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Sodium-dependent accumulation of 5-hydroxytryptamine by rat blood platelets

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1. The influence of sodium and potassium on the accumulation of 5-hydroxytryptamine (5-HT) by rat blood platelets was investigated.

2. An absolute dependence of 5-HT uptake on the sodium concentration in the medium was found.

3. Removal of potassium reduced the uptake by about 60%. High concentrations of potassium inhibited sodium-dependent accumulation.

4. The observations have been discussed in terms of a carrier-mediated transport process for 5-HT operating in the platelet membrane.

Blood platelets *in vitro* accumulate 5-hydroxytryptamine (5-HT) against a concentration gradient by a saturable process which can be described in terms of Michaelis-Menten kinetics (Humphrey & Toh, 1954 ; Born & Gillson, 1959 ; Hughes & Brodie, 1959). This accumulation is decreased by metabolic inhibitors (Sano, Kakimoto & Taniquchi, 1958 ; Born & Gillson, 1959 ; Weissbach & Redfield, 1960) and the uptake process shows structural specificity (Stacey, 1961). These findings have led to the hypothesis that the accumulation of 5-HT by blood platelets is an active process involving a hypothetical carrier, probably located in the platelet membrane.

Sodium has been shown to be a requirement for the active transport of non-electrolytes in various cells and tissues (for references see Crane, Forstner & Eichholz, 1965 ; Heinz, 1967), and the presence of sodium is an absolute requirement for the accumulation of noradrenaline by the heart (Iversen & Kravitz, 1966) and synaptosomes (Bogdanski, Tissari & Brodie, 1968). It thus seemed appropriate to investigate the role of sodium in the accumulation of 5-HT by blood platelets.

Methods

Preparation of platelets

Adult male rats of the Porton strain, weighing 300–400 g, were anaesthetized with ether. The chest was opened and blood collected by cardiac puncture into a plastic syringe containing 1·5 ml. of anticoagulant (1% ethylendiamine tetracetate, EDTA, in 0·9% NaCl solution). The blood was further diluted with 0·9% saline, 2 ml. to each 10 ml. of blood to increase the yield of platelets. Erythrocytes and white cells

were separated by centrifugation at 100 *g* for 40 min. The platelet-rich supernatant was removed and the platelets separated by centrifugation at 800 *g* for 30 min. The clear supernatant was decanted and the platelets washed by resuspension in the final incubation medium, and the platelets again precipitated by centrifugation. The washed pellet was stored on ice and suspended in incubation medium immediately before use.

Measurement of 5-HT uptake

The final platelet suspension was pre-warmed in an incubation bath at 37° C. Samples (2 ml.) of the suspension were transferred to pre-warmed plastic centrifuge tubes containing ^{3}H-5-HT (all 5-HT samples were contained in 0·2 ml. of 0·01 N HCl) and incubated by shaking, in air, for the time required. The ^{3}H-5-HT uptake was terminated by the addition of 2 ml. ice-cold incubation medium and immediate separation of the platelets by rapid centrifugation (B.T.L. micro-angle, 8,500 *g* in 80 sec) for 2 min. The supernatant was decanted, the inside of the tube thoroughly dried, and the pellet suspended in 0·5 ml. 0·1 N HCl. A 0·1 ml. sample was taken for liquid scintillation counting.

Protein determination

Total protein in the incubation medium was determined by the method of Lowry, Rosenbrough, Farr & Randall (1951). Choline chloride (see below) interfered with the protein determination, and to overcome this, samples of the incubation suspensions were centrifuged to precipitate the platelets, the supernatant decanted, and the platelet pellet suspended in a volume of water equal to that of the original sample ; aliquots of this were used for protein determination.

Calculation of 5-HT uptake

As rat blood platelets do not appear to contain monoamine oxidase (Paasonen, 1965), it was assumed that radioactivity was a measure of 5-HT accumulation. Uptake has been expressed either as c.p.m./mg protein or as a percentage of the uptake in the controls of individual experiments.

Composition of medium used

The standard incubation medium had the following composition (mM) NaCl, 116 ; KCl, 4·17 ; KH_2PO_4, 1·8 ; $MgSO_4$, 1·18 ; Tris-(hydroxymethyl)aminomethane-HCl (Tris) (pH 7·2), 24·87 ; glucose, 5·9. The Na^+-free medium contained 116 mM choline chloride. Medium containing low Na^+ or high K^+ was obtained by substituting choline chloride or KCl for NaCl to obtain the molarities shown in results.

Materials

^{3}H-5-hydroxytryptamine creatine sulphate (specific activity 300 mc/mole) was obtained from the Radiochemical Centre, Amersham. This preparation was diluted with 0·01 N HCl to the required concentrations for the determination of initial rates of 5-HT uptake. For the determination of 5-HT uptake at longer time intervals (usually 12 min) ^{3}H-5-HT was diluted with non-radioactive 5-HT to give a final concentration of 1 μg 5-HT/ml. incubation medium containing 0·1 μc tritium.

Results

Dependence of 5-HT uptake on sodium concentration

The rate of accumulation of 5-HT by rat blood platelets at 37° C in sodium medium (control) and sodium free medium is shown in Fig. 1a. The uptake was linear for the time over which it was determined and shows marked sodium dependence under these experimental conditions. This effect of sodium was further studied by progressively replacing NaCl by isomolar choline chloride. Figure 1b shows that up to 80% of the NaCl (92·8 mM) could be replaced by choline without markedly diminishing the 5-HT uptake, but further reductions in Na^+ concentration markedly impaired uptake. At 5·8 mM NaCl, the uptake was 48% of that occurring in 116 mM NaCl, and this was reduced to about 10% in the sodium free medium. This small " uptake " in the absence of sodium may represent the " background " of the amount of radioactivity trapped in the extra-cellular space or may mean that some part of the 5-HT uptake is independent of sodium.

These results suggest a direct relationship between the extracellular Na^+ concentration and the transport of 5-HT. Further evidence of a correlation between sodium concentration and 5-HT accumulation was obtained in another series of experiments, the results of which are shown in Fig. 2. The reciprocal of 5-HT uptake has been plotted against the reciprocal of the Na^+ concentration in the medium and the linear relationship obtained suggests that Na^+ and 5-HT are associated with the same transport system, and that one sodium ion functions as a co-

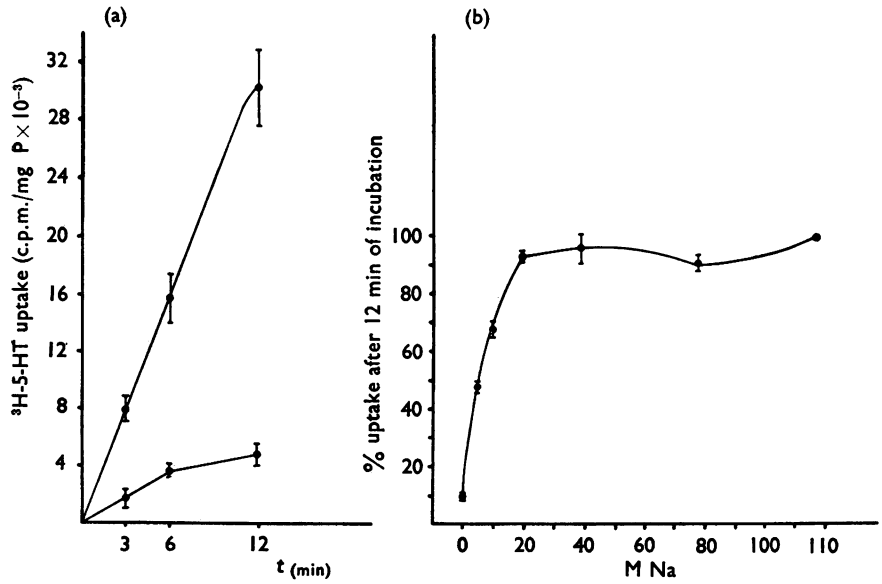

FIG. 1. a, Accumulation of ^{3}H-5-HT in the Na^+ containing medium and medium in which the NaCl has been replaced by isomolar choline chloride. Each point represents the mean ± S.E.M. of four separate experiments. b, Influence of Na^+ concentration on the accumulation of ^{3}H-5-HT. Each point represents the mean ± S.E.M. of the uptake occurring in six separate experiments. Blood platelets were incubated with ^{3}H-5-HT for 12 min. The variation between experiments has been reduced by expressing the uptake in the individual experiments as a percentage of that occurring in control medium.

substrate with one molecule of 5-HT for the carrier process. Similar relationships have been derived for the action of Na^+ on other uptake processes (Vidaver, 1964; Kipnis & Parrish, 1965).

As 5-HT uptake can be described in terms of Michaelis-Menten kinetics, where the *Km* value represents the concentration of 5-HT giving half maximal uptake and

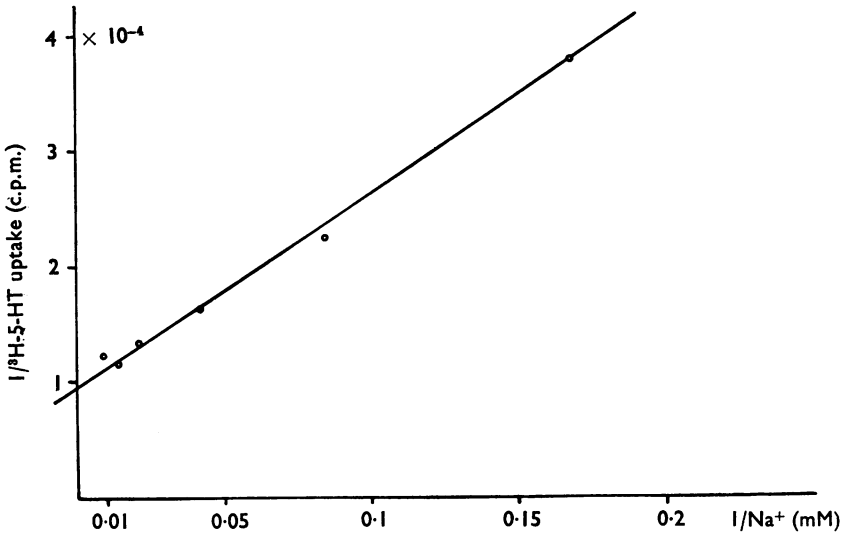

FIG. 2. The relationship between extracellular Na^+ and ^3H-5-HT uptake as shown by a double reciprocal plot of 1/5-HT uptake v $1/Na^+$ concentration. Each point represents the mean of 12 determinations obtained from two separate experiments.

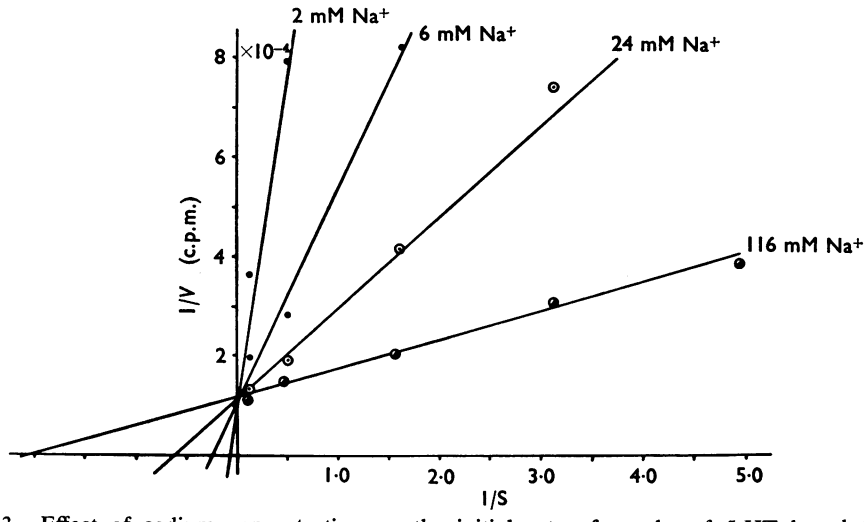

FIG. 3. Effect of sodium concentration on the initial rate of uptake of 5-HT by platelets. Initial velocity (V) is expressed as ^3H-5-HT uptake (c.p.m.) per mg protein after 2 min incubation. Abscissae are reciprocals of 5-HT concentration (μM) in the medium. Low levels of Na^+ were obtained by substituting choline chloride for NaCl in the incubation medium. Each point represents the mean of six experiments and the lines for 116 mM, 24 mM and 6 mM Na^+ were calculated by the method of least squares. The extrapolated *Km* values for 5-HT at the various Na^+ concentrations are given in the results.

V_{max}, is the maximal entry rate determined from the Lineweaver–Burke plot, the effect of different concentrations of Na^+ on the initial rates of 5-HT accumulation have been investigated. Platelet suspensions were prepared in medium containing 116 mM NaCl, 24·0 mM NaCl, and 6·0 mM NaCl, isomolarity being maintained with choline chloride. Initial rates of uptake were determined after 2 min of incubation at 37° C, and the amount of radioactivity in the pellet expressed as c.p.m./mg protein was taken as the velocity, V. Figure 3 shows that when the Na^+ concentration of the medium is lowered towards zero, the extrapolated maximal rate (intercept on the ordinate) remains constant, while the Km increases. (The extrapolated Km values for each Na^+ concentration were found to be:—116 mM NaCl, Km $4{\cdot}8 \times 10^{-7}$M ; 24 mM NaCl, Km $1{\cdot}4 \times 10^{-6}$M ; 6 mM NaCl, Km $4{\cdot}0 \times 10^{-6}$M.)

Effect of K^+

K^+ was not an absolute requirement for 5-HT uptake. The replacement of KCl by choline chloride and KH_2PO_4 by NaH_2PO_4, and the reintroduction of KCl to the incubation medium is shown in Table 1.

Removal of K^+ reduced the uptake by approximately 60% and the addition of even small amounts of KCl restored the uptake. With twice the normal concentration of K^+ (9·48 mM) there was a suggestion that inhibition of uptake may occur at high K^+. In order to investigate this possibility and to obtain as wide a variation in K^+ concentration as possible while mantaining the osmolarity of the incubation medium, the effect of K^+ was investigated using medium containing a fixed amount of Na^+ (11·8 mM) and substituting isosmolar KCl for choline chloride. Initial rates only were investigated, as Da Prada, Tranzer & Pletscher (1967) have shown that prolonged incubation in high K^+ medium leads to platelet damage. The results are shown in Fig. 4, each point being the mean of two experiments: Fig. 4 may be interpreted as showing that competition exists between the effects of K^+ and Na^+ ; the higher the K^+ concentration the more the Na^+ effect is reduced. The results also show that K^+ influences the Km of uptake in the opposite direction to Na^+, Na^+ decreasing the Km and K^+ (or Na^+ lack) increasing the Km.

Other Ions

Experiments were also carried out to investigate the role of the other constituents of the incubation medium on 5-HT uptake. Glucose, $MgCl_2$ and KH_2PO_4 were suc-

TABLE 1. *Effect of potassium on 5-HT uptake*

KCl concentration mM	% 5-HT uptake
0	41±2
0·59	69±5
1·18	64±5
2·39	73±3
4·47	100
9·48	71±6

Each value represents the mean±S.E.M. of ten values obtained in two separate experiments. Uptake was measured after 12 min of incubation and expressed as a percentage of that occurring in control medium (4·47 mM KCl).

cessively replaced with either choline chloride or sucrose. Uptake of 5-HT was measured after 12 min incubation and the results expressed as the percentage reduction in the 5-HT uptake occurring in the original medium (Table 2). Both removal of phosphate and glucose resulted in a small inhibition of uptake, and when Mg^{++} was absent the uptake was reduced by 30%.

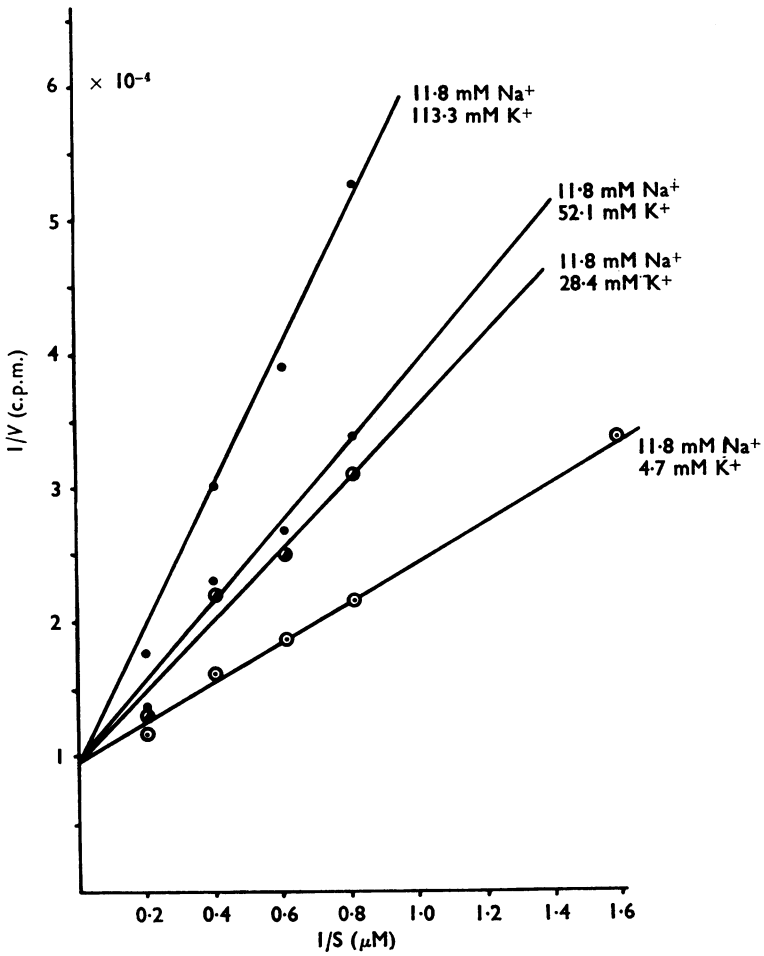

FIG. 4. Effect of potassium concentration on the initial rate of uptake of 5-HT by platelets, plotted as in Fig. 3. The medium contained 11·6 mM NaCl, osmolarity being maintained with choline chloride.

TABLE 2. *Inhibition of 5-HT uptake in the absence of glucose, $MgCl_2$, and KH_2PO_4*

Medium	% 5-HT uptake
Controls	100
Without glucose	82±3
Without $MgCl_2$	68±3
Without KH_2PO_4	92±2

Each value represents the mean±S.E.M. of eight determinations from two separate experiments. Uptake was measured after 12 min incubation and expressed as a percentage of that occurring in the controls.

Discussion

The uptake of 5-HT by blood platelets has been investigated by a number of workers (for references see Paasonen, 1965 ; Pletscher, 1968) and it appears to be a widely held belief that it is an active energy-requiring process, although the mechanisms by which this accumulation occurs are unknown. Born & Gillson (1959) suggested that 5-HT reacts with specific " receptors " located in the platelet membrane and that these " receptors " mediate 5-HT uptake. This hypothesis has been extended to explain the relationship between 5-HT uptake and platelet aggregation in terms of a mobile, membrane bound carrier, the movement of which results in a net accumulation of 5-HT by rabbit platelets (Baumgartner & Born, 1969). The present experiments demonstrate the Na^+ is obligatory for uptake to occur and that Na^+ is intimately related in some manner with the transport process.

An explanation of the action of Na^+ may be derived from the hypothesis suggested by Crane (1965) and Kipinis & Parrish (1965) for the Na^+-dependent transport of sugars and amino-acids in various tissues. It is suggested that the platelet membrane is practically impermeable to free 5-HT. It does, however, contain sites which are capable of binding 5-HT, but only in the presence of Na^+, and these binding sites act as mobile carriers which traverse the membrane. According to this concept the rate-limiting step in 5-HT transport is the binding of 5-HT to the carrier. The straight line obtained by plotting the reciprocal of 5-HT uptake against the reciprocal of the sodium concentration would be consistent with the suggestion that one sodium ion is bound to a site on the 5-HT carrier and that this facilitates the binding of one molecule of 5-HT. Similar graphs have been plotted for the effect of Na^+ on other uptake systems (Kipnis & Parrish, 1965 ; Horst, Kopin & Ramney, 1965), and Vidaver (1964), investigating glycine uptake by pigeon erythrocytes, found a similar relationship when the reciprocal of glycine concentration was plotted against the reciprocal of the square of the sodium concentration, from which he concluded that two sodium ions acted as co-substrates of glycine uptake at some step in the transport process.

According to Crane *et al.* (1965) the accumulation of non-electrolytes is intimately related to the asymmetric distribution of Na^+ and K^+ between the outside and inside of the cell, and the affinity of the substrate for the carrier depends on the ionic species to which it is exposed. It is postulated that a membrane carrier "*C*" has a high affinity for 5-HT on the outside of the membrane (high Na^+) and forms a substrate-carrier complex of the form 5-HT–*C*–Na, and that the loaded carrier traverses the membrane to a region of low Na^+ and high K^+. In order for accumulation of 5-HT to occur inside the cell, the 5-HT must dissociate from the carrier. The competition which exists between Na^+ and K^+ (Fig. 4) indicates that K^+ may compete with the Na^+ for a specific site on the carrier, K^+ altering the properties of the carrier so that it has a greatly decreased affinity for 5-HT (this is shown by the increase in *Km* in the presence of high K^+ concentration) and the 5-HT dissociates from the carrier inside the cell. The substrate-free carrier either in the form K–*C*– or –*C*– would now be available to traverse the membrane to the region of high Na^+ concentration, where it could bind more substrate. The results of Born (1967) demonstrating an increased exchange of K^+ by blood platelets incubated with 5-HT would add support to the suggestion that the carrier returns to the outside of the membrane associated with potassium. The concept of such a mobile carrier system

has been given theoretical treatment by Wilbrandt & Rosenberg (1961) and Stein (1967).

The question also arises as to the source of energy required for the accumulation of 5-HT. According to the hypothesis of Christensen, Riggs & Ray (1952), extended by Crane (1965), a cell may accumulate a substrate by utilizing the energy inherent in the spontaneous movement of cations down their respective concentration gradients across cell membranes. Thus the asymmetry of the ion distribution would drive the substrate-carrier complex 5-HT–*C*–Na to the region of low Na^+ (inside the cell), and energy would be required to maintain either the Na^+ gradients and/or the Na^+/K^+ ratio, the relative concentrations of cations being maintained by an outwardly directed, energy dependent Na^+ pump (Na^+, K^+ dependent ATPase). The possible role of such an Na^+ pump has not been investigated in the present experiments, but other workers (Sano *et al.*, 1958 ; Born & Gillson, 1959 ; Weissbach & Redfield, 1960) have shown that prolonged incubation of blood platelets with ouabain impairs their ability to accumulate 5-HT, an effect attributed to the inhibition of Na^+, K^+ ATPase (Pletscher, Burkard, Tranzer & Gey, 1967). The inhibition of the Na^+ pump by ouabain and the removal of Na^+ from the incubation medium would prevent the maintenance of a satisfactory Na^+ gradient, or K^+/Na^+ ratio, either of which would decrease the accumulation of 5-HT.

Experiments to study the action of inhibitors of 5-HT uptake and the role of intracellular ions in the accumulation of 5-HT are in progress and may be expected to yield more information concerning the mechanisms of active transport of 5-HT by blood platelets.

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