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STUDIES ON THE UPTAKE OF 5-HYDROXYTRYPTAMINE BY BLOOD PLATELETS

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The work to be described in this paper extends earlier work (Born, Ingram & Stacey, 1958; Born, Hornykiewicz & Stafford, 1958) which suggested that a study of the mechanism which allows platelets to accumulate 5-hydroxytryptamine (5-HT) and adrenaline might throw light on the nature of 'receptors' with which pharmacologically active amines are thought to react in living cells.

When freshly isolated from blood, mammalian platelets contain 5-HT (Rand & Reid, 1951; Zucker & Rapport, 1954; Zucker, Friedman & Rapport, 1954). When plasma containing platelets ('platelet-rich plasma') is incubated at 37° C the platelets take up added 5-HT, even when the concentration of 5-HT in the platelets greatly exceeds that in the plasma (Humphrey & Toh, 1954; Hardisty & Stacey, 1955; Zucker & Borrelli, 1956). Platelets retain their 5-HT even in plasma containing none (Udenfriend & Weissbach, 1954). On the basis of these observations it has become customary to say that 5-HT is 'bound' to platelets, but so far nothing is known of what this binding means in chemical terms.

Previous work showed that the 5-HT content of platelets as well as their capacity to take up 5-HT varies greatly (Hardisty & Stacey, 1955). This variation was correlated with a variation in the adenosine triphosphate (ATP) content of the platelets (Born, Ingram & Stacey, 1958). We now show that the amount of 5-HT which platelets take up depends also on the experimental conditions, and that the simple methods described in the literature are inadequate for determining the uptake capacity of platelets.

This paper is, therefore, divided into two parts: The first part describes experiments done to establish a method which enables platelets to accumulate at least as much 5-HT as the largest amount found *in vivo*, i.e. in platelets of cases of carcinoid tumour. This part also gives results obtained with this method. The second part describes experiments in which the method was not

required; they concerned the initial rate of uptake and the exchangeability of 5-HT in platelets. Reports of some of this work have appeared in the Proceedings of the Physiological Society (Born & Gillson, 1957, 1958).

METHODS

Preparation of platelet-rich plasma. All glassware with which platelets came into contact was coated with silicone (MS. 1107). Human platelets were used. An arm vein was punctured to obtain blood which flowed through the needle and through a short polythene tube into centrifuge tubes made of glass or of cellulose acetate. Each tube contained 10 ml. blood, which was mixed either with 0·8 ml. 19% (w/v) sodium citrate or with 1·2 ml. 4·5% (w/v) ethylene diamine tetra-acetate (EDTA). The final concentration of citrate was 12·9 mM and of EDTA 3·6 mM. The tubes were cooled to 0° C and centrifuged at 0–1° C and 500 *g* for 20 min.

The supernatant plasma which contained many platelets ('platelet-rich plasma') was sucked off with a capillary pipette. Duplicate samples of 0·1 ml. were mixed with 1·9 ml. of a solution containing 1% (w/v) formaldehyde in 3% (w/v) sodium citrate. The platelets in this diluted suspension were counted in haemocytometer chambers.

Methods used for exposing platelets to 5-HT in vitro. Two methods were used: (1) For experiments lasting only a short time, 5-HT was added to platelet-rich plasma in tubes made of siliconed glass or of cellulose acetate, according to the procedure of Hardisty & Stacey (1955); the tubes were incubated in a water-bath at 37° C. (2) For experiments lasting several hours the cellophane tube method was used; this is described later, together with the reasons for using it. Control experiments showed that 5-HT was not broken down when incubated in platelet-rich human plasma.

Isolation of platelets after incubation. Samples of platelet-rich plasma were transferred into centrifuge tubes at 0° C. Duplicate samples of 0·1 ml. were diluted for platelet counting. The rest of the plasma was centrifuged at 15,000 *g* and 1° C for 5 min. The supernatant plasma was carefully decanted and the inside of the tubes above the platelet sediment was wiped with filter paper to remove any remaining drops of plasma.

Effect of 'washing' platelets on their content of 5-HT. In experiments on uptake 5-HT was added to platelet-rich plasma. After centrifugation the supernatant plasma was removed as completely as possible, but some plasma remained in the platelet pellet. In order to obtain a true value for the amount of 5-HT in the platelets it was necessary either to wash away this plasma or to determine its volume.

According to Weissbach, Bogdanski & Udenfriend (1958), dog platelets can be centrifuged and resuspended repeatedly in isotonic saline without effect on their content of 5-HT as determined on the basis of platelet protein. Stacey (1958) found, however, that when human platelets were 'washed' in this way they always lost 5-HT.

We found that it was impossible to resuspend platelets after centrifugation in such a way that they could be counted; moreover, in relation to the *initial* count, washing brought about a loss of 5-HT. In one experiment platelet-rich plasma was incubated with 1·5 μg 5-HT/ml. for 50 min. After centrifugation the platelet pellet was gently dispersed in ice-cold 0·9% NaCl by means of a polythene rod and the suspension was centrifuged again, whereupon the platelets had lost 10% of their 5-HT. When the washing procedure was repeated two, four and six times the platelets lost 15, 26 and 34% respectively. Whether 5-HT leaked from intact platelets or whether they were progressively destroyed was not determined, but it was clear that platelets should not be washed with saline.

Determination of the volume of plasma in the platelet pellet. Experiments were made, therefore, to determine the volume of plasma which remained in the platelet pellet after centrifugation. This was done by mixing inulin with platelet-rich plasma at 0° C, centrifuging to sediment the platelets and determining inulin (Bacon & Bell, 1948) in the plasma and in the platelet pellet. Similar experiments were done in which human serum albumin labelled with ^{131}I was used instead of inulin. From the results the amount of plasma in the pellet was calculated. The values obtained

in six experiments with inulin were very similar to those obtained in four experiments with albumin: on the average there was 0·45 μl. plasma/10^8 platelets (range 0·27–0·83). With plasma containing 1·5 μg HT/ml. this volume corresponds to 0·7 ng 5-HT. In view of the results presented in Table 1 (see below, p. 479), it is clear that plasma left in the platelet pellet introduced no significant error in the calculation of the concentration of 5-HT in platelets.

5-hydroxytryptamine was used in two forms, non-radioactive and radioactive. The former was obtained from Roche Products, Ltd. 5-HT labelled with ^{14}C (5-hydroxy-3-indolyl-(ethyl-2-amine-1-^{14}C) creatinine sulphate monohydrate) was supplied by the Radiochemical Centre at Amersham and had a specific activity of 6·87 mc/m-mole.

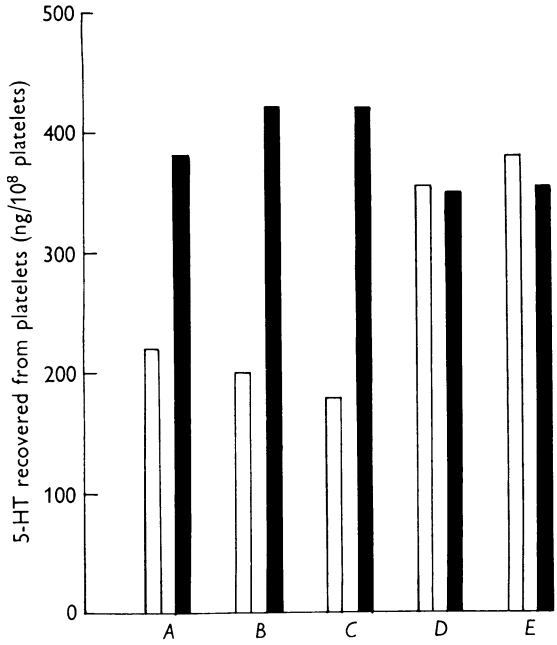

Fig. 1. Extraction of 5-HT from platelets using different procedures. Platelet-rich plasma was incubated at 37° C for 60 min with added radioactive 5-HT (1·5 μg/ml.). Platelets were separated by centrifugation and extracted as follows (for details, see text): *A*, with 90% acetone which was evaporated without a stream of air; *B*, *C*, with 90% acetone which was evaporated with the aid of a stream of air; *D*, *E*, with distilled water only. Open columns represent results of bioassay, solid columns those of ^{14}C counting.

Extraction and recovery of 5-HT. At first we used the method of Hardisty & Stacey (1955). The sedimented platelets were dispersed in 1 ml. water by means of a glass homogenizer (Potter & Elvehjem, 1936), the bulb of which fitted closely into the bottom of the centrifuge tubes. The suspension was transferred into glass-stoppered tubes, shaken with 9 vol. of acetone and kept at −10° C during the night. The tubes were centrifuged and the clear supernatant solutions were evaporated to dryness in round-bottomed flasks at 33–35° C *in vacuo*.

5-HT is completely extracted by 90% acetone. This is shown by the following experiment. Platelet-rich plasma was incubated with radioactive 5-HT until 88% was taken up by the platelets; when platelets and plasma were extracted with 90% acetone the total counts of ^{14}C recovered were equal to those added. On the other hand, the evaporation of the acetone extract brought about the break-down of a variable proportion of 5-HT. This was shown by adding radioactive

5-HT to platelets, extracting it as described and determining it by bioassay as well as by counting ^{14}C. Fig. 1 shows that, as measured by bioassay, only about half of the 5-HT was recovered, whether or not air passed through the extract during evaporation.

The figure shows that better results were obtained when acetone was not used. The platelets were merely dispersed in distilled water; the suspension was frozen, thawed and centrifuged to remove insoluble material. Samples of the supernatant solution were dried on planchettes for counting ^{14}C; other samples were used for bioassay after dilution with saline. Although only 80–90% was recovered, no 5-HT was broken down, since both methods of assay gave the same result.

Some of the earlier experiments were made before it was realized how much 5-HT was broken down during the evaporation of acetone extracts. All results were confirmed later by the method which avoided such break-down and by the use of radioactive 5-HT.

Determination of 5-HT

Non-radioactive 5-HT was determined by bioassay. At first we used the isolated uterus of the rat in oestrus (Gaddum, Peart & Vogt, 1949). The bath had a volume of 2·0 ml., otherwise the conditions were those used by Hardisty & Stacey (1955). Later, we used a strip of isolated stomach of the rat, as described by Vane (1957).

Radioactive 5-HT was determined on planchettes at infinite thinness in a helium gas flow counter which had an apparent counting efficiency of about 50%.

5-HT was added as the creatinine sulphate. The results in this paper are expressed in terms of the free base.

RESULTS

PART I. Development of method for measuring the capacity of platelets to take up 5-HT

The platelets of normal people contain 25–60 ng 5-HT/10^8 platelets (Hardisty & Stacey, 1955; see also Table 1 of this paper). The highest concentrations of 5-HT in human platelets occur in cases of carcinoid tumour, where values ranging from 80 to 340 ng/10^8 platelets have been reported, with an average of about 200 (Snow, Lennard-Jones, Curzon & Stacey, 1955; and unpublished results). Hardisty & Stacey (1955) got platelets to take up 5-HT by merely adding it to platelet-rich plasma which was incubated at 37° C in glass tubes pre-treated with silicone. When uptake stopped after 75–90 min the platelets contained from 127 to 355 ng/10^8 platelets with a mean of 221 ng (10 experiments). These values were similar to those in platelets of carcinoid tumours. The first question to be answered was, therefore, whether these concentrations represented the limit of the platelets' capacity to take up 5-HT, or whether this limit was imposed by experimental conditions.

Changes in platelet-rich plasma during incubation

When we began to use the method of Hardisty & Stacey we found that during incubation the pH of the plasma increased considerably; in four experiments the increase in 90 min was from 7·4–7·6 to 8·1–8·4. It was conceivable that such a change in the plasma might modify the uptake of 5-HT by platelets. Attempts were therefore made to maintain the pH more constant. In one experiment plasma containing 5-HT 1·5 μg/ml. was divided into two samples. One sample was incubated at 37° C for 90 min in an open glass tube;

in that time the pH changed from 7·5 to 8·4 and the concentration of 5-HT in the platelets rose from 20 to 75 ng/10^8 platelets. The other sample was incubated in the same way, except that a mixture of 95% O_2 and 5% CO_2 was bubbled through it. This reduced the rise in pH to 7·8, and brought about an increase in the uptake of 5-HT to 110 ng/10^8 platelets; but it had the disadvantage that it made the plasma froth. After various trials the following method was devised to maintain the pH of platelet-rich plasma constant and to provide the platelets as nearly as possible with physiological conditions for several hours.

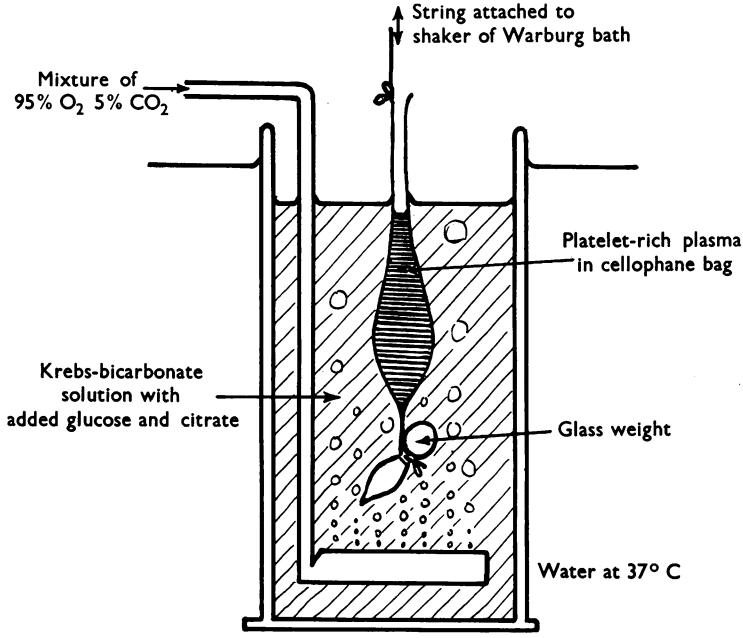

Fig. 2. Cellophane tube method for measuring the capacity of platelets to take up 5-HT; for description see text.

The cellophane tube method

This method is shown in Fig. 2. Platelet-rich plasma was introduced into a tube made of cellophane, as used for dialysis. The upper end of the tube was left open for sampling. The lower end was tied and a glass weight (*ca.* 10 g) was attached to it. The tube was immersed to just below the open upper end in Krebs's bicarbonate solution which was contained in a 250 ml. measuring cylinder. The cylinder stood in a water-bath at 37° C. The Krebs's solution contained anticoagulant at the same concentration as the plasma, and 0·2% (w/v) glucose; it was continuously gassed with a mixture of 95% O_2 + 5% CO_2. The cellophane tube was moved mechanically up and down in the Krebs's solution about once a second. This movement produced agitation in the

plasma and in the Krebs's solution and thereby facilitated the exchange of small molecules through the cellophane membrane. The volume of plasma was always less than 25 ml., so that the volume of the Krebs's solution exceeded that of the plasma by at least ten to one. 5-HT was added to the Krebs's solution only, or to the Krebs's solution and to the plasma in equal concentration; other substances to be tested were added in the same way.

The advantages of this method were as follows:

(1) 5-HT and other substances reached the platelets by diffusing through the cellophane membrane from the Krebs's solution into the plasma. A substance added to the solution was therefore present in such large amounts that if any was inactivated or taken up by platelets during the period of incubation its concentration was not significantly reduced.

(2) The platelets were left in their own plasma so that they remained in contact with approximately the same concentration of plasma proteins as in the circulating blood.

(3) Metabolites were able to diffuse away from platelets out of the plasma; glucose and oxygen were able to diffuse into the platelet-rich plasma.

The following control experiments were made with the cellophane tube method.

Effect on the platelet count. In fifteen experiments platelet-rich plasma from six different subjects was incubated for up to 22 hr; before incubating through the night penicillin and streptomycin were added to the Krebs's solution, each at a concentration of 0·2 mg/ml., to prevent the growth of micro-organisms. At different times the volume of the plasma and the platelet count were determined. During the first hour the plasma volume increased by about 5%; after that time it remained constant. Fig. 3 shows that, when corrected for this change in volume, the platelet count decreased progressively until about 50% were left after 22 hr. As time went on an increasing proportion of platelets became microscopically less refractile and more difficult to see.

Stability of 5-HT. 5-HT was not inactivated during incubation for up to 6 hr.

Rate of movement of 5-HT through the cellophane membrane. When only the Krebs's solution contained 5-HT the concentration in the plasma increased progressively for 90–120 min, when the concentrations reached the same level inside and outside the cellophane tube.

Effect on rate of uptake. The slowness with which 5-HT moved through the cellophane membrane made it likely that when 5-HT was added only to the solution outside the cellophane tube the rise in concentration inside might be so slow as to delay the uptake of 5-HT by platelets. This delay was demonstrated in two experiments. 5-HT (1·5 μg/ml.) was added in one cylinder to the Krebs's solution only and, in another, to the plasma as well at the same concentration, and the rates at which the platelets took up 5-HT were compared. When

5-HT was added to the plasma at the beginning the rate of uptake in the first 30 min was faster by about 30%.

Increased uptake with the cellophane tube method

When platelet-rich plasma was incubated by the cellophane tube technique the platelets took up several times as much 5-HT as they did in plasma incubated in glass tubes. This is shown in the following experiment. One sample of platelet-rich plasma containing 5-HT 1·5 μg/ml. was incubated at 37° C in an

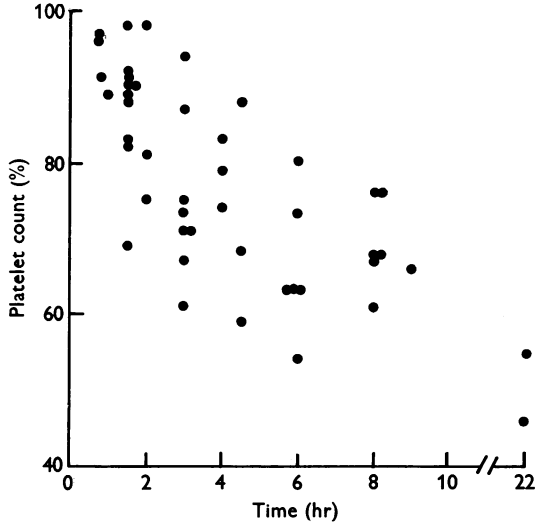

Fig. 3. Decrease in the platelet count of platelet-rich plasma incubated by the cellophane tube method. Ordinate, platelet count as percentage of the initial count; abscissa, time.

open glass tube. Two other samples which did not contain 5-HT were incubated by the cellophane tube method; the Krebs's solutions contained 5-HT 1·5 μg/ml. but only one of them was gassed.

Figure 4 shows the amounts of 5-HT taken up by platelets after 90 min, and also the change in pH of the plasma samples. With the cellophane tube method the uptake was much greater than with the glass tube method and the pH increased less. Furthermore, gassing the Krebs's solution increased the uptake considerably, although it had only a small effect on the pH. This suggests that the increased uptake was brought about not only by keeping the pH of the plasma more constant but also by providing the platelets with an adequate supply of oxygen.

Table 1 summarizes experiments in which the uptake of 5-HT by platelets was determined both by the glass tube method and by the cellophane tube method. The concentration of 5-HT in the medium was 1·5 μg/ml. The platelets ceased taking up 5-HT after periods of time ranging from 90 to 180 min. After incubation for 90 min in glass tubes the platelets contained a mean of

140 ng/10^8 platelets; after the same time in cellophane tubes they contained a mean of 350 ng/10^8 platelets, i.e. 2·5 times more. After 180 min in cellophane tubes the content rose to an average of 460 ng/10^8 platelets, and in one experiment to nearly 1000.

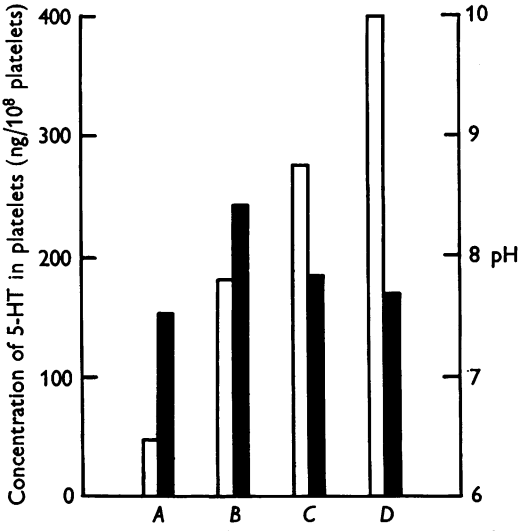

Fig. 4. Uptake of 5-HT by platelets under different conditions. Open columns indicate the concentration of 5-HT in platelets (ng/10^8 platelets). Solid columns indicate the pH of the plasma from which the platelets were obtained. *A*, values found with platelet-rich plasma before incubation; *B*, values found after the plasma containing 1·5 μg 5-HT/ml. had been incubated at 37° C for 90 min in an open glass tube pre-treated with silicone; *C*, values found after the plasma had been incubated at 37° C for 90 min by the cellophane tube method; the Krebs's solution contained 5-HT 1·5 μg/ml. and no gas was bubbled through it; *D*, values found when the conditions were the same as those for *C*, except that the Krebs's solution was gassed with 95% O_2 + 5% CO_2.

TABLE 1. Uptake of 5-HT by platelets by the glass tube method and by the cellophane tube method. Incubation at 37° C; the plasma contained 5-HT 1·5 μg/ml. and citrate as an coagulant. The results are expressed as ng/10^8 platelets

	Glass tube method 5-HT in platelets		Cellophane tube method 5-HT in platelets		
	Before incubation	After incubation for 90 min	Before incubation	After incubation for 90 min	After incubation for 180 min
Mean	25	140	38	350	460
Range	8–60	40–300	10–90	125–835	180–960
Variation	×7·5	×7·5	×9·0	×6·7	×5·3
No. of expts.	13	13	13	12	9

What determines the maximum amount of 5-HT taken up?

The following experiments were made to investigate some of the causes which might limit the uptake in amount and in time. All these experiments were made with the cellophane tube method.

Loss of 5-HT *from platelets.* It was possible that, when the concentration of 5-HT in platelets reached a certain level, a steady state was set up in which the rate of uptake was balanced by an equal rate of loss of 5-HT from the platelets. In four experiments platelets appeared unable to retain 5-HT which they had taken up, for they contained less after incubation for 6 hr than after incubation for 3 hr. However, in five other experiments the highest concentration of 5-HT reached in the platelets remained unchanged for at least 6 hr. The reason why some populations of platelets behaved differently from others

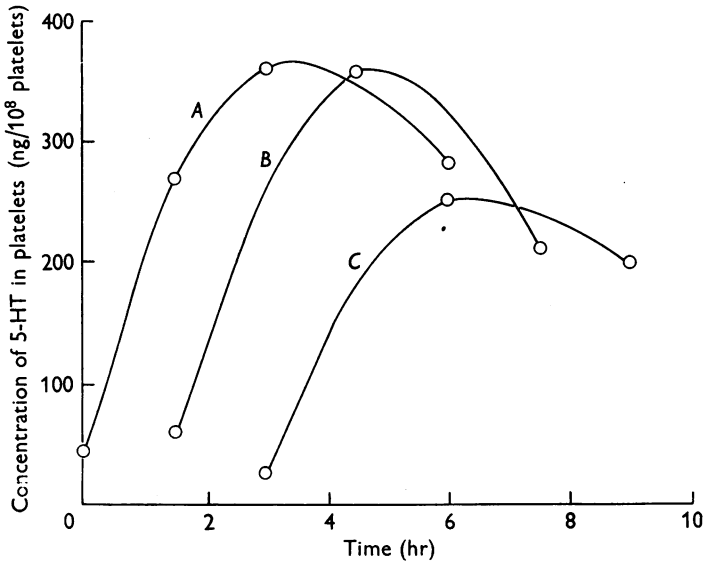

Fig. 5. Uptake of 5-HT by platelets using the cellophane tube method. *A* shows the results obtained when 5-HT (1·5 μg/ml.) was added to the Krebs's solution at the beginning of the incubation; *B*, when 5-HT was added after pre-incubation for 1·5 hr; *C*, when 5-HT was added after pre-incubation for 3 hr.

in this respect was not established, but it is clear that inability to retain 5-HT which has been taken up cannot be the general explanation for the limitation on the uptake.

Deterioration of platelets. Another possibility was that deterioration of the platelets during incubation brought the uptake of 5-HT to an end without affecting their ability to retain 5-HT. That this is not so is shown in Fig. 5. Three cylinders were set up, each containing Krebs's solution and platelet-rich plasma in a cellophane tube. 5-HT 1·5 μg/ml. was added to one solution at the beginning of incubation, to another 90 min later and to the third after 180 min. Platelets from each tube were analysed for 5-HT at different times. In the first cylinder platelets ceased taking up 5-HT after 180 min; yet after incubation for 90 min without 5-HT platelets in the second cylinder accumu-

lated 5-HT when it was added for about the same length of time and to the same extent. Even after preincubation for 180 min the rate of accumulation was only a little slower although the amount taken up was reduced by about one-third. Clearly the ability of platelets to accumulate 5-HT decreased only slowly under the conditions used.

Concentration of 5-HT. Hardisty & Stacey (1955) found that platelets took up maximal amounts of 5-HT when the plasma in which they were suspended contained 5-HT 1·5 μg/ml. We began by using this concentration of 5-HT in our experiments, but it was possible that under the conditions of the cellophane tube technique the concentration of 5-HT in the medium might limit

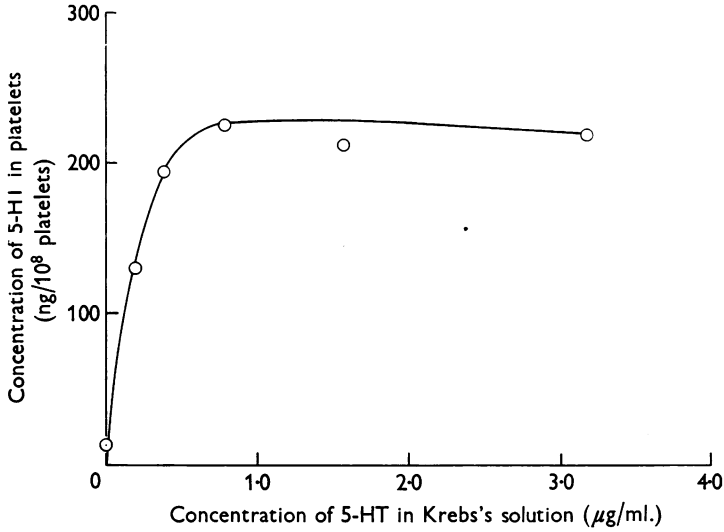

Fig. 6. Relation between concentration of 5-HT added to Krebs's solution and uptake of 5-HT by platelets, using the cellophane tube method; incubation at 37° C for 140 min.

the amount taken up by the platelets. Four experiments were made to test this; a representative result is shown in Fig. 6. In this experiment platelet-rich plasma was incubated for 140 min by the cellophane tube method in several cylinders of Krebs's solution, each containing a different concentration of 5-HT. The amount of 5-HT taken up by platelets increased with increasing concentrations of 5-HT in the medium, up to about 0·6 μg/ml.; higher concentrations brought about no further increase. The concentration in which platelets took up half of the maximum amount was about 0·2 μg/ml. These results show that the amount taken up was not limited by the concentrations of 5-HT which were used.

Effect of anticoagulants. To prevent the plasma from clotting it was found that the Krebs's solution had to contain an anticoagulant at the same concentration as the plasma. Since the presence of so much anticoagulant was

thought to be the most unphysiological element in the conditions under which the platelets were incubated, experiments were made to determine whether the anticoagulant had an effect on the uptake of 5-HT by platelets. Table 2 shows that during incubation for 90 min the platelets took up more than twice as much 5-HT when the anticoagulant was citrate than when it was EDTA. Other anticoagulants were not investigated; it is clear, however, that the substance used as anticoagulant may affect the uptake. Citrate was used in all other experiments.

TABLE 2. The effect of EDTA and of citrate on the uptake of 5-HT by platelets. The concentration of EDTA was 3·6 mM and that of citrate was 12·9 mM. Incubation at 37° C for 90 min. The concentration of 5-HT added to the medium was 1·5 μg/ml. The results are expressed as ng 5-HT/10^8 platelets (mean ± S.E.)

5-HT in platelets	EDTA	Citrate
Before incubation	51 ± 9·55	46 ± 10·8
After incubation	212 ± 27·7	454 ± 85
5-HT taken up by platelets	161 ± 21·9	408 ± 83·5
No. of expts.	7	7

The value of P for the difference in uptake lies between 0·02 and 0·01.

Effects of cyanide and iodoacetate

The cellophane tube method was used to observe the effects of sodium cyanide and sodium mono-iodoacetate on the uptake of 5-HT by platelets, because the effects became demonstrable only after a considerable time. The pH of the inhibitor solutions was adjusted to 7·0–7·3; each substance was added to plasma and to the Krebs's solution at the same concentration. Figure 7 shows that at a concentration of 5×10^{-2} M both substances brought about small reductions in the uptake after 45 min; but after 90 and 180 min cyanide had practically no effect, whereas iodoacetate reduced the uptake more after 90 min and nearly abolished it after 180 min. In this time neither substance brought about any significant alteration in the number or morphology of the platelets. In lower concentrations these substances had no significant effect on the uptake of 5-HT.

PART II. The rate of uptake and the exchangeability of 5-HT in platelets

In the experiments now to be described we measured the initial rate of uptake of 5-HT by platelets, and the rate and extent to which 5-HT in platelets is exchangeable with 5-HT in the medium. In these experiments the cellophane tube method was abandoned to avoid the diffusional delay associated with it; and we measured the rates in which we were interested directly by adding 5-HT to platelet-rich plasma, which was incubated in open centrifuge tubes made of cellulose acetate.

Relation between the concentration of 5-HT in the medium and the initial rate of uptake

The initial rate of uptake of 5-HT by platelets was determined with different concentrations of 5-HT in the plasma. Platelets rapidly clear plasma of 5-HT when the concentration is low. To prevent the rate of uptake being limited by this, few platelets and short periods of incubation were used. In one experiment, platelet-rich plasma was diluted with plasma free from platelets until it contained only $0{\cdot}67 \times 10^8$ platelets/ml. Samples were incubated for 6 min with different concentrations of 5-HT labelled with ^{14}C. The platelets were spun down and analysed for radioactive 5-HT. Under these conditions the platelets took up only 20% of the lowest concentration of 5-HT used, i.e. 0·1 μg/ml.

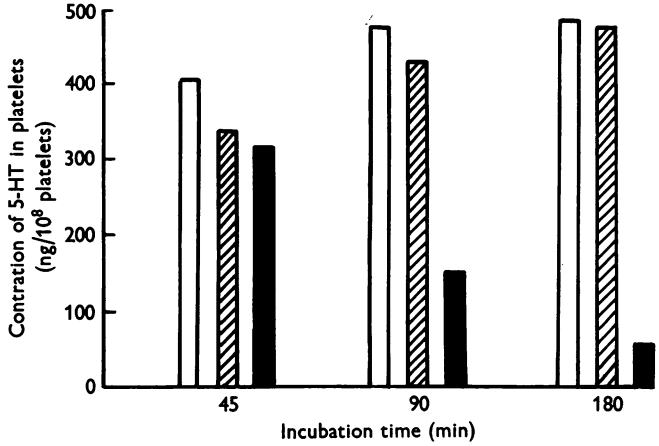

Fig. 7. Uptake of 5-HT by platelets, using the cellophane tube method (open columns); in the presence of 5×10^{-2}M sodium cyanide (shaded columns); and in the presence of 5×10^{-2}M sodium mono-iodoacetate (solid columns).

The concentration of radioactive 5-HT found in platelets after 6 min (C_i) was taken as a measure of the initial rate of uptake. When this was plotted against the concentration of 5-HT in the plasma (C_o) a curve was obtained which followed the Michaelis–Menten equation. This suggested that the rate of uptake was limited by a component of the platelets which became increasingly saturated with 5-HT as the concentration of 5-HT in the medium was raised. By plotting C_o/C_i against C_o according to the method of Lineweaver & Burk (1934), the straight line shown in Fig. 8 was obtained. Using this it was calculated that the concentration of 5-HT in the plasma producing half the maximal rate of uptake was 0·053 μg/ml.

Exchange of 5-HT in platelets with 5-HT in the medium

Using 5-HT labelled with ^{14}C, Udenfriend & Weissbach (1954) showed that in living animals the same molecules of 5-HT remain in a platelet for the whole

of the time during which the platelet exists in the circulation. This indicates that *in vivo* 5-HT in platelets does not exchange, either because it is 'bound' so firmly that it is unable to do so or because the concentration of 5-HT in plasma is so low that an exchange cannot be demonstrated.

The following experiment was made to decide between these alternatives. Two samples of platelet-rich plasma were incubated at 37° C, one without and the other with non-radioactive 5-HT 5 μg/ml. After 90 min radioactive 5-HT

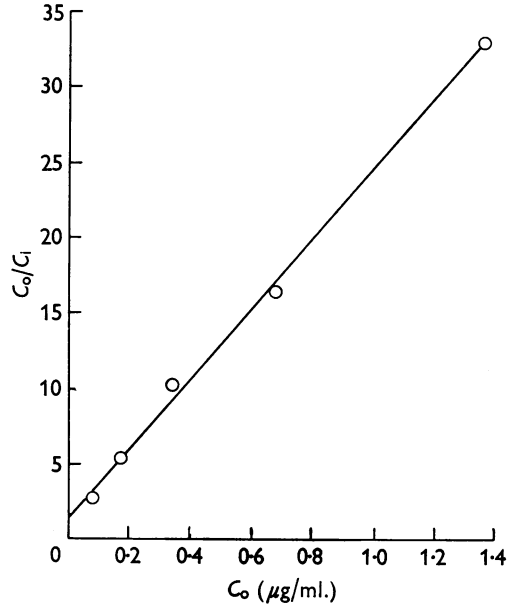

Fig. 8. Relation between the concentration of 5-HT in platelet-rich plasma (C_o) and the initial rate of uptake of 5-HT by platelets. The initial rate of uptake is taken as the increase in the concentration of 5-HT found in the platelets (C_i) after incubation at 37° C for 6 min. In the figure, C_o is plotted against C_o/C_i.

5 μg/ml. was added to both samples and incubation was continued. At different intervals samples of platelets were analysed for total and radioactive 5-HT. The results are shown in Fig. 9.

When platelets saturated with non-radioactive 5-HT were exposed to radioactive 5-HT they became increasingly radioactive, although their content of 5-HT remained constant. This showed that 5-HT in platelets was capable of exchanging with 5-HT in the medium. The rate of exchange was a little slower than the initial rate of uptake; but platelets pre-incubated without 5-HT took up amine added after 90 min as rapidly as at the beginning. Hence the slower rate of exchange was not due to deterioration of the platelets, unless the deterioration was brought about by the presence of 5-HT itself. In another experiment of this kind the rate of exchange was almost

exactly the same as the rate of uptake at the beginning. In both experiments the specific activity of 5-HT in the platelets approached that of 5-HT in the plasma after about 2 hr, showing that all 5-HT in platelets was exchangeable.

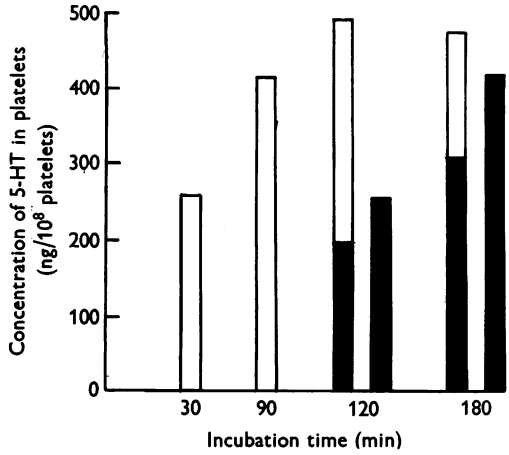

Fig. 9. Exchange of non-radioactive for radioactive 5-HT in platelets. Two samples of platelet-rich plasma were incubated at 37° C, one without and the other with non-radioactive 5-HT 5 μg/ml. After 90 min, radioactive 5-HT 5 μg/ml. was added to both samples and incubation was continued. Platelets separated by centrifugation were analysed for radioactive 5-HT by ^{14}C counting and for total 5-HT by bioassay. Open columns indicate results of bioassay, solid columns those of ^{14}C counting. Time of taking platelet samples is shown below the columns.

DISCUSSION

Comparison of uptake of 5-HT in vivo and in vitro

Platelets obtained from the blood of normal people contain only about 50 ng 5-HT/10^8 platelets; this is much less than the amounts of 5-HT which these platelets accumulated *in vitro*. It seems, therefore, that *in vivo* platelets never accumulate as much 5-HT as it is possible for them to do. One reason for this may be that the rate of uptake of 5-HT by platelets depends on the concentration of 5-HT in the plasma in which they are suspended; with low concentrations the rate is very low.

In vitro the maximum rate of uptake is reached when the concentration of 5-HT in the medium is greater than about 0·5 μg/ml. It seems that in the living animal platelets take up 5-HT as they pass through the capillaries of the intestine (Toh, 1954; Bülbring & Lin, 1958). In man the concentration of 5-HT in these capillaries is unknown; in the dog the plasma of the portal blood contains at most 0·006 μg/ml. (Toh, 1954). *In vivo*, therefore, platelets probably never come across concentrations of 5-HT approaching those which bring about maximal rates of accumulation *in vitro*. There is no reason to suppose that the relation between the concentration of 5-HT in plasma and

the rate of its uptake by platelets is different *in vivo* from what it is *in vitro*; and observations on rats made by Erspamer (1956) suggest that the uptake of 5-HT by platelets is just as slow *in vivo* as it is *in vitro*. Human platelets survive for about 8–9 days in the circulation (Leeksma & Cohen, 1955) and the fraction of this time which they spend in the intestinal capillaries may not be long enough for them to become saturated with 5-HT.

Slowness of the uptake of 5-HT by platelets

Even at its maximum rate the uptake of 5-HT by platelets is very slow when compared with the rate at which inorganic cations or amino acids are accumulated by other mammalian cells under comparable conditions. For example, the time taken for platelets to become half saturated with 5-HT was 30–60 min; the corresponding time for the uptake of glycine by Ehrlich ascites tumour of the mouse is less than 3 min (Heinz, 1954). To calculate the rate of uptake of 5-HT in absolute terms, it may be assumed that the shape of the normal platelet is approximately that of a thin disk with a radius of 1·5–2μ and a volume of about 10μ^3 (Bessis, 1956). Then the maximum rate of influx of 5-HT at 37° C works out at about 0·04 pmole/sec. cm^2 platelet surface. This value may be compared with the following fluxes at 37° C (in pmole/sec. cm^2 cell surface): K influx in human platelets, 0·68–1·13 (own unpublished results); K influx in erythrocytes, about 0·5 (calculated from Maizels, 1954) and glycine influx into ascites tumour, about 16 (calculated from Heinz, 1954).

The mechanism of the uptake of 5-HT

Hardisty & Stacey (1955) showed that the rate of uptake depends markedly upon the temperature and that the temperature coefficient between 25 and 37° C is higher than that for free diffusion. Our experiments on the rates of uptake of 5-HT by platelets showed that the initial rate is related to the concentration of 5-HT in plasma by the Michaelis-Menten equation. This suggests that 5-HT is taken up in combination with a constituent of the platelets which becomes increasingly saturated with 5-HT as the concentration in the medium is increased.

The observations indicate that 5-HT does not enter platelets by simple diffusion but in combination with a constituent of the platelets acting as a 'carrier'. This transport presumably needs energy supplied by the metabolism of the platelets, since it is inhibited by cyanide and by iodoacetate in concentrations which do not affect the morphological integrity of the platelets.

Reason for measuring uptake with the cellophane tube method

We measured the initial rates at which platelets took up 5-HT simply by adding 5-HT to plasma in which the platelets were suspended. These experiments did not last long enough for the 5-HT concentration in the plasma to be

reduced significantly or for the condition of the plasma to alter noticeably. In experiments made to determine the *maximum* amount of 5-HT which platelets were capable of taking up they had to be incubated for several hours. We found that previous methods used to study this problem resulted in platelets taking up less 5-HT than they could. The capacity of platelets to take up 5-HT is limited in some way; what this is can only be discovered when the experimental conditions do not themselves limit the uptake. We therefore developed the new method which has been described. Before discussing the results obtained with this method it will be useful to compare it with older methods.

Comparison of methods used for measuring uptake in vitro

Zucker & Borrelli (1956) and Weissbach *et al.* (1958) centrifuged platelet-rich plasma and resuspended the sedimented platelets in saline solutions which were then incubated with added 5-HT. Zucker & Borrelli found that only four out of nine samples of dog platelets and no human platelets treated in this way took up 5-HT. From Fig. 1 and Table 5 in the paper of Weissbach *et al.* (1958) one can calculate that the concentration of 5-HT in the platelets rose to only about twice that of the solution in which they were suspended. However, the concentration in the solution was so high that the calculation gives no indication of the ability of the platelets to concentrate 5-HT. In agreement with Stacey (1958), we found that it was impossible to resuspend platelets evenly in saline after they had been centrifuged, and that washing platelets in saline either destroyed them or reduced their ability to retain 5-HT.

Another method was used by Hardisty & Stacey (1955) and by Zucker & Borrelli (1956). Blood was centrifuged to sediment the red cells and the platelets were left suspended in plasma. When the plasma was incubated at 37° C and 5-HT was added it was taken up by the platelets. However, plasma exposed to the air during incubation becomes increasingly alkaline, presumably because it loses CO_2.

The cellophane tube method described in this paper was designed to maintain platelets in plasma under more constant conditions. This applied only in so far as the composition of Krebs's bicarbonate solution resembles human plasma. Clearly, substances present in plasma but not present in Krebs's solution and able to diffuse through cellophane were greatly diluted in the plasma. Whether this affected the survival or the behaviour of platelets was not investigated. The cellophane tube method permitted the continuous access of oxygen and glucose to the platelets and the continuous dilution of waste products; it maintained the pH of the plasma constant for periods of at least 6 hr; and the platelets remained in contact with almost the same concentration of plasma proteins as in the blood.

A grossly unphysiological condition was the presence of an anticoagulant in

high concentration. That the kind of substance used may have an effect on platelets was shown by the observation that in the presence of citrate platelets took up more than twice as much 5-HT as they did in the presence of EDTA. The reason for this difference was not discovered. In the presence of citrate the maximum uptake of 5-HT was, on the average, 2·6 times greater with the cellophane tube method than with the older method (Hardisty & Stacey, 1955) and the concentration gradient against which platelets accumulated 5-HT was also several times greater.

Comparison of uptake capacity in vivo and in vitro

The following considerations suggest that under the conditions of our experiments platelets accumulated 5-HT to the limit of their capacity. *In vivo* the highest concentrations of 5-HT in human platelets are found in patients with carcinoid tumours, i.e. up to 340 ng/10^8 platelets (Snow *et al.* 1955). *In vitro*, with our method, platelets accumulated up to 1050 ng/10^8 platelets. Furthermore, results obtained by Weissbach *et al.* (1958) show that when large amounts of 5-HT are infused into a dog the highest concentration found in circulating platelets is about 1200 ng/10^8 platelets; this is about the same as the highest concentration found in human platelets *in vitro*.

Variability in the capacity of platelets to take up 5-HT

Platelets freshly isolated from healthy people differ greatly in their content of 5-HT; some samples contain up to nine times more than others. The maximum amount of 5-HT which different samples of platelets can take up also varies, the range being about fivefold. Although with the cellophane tube method platelets took up, in the mean, 2·5 times more 5-HT than with the older method, the variation in maximum uptake was similar. This suggests that the cellophane tube method allowed the platelets to make better use of their uptake capacity but that the capacity varied in different samples of platelets.

Uptake capacity

What determines the amount of 5-HT that platelets can take up? One possibility is that as platelets accumulate 5-HT the amine leaks back into the plasma at an increasing rate, until a steady state is established in which the rate of this diffusion down the gradient of concentration is equal to the rate of uptake. Steady states of this kind determine, for example, the intracellular accumulation of certain amino acids (Heinz, 1957) and of the potassium ion (Hodgkin, 1951). Such an explanation presupposes that at least a part of the 5-HT in platelets is free in solution. We do not know whether this is so. The following considerations suggest, however, that the amount of 5-HT which platelets can take up is not determined solely by such a simple dynamic equilibrium.

Amino acids, such as glycine, and potassium ions are accumulated by mammalian cells until the intracellular concentration is about twenty times greater than the extracellular concentration. 5-HT is accumulated by platelets against concentration gradients of up to a thousand to one. Furthermore, the same molecules of 5-HT are retained by platelets as long as they survive in circulating plasma (Udenfriend & Weissbach, 1954) which does not contain 5-HT in measurable concentrations. *In vitro*, too, platelets can retain for at least 6 hr all the 5-HT which they have taken up, and they are able to clear plasma completely of 5-HT.

These observations make it unlikely that the uptake capacity is limited by the rate at which 5-HT leaks out of platelets. It seems more likely that the capacity depends on the concentration of something in platelets which has a high affinity for 5-HT and to which 5-HT attaches itself after it has moved into the platelets. The suggestion has been made that the uptake capacity depends upon the concentration of ATP in platelets and that 5-HT might form a chemical complex with ATP (Born, 1958*b*), similar to the complex which the catechol amines are believed to form with ATP in intracellular granules of the adrenal medulla (Blaschko, Born, D'Iorio & Eade, 1956; Hillarp, 1958). This suggestion was based on the observations that platelets contain extraordinarily high concentrations of ATP (Born, 1956); that the amount of 5-HT which different samples of platelets are able to take up is proportional to the amount of ATP which they contain (Born, Ingram & Stacey, 1958); and that, under the best conditions so far devised, platelets can be made to contain 1–2 molecules of 5-HT for each molecule of ATP (Born & Gillson, 1957). Moreover, platelets present in plasma during clotting rapidly release 5-HT into the serum and at the same time the greater part of their ATP disappears (Born, 1958*a*).

These results suggest that platelets contain a cationic exchange system with a high affinity for 5-HT, and that the negative charges of the system are provided by ATP. The intracellular association of 5-HT with a cation exchange system is also compatible with the observation that 5-HT in platelets can exchange completely with 5-HT in the plasma in which they are suspended.

The accumulation of 5-HT on a cation exchange system should be accompanied by the displacement from platelets of an equivalent amount of one or more other cations. Work with cells capable of accumulating dibasic amino acids has suggested that K might be displaced in this way (Christensen, Riggs, Fischer & Palatine, 1952). The concentration of K in platelets was determined before and after they had accumulated the maximum amount of 5-HT, but no significant difference was found. Calculation showed that no more than 3–4% of platelet K could be expected to be displaced. Since this was also the size of the experimental error the possibility that K is displaced from platelets when 5-HT is taken up was not excluded.

A working hypothesis which explains all the results so far obtained is that platelets accumulate 5-HT by an active process and that they retain it in ionic combination with ATP. If the immediate source of energy needed for the active process is also ATP it would provide a link between uptake and retention.

SUMMARY

1. A method is described which allows platelets suspended in plasma to take up 5-hydroxytryptamine *in vitro* against concentration gradients of up to one thousand to one. Platelets can be made to contain two to three times more 5-hydroxytryptamine than the amounts found in the platelets of patients with carcinoid tumours.

2. Platelets take up more 5-hydroxytryptamine when the anticoagulant in the plasma is sodium citrate than when it is sodium ethylene diamine tetraacetate.

3. At 37° C platelets continue to take up 5-hydroxytryptamine from plasma for 90–180 min.

4. The rate of uptake increases with increasing concentration of 5-hydroxytryptamine in the plasma up to concentrations of about 0·5 μg/ml. The relation between the initial rate of uptake and the external concentration of 5-hydroxytryptamine can be described by the Michaelis–Menten equation.

5. 5-Hydroxytryptamine present in platelets can exchange completely with 5-hydroxytryptamine in the plasma in which the platelets are suspended, and the rate of exchange is the same as the rate at which platelets take up 5-hydroxytryptamine.

6. Sodium cyanide and sodium mono-iodoacetate (5×10^{-2} M) decrease the rate of uptake, although neither substance increases the rate at which platelets disintegrate.

7. The result suggest that platelets take up 5-hydroxytryptamine by an active process.

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