Br. J. Pharmac. (1974), 52, 237-244

EFFECT OF SELECTIVE PROTEOLYSIS ON THE ACCUMULATION OF 5-HYDROXYTRYPTAMINE BY INTACT RAT BLOOD PLATELETS

J.M. SNEDDON & K.I. WILLIAMS
Department of Pharmacology, The Medical School, University Walk, Bristol BS8 1TD

1 The effect of perturbation of intact blood platelets with proteolytic enzymes was studied with respect to 5-hydroxytryptamine (5-HT) transport, adenine transport and intracellular Na^+ and K^+ levels.

2 Leucine aminopeptidase and thrombin reduced 5-HT transport, released 5-HT from pre-labelled platelets and disturbed the gradient to monovalent cations. Leucine aminopeptidase, but not thrombin, inhibited adenine transport.

3 α-Chymotrypsin and carboxypeptidases A and B were without effect on the parameters studied.

4 Trypsin selectively reduced 5-HT transport. It did not release 5-HT from blood platelets or inhibit adenine transport.

5 The action of proteolytic enzymes is discussed in terms of proteins localized in the external membrane that may function in part as membrane carriers.

Introduction

The general characteristics of the 5-hydroxytryptamine (5-HT) transport system in blood platelets are very similar to the biogenic amine transport systems reported for nervous tissue (Sneddon, 1973). The kinetic properties of 5-HT transport in blood platelets have been extensively investigated; it appears to be a saturable, energy dependent process obeying Michaelis-Menten kinetics (Humphrey & Toh, 1954; Born & Gillson, 1959; Lingjaerde, 1969). Sneddon (1969) demonstrated that 5-HT transport by blood platelets is a Na^+-dependent process and the available data are consistent with a mechanism in which the binding of 5-HT to the transport carrier is activated by Na^+. In addition to the 5-HT transport carrier, the platelet membrane possesses additional carriers for other non-electrolytes, the best characterized of which appears to be that for adenine (Sixma, Holmsen & Trieschnigg, 1973).

To date little progress has been made towards elucidating the molecular basis of the reactions underlying these transport mechanisms, and as a step towards a solution of this problem we have attempted to characterize the membrane components which are essential to the carrier mediated transport of 5-HT. The present paper analyses the consequences of selective proteolysis of intact blood platelets on the transport of 5-HT.

Methods

Blood platelets were obtained from adult male rats as previously described (Sneddon, 1969).

Measurement of [^{3}H]-5-hydroxytryptamine uptake

The platelet pellet was resuspended in 12 ml of incubation medium at 36°C (composition (mM): NaCl 121, KCl 4.1, KH_2PO_4 1.18, $MgSO_4$ 1.18, TRIS (hydroxymethyl) methylamine-HCl (pH 7.2) 24.87, disodium edetate (EDTA) 5.37). The pH was adjusted to 7.2 unless specified otherwise. Some of the enzymes used in this study are inhibited by chelating agents, thus where necessary, EDTA was omitted. The platelet suspension was distributed in 1 ml aliquots to plastic centrifuge tubes containing a mixture of 5-HT and [^{3}H]-5-HT at a final concentration of 2.47×10^{-6} M, with the appropriate concentration of the enzyme under investigation, and incubated for 4 minutes. [^{3}H]-5-HT uptake was

stopped by rapid centrifugation, the supernatant decanted and the inside of the tubes wiped dry with tissue paper. The platelet pellet was re-suspended in 1 ml of 1% sodium lauryl sulphate. Samples (0.1 ml) were taken for liquid scintillation counting.

[^{3}H]-adenine uptake

[^{3}H]-adenine uptake was measured in an identical manner to that for [^{3}H]-5-HT. The final concentration of adenine was 2.97×10^{-7} M. All values for the uptake of [^{3}H]-5-HT or [^{3}H]-adenine are expressed as μmol or nmol/10^6 platelets.

Determination of intracellular Na^+ and K^+

Intracellular Na^+ and K^+ were determined as previously described (Sneddon, 1971) except that determinations were made with an atomic absorption spectrophotometer.

Release of [^{3}H]-5-hydroxytryptamine

Platelets were pre-labelled by incubating platelet rich plasma with [^{3}H]-5-HT (2.47×10^{-6} M) for 30 min at 36°C. The platelets were separated from the plasma by centrifugation and resuspended in the appropriate incubation medium. Samples (1 ml) were added to centrifuge tubes containing varying concentrations of the enzyme under investigation and incubated for 4 minutes. Platelets were separated by centrifugation and the amount of radioactivity present in the supernatant and the platelet pellet determined. The percentage of radioactivity appearing in the extracellular phase (supernatant) was taken as an index of release.

Platelet counts

The number of platelets in each suspension was measured by means of a thrombocounter (Coulter Electronics Ltd).

Reagents

All solutions were made up in the appropriate incubation medium. Adenine-[8-^{3}H] (sp. act. 15 Ci/mmol) and 5-hydroxytryptamine-[^{3}H3] (G) creatine sulphate (sp. act. 500 mCi/mmol) were obtained from the Radiochemical Centre, Amersham. The following enzymes were obtained from Boehringer: trypsin, E.C. 3.4.4.4. sp. act. 9000 u/mg; α-chymotrypsin, E.C. 3.4.4.5. sp. act. 10,000 u/mg; leucine aminopeptidase, E.C. 3.4.11. sp. act. 100 u/mg; carboxypeptidase A., E.C. 3.4.2.1. sp. act. 35 u/mg; carboxypeptidase B., E.C. 3.4.2.2. 150 u/mg. Thrombin (bovine origin) was obtained from Parke-Davis and purified on sulphoethyl-sephadex C-50 (Lundblad, 1971).

Results

Effect of trypsin on the accumulation of [^{3}H]-5-hydroxytryptamine and [^{3}H]-adenine

[^{3}H]-5-hydroxytryptamine uptake The initial investigations into the action of trypsin on the accumulation of [^{3}H]-5-HT were carried out by preincubation of platelet suspensions with various concentrations of trypsin (10-100 u/ml) for 10 minutes. The proteolytic action of trypsin was terminated by the addition of sufficient soybean trypsin inhibitor to inhibit the highest concentration of enzyme used. [^{3}H]-5-HT was then added to the platelet suspension and the incubation continued for a further 10 minutes. These experiments gave variable results probably due to the relatively long incubation time in the presence of EDTA which may have resulted in damage to the platelet membrane in addition to proteolysis due to trypsin. Consequently the experimental approach was simplified so that the enzyme and [^{3}H]-5-HT were present simultaneously during the incubation period. The results obtained from such experiments are shown in Figure 1.

During the 6 min incubation period the accumulation of [^{3}H]-5-HT was significantly reduced by low concentrations of trypsin which exerted its inhibitor effect within 1 min of being added to the platelet suspension, and this inhibition appeared to be progressive as it is much more marked after 6 min incubation, especially at a trypsin concentration of 100 u/ml. Further studies were carried out in an attempt to analyse this inhibitory action of trypsin. A kinetic analysis of the effect of different concentrations of trypsin on the initial rates of [^{3}H]-5-HT uptake indicated that treatment with trypsin changed both the apparent V_{max} and Km when the initial rates of [^{3}H]-5-HT uptake were expressed in terms of the Michaelis-Menten equation (Figure 2).

In order to demonstrate that trypsin, or any other enzyme, is exerting a possible selective action on [^{3}H]-5-HT uptake it was necessary to perform a number of control experiments. These were, the effect of proteolysis on intracellular Na^+ and K^+, on the release of [^{3}H]-5-HT from pre-labelled platelets, and effect on [^{3}H]-adenine uptake. For the rationale of these control experiments see the discussion section.

[^{3}H]-adenine uptake The effect of trypsin on the uptake of [^{3}H]-adenine was determined in the

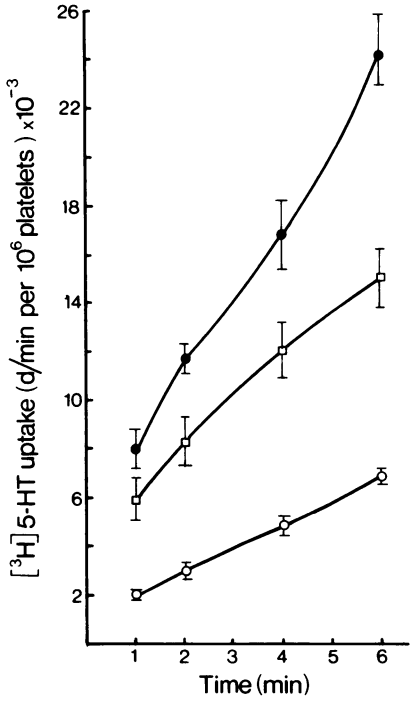

Fig. 1 The effect of trypsin on the accumulation of [^{3}H]-5-hydroxytryptamine (5-HT) by rat blood platelets during a 6 min incubation period. Controls (●); trypsin 50 u/ml (□); trypsin 100 u/ml (○). Each point represents the mean from four experiments. Vertical bars indicate s.e. mean.

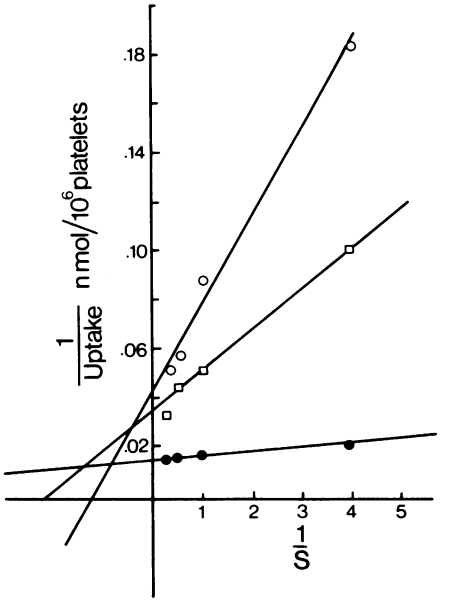

Fig. 2 Effect of trypsin on the initial rate of 5-hydroxytryptamine (5-HT) uptake by platelets. Initial velocity V is expressed as μmol/10^6 platelets after 3 min incubation. Abscissae are reciprocals of 5-HT concentration (μM) in the medium; (●) controls; (□) trypsin 75 u/ml; (○) trypsin 150 u/ml. Each point represents the mean of 12 determinations from 3 experiments.

same experiments as the uptake of [^{3}H]-5-HT. The results of these experiments are shown in Table 1 and, for comparison, the values of [^{3}H]-5-HT uptake at 4 min are also shown. As can be seen from the table, concentrations of trypsin which produce significant inhibition of [^{3}H]-5-HT uptake had no significant effect on the carrier mediated uptake of [^{3}H]-adenine.

Intracellular Na^+ and K^+ levels As the accumulation of 5-HT by rat blood platelets is related to the relative magnitudes of intracellular Na^+ and K^+ (Sneddon, 1971) it was considered essential that the enzymes used in this study should not disturb the intracellular concentrations of Na^+ and K^+. To investigate this possibility blood platelets were incubated with [^{3}H]-5-HT with and without trypsin 100 u/ml for 6 minutes. Although the [^{3}H]-5-HT uptake was markedly inhibited under

Table 1 Effect of trypsin on the transport of [^{3}H]-5-hydroxytryptamine and [^{3}H]-adenine in rat blood platelets

Concentration of trypsin	*Uptake*	
	[^{3}H]-5-HT (μmol/10^6 platelets)	*[^{3}H]-adenine (nmol/10^6 platelets)*
50 u/ml	1.195 ± 0.119 ($P > 0.5$)	0.2732 ± 0.047 (NS)
100 u/ml	0.489 ± 0.084 ($P < 0.001$)	0.2633 ± 0.195 (NS)
Controls	1.684 ± 0.148	0.3140 ± 0.055

Uptake of both compounds measured after incubation for 4 min at 36°C. Values are the mean with s.e. mean from at least 3 separate experiments. Significance calculated by Student's t test.

these conditions no significant change in the levels of intracellular Na^+ and K^+ were found. The values for intracellular ions were (μmol/10^6 platelets): trypsin-treated platelets, Na^+ 0.479 ± 0.043, K^+ 2.200 ± 0.264; controls Na^+ 0.465 ± 0.043, K^+ 2.304 ± 0.244, for 10 determinations from 3 separate experiments.

[^{3}H]-5-hydroxytryptamine release The ability of trypsin to release 5-HT was investigated by incubation of blood platelets pre-labelled with [^{3}H]-5-HT with increasing concentrations of trypsin under identical conditions to those used in the uptake experiments. The release of [^{3}H]-5-HT by trypsin in concentrations up to 200 u/ml did not exceed 7% of the total radioactivity present in the platelet suspension, a value similar to that obtained from pre-labelled platelets resuspended in incubation medium without trypsin. Higher concentrations (500 and 1000 u/ml) of trypsin were also studied. Although these concentrations resulted in a greater inhibition of [^{3}H]-5-HT uptake than that reported for 100 u/ml, this inhibition was associated with proportionately more [^{3}H]-5-HT being released from the platelets by the enzyme, and at 1000 u/ml by marked disturbances in the ionic gradients for Na^+ and K^+.

Effect of pH on the action of trypsin In a comparison made over 4 experiments essentially identical results on the trypsin inhibition of [^{3}H]-5-HT uptake were obtained at pH 5.5 and pH 7.2-7.3. At the lower pH there was no significant effect on [^{3}H]-adenine uptake or the proportion of [^{3}H]-5-HT released from pre-labelled platelets.

Effect of thrombin on the accumulation of [^{3}H]-5-hydroxytryptamine and [^{3}H]-adenine

The action of low concentrations of thrombin on the uptake of [^{3}H]-5-HT and [^{3}H]-adenine are shown in Table 2. Thrombin in concentrations as high as 5 u/ml did not inhibit the accumulation of [^{3}H]-adenine, but over the same range of concentrations thrombin significantly reduced the uptake of [^{3}H]-5-HT.

In addition to the inhibition of [^{3}H]-5-HT uptake thrombin also released [^{3}H]-5-HT from pre-labelled platelets in the presence of EDTA (5.37 mM) at pH 7.2. The release at the lower concentrations (1.75 and 2.5 u/ml) was 12 to 15% of the total radioactivity present in the total platelet suspension and this increased to 22% following incubation with 5 u/ml thrombin for 4 minutes. This releasing activity of thrombin did not appear to be directly related to the action on [^{3}H]-5-HT uptake and at a concentration of 1.75 u/ml 12% of the radioactivity was released (controls without thrombin released 6%) but the inhibition of uptake was approximately 50%, and at 5 u/ml the values were 22% and 67% respectively. If the incubations were carried out at pH 5.5 instead of pH 7.2 thrombin failed to release significant amounts of [^{3}H]-5-HT from pre-labelled platelets and did not reduce the accumulation of either [^{3}H]-5-HT or [^{3}H]-adenine. The action of thrombin in producing inhibition of [^{3}H]-5-HT uptake, or [^{3}H]-5-HT release was associated with disturbances of the intracellular Na^+ and K^+ levels. At the highest concentration of thrombin used (5 u/ml) there was an approximate 50% decrease in intracellular K^+ levels, and a proportional increase in intracellular Na^+ after 4 min incubation with thrombin in the presence of EDTA.

Effect of leucine aminopeptidase on the uptake of [^{3}H]-5-hydroxytryptamine and [^{3}H]-adenine

These experiments were carried out in the absence of EDTA as leucine aminopeptidase is inhibited by chelating agents probably due to the removal of Mn^{++} which is an essential component of this

Table 2 Effect of thrombin on the transport of [^{3}H]-5-hydroxytryptamine (5-HT) and [^{3}H]-adenine in rat blood platelets

Thrombin concentration (u/ml)	*Uptake*	
	[^{3}H]-5-HT (μmol/10^6 platelets)	*[^{3}H]-adenine (nmol/10^6 platelets)*
0.5	1.193 ± 0.100 (NS)	0.375 ± 0.014 (NS)
1.75	0.683 ± 0.039 (P = 0.001)	0.352 ± 0.021 (NS)
2.50	0.558 ± 0.028 (P = 0.001)	0.314 ± 0.023 ($P < 0.2$)
5.00	0.471 ± 0.031 (P = 0.001)	0.360 ± 0.027 (NS)
Controls	1.416 ± 0.118	0.405 ± 0.030

Determinations made after 4 min incubation. Values are the mean with s.e. mean from 4 or more experiments. Significance calculated by Student's t test.

Table 3 Effect of leucine aminopeptidase on the transport of [^{3}H]-5-hydroxytryptamine (5-HT) and [^{3}H]-adenine in rat blood platelets

Leucine aminopeptidase (u/ml)	*Uptake* [^{3}H]-5-HT (μmol/10^6 platelets)	*Uptake* [^{3}H]-adenine (nmol/10^6 platelets)
5	2.266 ± 0.114 (NS)	0.41
10	1.765 ± 0.136 (NS)	0.34
50	1.349 ± 0.133 (NS)	0.25
Controls	2.704 ± 0.597	0.52

Determinations were made after 4 min incubation. For [^{3}H]-adenine uptake values are the average of two determinations from separate experiments.

Table 4 Effect of carboxypeptidase A and B on the transport of [^{3}H]-5-hydroxytryptamine (5-HT) and [^{3}H]-adenine by rat blood platelets

Enzyme concentration	*Uptake* [^{3}H]-5-HT (μmol/10^6 platelets)	*Uptake* [^{3}H]-adenine (nmol/10^6 platelets)
Carboxypeptidase A		
9 u/ml	1.112 ± 0.184 (NS)	0.511
18 u/ml	1.073 ± 0.122 (NS)	0.477
45 u/ml	1.251 ± 0.132 (NS)	0.518
Controls	1.113 ± 0.245	0.399
Carboxypeptidase B		
7.5 u/ml	1.092	–
15.0 u/ml	1.069 ± 0.036 (NS)	0.3861 ± 0.008 (NS)
30.0 u/ml	1.017 ± 0.106 (NS)	0.3863 ± 0.025 (NS)
Controls	0.927 ± 0.451	0.4210 ± 0.075

Uptakes measured after 4 min incubation. Values are mean with s.e. mean of three separate experiments. Other values are the average of two determinations from separate experiments. NS = not significantly different from controls.

enzyme. The effect of incubation with increasing concentrations of leucine aminopeptidase on [^{3}H]-5-HT and [^{3}H]-adenine transport is shown in Table 3. The enzyme inhibited the accumulation of both solutes to approximately the same extent.

Leucine aminopeptidase also increased the release of [^{3}H]-5-HT from pre-labelled platelets. At a concentration of 5 u/ml the enzyme released approximately 20% of the total platelet radioactivity in 4 min, and this increased to approximately 40% at an enzyme concentration of 50 u/ml. These values are comparable with the degree of inhibition of [^{3}H]-5-HT and [^{3}H]-adenine uptake observed at the same enzyme concentrations. In addition there was a measurable loss of intracellular K^+ at all enzyme concentrations studied.

Effect of α-chymotrypsin on the uptake of [^{3}H]-5-hydroxytryptamine and [^{3}H]-adenine

In the presence of EDTA (5.37 mM), pH 7.2-7.3, α-chymotrypsin at concentrations of 50, 100, 500 or 1000 u/ml did not reduce the uptake of either [^{3}H]-5-HT or [^{3}H]-adenine when the uptake was measured after 4 min incubation at 36°C, nor did it stimulate the release of [^{3}H]-5-HT from pre-labelled platelets. In one experiment similar results were obtained in the absence of EDTA indicating that, under the conditions of the experiment, α-chymotrypsin exerted no significant effect on the parameters studied. Essentially similar results were obtained with carboxypeptidases A and B (Table 4).

Discussion

The uptake of 5-HT by blood platelets is believed to be a Na^+-dependent, carrier mediated process (Humphrey & Toh, 1954; Born & Gillson, 1959; Sneddon, 1969). This implies that there is a specific membrane component which selectively transports solutes across an otherwise impermeable diffusion barrier (Pardee, 1968). To date, no

positive attempt has been made to elucidate the molecular basis underlying the specific transport of monoamines across cell membranes, and attempts in this laboratory to isolate the membrane components essential for these transport systems have not been successful. An alternative approach is to attempt to study these membrane components *in situ*. In recent years various enzymes have been employed to elucidate the structure-function relationships in soluble proteins and it appeared to us that enzymatic probes may be used to investigate the characteristics of surface located groups in blood platelets which may be important in 5-HT transport.

Platelet proteins are susceptible to the proteolytic enzymes thrombin, trypsin, papain and chymotrypsin (Davey & Luscher, 1967; Ganguly, 1969), but such extensive proteolysis can induce changes in the conformation of the membrane, bring buried protein to the surface for further proteolysis, or even release proteins from inside the cell (Baenziger, Brodie & Majerus, 1972). For such reasons, the use of proteolysis to probe the surface of the platelet must be subject to various interpretations, and it is essential that proteolysis should induce only minor changes so that the total biological activity of the cell is altered as little as possible.

The present study explores the consequences of peptidolysis of intact rat blood platelets in an effort to identify possible essential structures of the 5-HT carrier. This was achieved by selectively attacking various protein components of the membrane with specific endopeptidases such as trypsin, α-chymotrypsin and thrombin rather than peptidases with a wider spectrum of activity such as protease or papain. In order to demonstrate that a particular enzyme treatment was exerting a selective action on 5-HT uptake it was considered essential to perform a number of control experiments in an attempt to invalidate any observations made on 5-HT uptake. These controls were:

The ability of proteolytic enzymes to release [^{3}H]-5-hydroxytryptamine from pre-labelled platelets

Extensive observations have shown that a large number of proteolytic enzymes are capable of triggering the platelet release reaction (Markwardt, 1967; Davey & Luscher, 1967; Holmsen, Day & Stormorken, 1969). This reaction is characterized by the selective release of 5-HT, adenine nucleotides and other biologically active substances from blood platelets, and effectively destroys the intracellular storage sites for 5-HT. This would reduce the 'uptake' of 5-HT by preventing the intracellular accumulation of 5-HT after it had traversed the cell membrane, and the process of exocytosis may also be expected to cause gross malformation of the plasma membrane. The incidence of the platelet release reaction can be reduced by chelating divalent cations with EDTA and where possible this was carried out in the present experiments.

Intracellular Na^+ and K^+ levels

The accumulation of 5-HT by rat blood platelets is related to the relative magnitude of the gradients for Na^+ and K^+ (Sneddon, 1971). Thus, enzyme treatment could inhibit 5-HT uptake by destroying the energy producing systems of the cell and not by a direct effect on the 5-HT 'carrier'. Any enzyme which was found to inhibit 5-HT uptake was further investigated for its action on intracellular Na^+ and K^+ and if it was found to produce disturbances in the gradients of these ions its inhibitory action is not attributable to a direct action on the membrane carrier.

Intracellular ions are also an indirect measure of the integrity of the plasma membrane as non-specific damage to the membrane may be expected to result in the leakage of intracellular K^+.

Adenine transport

In addition to 5-HT the platelet membrane has the ability to transport other solutes such as adenine. Some of these solutes are transported by carriers distinct from that for 5-HT and as adenine does not influence the accumulation of 5-HT or *vice versa*, this is indicative of discrete carriers for the two solutes. Thus a measure of adenine transport during treatment with the enzymes gives a measure of the selectivity of the proteolytic perturbation of the 5-HT carrier.

When the results are analysed in terms of these control studies it is found that the proteolytic enzymes used in the present study fall into several categories. The first is represented by leucine aminopeptidase which inhibits the uptake of [^{3}H]-5-HT and [^{3}H]-adenine to the same degree, indicating no selective action on 5-HT transport. This lack of specificity is further characterized by the ability of the enzyme to release 5-HT from pre-loaded platelets and to reduce intracellular K^+ levels, perhaps indicating that the inhibitory effect on solute transport represents non-specific hydrolysis of the cell membrane. The second category is represented by thrombin which shares with leucine aminopeptidase the ability to inhibit [^{3}H]-5-HT uptake and also release [^{3}H]-5-HT from pre-labelled platelets. However, thrombin does not appear to inhibit [^{3}H]-adenine uptake,

even in concentrations that significantly inhibit [^{3}H]-5-HT uptake and result in a loss of intracellular K^+ and a gain in Na^+. Such results imply that thrombin exerts an unselective action on the 5-HT transport system and that the degree of inhibition produced by this enzyme is increased by the destruction of the ionic gradients. From these results it would also appear that the accumulation of [^{3}H]-adenine is not dependent upon the asymmetric distribution of Na^+ and K^+ as suggested for 5-HT transport although further experiments would be required to clarify this point.

The third category of proteolytic enzyme is represented by trypsin. This enzyme exhibited the most selective action on [^{3}H]-5-HT uptake. At all concentrations studied it significantly reduced the accumulation of 5-HT but had no measurable effect on either [^{3}H]-adenine accumulation or the levels of intracellular Na^+ and K^+.

The other enzymes used in this study, α-chymotrypsin and carboxypeptidases A and B, had no detectable action on any of the three parameters investigated.

These results indicate that the accumulation of [^{3}H]-5-HT by rat blood platelets is susceptible to manipulation by selective proteolysis by trypsin and perhaps also by thrombin. Naturally the question arises as to the identity of the hypothetical surface located carrier. We have no direct evidence to suggest which polypeptide chains are being attacked by trypsin, but there appears to be no doubt that trypsin, and other proteolytic enzymes, are capable of interacting with proteins on the outer surfaces of intact cells. The evidence has recently been reviewed by Wallach (1972). Winzler, Harris, Pekas, Johnson & Webet (1967) demonstrated that trypsinization of human erythrocytes results in hydrolysis of surface located glycopeptides. Martin (1970) further demonstrated that incubation of intact human erythrocytes with pronase, trypsin, and chymotrypsin destroyed the cholinesterase activity, but produced no alteration of choline or Na^+ transport. In a similar manner, trypsin appears to hydrolyse surface located proteins in blood platelets. Behnke (1968) reported that trypsin removed the ruthenium-red stainable material from the surface of both rat and human platelets, but had no effect on the electron dense staining produced by incubation with either colloidal iron or thorotrast. Pepper & Jamieson (1970) found that pretreatment of platelets with trypsin released glycoprotein fragments from the platelet membrane; similar treatment also decreased the sialic acid content of the platelet membranes (Barber & Jamieson, 1971). Phillips (1972) using the lactoperoxidase iodination technique, which specifically iodinates exposed surface proteins, has shown that intact blood platelets possess at least seven exposed polypeptides in the surface membrane, and that the major proportion of these polypeptides is represented by three separate glycoproteins. Pretreatment of intact platelets with trypsin hydrolysed the three glycopeptides but had no effect on other surface located proteins. Similar results were reported by Nachman & Ferris (1972) who later showed that thrombin, unlike trypsin, did not alter the pattern of surface labelling with radioiodine following incubation with lactoperoxidase (Nachman, Hubbard & Ferris, 1973). The possibility remains, however, that trypsin may be acting in a purely physical manner by adsorption of the enzyme on to the carrier. Although we cannot eliminate this possibility we consider it unlikely as the effect of trypsin was completely prevented by soybean trypsin inhibitor, and after perturbation of the platelets with trypsin it was not possible to restore the control uptake by washing the cells in standard incubation medium in the presence or absence of soybean trypsin inhibitor.

Thus it appears highly probable that under our experimental conditions proteolytic enzymes, and trypsin in particular, are capable of hydrolysing surface located proteins in intact blood platelets. Trypsin reduced 5-HT transport without affecting either adenine or Na^+ transport perhaps indicating a selective action on the surface located carrier. Born & Gillson (1959) suggested that there were 10^4 5-HT carrier molecules per platelet and in the present experiments this would indicate an average of 10^{10} carriers/10^6 platelets. This number of molecules must represent an infinitely small proportion of the total platelet protein, but it may represent a significant proportion of the surface orientated membrane located polypeptides. Whether or not the hypothetical 5-HT carrier is located in the trypsin sensitive glycoproteins cannot be determined from the present experiments, but studies investigating the action of enzyme probes on membrane polysaccharide structures may help to elucidate this point.

K.I.W. is in receipt of a post-doctoral fellowship from the Wellcome Trust. We are indebted to Miss J. Glanville for technical assistance.

References

BAENZIGER, N.L., BRODIE, G.N. & MAJERUS, P.W. (1972). Isolation and properties of a thrombin sensitive protein of human platelets. *J. Biol. Chem.*, **247**, 2723-2731.

BARBER, A.J. & JAMIESON, G.A. (1971). Isolation of glycopeptides from low- and high-density platelet plasma membrane. *Biochemistry*, **10**, 4711-4717.

BEHNKE, O. (1968). Electron microscopical observations on the surface coating of human blood platelets. *J. Ultrastruc. Res.*, **24**, 51-69.

BORN, G.V.R. & GILLSON, R.E. (1959). Studies on the uptake of 5-hydroxytryptamine by blood platelets. *J. Physiol., Lond.*, **146**, 472-491.

DAVEY, M.G. & LUSCHER, E.F. (1967). Actions of thrombin and other coagulant and proteolytic enzymes on blood platelets. *Nature, Lond.*, **216**, 857-858.

GANGULY, P. (1969). Studies on human platelet proteins. II Effect of thrombin. *Blood*, **33**, 590-597.

HOLMSEN, H., DAY, H.J. & STORMORKEN, H. (1969). The blood platelet release reaction. *Scand. J. Haematol.* Suppl. **8**, 1-26.

HUMPHREY, J.H. & TOH, C.C. (1954). Absorption of serotonin (5-hydroxytryptamine) and histamine in dog platelets. *J. Physiol., Lond.*, **124**, 300-304.

LINGJAERDE, O. (1969). Uptake of serotonin in blood platelets: dependence on sodium and chloride and inhibition by choline. *FEBS Letters*, **3**, 103-106.

LUNDBLAD, R.L. (1971). A rapid method for the purification of bovine thrombin and the inhibition of the purified enzyme with phenylmethyl-sulfonyl fluoride. *Biochemistry*, **10**, 2501-2505.

MARKWARDT, F. (1967). Studies on the release of biogenic amines from blood platelets. In *Biochemistry of Blood Platelets*, eds Kowalski, E. & Niewiarowski, S., pp. 105-116. London: Academic Press.

MARTIN, K. (1970). The effect of proteolytic enzymes on acetylcholinesterase activity, the sodium pump and choline transport in human erythrocytes. *Biochim. biophys. Acta*, **203**, 182-184.

NACHMAN, R.L. & FERRIS, BARBARA (1972). Studies on the proteins of human platelet membranes. *J. biol. Chem.*, **247**, 4468-4475.

NACHMAN, R.L., HUBBARD, ANN & FERRIS, BARBARA (1973). Iodination of the human platelet membrane. Studies of the major surface glycoprotein. *J. biol. Chem.*, **248**, 2928-2936.

PARDEE, A.B. (1968). Membrane transport proteins. *Science*, **162**, 632-637.

PEPPER, D.S. & JAMIESON, G.A. (1970). Isolation of a macroglycopeptide from human platelets. *Biochemistry*, **9**, 3706-3713.

PHILLIPS, D.R. (1972). Effect of trypsin on the exposed polypeptides and glycoproteins in the human platelet membrane. *Biochemistry*, **11**, 4582-4588.

SIXMA, J.J., HOLMSEN, H. & TRIESCHNIGG, A.C.M. (1973). Adenine nucleotide metabolism of blood platelets. VIII. Transport of adenine into human platelets. *Biochim. biophys. Acta*, **298**, 460-488.

SNEDDON, J.M. (1969). Sodium-dependent accumulation of 5-hydroxytryptamine by rat blood platelets. *Br. J. Pharmac.*, **37**, 680-688.

SNEDDON, J.M. (1971). Relationship between internal Na^+/K^+ and the accumulation of ^{14}C-5-Hydroxytryptamine by rat platelets. *Br. J. Pharmac.*, **43**, 834-844.

SNEDDON, J.M. (1973). Blood platelets as a model for monoamine-containing neurones. In *Progess in Neurobiology*, Vol. **1**, ed. Kerkut, G.A. & Phillis, J.W., pp. 151-198. Oxford: Pergamon Press.

WALLACH, D.F.H. (1972). The dispositions of proteins in the plasma membranes of animal cells: Analytical approaches using controlled peptidolysis and protein labels. *Biochim. biophys. Acta*, **265**, 61-83.

WINZLER, R.J., HARRIS, E.D., PEKAS, D.J., JOHNSON, C.A. & WEBER, P. (1967). Studies on glycopeptides released by trypsin from intact human erythrocytes. *Biochemistry*, **6**, 2195-2202.

(Received January 25, 1974)