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Mechanisms by which human blood platelets accumulate glycine, gaba and amino acid precursors of putative neurotransmitters

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Summary

1. We have examined the accumulation by human blood platelets of amino acids that are believed to be involved in neurohumoral transmission in the central nervous system.

2. Platelets were incubated in Ca^{++}-free Krebs solution at 37° C with radioactive amino acids for various times and then the platelets were analysed for the radioactive substance and its metabolites.

3. L-Phenylalanine, L-DOPA, L-tryptophan and L-tyrosine were rapidly accumulated, the equilibrium tissue/medium concentration ratio (C_i/C_o) being greater than 10:1 when the concentration of amino acid in the medium was 10^{-7}M or lower. Glycine and γ-aminobutyric acid (GABA) accumulation was less, C_i/C_o being lower than 3:1 when C_o was 10^{-7}M.

4. Uptake of L-phenylalanine, L-DOPA and L-tryptophan were all decreased or abolished by incubation at 4° C, or with metabolic inhibitors or by disruption of the platelet membrane prior to incubation, while L-tyrosine accumulation was not affected.

5. It is considered that L-phenylalanine, L-DOPA and L-tryptophan are accumulated by saturable, energy-dependent processes; that glycine and GABA diffuse into the platelet, and L-tyrosine accumulates as a result of diffusion and intracellular binding.

6. None of the amino acids examined showed any significant metabolism during a 20 min incubation. However, some evidence for tyrosine binding to soluble protein was obtained.

7. Results are compared to reports of accumulation of these amino acids by the central nervous system.

Introduction

The blood platelet is able to accumulate various substances which may function as neurohumoral transmitters in nerve cells. These include 5-hydroxytryptamine (Humphrey & Toh, 1954; Hardisty & Stacey, 1955), noradrenaline (Born &

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Hornykiewicz, 1957 ; Born, Hornykiewicz & Stafford, 1958) and dopamine (Boullin & O'Brien, 1970 ; Solomon, Spirt & Abrahms, 1970). Because of this similarity between blood platelets and neurones, it has been suggested that the platelet might provide a useful model for the brain nerve ending (Paasonen, 1968 ; Page, 1968 ; Pletscher, 1968). Since several putative neurotransmitters in the brain are formed from amino acid precursors and some amino acids may function as neurotransmitters in their own right (for review see Iversen, 1971), we have studied the accumulation and metabolism of appropriate compounds which are of interest to the neuropharmacologist from this viewpoint. A preliminary account of some of this work has already been given (Green, Votavova, & Boullin, 1971).

Methods

Blood was obtained by venipuncture from normal adult volunteers of either sex. Subsequent platelet isolation was made by the method of Boullin & O'Brien (1969) with polycarbonate laboratory ware. The number of platelets per ml of plasma was determined with a Model B Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida). All experiments were carried out with platelets resuspended in Krebs solution (Ca^{++} free), as described by Umbriet, Burris & Stauffer (1964). The platelet rich plasma (PRP) was centrifuged for 10 min at 8,000 *g* in a refrigerated centrifuge at 0° C (IEC Model PR-6 ; International Equipment Co., Needham Heights, Mass.). We decanted the supernatant platelet-free plasma and re-suspended the cells derived from approximately 15 ml of whole blood by adding 1 drop of EDTA (1 g/100 ml 0·7 M NaCl) per 1 ml of PRP, and sufficient Krebs solution to restore the volume of fluid to the original value. Platelet volumes were calculated by use of a thrombocytocrit (Hardisty & Stacey, 1955). However, since the re-suspended cells did not sediment well in the thrombocytocrit, platelet volume was measured with PRP and the volume of platelets resuspended in Krebs solution was estimated from the Coulter Count of the PRP and of the resuspended platelets.

Accumulation experiments

One ml of re-suspended platelet solution was incubated at 37° C with various concentrations of ^{14}C-labelled amino acids, added in a volume of 10 μl. After varying periods as stated in **Results**, incubation was terminated and platelets separated by centrifugation of the incubation tube at 20,000 *g* for 3 min at 0° C, which removed more than 99·5% of the platelets from the medium. The medium was decanted and saved for radiochemical assay. Traces of fluid remaining in the tube were removed by cotton-tipped applicators covered with paper tissue. The platelet pellet was lysed by sonification in 1·0 ml of H_2O using a Biosonik 2 sonifier, fitted with a microtip (Bronwill Scientific, Rochester, N.Y.), at a setting of 70 and the resulting fluid assayed for total radioactivity as described below. In all experiments, allowance was made for radioactive amino acids trapped in the interstices between cells by incubation of samples of re-suspended platelets with ^{14}C-carboxylic acid-inulin (specific activity 3·08 mCi/mg ; New England Nuclear Corp. Boston, Mass.) at 37° C for 5–20 minutes. The range of concentrations of amino acids studied was limited by low specific activity in the case of experiments with phenylalanine and DOPA.

Inhibition experiments

Platelets were incubated for 1 h in the presence or absence of 10^{-3}M dinitrophenol (DNP) plus 10^{-3}M iodoacetic acid (IAC) added in a total volume of 20 μl. Platelets were then incubated for a further 20 min in the presence of the appropriate radioactive amino acid in a concentration of 10^{-7}M. When experiments were made at 4° C, platelets were incubated for 20 min with 10^{-7}M ^{14}C-labelled amino acid in tubes resting in a bucket cooled with crushed ice.

In experiments where the membrane was disrupted ultrasonically, 1 ml samples of the re-suspended platelet medium were preincubated at 37° C for 5 min followed by several periods of 3–4 s sonification to a total time not exceeding 15 seconds. Then radioactive amino acid was added and 30 s thereafter the platelet debris centrifuged and debris and medium subjected to radiochemical assay, to determine the binding of amino acids onto platelet proteins.

Metabolism experiments

Re-suspended platelet solution (3 ml) was incubated for various times with ^{14}C-amino acid (see **Results**). Platelets were separated from the incubation medium as described above and the platelet pellet sonified in 0·1 ml H_2O containing 20 μg/ml of the appropriate unlabelled amino acid or 20 μg/ml of various known metabolites as chromatographic markers. The sonified material was centrifuged at 20,000 *g* for 3 min and 10–60 μl of the clear supernatant fluid was spotted quantitatively on to a thin layer chromatography (TLC) plate coated with 250 μ silica gel (H) (Analtech Inc., Newark, Delaware). In the experiments with tryptophan, the platelet pellet was sonified in acetone to extract any indole derivatives. The thin layer chromatography plates were developed in the following solvents (v/v): (1) phenylalanine and glycine; ethanol:water, 96:4; (2) tryptophan; ethyl acetate:2-propanol:ammonia (25% v/v), 45:35:20; (3) tyrosine; l-butanol: glacial acetic acid:water, 120:30:50; (4) γ-aminobutyric acid (GABA); phenol saturated with water. Dihydroxyphenylalanine (DOPA) was spotted on to a thin layer plate coated with 500 μ cellulose (Macherey Nagel) and developed in 1-butanol:ethanol:acetic acid (1 N), 35:10:10 (Johnson & Boukma, 1967). After development in the solvent, spots were identified with ninhydrin spray (Sigma, St. Louis Mo.), cut out and transferred to a counting vial. The remainder of the plate coating was cut up into 2 × 2 cm areas and placed in counting vials. Toluene–triton × 100 based scintillant (10 ml) (Boullin, Green & Price, 1972) was added and the radioactivity of each area measured with a Beckman LS 250 liquid scintillation spectrometer. Quench corrections were made by means of internal standards.

Drugs

We used radioactive amino acids with the following specific activities (SA). Glycine-^{14}C, 50 mCi/mmol; L-phenylalanine-^{14}C 25 mCi/mmol; L-tryptophan (methylene-^{14}C), 56·5 mCi/mmol; L-tyrosine-^{14}C, 344 mCi/mmol, L-3-(3,4-dihydroxyphenyl) alanine-3-^{14}C, 9·7 mCi/mmol, all obtained from Amersham/Searle, Arlington Heights, Ill., γ-aminobutyric acid 2C/mmol (New England Nuclear Corp, Boston, Mass.). Dinitrophenol and iodoacetic acid (Calbiochem, Inc., Los Angeles, California). All non-radioactive amino acids and metabolites were obtained from Mann Research Laboratories, New York.

Results

Accumulation of amino acids

We found that accumulation of all amino acids was very rapid and equilibrium platelet concentrations (C_i) were attained after 5–20 min incubation at 37° C; this was true for a range of medium concentrations (C_o). Uptake has been expressed in terms of the relationship between the platelet/medium concentration ratio (C_i/C_o) and time of incubation, where C_i is concentration of amino acid inside the platelet and C_o is concentration of amino acid in the medium, both at the end of incubation. C_i/C_o was low for both glycine (Fig. 1a) and GABA (Fig 1b) at all medium concentrations studied, suggesting that both compounds might be diffusing into the platelet. In contrast C_i/C_o for L-DOPA (Fig. 1c) and L-tryptophan (Fig. 1d) was greater than 10:1 when the initial concentration of both compounds in the medium was 10^{-6}M or less. The time-course of accumulation of L-phenylalanine (Fig. 2a) resembled that of DOPA or tryptophan; in each instance C_i/C_c at equilibrium was inversely related to C_o.

Fig. 1. (a)

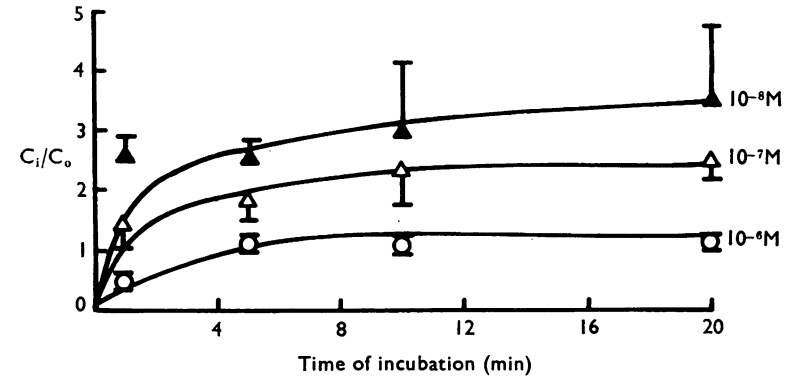


Fig. 1. (b)

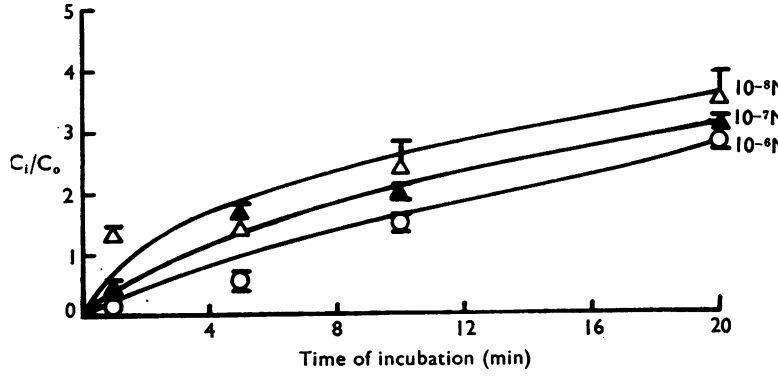


FIG. 1. Accumulation of glycine (a), γ-aminobutyric acid (b), L-DOPA (c), and L-tryptophan (d) by human blood platelets at various initial concentrations of amino acid in the incubation medium. Plot of tissue/medium concentration ratio (C_i/C_o) against time of incubation (min). Each point shows mean ± 1 S.E.M. of 3–4 determinations; each on a different subject.

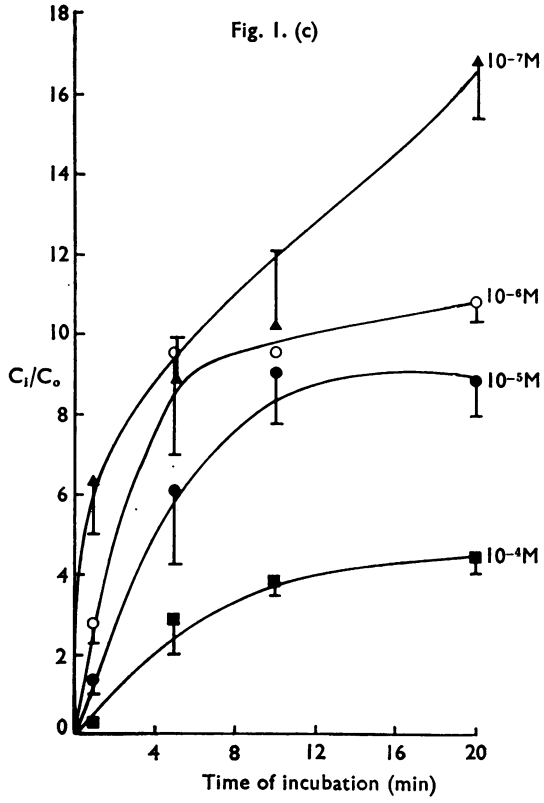


Fig. 1. (c)
10^{-7}M
10^{-6}M
10^{-5}M
10^{-4}M
C_i/C_o
Time of incubation (min)

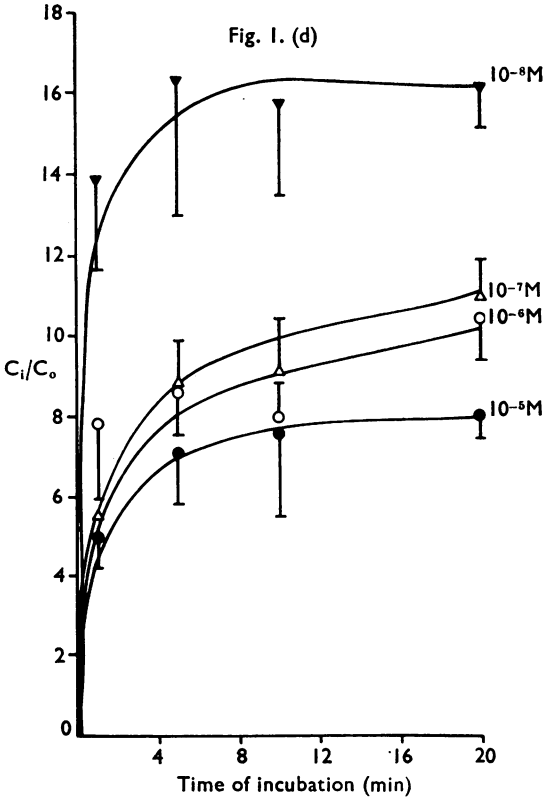
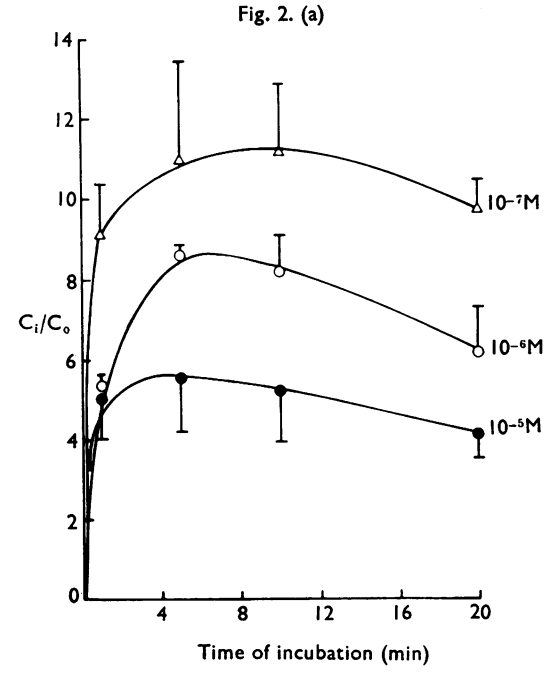


Fig. 1. (d)
10^{-8}M
10^{-7}M
10^{-6}M
10^{-5}M
C_i/C_o
Time of incubation (min)



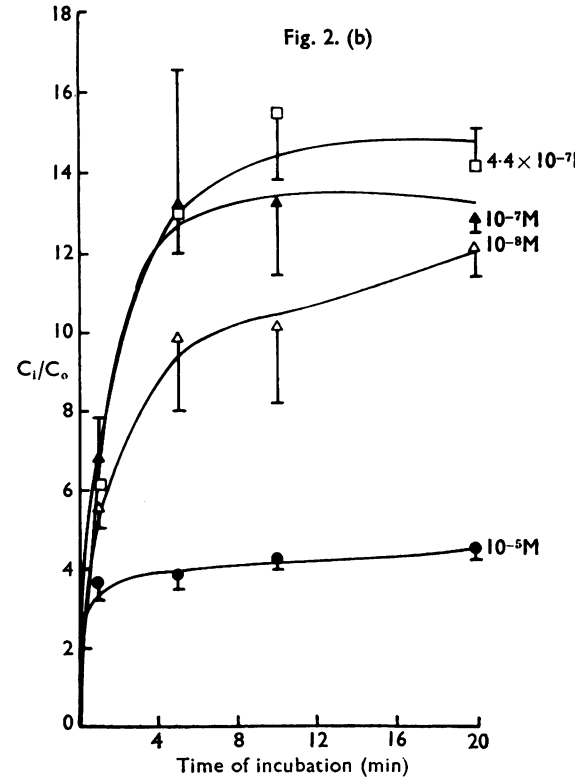


FIG. 2. Accumulation of L-phenylalanine (a) and L-tyrosine (b), by human blood platelets at various initial concentrations of amino acid in the incubation medium. Plot of tissue/medium concentration ratio (C_i/C_o) against time of incubation (min). Each point shows mean $\pm$1 S.E.M. of 3 to 4 determinations; each on a different subject.

Unlike the other amino acids C_i/C_o, for L-tyrosine uptake was not obviously related to the initial concentration of amino acid in the medium (Fig. 2b), accumulation being similar between $4{\cdot}4 \times 10^{-7}$M and 10^{-8}M. It appeared, therefore, that DOPA, phenylalanine and tryptophan were probably being accumulated by a saturable transport mechanism, but tyrosine accumulation involved some other process. Further evidence for these views was obtained by plotting the relationship between C_i/C_o at equilibrium (20 min) and log C_o. In the case of DOPA, tryptophan and phenylalanine C_i/C_o is inversely related to log C_o in a manner reminiscent of saturable transport mechanisms (Fig. 3a). With tyrosine and GABA there was no obvious relationship (Fig. 3b) and while glycine C_i/C_o did increase with decreasing medium concentrations, the ratio was very low and the significance of this result is questionable (Fig. 3b).

Mechanism of accumulation

To determine whether uptake was by energy dependent processes, we observed the effect of low temperature (4° C) or the metabolic inhibitors, dinitrophenol plus iodoacetic acid (both 10^{-3}M) on transport (see **Methods**). Low temperature

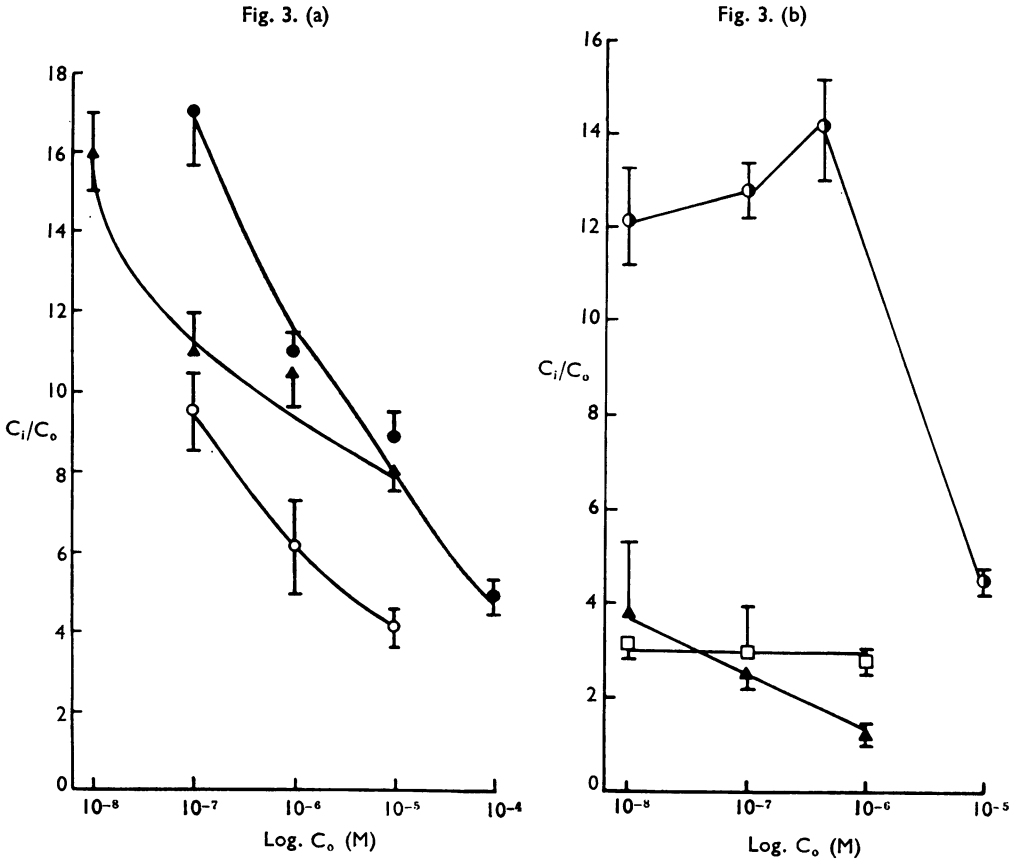


FIG. 3. Relationship between the equilibrium (20 min) tissue/medium concentration (C_i/C_o) of amino acid in human blood platelets and the log of the initial medium concentration of amino acid (log C_o). Figure 3a: ●, L-DOPA; ▲, L-tryptophan; ○, L-phenylalanine; Figure 3b: ◑, L-tyrosine; □, γ-aminobutyric acid; ▲, glycine. Each point shows mean ± 1 S.E.M. of 3 to 4 determinations; each on a different subject.

decreased the uptake of all amino acids except tyrosine (Table 1). The metabolic inhibitors produced a similar pattern of inhibition but in addition to tyrosine the uptake of GABA and glycine was also unaffected. In an attempt to obtain further information regarding the mechanisms of uptake, the resuspended platelet solution was subjected to sonification before addition of the amino acid (see **Methods**). The purpose of this was to fragment the plasma membrane and other subcellular structures. We supposed that if any of the amino acids were merely binding to platelet proteins, this would be detected after sonification, because particle-bound radioactivity would either remain unchanged or actually be increased. Following disruption of the membrane, glycine and GABA accumulation was completely abolished and that of tryptophan, DOPA and phenylalanine severely reduced (Table 1). Tyrosine accumulation on to the platelet debris, however, did not significantly change (Table 1).

Kinetics of amino acid accumulation

Our results indicated that tryptophan, phenylalanine and DOPA were accumulated by a saturable transport process. Therefore, we determined the kinetics of the uptake processes (K_m and V_{max}) making the assumption that they fulfilled the criteria for reactions described by Michaelis Menten for saturable enzyme/substrate interactions. Results are given in Table 2 and will be discussed.

TABLE 1. *Effect of incubation at 4° C, pre-incubation with dinitrophenol (DNP) and iodoacetic acid (IAc) or sonification on amino acid accumulation by human blood platelets*

Amino acid	Incubation for 20 min at 37° C	Incubation for 20 min at 4° C	Incubated 20 min at 37° C after 60 min preincubation with DNP/IAc (10^{-3}M)	Sonification for 15 s and incubation for 30 s
L-Tryptophan	10·9±1·1 (5)	4·3±0·9 (4)	5·3±0·4 (4)	2·4±0·3 (3)
L-DOPA	17·0±1·5 (3)	1·6±0·3 (4)	4·3±0·6 (4)	4·0±0·4 (4)
L-Phenylalanine	9·5±1·0 (4)	5·8±0·6 (4)	4·6±0·3 (4)	1·6±0·3 (3)
L-Tyrosine	12·9±0·6 (3)	10·4±1·5 (4)	11·6±1·6 (4)	10·2±1·4 (3)
Glycine	2·5±0·2 (4)	0·8±0·3 (4)	2·6±0·1 (4)	0 (3)
GABA	3·2±1·1 (3)	0·6±0·2 (4)	4·1±0·5 (4)	0 (3)

Platelets were incubated with one amino acid (10^{-7}M) for 20 min at 37° (Col. 2) or 4° (Col. 3). In the experiments with metabolic inhibitors, DNP+IAc (both 10^{-3}M) were added 60 min before the appropriate amino acid. When the platelets were fragmented by sonification (Col. 5) the amino acids were added 30 s before the centrifugation as described in **Methods**. Concentration of amino acids: 10^{-7}M in all experiments. Results expressed as mean % C_i/C_o±S.E.M. Figures in parentheses show number of experiments performed, each on a different subject.

TABLE 2. *Kinetics of amino acid uptake by blood platelets*

Amino acid	$K_m \times 10^{-6}$M	V_{max} (nmol/min)/10^{11} platelets)
L-Tryptophan	0·2±0·02	7·1± 6·4
L-Phenylalanine	7·7±3·3	131·6± 50·6
L-DOPA	25·0±0·0	303·0±103·0

Results (mean ± S.E.M.) were obtained from determinations on 4–6 subjects. Platelets were incubated with ^{14}C-amino acid for 5 min at concentrations used in the accumulation experiments.

Metabolism of amino acids by the platelet

The amount of unchanged ^{14}C-amino acid in the platelet at the end of a 20 min incubation was investigated. There was little metabolism after a 20 min incubation (Table 3). Since L-tyrosine gave a slightly lower recovery as unchanged amino acid than the other compounds we made further studies up to 120 minutes. The recovery of counts as authentic tyrosine decreased steadily during this time while counts at the ninhydrin-positive origin of the TLC plate increased, suggesting possible binding of this amino acid to soluble protein. Recovery of radioactivity of all amino acids added to the plate was good (Table 3).

Discussion

Our results with tryptophan, DOPA and phenylalanine suggest that these amino acids are accumulated by the human blood platelet by a mechanism that is saturable and energy dependent. Furthermore, the intact plasma membrane is necessary for accumulation since uptake was almost abolished when the membrane was disrupted. The fact that it was not completely abolished would suggest that some intracellular binding occurred and this may explain why uptake was only inhibited by about 80% with dinitrophenol and iodoacetic acid, since binding and passive diffusion would still be occurring in their presence.

The finding with glycine is in agreement with Zieve & Solomon (1968) who observed diffusion of glycine into the platelet. The low C_i/C_o ratio and the small accumulation of GABA is consistent with the fact that GABA is transported from plasma to brain only with difficulty (Roberts, Lowe, Guth & Jelinek, 1958). If both compounds diffuse into the platelet it is surprising to find that incubation in the cold decreases accumulation. However, exposure of the platelet to low temperatures leads to deformation of the outer membrane (Bull & Zucker, 1965) and this might well alter its permeability.

The mechanism of tyrosine accumulation is different from that of all other amino acids studied here and also arginine and leucine (Boullin, 1972 ; Boullin, Votavova & Green, unpublished). Tyrosine is accumulated rapidly against the concentration gradient, but transport cannot be inhibited by dinitrophenol and iodoacetic acid or by cold. Furthermore the fact that uptake is not concentration dependent (Figs. 2b & 3b) shows that accumulation is not by a saturable process. As fragmentation of the plasma membrane by ultrasound did not alter accumulation of tyrosine when the amino acid was added to the samples for an incubation period of only 30 s, this suggests that in the intact platelet the accumulation process involves cellular penetration by diffusion followed by rapid intracellular binding.

TABLE 3. *Recovery of unchanged ^{14}C-labelled amino acid in the platelet after 20 min incubation*

Amino acid	% Recovery of radioactivity as unchanged ^{14}L-amino acid	% Recovery of total radioactivity added to chromatography plate
L-Tryptophan	94	96
L-Phenylalanine	97	96
L-DOPA	87	82
L-Tyrosine	85	96
Glycine	89	95
GABA	98	89

Results expressed as mean of 2–3 determinations performed in duplicate on different subjects.

There was no evidence for formation of ^{14}C-DOPA or ^{14}C-noradrenaline from tyrosine, ruling out the possibility that low intracellular concentrations of amino acid were due to formation of metabolites and their subsequent efflux from platelets. The detection of increasing amounts of radioactive material at the ninhydrin-positive origin of the TLC plates with time of incubation is a further indication of binding of tyrosine by protein-like material. We have no information on the actual binding sites but radioautography might prove helpful in this regard.

Little previous work has been done on amino acid accumulation by the platelet. Zieve & Solomon (1968) studied glycine, glutamic acid, and γ-aminobutyric acid accumulation with media containing 10^{-3}M–10^{-5}M, but the present studies suggest that any active transport systems present might well be saturated at these concentrations. Cooper & Firkin (1970) showed transport of some amino acids into platelets, and subsequent incorporation into platelet protein but did not include the amino acids used here. Unfortunately, their results were reported as counts per minute accumulated or incorporated and it is therefore not known whether any metabolism took place.

The report of Warshaw, Laster & Shulman (1967) showing leucine accumulation by the platelet has been confirmed (Boullin, 1972 ; Boullin, Votavova & Green, unpublished) and we have shown that leucine accumulation resembles that described here for DOPA, tryptophan, and phenylalanine, being saturable, energy-dependent, and requiring the intact cell membrane. Whether all these compounds utilize a similar transport system will require further elucidation. Regarding tyrosine, the mechanism of accumulation by the platelet appears unique to this cell.

The role of the amino acids in platelet physiology is unknown at present, but as the various essential amino acids are present in platelets in high concentrations (Barber & Jamieson, 1970) and as protein synthesis occurs in platelets to some degree (Warshaw, Laster & Shulman, 1967 ; Boullin 1972 ; Boullin, Votavova & Green, unpublished), these substances may play a role in the synthesis of the contractile protein thrombasthenin (Booyse & Rafelson, 1967). Perhaps amino acid uptake regulates the rate of synthesis of the protein.

Our studies lead us to question the validity of the concept of the platelet as a neuronal model for studying accumulation and metabolism of amino acids. First, the accumulation of GABA and glycine by platelets is very different from neurones in that the platelet concentrates these amino acids only slightly, whereas the spinal cord, for example, accumulates glycine to high concentrations by active transport, and then stores it for use as a neurohumoral transmitter (Neal & Pickles, 1969). Second the mechanism of tyrosine accumulation is quite different from that observed in brain slices (Guroff, King & Udenfriend, 1961), or other tissues investigated to date, as it involves passive diffusion and intracellular binding in platelets rather than energy-dependent transport. Third, we find no evidence for transformation of DOPA, phenylalanine, or tyrosine to the appropriate catechol or indole amines.

Finally, the kinetics of transport of amino acids may be different for platelets and neurones. In general, the affinity of amino acids for platelet transport systems is higher than in neurones. Thus the K_m for tryptophan transport is lower in platelets ($2{\cdot}5 \times 10^{-7}$M ; Table 3) than in synaptosomes (1×10^{-3}M, Grahame-Smith & Parfitt, 1970), and the results of Blasberg (1968) indicate that the K_m of accum-

ulation of some amino acids in brain slices is in the range 10^{-4} to 10^{-3}M. On the other hand Logan & Snyder (1971) have recently found both low and high affinity uptake systems for aspartic and glutamic acids in the spinal cord and cerebral cortex of rats. As the K_m for aspartic acid transport into platelets ($1{\cdot}3 \times 10^{-4}$, Boullin, 1972) approximates to the K_m for the *low affinity* system of Logan & Snyder ($3{\cdot}7 \times 10^{-4}$M) and the K_m for platelet transport of glutamic acid is almost identical to the values for the *high affinity* system in cortex (both values approximately 4×10^{-5}M) (Logan & Snyder, 1971; Boullin, 1972) it is difficult to reach a conclusion on this matter.

Nevertheless we do feel that in general the platelet is not a suitable model for studying uptake of amino acids by neurones. However, there is much evidence to show that platelet accumulation of neurohumoral transmitter amines may have relevance to neuronal amine accumulation (Boullin & O'Brien, 1970, 1971; Boullin, Coleman & O'Brien, 1970) and this view is not invalidated by our current study.

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