Biochemical Pharmacology, Vol. 23, 351–367. Pergamon Press, 1974. Printed in Great Britain.

SUBCELLULAR DISTRIBUTION OF RESERPINE AND 5-HYDROXYTRYPTAMINE IN BLOOD PLATELETS AFTER TREATMENT WITH RESERPINE *IN VITRO* AND *IN VIVO*

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(*Received* 2 *April* 1973; *accepted* 19 *July* 1973)

Abstract—Using subcellular fractionation procedures with sucrose density gradients the distribution of reserpine and 5-HT in pig platelets has been investigated following exposure of the cells to the agents *in vitro* and *in vivo*. Pig platelets release about 50 per cent of their 5-HT content during 1–2 hr incubation at 37° with reserpine at levels to 0·2–1·0 μg/ml. The release is accompanied by a concomittant increase in the concentration of amine in the surrounding medium with little evidence of metabolic degradation. The platelets readily absorb 5-HT from the surrounding medium and will increase their content about 2-fold. This effect is completely blocked by the presence of reserpine at an incubation level of 1 μg/ml. Subcellular fractionation of platelets after exposure to reserpine revealed that the fraction containing granular organelles is most affected and total release from this store takes place even when the cells are preloaded with 5-HT before the reserpine exposure. Analysis of the reserpine content of the fractions showed binding to both the membranous and granular organelles and the proportion bound increases almost linearly with reserpine concentration, in the incubation media, in the range 1–4 μg/ml. Although reserpine levels as low as 0·2 μg/ml effect significant 5-HT release from the whole cell, concentrations as high as 15 μg/ml are required for significant release from isolated 5-HT granules. Rabbit platelets differ from the pig platelets in that they will absorb 5-HT both *in vitro* and *in vivo* after reserpinization in spite of the fact that reserpine induced 5-HT release is similar for both species. It seems that although the surface membrane of the platelet and the limiting membrane of the storage granule are both targets for reserpine and show similar and significant binding affinities for the alkaloid they differ in the magnitude of their response suggesting that high intracellular local concentrations of reserpine are required for granule release. However, since reserpine release from the whole cell primarily induces granule bound 5-HT depletion, the intracellular equilibrium between the tightly stored component and the more diffusable soluble phase pool is of considerable importance in the way in which the membrane responds to the agent by alterations in 5-HT flux.

RESERPINE, one of the *Rauwolfia* alkaloids, is a powerful agent for releasing 5-hydroxytryptamine (5-HT) and other amines from tissue storage sites. The principal sites of storage of 5-HT in the body are the brain, the intestinal tract and the blood platelets. Blood platelets differ from other storage tissues in that they do not appear to be involved in the biosynthesis of 5-HT.[1] They feature little in its overall metabolism and merely absorb the amine from the external plasma environment, transport it, and release it, either for functional purposes or for enzymatic degradation. The absorption of 5-HT into the platelet has been shown to occur against concentration gradients as high as 1000:1,[2] and it is now believed that this uptake may proceed by two quite distinct processes (a) by an active transport mechanism which predominates when the external concentration of amine is below about 2–5 μg/ml and (b) by passive diffusion

which proceeds at 0° and operates when the external amine concentration is greater, 5–10 μg/ml.[3] From our own studies on the subcellular localization of 5-HT within the pig platelet,[4,5] it appears that there may be two separate 5-HT storage compartments within the cell, a tightly bound component associated with the granular particles of the cell and a more freely diffusable pool located within the extragranular cytoplasm. The dynamic relationship between these two storage pools and particularly the level of saturation of the granule storage compartment are factors which are believed to be important in controlling the rate of absorption of amine into the cell and its release into the extracellular environment. Previous studies[6] with human platelets, showed that the administration of reserpine to volunteers caused release of about 50 per cent of the platelet 5-HT but did not promote a simultaneous release of ATP or its catabolites. They also found that platelets pretreated with reserpine did not readily absorb 5-HT from the surrounding medium. Päasonen and Pletscher[7] showed that the amine could be released from platelets by the action of reserpine even in the presence of a monoamine oxidase inhibitor and concluded that the releasing action of reserpine was independent of amine degradative enzyme systems of the cell. Pletscher *et al.*[8] found that the 5-HT outflow from platelets after treatment with amine-releasing drugs, was very much faster at 37° than at 10° and they suggested that the cell membrane might become more permeable to the passage of 5-HT at higher temperatures.

Although the release of the amine from the cell by reserpine is a relatively slow process[9] it would appear that the inhibition of 5-HT absorption is almost immediate, upon the addition of reserpine to the incubation media.[10] These latter workers proposed that the primary effect of reserpine in depleting the platelets of their 5-HT was by the immediate inhibition of active transport into the cell at the membrane surface, whilst still allowing normal diffusion of the amine out of the cell. This results in a net depletion of 5-HT in the cell simulating a specific releasing process.

Little attention has been focussed upon the chemical nature of the subcellular changes which accompany reserpine-induced release from the platelet and the exact nature of the release process and the mechanisms which may operate at the molecular level in or at the surface of the cell, or the reserpine sensitive intracellular storage compartments has not been clearly established. Electron microscopic studies by Tranzer *et al.*[11] have shown that rabbit platelets when exposed to reserpine either *in vitro* or *in vivo* show a marked decrease in the number of the small dense osmiophilic granules in the cytoplasm coincident with the loss of 5-HT from the whole cell. These granules are considered by Pletscher and his colleagues[12,13] to be directly involved in the storage of the amine and to be distinct from the larger alpha granules whose function is as yet unknown. However, the exact chemical nature of the smaller 5-HT-storage bodies and whether they are in fact of an entirely different morphological origin from the α-granules is still somewhat controversial.[14]

The experiments in this series of investigations, were concerned with a study of the subcellular changes in the distribution of 5-HT and of reserpine within the platelet following treatment with reserpine. The pig platelet was chosen because of its availability in quantity and its structural and enzymatic similarity to the human cell. Although the serotonin content of pig platelets is about 3–4 times higher than in the human platelet, this level is considerably lower than those found in the rabbit. (Rabbit platelet 5-HT concentrations may reach values 15–20 times higher than found

in normal human platelets.) Moreover from the studies of Sano *et al.*[15] and Hughes and Brodie,[16] it appears that rabbit platelets because of their high content may have a slightly restricted ability to absorb 5-HT from the surrounding medium than platelets of other animal species. Some *in vivo* experiments with rabbits have been included in the present study to explore this point.

MATERIALS AND METHODS

The isolation of platelet rich plasma. Pig blood was provided by the local abattoir. The full procedure for the isolation of platelets has been described in detail earlier.[17] For the studies they were washed twice with a solution containing 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.

Incubation conditions. For incubation experiments, either platelet rich plasma was used or the washed cells were carefully resuspended in a solution containing 0·154 M NaCl 0·077 M Tris buffer, pH 7·4 (90:10 v/v).

All incubations were conducted in a metabolic shaking water bath at 37°. Incubations were terminated by immersing the incubation vessels in an ice bath and the cells then sedimented by centrifugation at 5000 *g* for 5 min at 4 .

All experiments with whole cells were carried out with approximately the same cell/volume conditions by prior adjustment to 200,000–300,000 platelet/mm^3.

Homogenization of platelets. Washed platelet pellets were resuspended in a volume of buffered sucrose (0·25 M sucrose in 0·3 M imidazole buffer containing 1 mM EDTA) approximately equal to one-hundredth of the volume of the platelet-rich plasma from which the cells were derived. The resuspended platelets were homogenized for 3 min in a MSE blender (Cat. No. 7700-A) with the blade speed set at approximately 3000 rev/min. The homogenization vessel was surrounded by an ice–water mixture and the 3 min homogenization period excluded short intervals for re-cooling.

The unbroken platelets and large cell fragments were removed by centrifugation at 5000 *g* for 20 min at 4° and the homogenate supernatant was used for subcellular fractionation on sucrose density gradients.

Preparation of sucrose gradients. All sucrose solutions for the preparation of the gradients contained 1 mM disodium EDTA. The addition of EDTA to the gradient was found to give a better preservation of α-granules and mitochondria as indicated by electron microscopy and marker enzymes. The gradients were prepared as previously described[16] and were allowed to diffuse overnight at 4° before use. This produced an almost linear gradient with only slight distortion at the very high and very low density ends. One ml aliquots of homogenate were layered on to the upper surface of the gradients and the tubes centrifuged at 100,000 *g* for 90 min at 4° in a 3 × 10 ml swing-out rotor in a MSE 40 refrigerated centrifuge. The subcellular fractions were removed from the gradient with a "J" type Pasteur pipette according to the visible separation produced. This gradient fractionation procedure consistently produced two major particulate zones, A and C (Fig. 1). Fraction A from the low density region of the gradient (between 1·12 and 1·15) contained sheet and vesiculated membrane structures and was free from any identifiable granular organelles. Fraction C (density range 1·205–1·230) was a heterogenous mixture of large α-granules, small dense bodies and occasional mitochondria. The intermediate zone B though slightly turbid contained no sedimentable material (100,000 *g* for 90 min) and fraction

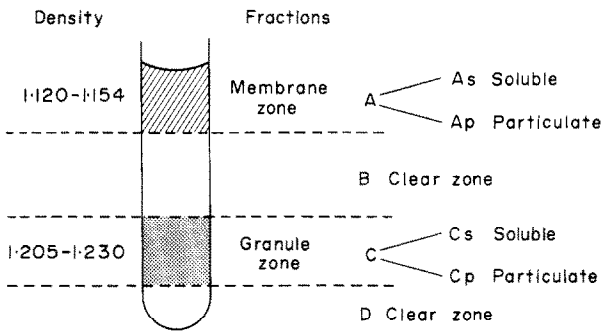

FIG. 1. Diagram of sucrose density gradient after centrifugation showing position of the two major particulate zones.

D at the bottom of the gradient was crystal clear. Full details of the homogenization and gradient fractionation procedures have been presented earlier,[17,18] together with electron micrographs of the major fractions.

Protein estimations. The method used was that of Lowry *et al.*[19] as modified by Price.[20] Bovine plasma albumin was used to prepare the standard curves and assays were adjusted to fall within the range 5–60 μg protein.

Reserpine assay. Reserpine was determined by the method of Glazko *et al.*[21] The extracted reserpine was measured in a Farrand spectrophotofluorimeter at an activating wavelength of 402 nm and fluorescent wavelength setting of 495 nm. Standards in the range of 50–400 ng reserpine were taken through the entire procedure with appropriate blanks. Linearity extended to at least 1000 ng reserpine and recoveries were of the order 100 ± 3 per cent for 50–400 ng range of reserpine added to biological materials. Standard solutions of reserpine were calibrated using an extinction coefficient of 0·270 at 268 nm for an aqueous solution containing 10 μg/ml.

Assay of 5-hydroxytryptamine (5-HT). The method used for the determination of whole platelet and homogenate 5-HT was that of Crawford.[9] However, for the estimation of 5-HT in the presence of higher molarities of sucrose present in the gradient subcellular fractions a modification of this procedure was necessary. The more sensitive ninhydrin fluorescence reaction of Jepson and Stevens[22] later adapted by Vanable[23] was incorporated into the assay procedure. A final extraction solution of 0·05 M phosphate buffer (pH 7·0) was substituted for the 3 N HCl used in the Crawford[9] method and after removal of the trimethyl pentane phase, the fluorophor was developed by the addition of 0·6 ml of 0·24 per cent ninhydrin solution to the aqueous phase. The mixture was heated at 70° for 30 min and allowed to cool for 60 min at room temperature. For the readings a Farrand spectrophotofluorimeter was used with an activation wavelength set at 385 nm and fluorescent wavelength set 500 nm.

Assay of 5-hydroxyindole acetic acid (5-HIAA). For the determination of the daily urinary excretion of 5-HIAA in the animal experiments the method of McFarlane *et al.*[24] was used, modified for smaller volumes of urine.

Radioactive measurement. 0·2 ml vol. of sample were added to 10 ml of scintillation fluid. The scintillation fluid contained 6 g of 2·5-diphenyloxazole, 0·12 g of 2-*p*-phenylene bis (5-phenyl oxazole) dissolved in 1000 ml xylene and was diluted for use, 2:1 by volume with Triton X-100. Samples were counted in a Packard Scintillation

Spectrometer (Model 527) for 10 min or 10,000 counts and the counts corrected to 100 per cent efficiency by the channel ratio method.

Platelet counts. Platelets were counted microscopically on dilutions of platelet rich plasma using improved Neubauer counting chambers.

RESULTS

The effect of reserpine on the 5-HT concentration of pig platelets. Figure 2 shows the effect of reserpine on the 5-HT content of whole platelets when samples of pig platelet rich plasma were incubated with reserpine for varying periods up to 90 min at 37°. The level of reserpine in the surrounding plasma was 0·2 μg/ml. It can be seen that there is a rise in the plasma 5-HT level, concommitant with a fall in platelet 5-HT. The majority of the 5-HT lost from the platelets was recovered from the plasma as unmetabolized base. The platelets were found to have lost about 40–50 per cent of their 5-HT after 75 min incubation with reserpine.

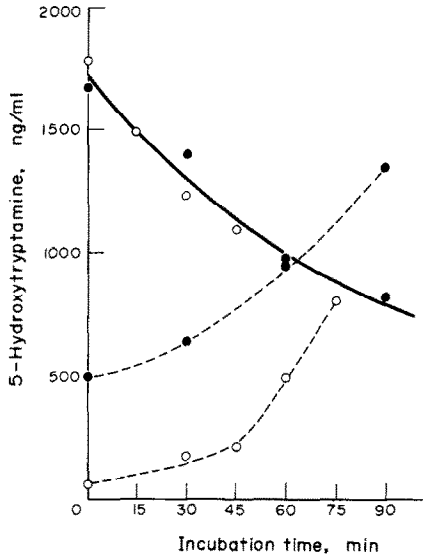

FIG. 2. 5-Hydroxytryptamine content of whole platelets and plasma after varying periods of exposure to reserpine (0·2 μg/ml) at 37°. Platelet levels (○—○) and (●—●) (two experiments); plasma levels (○- - ○) and (●- - ●).

Figure 3 shows in two experiments with different platelet samples, the effect of reserpine at a higher level of concentration (1 μg/ml). The rate of loss of 5-HT from the platelets was approximately the same as for the 0·2 μg/ml level of reserpine and the platelets lost between 50–60 per cent of their 5-HT content after 80 min incubation.

The effect of reserpine on the uptake of 5-HT by pig platelets. Figure 4 shows the effect of reserpine on the absorption of 5-HT by normal pig platelets. When reserpine was present at a concentration of 1 μg/ml in the surrounding media the platelets did not take up any significant quantity of 5-HT with an amine concentration of 2 μg/ml. In the absence of reserpine, 5-HT is taken up quite readily by the control cells and a concentration is reached within the platelets approaching twice the preincubation level in about 40 min.

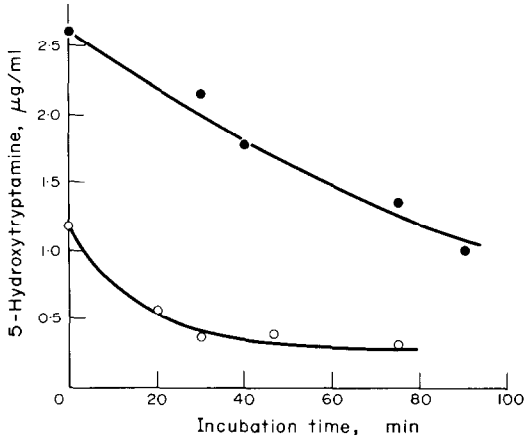

FIG. 3. Effect of reserpine on the 5-HT content of platelets (two experiments). Reserpine concentration 1 μg/ml/plasma.

The change in the subcellular distribution of 5-HT after treatment of the whole cell with reserpine. After high speed centrifugation of platelet homogenates on sucrose density gradients the four major zones were visible (Fig. 1).

Figure 5 shows for three separate experiments, the distribution of 5-HT (ng/mg of protein) in the membrane and granular subcellular fractions A and C of homogenates prepared before and after incubation of platelets for a period of 1 hr with different reserpine concentrations. The conditions for these experiments are included in the legend to Fig. 5. All incubations were carried out at 37° and the control and reserpinized platelets from each experiment were separated by centrifugation and then homogenized and subfractioned by sucrose density gradient centrifugation. The whole membrane layers (fractions A, which also included the soluble phase of the cell) and the granular layers (fractions C) were removed from the gradient for analysis. In all three experiments the fraction most affected by reserpine was the granular fraction, fraction C. No 5-HT could be detected in this fraction after incubation of the cells with reserpine in any of the three experiments. Fraction A in all experiments was little affected by the reserpine treatment. In a further experiment (Table 1) the

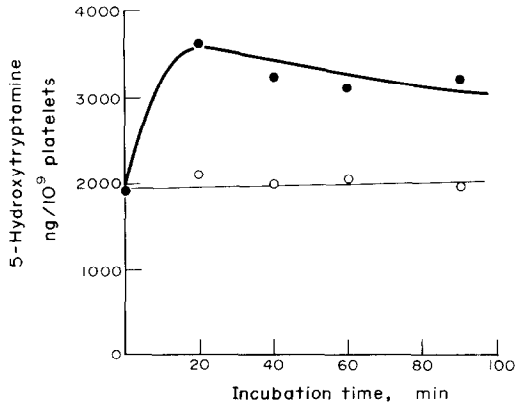

FIG. 4. Uptake of 5-HT (plasma concentration 2 μg/ml) in the presence of (○) and absence of (●) reserpine (1 μg/ml/plasma).

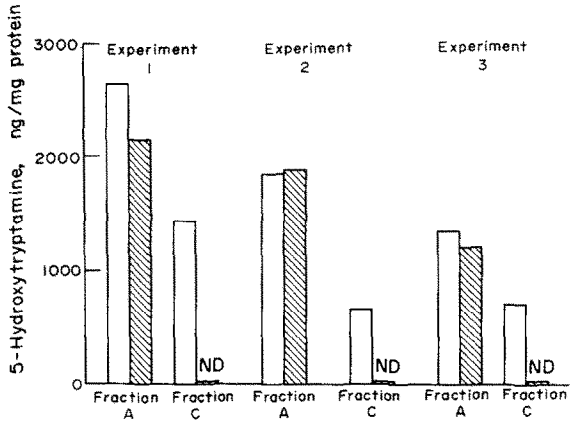

FIG. 5. Concentration of 5-HT (ng mg protein) in the membrane (A) and granular (C) fractions of platelets prepared from homogenates separated by sucrose density gradient centrifugation. Open diagrams = controls untreated. Hatched diagrams = platelets exposed to reserpine before homogenization. Expt. 1: 0·2 μg/ml reserpine 60 min at 37°. Expt. 2: platelets from another blood collection preincubated with 5-HT (2 μg/ml, 60 min), and then exposed to reserpine at a concentration of 0·2 μg/ml for 60 min at 37°. Expt. 3: Platelets from same collection as expt 2 incubated with reserpine 1 μg/ml 60 min at 37°. ND = not detected (i.e. below the sensitivity limits of the assay *ca.* 2–5 ng 5-HT).

distribution of protein and 5-HT was determined in the four major subcellular fractions prepared from platelets taken before and after incubation for 1 hr with reserpine at a concentration of 0·2 μg/ml. The recovery of 5-HT with respect to homogenate for these gradients was 84 per cent for the control gradient and 82 per cent for the gradient prepared from reserpinized platelets. Although the protein recoveries for these gradients with respect to homogenate applied were 94 and 109 per cent respectively, the homogenization conditions produced very different total protein yields for the control and reserpine gradients. However, since the percentage distribution of protein between the test and control subfractions was essentially the same, the results can be directly compared.

TABLE 1. PER CENT DISTRIBUTION AND CONCENTRATION OF PROTEIN AND 5-HYDROXYTRYPTAMINE IN THE FOUR SUBCELLULAR FRACTIONS

	Protein				5-Hydroxytryptamine		
	mg/Fraction		Distribution (%)		Protein (μg/mg)		Decrease (%)
Fraction	Control	RES	Control	RES	Control	RES	(C − R/C × 100)
A	7·35	2·38	81·6	80·1	2650	2140	19·2
B	0·46	0·21	5·1	7·1	1780	1530	16·3
C	1·20	0·38	13·3	12·8	1420	Nil	100
D	Nil	Nil	Nil	Nil	Nil	Nil	—
Recovery with respect to homogenate payload (%)			94	109	84	82	

For details of homogenization and gradient fractionation see text. Control platelets subfractionated before incubation with reserpine. Test platelets subfractionated after incubation with reserpine for 60 min at 37° at a concentration of 0·2 μg/ml plasma.

The concentration of 5-HT in fractions A and B expressed as ng/mg protein decreased during reserpine treatment by 19 and 16 per cent respectively. The concentration of 5-HT in the granule fraction, (fraction C) fell from 1420 ng/mg protein to a concentration below the detectable limits of the 5-HT assay (*ca.* 2 ng).

The distribution of reserpine in the platelet subcellular fractions after incubation of whole platelets with reserpine. In this experiment, 2 samples of platelet rich plasma from different pig blood collections were incubated with reserpine at a concentration of 0·2 μg/ml for 1 hr at 37°. Similarly, three other platelet rich plasma samples from different collections were incubated under the same conditions but with a concentration of reserpine of 1 μg/ml. The platelets, after incubation, were quickly sedimented, washed, homogenized and applied to sucrose density gradients. In each experiment fractions A and C were taken from the gradient and a small aliquot analyzed for reserpine by spectrophotofluorimetry. The remainder of the fractions were subjected to high-speed centrifugation (1,000,000 *g*, 90 min) to separate the particulate and soluble components. These two sub-fractions of fractions A and C were analyzed for their reserpine content and the per cent distribution between the soluble and particulate phases of each fraction calculated. The concentrations of reserpine in the subfractions have also been expressed as μg/ml for the soluble phase of each fraction and μg/particles derived from 1 ml of the fraction. The results of these five experiments are presented in Table 2. Although the absolute concentrations of reserpine associated with the major fractions A and C in the five experiments varied widely (between 50–1371 ng/ml for fraction A and between 3–100 ng/ml for fraction C), in the two experiments using 0·2 μg/ml reserpine 27 and 53 per cent of the reserpine content of fraction A (the membrane fraction) appeared in the soluble phase and 73 and 47 per cent was found to be firmly associated with the particulate material. With higher levels of reserpine during the incubation, between 4 and 19 per cent of the reserpine was particulate bound and between 81 and 96 per cent located in the soluble fractions of fraction A. Despite the wide variation in reserpine concentration associated with the whole fraction A, the absolute concentrations of particulate bound reserpine expressed as ng reserpine associated with particles from 1 ml of the

TABLE 2. DISTRIBUTION OF RESERPINE IN THE SOLUBLE AND PARTICULATE COMPONENTS OF FRACTIONS A AND C AFTER INCUBATION WITH RESERPINE

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
Fraction A					
Total reserpine (ng/ml)	75 (100)	50 (100)	113 (100)	1371 (100)	139 (100)
Soluble reserpine (ng/ml)	40 (53)	14 (27)	98 (87)	1316 (96)	112 (81)
Particulate reserpine (ng/ml)	35 (47)	36 (73)	15 (13)	55 (4)	27 (19)
Fraction C					
Total reserpine (ng/ml)	100 (100)	3 (100)	—	67 (100)	50 (100)
Soluble reserpine (ng/ml)	65 (65)	0 (0)	—	51 (76)	0 (0)
Particulate reserpine (ng/ml)	35 (35)	3 (100)	—	16 (24)	50 (100)

The five experiments refer to different platelet pools. In experiments 1 and 2 the platelets were incubated for 1 hr at 37° in the presence of reserpine 0·2 μg/ml. In experiments 3, 4 and 5 the external reserpine concentration was 1·0 μg/ml. For details of homogenate preparation, gradient fractionation, preparation of soluble and particulate fractions and the reserpine assay, see methods section. Figures in parentheses indicate the per cent of reserpine associated with the soluble and particulate subfractions prepared from the major fractions A and C.

fraction were not significantly different after exposure to low and high level of exogenous reserpine (35 and 36 ng/ml at the 0·2 μg/ml reserpine concentration and 15, 27 and 55 ng/ml at the 1 μg/ml reserpine incubation level). With regard to the distribution of reserpine in the soluble and particulate components of fraction C (the granular layer), in two experiments (experiments 1 and 4) 24 and 35 per cent of the total reserpine content was found to be associated with the sedimentable material. In the remaining two experiments (experiments 2 and 5) no reserpine could be detected in the soluble phase of the fractions and all the reserpine found in these fractions appeared to be firmly bound to the particulate deposits.

TABLE 3. PER CENT DISTRIBUTION OF RESERPINE IN THE FOUR MAJOR SUBCELLULAR FRACTIONS OF PLATELETS AFTER INCUBATION WITH RESERPINE

Fraction	Cells incubated with 1·0 μg/ml reserpine					Mean distribution (%)
	Expt 1	2	3	4	5	Expts 2–5
A	87·5	71·8	70·8	66·5	61·3	67·6
B	7·5	10·9	15·5	24·8	14·4	16·4
C	1·2	9·9	13·7	4·1	22·0	12·4
D	3·8	7·7	0	4·4	2·3	3·6

Platelets incubated for 60 min at 37° in presence of reserpine for preparation of the four subcellular fractions see "Methods and Materials" section. Reserpine assayed by spectrophotofluorimetry.

In a further series of experiments (Table 3) the overall percentage distribution of reserpine in the four major subcellular fractions was determined. The fractions were prepared from platelets which had been exposed to reserpine for 60 min at 37°. Five different platelet pools were used and in four experiments the concentration of reserpine in the external medium was 1 μg/ml and in one experiment 0·2 μg/ml. For cells incubated with 0·2 μg/ml reserpine, 87·5 per cent of the platelet reserpine located with fraction A and only about 1 per cent with the granule fraction C. However after incubation with 1·0 μg/ml reserpine, fraction C accounted for between 4 and 22 per cent of the cell total reserpine content.

The binding of reserpine to the particles of fractions A and C after incubation of isolated fractions with reserpine. Fractions A and C were isolated from three different platelet homogenates and pooled. Two ml aliquots of the fraction A pool and of the fraction C pool were incubated for 60 min at 37° with concentrations of reserpine in the range 1, 2 and 4 μg/ml.

Following incubation, the aliquots were centrifuged at 100,000 *g* for 30 min to separate the soluble and particulate components. The particulate material from each fraction at each level of reserpine was analyzed for bound reserpine content. The results are presented in Fig. 6 and show that the reserpine associated with the particulate material of both fractions A and C increases with increasing concentrations of reserpine in the incubation medium.

In a second series of experiments, the granular fraction C was isolated from gradients prepared from three different platelet homogenates. These were pooled and divided into 4 ml samples. The samples were incubated for 1 hr at 37° with reserpine

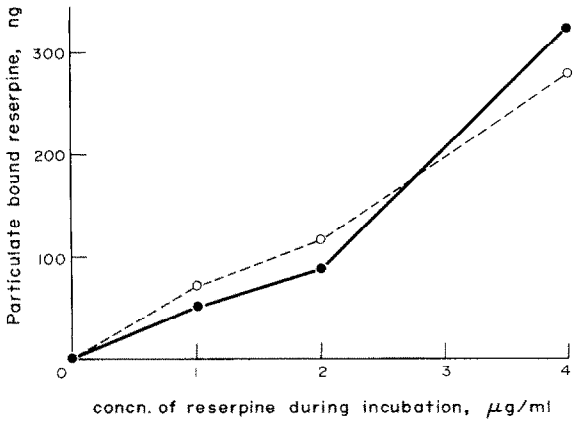

FIG. 6. Binding of reserpine to particulate components of fractions A (●) and C (○) after incubating fractions with reserpine (60 min at 37°) in the concentration range 1–4 μg/ml.

at external concentrations of 0, 3·75, 7·5 and 15·0 μg/ml and afterwards particles were separated by high speed centrifugation. The reserpine content of the particulate material was again assayed and in addition the amount of 5-HT present in the soluble phase also determined. These findings are presented in Fig. 7. The granule bound reserpine increased steadily with the increasing concentrations of reserpine in the incubation medium. However, release of 5-HT was minimal or nil at the 3·75 and the 7·5 μg/ml reserpine concentration and a significant release only took place from the particles after incubation with reserpine for 1 hr at 37° at a concentration of 15 μg/ml, a concentration many times higher than that required for a measureable release from whole platelets.

The absorption and distribution of serotonin in rabbit platelets following in vivo *exposure to reserpine.* For these *in vivo* experiments, laboratory-bred rabbits of approximately 3 kg wt were used. These animals (rabbits A, B and C) were maintained on a standard Oxoid diet of 100 *g* a day with water *ad lib.* They were housed in metabolism cages to allow complete daily urine collections. After a short period of

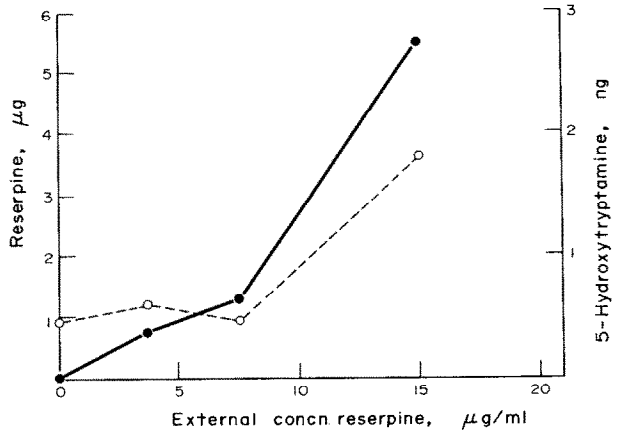

FIG. 7. Levels of bound reserpine and 5-HT content of isolated granules after incubating with reserpine (60 min at 37°) in the range 0–15 μg/ml reserpine (●) 5-HT (○).

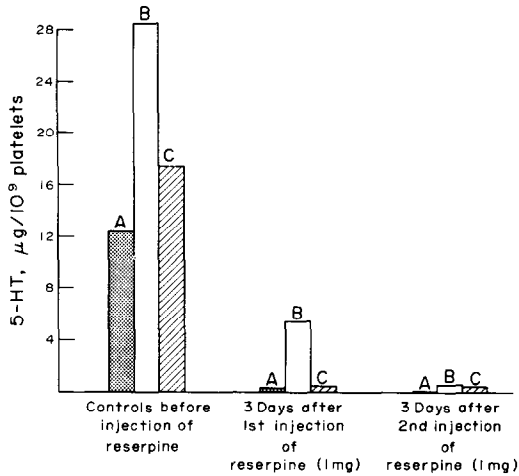

FIG. 8. Circulating platelet 5-HT content, ($\mu g/10^9$ cells), of three rabbits, A, B, C before and after injection of reserpine (1 mg).

acclimatization (2–3 days), control blood samples were taken from a marginal ear vein and intravenous injections of reserpine given in the opposite ear. All injections were made as an aqueous suspension containing 1 mg in 0·5 ml of 0·15 M NaCl. Blood samples were taken 3 days after this first injection and a further injection of 1 mg of reserpine immediately given. Three days after the second injection of reserpine further blood samples were taken. The platelet serotonin values in the blood samples of three rabbits taken during control and experimental periods are shown in Fig. 8. The 5-HT content of the cells has been expressed as $\mu g/10^9$ platelets and control values of between 12 and 28 $\mu g/10^9$ cells were recorded. Each rabbit showed a very pronounced decrease in circulating 5-HT during the period following the first reserpine injection. After two injections of reserpine the platelets of all three rabbits were almost completely depleted of 5-HT. The daily urinary excretion of 5-hydroxyindole acetic acid (5-HIAA) by the three rabbits was measured over a 7-day period which included 2 control days before the injection of reserpine. All three rabbits showed a significant though only transient increase in the output of 5-HIAA on the third day following the reserpine injection (Fig. 9), that is, on the day of the second injection of reserpine. Unfortunately, due to urine collection difficulties the analyses were not continued on throughout the second reserpine post injection period but the findings for two of the rabbits suggest that the output of 5-HIAA returns to the control level immediately following the transitory increase occurring on the third experimental day.

Absorption of 5-*HT* in vitro *by* in vivo *reserpinized platelets.* In another experiment one rabbit (rabbit D) was given an injection of 1 mg of reserpine and this was followed 3 days later by a second injection, also of 1 mg. On the day following this second injection the rabbit was killed and bled out by heart cannulation. The platelet-rich plasma was prepared and the entire sample volume (70 ml) incubated for 60 min at 37° with 5-HT at a concentration of 100 μg/ml. The added 5-HT contained a proportion of radioactive 5-HT in the ratio ^{14}C 5-HT:carrier 5-HT of 1:19. The specific activity of the ^{14}C 5-HT from which the solution was prepared was

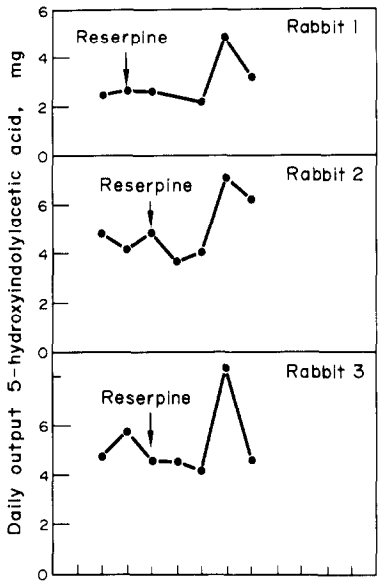

FIG. 9. Daily urinary output of 5-hydroxyindole acetic acid by three rabbits, before and after reserpine injection (1 mg).

39·7 mCi/m-mole. After the incubation period 1·0 ml aliquots were taken from the plasma, the platelets removed by centrifugation and the total cell 5-HT content ($\mu g/10^9$ cells) determined by spectrophotofluorimetry. The remaining platelet rich plasma was centrifuged to sediment the cells and after washing once, the platelets were homogenized and the fractions separated by density gradient centrifugation. The major gradient zones A, B, C and D were removed and the total 5-HT and the ^{14}C 5-HT determined in each fraction. From the ^{14}C analyses of each fraction the amount of absorbed 5-HT was calculated and the endogenous 5-HT was calculated by subtraction of the radioactive component from the total 5-HT determined by spectrophotofluorimetry. The distribution of the total and absorbed 5-HT in the platelet gradient fractions A, B, C and D for this rabbit are presented in Table 4.

TABLE 4. DISTRIBUTION OF 5-HT IN RABBIT PLATELET SUBCELLULAR FRACTIONS AFTER INCUBATION OF *in vivo* RESERPINIZED PLATELETS WITH 5-HT *in vitro*

		Total 5-HT (fluorimetric assay)			Absorbed 5-HT (^{14}C 5-HT assay)			Inherent 5-HT (fluo − ^{14}C)		
Fraction	Protein (mg)	(μg)	(%)	Protein (μg/mg)	(μg)	(%)	Protein (μg/mg)	(μg)	(%)	Protein (μg/mg)
A	16·95	206·2	78·0	12·2	190·5	81·2	11·2	15·7	52·6	0·9
B	0·85	38·3	14·6	45·1	27·6	11·8	32·4	10·7	35·8	12·6
C	1·85	16·9	6·3	9·2	13·8	5·9	7·5	3·1	10·6	1·7
D	0·34	2·9	1·1	8·4	2·6	1·1	7·6	0·3	1·0	0·8
Total	19·99	264·3	100·0	13·2	234·5	100·0	11·7	29·8	100·0	1·5

For details of the homogenization and density gradient fractionation see "Methods and Materials" section. This rabbit was given an intravenous injection of 1 mg reserpine and 3 days later a further 1 mg. Blood for these analyses was taken on the day following the second intravenous reserpine injection. Recovery of protein with respect to homogenate was 110 per cent. Platelet rich plasma incubated for 60 min at 37° with 5-HT (100 μg/ml). (Radioactive 5-HT/Carrier 5-HT ratio 1:19.)

It can be seen from this table that the 264 μg 5-HT found in the total homogenate (i.e. the sum of the four major gradient fractions) 234 μg were absorbed during the *in vitro* incubation with 5-HT. Of this absorbed 5-HT, the largest proportion 81 per cent (190 μg), was found to be associated with fraction A (the membrane and soluble phase fraction) and a further 12 per cent (28 μg) with the non-particulate intermediate zone, fraction B. Only 6 per cent (14 μg) located with the granule fraction C. Although the platelets had been well depleted of 5-HT by the *in vivo* reserpine exposure, their capacity for absorbing the amine into the cell during the *in vitro* incubation was apparently not impaired.

Aliquots of the membrane fraction A and the granule fraction C were taken for high speed centrifugation (1000,000 *g* for 90 min) to separate the particulate (A_p and C_p) and soluble (A_s and C_s) components. These four subfractions were analyzed for absorbed 5-HT by ^{14}C 5-HT assay method.

TABLE 5. DISTRIBUTION OF ABSORBED 5-HT BETWEEN THE SOLUBLE AND PARTICULATE COMPONENTS OF THE MAJOR GRADIENT ZONES A AND C OBTAINED FROM *in vivo* RESERPINIZED PLATELET EXPOSED TO 5-HT *in vitro* (RABBIT D) AND *in vivo* (RABBIT E)

	Rabbit D (*in vitro* 5-HT) expt		Rabbit E (*in vivo* 5-HT) expt	
	μg 5-HT	%	μg 5-HT	%
Fraction A (membrane fraction)				
Soluble (A_s)	191	100	13·6	100
Particulate (A_p)	Nil	Nil	Nil	Nil
Total	191	100	13·6	100
Fraction C (granule fraction)				
Soluble (C_s)	10	71	1·5	68
Particulate (C_p)	4	29	0·7	32
Total	14	100	2·2	100

The results for the distribution of ^{14}C–5-HT in the platelet subfractions of A and C in Table 5 demonstrate that all of the absorbed 5-HT present in fraction A could be accounted for in the soluble component (A_s) of that fraction and no 5-HT could be associated with the membrane particles (A_p). However in fraction C, 29 per cent of the absorbed 5-HT associated with this fraction, was firmly bound to the granular components.

Uptake of 5-HT in vivo *by* in vivo *reserpinized rabbit platelets.* In this experiment one rabbit (rabbit E) was given two injections of 1 mg of reserpine with 3 day intervals between doses. On the first day following the second injection a slow intravenous injection of 5-HT was given. The solution contained 15 mg 5-HT and 75 μg of ^{14}C 5-HT (39·7 mCi/mM) dissolved in 2·0 ml of 0·85 per cent saline. One hour after the 5-HT injection the rabbit was killed and bled by heart cannulation. A platelet rich plasma sample was prepared by low speed centrifugation and a aliquot taken and the platelets removed for total 5-HT assay by spectrofluorimetry. A further aliquot was taken for determination of absorbed 5-HT by radioactive counting. The platelets were

TABLE 6. THE DISTRIBUTION OF SEROTONIN IN PLATELET SUBCELLULAR FRACTIONS AFTER *in vivo* RESERPINIZATION AND EXPOSURE TO 5-HT *in vivo* (RABBIT E)

		Total 5-HT fluorimetric assay			Absorbed 5-HT ^{14}C assay		
Fraction	Protein (mg)	(μg)	(%)	Protein (μg/mg)	(μg)	(%)	Protein (μg/mg)
A	13·66	13·04	73·0	0·95	13·62	76·0	0·99
B	0·63	0·81	4·5	1·28	1·67	9·3	2·65
C	0·57	1·69	9·4	2·97	2·15	11·9	3·78
D	0·48	2·38	13·1	4·98	0·52	2·8	1·08
Total	15·33	17·92	100	1·17	17·97	100	1·19

The recovery of protein, total 5-HT (fluorimetric assay), and radioactive 5-HT each calculated with respect to homogenate was 110, 84 and 98 per cent respectively.

then separated from the remaining platelet rich plasma washed once, homogenized, and applied to sucrose density gradients for fractionation. Aliquots of the major gradient zones A, B, C and D were taken for analysis of total and absorbed 5-HT. The results of this experiment are presented in Table 6 and it can be seen that although the injection dose of 5-HT was quite high, (15 mg) and had been estimated to produce *in vivo* an initial plasma concentration approximately equivalent to the concentration of 5-HT to which rabbit platelets were exposed to in the previous *in vitro* experiment, the 5-HT concentration in the cells calculated from the sum of the fractions and expressed per mg protein (1·17 μg/mg protein) was less than one tenth of that recorded for the *in vitro* 5-HT incubation study (13·2 μg/mg protein). This large difference cannot be accounted for by the minor variations in homogenization procedures between the two experiments.

When the two major particle-containing fractions A and C from this experiment were subfractionated into their soluble and particulate components A_s, A_p and C_s, C_p and the absorbed 5-HT determined by radioactive analyses it was found too, that as with the *in vitro* incubation experiment (rabbit D) the absorbed 5-HT found in fraction A was entirely associated with the soluble subfraction A_s and in fraction C the granule zone the distribution of 5-HT between the soluble and particulate components was almost the same in both experiments (Table 5). After *in vivo* reserpinization 32 per cent of the absorbed 5-HT was particulate bound in the granule fraction C compared with 29 per cent in the same subfraction prepared from *in vivo* reserpinized platelets exposed to 5-HT *in vitro*. (Also shown Table 5.) Comparison in the *in vivo* experiment (Table 6) of the distribution of the total 5-HT content (endogenous and absorbed) and the absorbed 5-HT (calculated from ^{14}C assays) between the major gradient zones A, B, C and D reveals that there is no significant difference between the total 5-HT and absorbed 5-HT for the whole gradient (17·97 μg and 17·92 μg, respectively) calculated from the sum of all the fractions. The values for total and absorbed 5-HT for fraction A were 13·05 and 13·62 μg respectively. In comparing the much lower levels of total and absorbed 5-HT in the fractions B and C these gave values for the absorbed component unaccountably slightly higher than the total 5-HT content of these fractions. It will be seen however (Table 6) that the overall recovery of 5-HT from these gradients, calculated with respect to homogenate in each case, is different for the two analytical approaches (84 per cent

for the total 5-HT by spectrofluorimetric analysis and 98 per cent for the absorbed component calculated from the radioactive assays). It is considered therefore that some differential recovery differences between the low 5-HT containing fractions in the two procedures may account for these discrepancies.

DISCUSSION

Pig platelets appear to respond to reserpine *in vitro* at 37° in a manner similar to platelets from other species, releasing on exposure to the agent 40–50 per cent of their 5-HT content into the surrounding media. In the present experiments two different concentrations of reserpine have been used for the *in vitro* experiments (0·2 μg/ml and 1·0 μg/ml). At the former concentration about 40 per cent of the total cell 5-HT content was released in 75 min compared with about 50 per cent at 90 min with the higher concentration of the alkaloid. These findings relate fairly well to those of Paasonen[25] who used rabbit platelets exposed to 2·5 μg/ml reserpine and showed 22 per cent loss after 30 min and 56 per cent loss after 120 min. The rate of loss of 5-HT by pig platelets was very similar at both reserpine concentrations.

The reserpine-induced liberation of 5-HT from pig platelets *in vitro* is accompanied by a concomitant increase in 5-HT concentration in the surrounding medium. There appears to be no significant metabolic degradation of the amine. Pig platelets readily absorb 5-HT from the surrounding media when incubated at 37° with the amine, reaching a cell concentration about twice that of the preincubation control level in 40 min. However, the effect of reserpine added to the medium at a concentration of 1 μg/ml was to completely block this uptake. Using subcellular fractions prepared by sucrose density gradient centrifugation, the distribution of platelet endogenous 5-HT before and after incubation with reserpine has been compared. At a concentration of reserpine of 0·2 μg/ml and 1 μg/ml and exposure for 60 min at 37°, fraction C, the granule fraction contained the organelles most affected by reserpine treatment. No 5-HT could be detected in this fraction after reserpine exposure at the two concentrations. Even a preliminary exposure of the platelets to 5-HT (2 μg/ml for 60 min) before reserpine was added, resulted in the same finding; no 5-HT was detected in the granular fractions. The 5-HT content of fraction A (containing the platelet membranes) was not significantly affected by reserpine exposure. In a study of the distribution of intracellular reserpine between the subcellular fractions prepared from whole platelets previously exposed to reserpine (0·2 μg/ml and 1·0 μg/ml for 60 min at 37°) *in vitro*. Fraction A, the membrane containing zone, and fraction C containing the granular organelles were further subfractionated into soluble and particulate components by high speed centrifugation. A proportion of the cell reserpine content was always found to be tightly bound to the particulate elements of both these fractions although the per cent distribution varied considerably. The particulate material of both fractions showed an almost linear increase in bound reserpine when the isolated fractions were exposed to the alkaloid over the range 1–4 μg/ml. However, although low levels of reserpine (< 1 μg/ml) induce significant release of the 5-HT from the whole cell reflected in losses from both the granule and the soluble phase compartments, release of the amine from the isolated granules was only significant at the 15 μg/ml level. These findings would suggest that during exposure of platelets to reserpine the alkaloid readily enters the cytoplasmic compartment of the cell affecting active transport across the cell surface membrane. However,

for release of amine from intracellular storage granules a much higher local concentration of reserpine must be reached before an effect upon the limiting membrane of the 5-HT storage granule takes place. In their studies of neuronal amine storage, Berti and Shore[26] suggested that reserpine interfered with the very specific amine concentrating mechanism of the intraneuronal storage granule but did not affect the non-specific uptake mechanism which they believed was linked to an ATPase enzyme. It would appear that in the platelet, the alkaloid may act at both the surface and granule membranes but that the sensitivity of these towards reserpine is quite different. It may also of course be necessary for a shift in the equilibrium existing between the cytoplasmic store and granule-stored amine to take place before granule release can be affected. The findings presented here do not conflict with the views of Hughes *et al.*[10] that the slow release of 5-HT from platelets reflects a blockage of 5-HT transport into the cell whilst still allowing normal passive diffusion out, with the net result being a simulated release process. In the studies using rabbits bled before and at intervals after reserpine injection, a significant decrease in circulating platelet 5-HT was detectable after a single injection (1 μg). After a second injection 3 days later, the platelets of all these rabbits were almost completely depleted of 5-HT. Serial assays of urinary 5-hydroxyindole acetic acid revealed a significant though only transient increase in output of the acid metabolite on the third day following the first injection of reserpine. Incubation of 5-HT-depleted rabbit platelets, collected after two injections of reserpine, with 5-HT *in vitro*, indicated that their capacity for absorbing the amine was not significantly impaired. Most of the absorbed ^{14}C serotonin was located within the soluble phase of the membrane fraction (*ca.* 80 per cent) although each subcellular fraction showed a significant increase above the endogenous level calculated by subtracting the radioactively labelled absorbed component from the total values estimated fluorimetrically.

The findings that rabbit platelets differ from pig platelets in that they will still take up small quantities of 5-HT *in vivo* and *in vitro* after *in vivo* reserpine action is particularly interesting, since the platelet surface membrane of both species displays a sensitivity towards reserpine in the release of amine. At the very high 5-HT concentrations encountered in rabbit platelets both the cytoplasmic and granule stored compartments would appear to be at saturation. An investigation of the character of the reserpine receptor protein in these two membranes may reveal some significant differences. Both the membrane and granule fractions of the cell tightly bind reserpine but the heterogeneity of the fractions isolated in this study does not allow firm conclusions to be made about the respective binding affinities of the surface and 5-HT granule membrane for the alkaloid.

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