASE AND SUCCINATE DEHYDROGENASE IN SUCROSE DENSITY GRADIENT FRACTIONS OF PIG PLATELETS

		Distribution (%)			
Fraction	Morphology	Protein (6)	5′ Nucleotidase (5)	Glucose-6-phosphatase (4)	Succinate dehydrogenase (5)
A	contains membranes	86·6 ± 2·5	95·7 ± 3·4	86·6 ± 3·3	32·6 ± 7·7
B	clear	2·6 ± 1·0	0	0	1·5 ± 1·7
C	contains granules	9·8 ± 2·6	4·3 ± 2·6	13·4 ± 3·3	65·9 ± 6·8
D	clear	1·0 ± 2·5	0	0	0

Homogenization by "nil clearance" Teflon pestle: for nomenclature of fractions see text and Fig. 3. Results are expressed as percentage of total recovered component present in each fraction. Values are means ± S.D. of the number of experiments given in parentheses. Recovery of protein with respect to homogenate (six gradients) 103·5 ± 5·6%.

In a further series of experiments in which homogenization was performed with the MSE blendor, fractions A and C were subsequently subdivided into soluble and particulate components (Table 2). The distribution of total protein and the activity of the marker enzymes was very similar to the previous distribution. However of the 94 per cent of the 5′-nucleotidase activity recovered in fraction A, 83 per cent of this was located in the soluble component of this fraction. Fraction C accounted for approximately 6 per cent of the activity of this enzyme all of which was associated with particulate material. A similar result was found for glucose-6-phosphatase but here slightly more was particulate with approximately 72 per cent of the total activity

TABLE 2. DISTRIBUTION OF PROTEIN, 5′ NUCLEOTIDASE, GLUCOSE-6-PHOSPHATASE AND SUCCINATE DEHYDROGENASE IN THE PARTICULATE AND SOLUBLE COMPONENTS OF THE MEMBRANE AND GRANULE FRACTIONS OF PIG PLATELETS

				Distribution (%)				
Fraction	Protein (3)			5′ Nucleotidase (1)	Glucose-6-phosphatase (2)		Succinate dehydrogenase (2)	
A (soluble)	78·1	81·3	81·0	82·8	72·4	72·2	19·4	15·7
A (particulate)	7·0	8·1	5·6	10·9	16·9	10·9	8·7	12·1
B	3·7	2·8	2·0	0	0	0	4·7	0
C (soluble)	4·2	2·5	4·1	0	0	0	0	0
C (particulate)	5·2	3·2	5·3	6·3	13·0	10·9	67·2	72·3
D	1·8	1·5	2·0	0	0	0	0	0
Recovery per cent homogenate	107·5	98·4	102·2	96·4	87·0	114·8	109·2	94·6

Homogenization was by MSE blender. For details of gradient preparation and subfractionation of major zones A & C see text. Results expressed as percentage distribution (protein) and percentage recovered enzyme activity. Number of experiments in parentheses.

locating in the low density soluble fraction of zone A. Approximately 70 per cent of the succinate dehydrogenase activity was associated with the particulate material in fraction C.

Absorption of 5-hydroxytryptamine by whole pig platelets

In preliminary experiments it was found that pig platelets incubated at 37° for varying periods of time in the presence of 5-hydroxytryptamine (range 1–50 μg/ml) reach a steady state concentration after about 40 min. The plateau extends for 20–30 min and thereafter a slight loss of 5-hydroxytryptamine from the cells occurs, presumably due to the mechanical effects of agitation in the metabolic water bath. Accordingly, 60 min was chosen as the most suitable incubation time. One ml samples of freshly prepared pig platelet-rich plasma were incubated for 60 min at 37° in the presence of added 5-hydroxytryptamine. The concentrations of the amine in the external media were adjusted to cover the range of 10–100 μg 5HT/ml by the addition of equal volumes of varying strength solutions of 5-hydroxytryptamine in 0·85% NaCl. After incubation, the platelet pellets were collected by centrifugation in the cold,

washed once with ice-cold saline and the pellets resuspended in 1 ml saline. One ml of distilled water was added to partially disrupt the cells, followed by 2 ml 20% trichloroacetic acid. The platelet protein was allowed to aggregate and sedimented by centrifugation. The 5-hydroxytryptamine content of the protein-free supernatant was determined by spectrophotofluorimetry. Figure 6 shows a curve of the increase in content of 5-hydroxytryptamine in pig platelets after 60 min incubation plotted against the external concentration of 5-hydroxytryptamine to which the cells were exposed.

The circulating level of 5HT in pig platelets (1000–2500 ng/10^9 cells) though much lower than in the rabbit is considerably higher than the concentration found in human platelets (336 $\pm$ 94 ng/10^9 cells).[9] Unlike human platelets however, pig platelets absorb

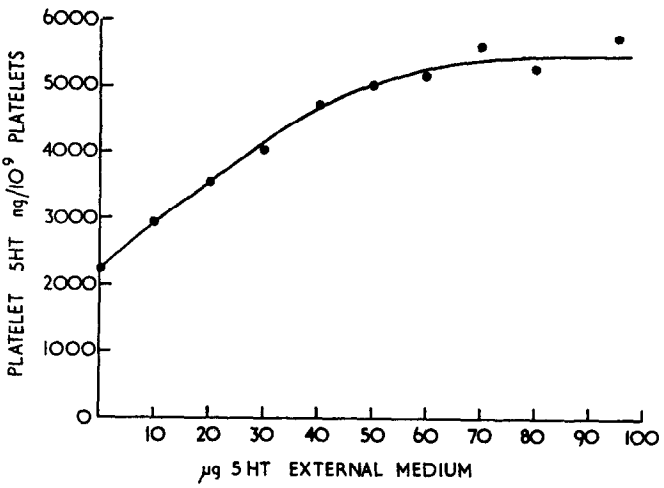

FIG. 6. Platelet 5HT content after 60 min incubation in media containing 5HT (range 0–100 μg/ml). Each plot: mean of seven experiments with different platelet rich plasma samples.

only very small quantities of the amine from media containing low 5HT levels (ca. 1–2 μg/ml). At higher external 5HT concentrations (5–50 μg/ml), the present studies have shown that pig platelets reach a concentration after 60 min incubation at 37° which is linearly related to the concentration of 5HT to which they have been exposed. This linear relationship ceases at around 50 μg/ml external 5HT concentration and above this level further uptake is minimal. A concentration of about 5000–6000 ng 5HT/10^9 cells represents the saturation capacity under the present experimental conditions.

The distribution of protein and 5-hydroxytryptamine in pig platelet subcellular fractions before and after incubation of the platelets with 5-hydroxytryptamine (50 μg/ml)

Pig platelet-rich plasma samples were incubated with 5-hydroxytryptamine (50 μg/ml) for 60 min and after removal of the platelets from the incubation medium by centrifugation the cells were washed once with cold saline, homogenized and fractionated by sucrose density gradient centrifugation. The four major subcellular fractions A, B, C and D were analysed for protein and 5-hydroxytryptamine. Unincubated platelets taken through the same homogenization and fractionation procedure served

as a control. (The results are shown in Table 3.) There was no significant difference between the subcellular distribution of 5-hydroxytryptamine, expressed as a percentage of the total content, in control cells and those which have been incubated with 5-hydroxytryptamine for 60 min at a concentration of 50 μg/ml 5HT. Between 70–80 per cent of the cell amine is associated with the low density region of the gradients (fraction A containing the membranes), and 8–9 per cent with the higher density zone (fraction C containing granules). In four experiments with control and incubated platelets the membrane and granule-containing fractions A and C were diluted to sucrose concentrations of approximately 0·3 M and centrifuged at 100,000 *g* for 60 min. The

TABLE 3. THE DISTRIBUTION OF PROTEIN AND 5 HYDROXYTRYPTAMINE IN THE SUCROSE DENSITY GRADIENT FRACTIONS FROM PIG PLATELETS BEFORE AND AFTER INCUBATION WITH 5HT AT A CONCENTRATION OF 50 μg/ml

Fractions	Protein		5 Hydroxytryptamine	
	Controls (10)	Incubated with 5HT (6)	Controls (10)	Incubated with 5HT (6)
A	71·3 ± 14·0	62·8 ± 16·3	78·8 ± 12·0	75·6 ±5·9
B	10·3 ± 7·3	17·4 ± 11·0	8·4 ± 5·1	12·8 ± 7·8
C	13·3 ± 6·4	14·9 ± 5·0	9·5 ± 6·7	8·3 ± 4·2
D	3·7 ± 3·9	3·4 ± 2·2	3·0 ± 1·7	3·4 ± 2·2

The results are expressed as Mean ± S.D. of the percentage of the total constituent recovered. The number of samples analysed is given in parentheses [Recovery-Protein (16 experiments) 106 ±19·4; 5HT (16 experiments) 81·3 ± 17·9].

supernatant fractions were analysed for 5-hydroxytryptamine and the particle bound amine calculated by difference from the total fraction assays. In the membrane-containing fractions (A) which were subjected to this further differential subdivision it was found that in both the control and incubated cells all the 5-hydroxytryptamine in these fractions could be accounted for in the particle-free supernatant with none associated with the particles. In the granule fractions, however (Table 4), between 38 and 83 per cent of the 5-hydroxytryptamine was bound to the particulate material in the control cells (four experiments) and between 36 and 77 per cent associated with the particles in the cells pre-incubated with 50 μg/ml 5-hydroxytryptamine (three experiments).

Distribution of total 5-hydroxytryptamine in fractions after incubation with increasing concentrations of 5HT (0–100 μg/ml)

In these experiments samples of platelet-rich plasma were incubated at 37° for 1 hr with 5-hydroxytryptamine at the following concentration levels: 0, 1, 5, 10, 20, 40, 50, 75 and 100 μg/ml. The incubations were terminated by immersion in ice and the platelets from each experiment collected by centrifugation. The cells were washed quickly with ice-cold saline, centrifuged, the packed cells suspended in 0·3 M sucrose

TABLE 4. THE DISTRIBUTION OF 5HT BETWEEN THE SOLUBLE PHASE AND THE PARTICLES OF THE GRANULE FRACTION (C)

	Control platelets (%)		Platelets incubated with 5HT (%)	
Experiment	Soluble	Particles	Soluble	Particles
1	17	83	23	77
2	67	33	64	36
3	60	40	48	52
4	62	38	—	—

Incubation conditions: 50 μg/ml 5HT 60 min at 37°. 5HT was determined in the whole fraction and in the supernatant phase after centrifugagation (100,000 *g* for 60 min). The particle bound 5HT was calculated by difference. For details of gradient separation and subfractionation see text.

and homogenized in the "nil clearance" Teflon homogenizer for 3 min. After a short period of centrifugation (7500 rev./min for 20 min) to remove unbroken cells, the homogenates were subjected to sucrose density gradient centrifugation and again subdivided.

Samples from the membrane and granular layers (A and C) were taken for the determination of 5-hydroxytryptamine and protein. The results have been expressed as ng 5HT/mg protein for each fraction at each level of external 5HT, and are presented in Fig. 7. Both fractions showed an initial fall in concentration. With increasing external 5HT levels fraction A increased in concentration, eventually reaching a level approximately twice that of the same fraction from cells incubated without added 5HT. After an initial fall fraction C also increased in concentration throughout the range 1–100 μg/ml 5HT but the final concentration reached did not differ significantly from the value recorded for fraction C from the control, unincubated cells.

The subcellular localization of absorbed 5-hydroxytryptamine after incubation of platelet with [^{14}C] *5HT in the range* 0–50 μg/ml

Samples of pig platelet rich plasma were incubated for 1 hr at 37° with [^{14}C] 5-hydroxytryptamine (0·397 mc/mM) at external 5HT levels of 1, 2, 5, 10, 25 and 50 μg/ml. The [^{14}C] 5HT solutions were prepared in 0·85% sodium chloride and for each concentration in the range studied the solutions also contained non-radioactive 5HT in the proportion 1:99 ([^{14}C] 5HT–carrier 5HT). Following incubation the cells were deposited and homogenized and fractionated as in the non-radioactive experiments. The four major subcellular fractions A, B, C and D were removed and aliquots taken for radioactive counting. The amount of absorbed 5-hydroxytryptamine determined and expressed as ng/mg protein for each major fraction. Figure 8 shows a plot of the increase in absorbed [^{14}C] 5-hydroxytryptamine for the two major subcellular fractions A and C in relation to the concentration of 5HT in the external medium during incubation.

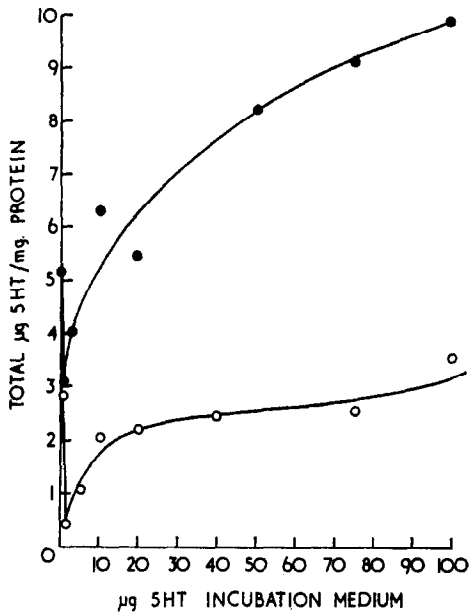

FIG. 7. Total 5HT concentration (inherent + absorbed) of fraction A and C after incubation of whole cells with 5HT (range 0–100 μg/ml).
fraction A. ●——●
fraction C. ○——○

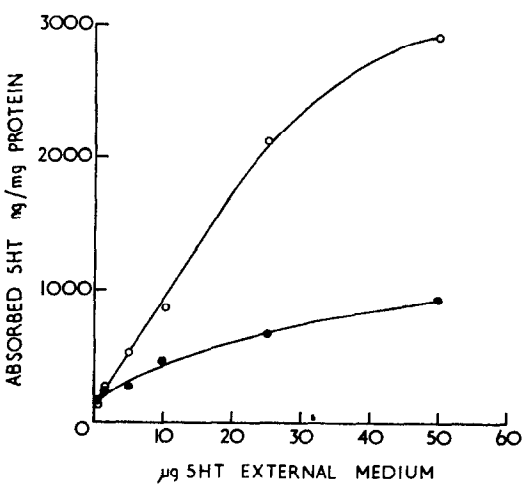

FIG. 8. Increase in absorbed 5HT (^{14}C 5HT) fractions A and C after incubation of whole cells with 5HT (range 0–60 μg/ml).
fraction A. ○——○
fraction C. ●——●

In one experiment (three gradients) the four major subcellular fractions were pooled from each gradient and dialysed against 0·154 M Tris buffer pH 7·4 at 4°. Four changes of buffer, using volumes of 500 ml, had previously been found sufficient to produce a dialysate free from radioactivity. The dialysed fractions were centrifuged (100,000 *g* for 30 min) and the particulate deposit from each fraction assayed for absorbed 5HT by radioactive counting. The results from this experiment are shown in Table 5. Although the majority of the total absorbed 5HT before dialysis was found in the fraction A pool (9620 μg) none of this was associated with the particles sedimented from the fraction after dialysis. Of the 3070 μg 5HT found in the fraction B pool before dialysis, the particles after dialysis accounted for a negligible amount (0·4 per cent).

TABLE 5. THE ABSORBED 5HT CONTENT OF POOLED FRACTIONS FROM PLATELETS INCUBATED WITH [^{14}C] 5HT (RANGE 1–50 μg/ml) AND THE 5HT CONTENT OF THE PARTICULATE DEPOSITS ISOLATED FROM THE FRACTIONS AFTER DIALYSIS

Fractions	Total 5HT/fraction before dialysis (μg)	Particle 5HT after dialysis (μg)	Total 5HT bound to particles (%)
A	9620	nil	nil
B	3070	12	0·4
C	960	396	41·2
D	140	24	17·1
Sum fractions	13,790	432	3·1

The pooled fractions were dialysed extensively against 0·154 M Tris buffer pH 7·4 at 4° and the particles separated by centrifugation 100,000 *g* 30 min.

In the fraction C pool containing the granules and accounting for 960 μg of total 5HT, over 40 per cent of this 5HT remained tightly bound to the granule particles after dialysis of the fraction. Fraction D which included a small deposit of unbroken cells and large cell fragments which had survived homogenization contained 140 μg 5HT before dialysis and 17 per cent of this was bound to sedimentable material.

The distribution of 5-hydroxytryptamine in the subfractions C_1, C_2 and C_3 after incubation with 5-hydroxytryptamine

In the previous experiments, fraction C was removed as a broad band although on several occasions two and sometimes three separate bands could be distinguished in gradients from platelets which had been incubated with 5-hydroxytryptamine. (A clear splitting of band C occurred only rarely with homogenates from non-incubated cells.) Since with pig platelets very little 5-hydroxytryptamine is absorbed during incubation with low concentrations of the amine (ca. 1 μg/ml), the concentration in the cell after incubation at this level can be taken as approximately the same as the

TABLE 6. INCREASE IN 5 HYDROXYTRYPTAMINE CONCENTRATION OF THE MAJOR SUBCELLULAR FRACTIONS AND SUBFRACTIONS C_1 C_2 + C_3 AFTER INCUBATION WITH 20 μg/ml 5 HYDROXYTRYPTAMINE

Fraction	Protein mg/fraction 1 μg	Protein mg/fraction 20 μg	5HT mg/fraction 1 μg	5HT mg/fraction 20 μg	5HT ng/mg protein 1 μg	5HT ng/mg protein 20 μg	Ratio: cells incubated with 20 μg 5HT: cells incubated with 1 μg/ml 5HT
A	1·61 (37·5)	1·53 (34·0)	5870 (77·5)	8300 (47·5)	3660	5450	1·5
B	0·33 (7·7)	0·52 (11·5)	660 (8·7)	4200 (24·1)	1910	8150	4·2
C_1	0·70 (16·3)	0·51 (11·3)	452 (6·0)	1845 (10·6)	695	3740	5·4
C_2	0·59 (13·8)	0·61 (13·6)	440 (5·9)	1930 (5·9)	750	1685	2·2
C_3	0·88 (2·6)	0·98 (21·8)	144 (1·9)	1610 (9·2)	193	1660	8·6
$C_1 + C_2 + C_3$	2·17 (50·7)	2·10 (46·7)	1036 (13·8)	4485 (25·7)	412	2140	5·2
D	0·17 (4·1)	0·35 (7·8)	0 0	481 (2·7)	0	1370	—
Sum of all fractions	4·28 (100)	4·50 (100)	7566 (100)	17,466 (100)	—	—	

For details of density gradient fractionation and assay procedures see text. Figures in parentheses represent percentage distribution. Fractions pooled from two gradients. 5HT found after incubation with 1 μg 5HT/ml regarded as "endogenous". See text.

endogenous level. The advantage is that band C can then be collected as three fractions from cells with low as well as high total 5HT content. In the following experiments freshly prepared pig platelets were incubated for 60 min at 37° with 5-hydroxytryptamine at a concentration of 20 μg/ml and control cells incubated for the same period of time at 1 μg/ml 5HT. The platelets from both series were homogenized with the Teflon pestle and separated into fractions by sucrose density gradient centrifugation and in both, a clear visible subdivision of fraction C into 3 bands occurred. This granule zone was collected as three separated bands C_1, C_2 and C_3 with C_1 representing the lowest and C_3 the highest density component. Fraction A and the subdivided C fractions were analysed for protein and 5-hydroxytryptamine. In both series i.e. the low and high 5HT gradients the 5-hydroxytryptamine levels for each subfraction have been expressed as ng/mg protein. The results appear in Table 6.

In a second experiment a slightly different approach was tried. Platelets were incubated with [^{14}C] 5-hydroxytryptamine at a level of 50 μg/ml. Incubations were carried out for 60 min at 37° and the platelets isolated, homogenized in the MSE blendor and subjected to density gradient centrifugation. The major subcellular fractions and subfractions C_1, C_2 and C_3 were analysed for protein, total 5HT content by spectrophotofluorimetry and absorbed 5HT by radioactive counting. The endogenous 5-hydroxytryptamine was calculated by subtracting the absorbed [^{14}C] 5HT from the total 5HT for each fraction. The results from these experiments are presented in Table 7. Figure 9 illustrates the affinity of 5-hydroxytryptamine absorbed into the cell for the major fractions and subfractions by a comparison of the ratios of absorbed 5-hydroxytryptamine concentrations to the endogenous concentrations for each fraction in these two series of experiments. Figure 10 is an electron micrograph of subfraction C_3 from one of these experiments and shows that it contains small electron dense bodies considerably smaller than α-granules (see Fig. 5a). These organelles are however more distorted than those seen in whole cell sections.

DISCUSSION

In the present investigations, since pig platelets show little, if any, absorption of 5HT at low external concentrations it has not been possible to follow the subcellular distribution of the amine under conditions favourable for the active transport mechanism. The majority of the incubation studies have therefore been made under external 5HT concentration conditions in which a passive diffusion process would predominate. This property of 5HT absorption though little studied with pig platelets, has been extensively investigated with rabbit cells by Pletscher *et al.*[7,8,18] These workers hold the view that transfer of 5HT across the platelet membrane may involve both an "active", energy-requiring process and passive diffusion. The former mechanism is believed to operate at low environmental levels of 5HT such as may occur naturally *in vivo* and the latter process when the concentrations are higher > 5 μg/ml. In their studies with rabbit platelets a saturation level was not reached even with external 5HT concentrations as high as 300 μg/ml.[32] With human platelets there is evidence that the capacity of the cells *in vitro* for 5HT absorption is exceeded at lower external levels than for the rabbit,[9] and *in vivo* saturation has also been demonstrated in patients with 5HT secreting tumours,[9] and in human volunteers after about 8 days of orally administered 5HT.[33] It would seem, therefore, that with respect to the manner of 5HT absorption

Table 7. Increase in 5 hydroxytryptamine concentration of the major subcellular fractions and subfractions C_1 C_2 + C_3 after incubation with 50 μg 5HT/ml (^{14}C 5HT)

Fraction	Protein mg/fraction	Total 5HT/ fraction ng (fluorimetric) assay	Absorbed 5HT ng/fraction (^{14}C assay)	Inherent 5HT (fluorimetric — ^{14}C 5HT) ng/fraction	Inherent 5HT ng/mg protein	Absorbed 5HT ng/mg protein	Total 5HT ng/mg protein	Ratio total 5HT: inherent 5HT (ng/mg protein)
A	2·64 (73·8)	21,320 (76·7)	12,342 (76·7)	8978 (75·5)	3400	4675	8076	2·4
B	0·23 (6·4)	1550 (5·6)	778 (4·8)	772 (6·6)	3557	3383	6739	2·0
C_1	0·31 (8·7)	989 (3·5)	626 (3·8)	363 (3·4)	1171	2019	3190	2·7
C_2	0·23 (6·4)	1676 (6·0)	879 (5·5)	797 (6·8)	3465	3821	7287	2·1
C_3	0·04 (1·1)	900 (3·2)	646 (4·0)	254 (2·4)	6350	16,150	22,500	3·6
$C_1 + C_2 + C_3$	0·58 (16·2)	3565 (12·7)	2151 (13·3)	1414 (12·6)	2431	3707	6147	2·3
D	0·13 (3·6)	1368 (5·0)	798 (5·2)	570 (5·3)	4384	6138	10523	2·4
Sum of all fractions	3·58 (100)	27,803 (100)	16,069 (100)	11,734 (100)	3277	4460	7765	2·4
Homogenate	3·66	24,450	17,900					
Recovery % homogenate	97·8	114·0	111·4					

For details of density gradient fractionation and assay procedures see text. Fractions pooled from three gradients Inherent 5HT = Total (Fluorimetric) — ^{14}C 5HT Absorbed. Figures in parentheses represent percentage distribution in the fractions.

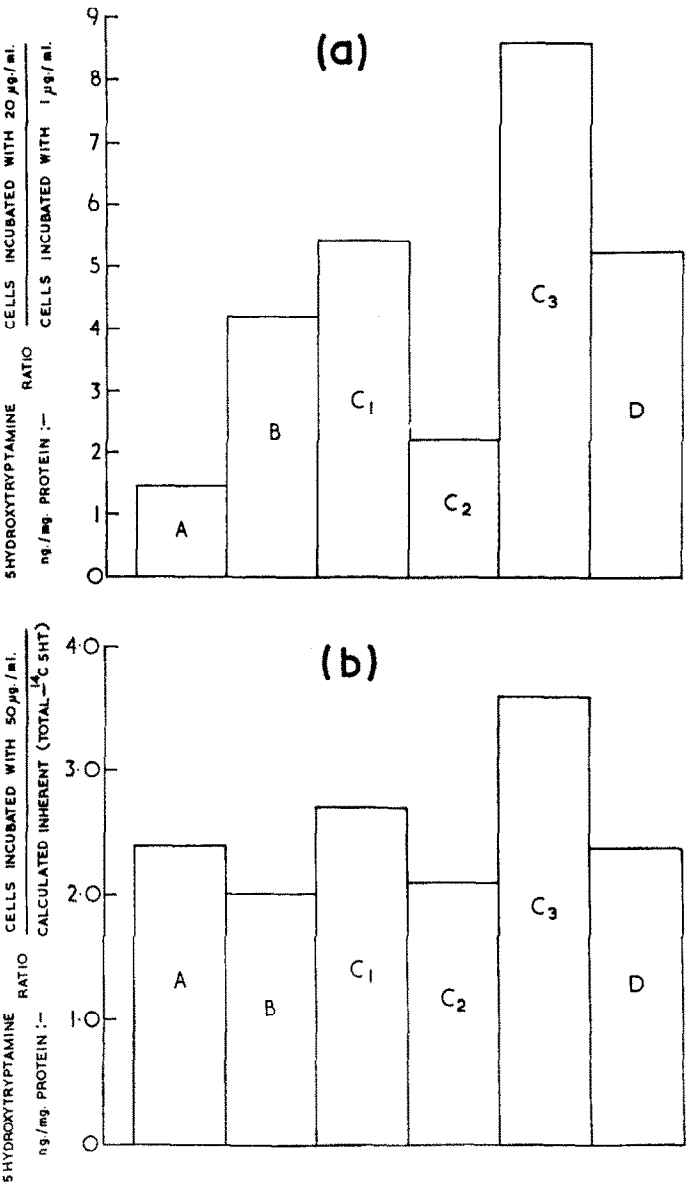

FIG. 9. Ratio 5HT concentration—absorbed to endogenous in the major subcellular zones A, B and D and in the subfractions C_1 C_2 and C_3. (a) Cells incubated with 5HT 20 µg/ml and 1 µg/ml ("endogenous") (b) Cells incubated with [^{14}C] 5HT 50 µg/ml. Endogenous 5HT calculated by subtraction of [^{14}C] 5HT content from total content determined by spectrophotofluorometry.

and the saturation capacity of the cell for the amine, pig platelets lie in an intermediate position between those of the human and the rabbit.

The conditions for homogenization and subcellular fractionation used in the present studies, although not ideal, produce two particulate fractions which under the electron microscope appear relatively free from cross contamination. The major proportion, about 70 per cent, of the particulate bound mitochondrial enzyme, succinate dehydro-

genase, locates in the low density granule-containing zone of the gradient (fraction C). However, some activity of this enzyme, 8–12 per cent, is associated with particulate material in the low density region of the gradient and it must be assumed that small mitochondrial fragments are present in this fraction. Although approximately 95 per cent of the 5'nucleotidase activity of the cells is associated with fraction A, only about 10 per cent of this is particulate bound, the remainder occurring as a soluble component. Similarly over 80 per cent of the total glucose-6-phosphatase activity locates with the major fraction A but here again less than 20 per cent associated with sedimentable material. It would seem therefore that in addition to the two major particulate zones containing membranes and granules, the fractionated cell has a large soluble component under the present experimental conditions. This soluble cell fraction accounts for about 80 per cent of the cell protein and in this study it has not been possible to determine what proportion of this soluble material represents a valid component of the whole cell and how much of it may result as a solubilization artefact during the homogenization and gradient procedures.

In earlier experiments (not included in this study) isolation of the soluble phase from the particles before application to the gradient has proved unsatisfactory. Considerable leakage takes place from the granular organelles during separation and gradient centrifugation and mitochondrial and lysosomal enzymes are found distributed throughout the gradient. It seems that removal of these organelles from their colloidal environment confers an increased sensitivity to osmotic shock and studies are being made using the addition of serum albumin and inert high molecular weight polymers to the particles in order to find an optimum environment for their isolation. In our fractionation procedures the soluble fraction appears to be the major 5-hydroxytryptamine store for both control platelets and platelets which have been incubated with 50 μg/ml 5HT. This 5HT fraction locating in the low density areas of the sucrose gradient in the region of the membrane particles is in a dialysable form and represents 70–80 per cent of the total cell 5HT content. This distribution of soluble 5HT is fairly constant (Table 3), fraction A representing 78·8 $\pm$ 12·0 per cent for controls and 75·6 $\pm$ 5·9 per cent for cells incubated with 50 μg/ml. These figures are from 10 different pig platelet pools subjected to slight variations in homogenization procedure and their constancy supports our view that a considerable proportion of the soluble 5HT is stored unassociated with any intracellular organelle, is a valid component of the cell and unlikely to be entirely accounted for by redistribution during the fractionation procedures. When the concentrations of 5HT in the platelet fractions A and C are determined following incubation with 5HT in the range 0–100 μg/ml external 5HT concentration (Fig. 7) both fractions showed an initial fall in concentration. However, whereas fraction C increases only to a level of approximately that found in control non-incubated cells, fraction A rises to twice that of the corresponding control fraction. This suggests that the granular compartment of stored amine is probably at saturation in the normal pig platelet and much of the absorbed amine locates as a soluble component. When [^{14}C] 5HT was used in a series of absorption experiments both fractions A and C showed a progressive increase in [^{14}C] 5HT (Fig. 8) but fraction A accounts for the majority of the absorbed amine. This finding suggests that since the total 5HT concentration in fraction C does not significantly increase some degree of intracellular exchange or equilibration of absorbed 5HT must take place between the granule bound component and the soluble cytoplasmic pool.

In the experiments in which direct comparison was made between subcellular fractions prepared from the same platelet pool after incubation at 1 and 20 μg/ml 5HT, fraction C could be subdivided into its three visible zones C_1, C_2 and C_3. It was found that if the distribution of 5HT in the cells incubated with 1 μg/ml 5HT can be regarded as the normal endogenous situation (absorption is minimal or almost nil with pig platelets at this external level), then all fractions show a considerable concentration increase following incubation with 20 μg 5HT/ml. The ratios of incubated to endogenous concentrations as ng/mg protein suggest, however, that fraction C_3 has a higher affinity for the amine than any of the other subfractions of the granule compartment or the remaining major zones A, B and D (Fig. 9a). A similar ratio expression was calculated for the study using [^{14}C] 5HT. Here the endogenous level was obtained by subtracting the absorbed [^{14}C] 5HT from the total content in each fraction and these results also reveal a higher affinity for 5HT by the subfraction C_3 (Fig. 9b). Electron microscopy of the three subfractions of the major granule zone C showed small dense bodies in fraction C_3 of the kind Pletscher and his colleagues have associated with 5HT storage in rabbit platelets. Conclusive evidence for their specific localization in the lowest density region of fraction has not however been obtained. The dense osmiophilic bodies of the pig platelet though smaller than the α-granules are larger, often more elongated and far less numerous than those described by Pletscher *et al.*[31,32,34] in rabbit platelets. The rather heterogeneous nature of the granule fractions from pig platelets prepared by the present gradient procedures make it unlikely that a definite identification of the specific 5HT storage organelle can be made.

In earlier investigations involving the subcellular fractionation of the human platelet Schultz *et al.*[12] showed that 95 per cent of the 5HT appeared in the "hyalomere" or non-granular fraction. Their technique however depended upon freezing and thawing to rupture the cells, a procedure shown later to liberate the amine from isolated storage granules.[15] Buckingham and Maynert[14] using differential centrifugation of platelet homogenates prepared by ultrasonics produced evidence of some particulate association of 5HT but the 5HT content of their soluble component increased with increasing time of sonication. Wurzel *et al.*[15] established the presence of 5HT in a granule-like structure and also showed a soluble 5HT in the low density regions of their gradients. Their assay procedure for 5HT using aortic strip contraction did not allow a good quantitative assessment of the 5HT distribution. Day *et al.*[17] in a comparison of both pestle and blendor homogenization procedures applied to human platelets, showed that a proportion of the 5HT was particulate-bound and this located in the high sucrose density region of their gradients. They too demonstrated a large soluble 5HT component, which varied to some extent with the homogenization procedure. Because of the considerable technique variations in these earlier reports a direct comparison of the findings with the present studies would be inappropriate.

It is believed however, from the present investigations that the pig platelet has a specific storage organelle for 5HT capable of holding the amine in a tightly bound form. These structures seem to have a high affinity but low capacity for 5-hydroxytryptamine but unlike the human platelet this storage compartment is either at or almost at capacity, and an extra-granular cytoplasmic pool probably accounts for a good proportion of the total cell 5HT content even in normal circumstances. In pig cells with a high 5HT content, for example after exposure to high external 5HT levels under con-

ditions favourable for transit into the cell by passive diffusion, this cytoplasmic compartment probably accounts for the majority of the absorbed material. Some intracellular exchange may of course take place between the tightly bound and freely diffusable pools.

It is considered that further study of these intracellular storage compartments, the equilibrium between them, and their relationship to the external plasma milieu may be useful, particularly in relation to drugs which may specifically affect the surface and intracellular membranes and influence the uptake, and release of 5-hydroxytryptamine and thus its functional role *in vivo*.

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