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ABNORMALITIES OF 5-HYDROXYTRYPTAMINE UPTAKE AND BINDING BY BLOOD PLATELETS FROM CHILDREN WITH DOWN'S SYNDROME

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SUMMARY

1. Blood platelets from normal children and children with the trisomy 21 form of Down's syndrome (mongolism) were studied to determine the cause of the well established reduction in platelet 5-HT in the disease.

2. Concentrations of endogenous 5-HT in the platelets from mongols were 25·3 % of the concentrations found in normal children.

3. The net accumulation of 5-HT in the mongol cells was decreased to 52·7 % of normal. This reduction was probably due, in part, to a defect in 5-HT transport, because the initial rates of 5-HT uptake at plasma concentrations of 10^{-6} and 10^{-5} M were significantly slower.

4. Experiments on the efflux of 5-HT from mongol platelets loaded with amine showed that the rate of loss was initially 2·6 and later 7·8 times faster than normal.

5. Platelet ATP in mongol cells was 26 % of normal, and the reduction of ATP and 5-HT was in the molar ratio of 3:1.

6. It is considered that the low platelet 5-HT in Down's syndrome is due to a defective 5-HT transport mechanism and impaired 5-HT binding, resulting from a reduction in the essential binding substance, ATP.

INTRODUCTION

The possibility of using the human platelet as a model for the 5-hydroxytryptaminergic neurones in the brain has been considered recently by several workers (Pletscher, 1968; Page, 1968). The analogy is based on evidence that, although platelets do not synthesize 5-hydroxytryptamine (5-HT), platelets and neurones both possess similar mechanisms for accumulation of 5-HT, for storage of amine in electron-dense subcellular organelles (Davis & White, 1968) and for formation of the deaminated metabolites, 5-hydroxyindolylacetic acid and 5-hydroxytryptophol.

In cases of Down's syndrome blood 5-HT is reduced (Rosner, Ong, Paine & Mahanand, 1965; Tu & Zellweger, 1965; Jerome & Kamoun, 1967) and the muscular hypotonia is reversed following administration of oral 5-hydroxytryptophan (5-HTP) (Bazelon, Paine, Cowie, Hunt, Houck & Mahanand, 1967). We have investigated the uptake, binding, and release of 5-HT from platelets isolated from mongol children, with the view that, if the platelet is indeed a valid model for 5-hydroxytryptaminergic neurones, any changes observed in platelets would suggest that similar effects may occur within the brain, and that direct experiments using brain tissue should be the next step.

We report here defects in the uptake and binding of 5-HT by platelets from mongol children, accompanied by a reduction in platelet ATP. Some of these results have been reported at the July, 1969, meeting of the Physiological Society (Boullin, Coleman & O'Brien, 1969).

METHODS

Fifty mongoloid children of either sex, aged 3–16 yr, were subjects of the investigations, the majority being between 6 and 12 yr old. All were karyotyped as trisomy 21. Fifty normal school children of similar age were studied as controls, including normal siblings where possible.

Blood collection and platelet isolation was made by the method of Boullin & O'Brien (1969), using pipettes and other laboratory utensils made of polycarbonate. The packed platelet volume was determined with thrombocytocrits (Hardisty & Stacey, 1955); the number of platelets per ml. plasma was measured with a Model B Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida).

In all experiments involving studies of the accumulation of 5-HT by platelets, [3-^{14}C]5-HT creatinine sulphate (specific activity 56 mc/m-mole; Radiochemical centre, Amersham) was used. The final concentration of 5-HT in plasma at the beginning of incubation was 10^{-5} M. 1–3 ml. samples of platelet-rich plasma were incubated for 10 min at 37° C in 5 % CO_2 in O_2 before addition of 5-HT. Incubation was then contained for 5–120 min. 5-HT accumulation was stopped by cooling the incubation tubes to 2° C. The platelets were separated from the plasma by centrifugation at 20,000 *g* for 5 min at 2° C, or at 8000 *g* for 5 min if the cells were to be subsequently resuspended. Both centrifugation procedures removed more than 99·5 % of the platelets from the plasma. The resulting platelet-poor plasma was decanted and saved for radiochemical or spectrophotofluorimetric analysis (see below).

Traces of plasma remaining in the incubation tube were removed with a cotton-tipped applicator covered with a paper tissue. The platelet pellet was lysed either by freezing in solid CO_2 and thawing, or by ultrasonics using a Bronwill 'Biosonic II' Sonifier at a setting of 50 (Bronwill Scientific, Rochester, New York).

The platelet 5-HT content was determined by spectrophotofluorimetry (Bogdanski, Pletscher, Brodie & Udenfriend, 1956) and by liquid scintillation spectrometry.

In experiments involving measurement of the efflux of 5-HT, the cells were first incubated with 10^{-5} M-[^{14}C]5-HT for 90 min, and then separated from plasma by centrifugation at 8000*g* for 5 min. The platelet-poor plasma was decanted as described above. 0·05 ml. of 0·3 M disodium edetate/ml. plasma was added together with sufficient plasma to restore the plasma volume to the original value. The cells were

then resuspended at 2° C, by agitation on a Vortex mixer until no platelet aggregates were visible. In all experiments, allowance was made for the quantity of 5-HT trapped in the interstices between cells by incubation of samples of platelet rich plasma with [^{14}C]5-HT at 0° C or [^{14}C]carboxylic acid-inulin (specific activity 3·08 mc/g, New England Nuclear Corporation, Boston, Mass.) at 37° C for 5–120 min. The quantity of radioactivity recovered in the platelet pellet was taken as the 'trapped cell volume' content.

For ATP determinations, platelets were isolated and lysed by sonification as described above. ATP was determined in the platelet lysate by the method of Holmsen, Holmsen & Bernhardsen (1966).

RESULTS

Number and volume of platelets in mongols. In fifty mongol children, the number of platelets was just significantly greater than in controls, although the volume of cells/ml. plasma was not different (Table 1). The abnormalities to be described later in this paper cannot be accounted for by the differences in platelet count between normal and mongol children.

TABLE 1. Number, volume and 5-HT content of platelets from normal and mongol children

	Platelets/ml. plasma 10^8	μl. platelets/ml. plasma	n-mole 5-HT/10^{11} platelets
Normal	3·78 ± 0·14	6·34 ± 0·25	1064 ± 95
n	50	50	14
Mongol	4·10 ± 0·14*	6·68 ± 0·28	269·5 ± 18·2**
n	50	50	14

Values are the mean ± S.E. of mean of the number of observations (n) indicated. * $P < 0·05$ mongol *vs.* normal: ** $P < 0·01$ mongol *vs.* normal.

Endogenous 5-HT in mongol platelets. The 5-HT content of the platelets was reduced by 64% compared to normal children of the same age (Table 1). This confirms the earlier findings of Rosner *et al.* (1965); Tu & Zellweger (1965), Jerome & Kamoun (1967, 1968) and McCoy, Rostafinsky & Fishburn (1968).

Uptake of 5-HT by normal and mongol platelets. In our first experiments we incubated normal and mongol platelets with varying concentrations of 5-HT for up to 120 min. The increases in platelets 5-HT concentration when the initial plasma concentration was 10^{-5} M are shown in Fig. 1.

Accumulation reached equilibrium after about 90 min, as seen previously in normal platelets (Stacey, 1961), but the concentrations were much less in the mongol platelets than in normal cells. The net uptake of amine is given in Fig. 2; the platelet 5-HT concentration was less in the mongol platelets at all incubation times studied.

Initial rates of uptake were also calculated from results obtained by incubating platelets with 5×10^{-8}–10^{-5} M 5-HT for 5 min (Table 2). Comparable rates of uptake were obtained for both groups of platelets with the

lower plasma concentrations of 5-HT, but the rates were significantly slower in mongol platelets with the two higher concentrations.

These results suggest an abnormality in 5-HT uptake into mongol platelets at high plasma concentrations. Additionally, as the equilibrium platelet concentrations were greatly reduced, this might involve defects in 5-HT

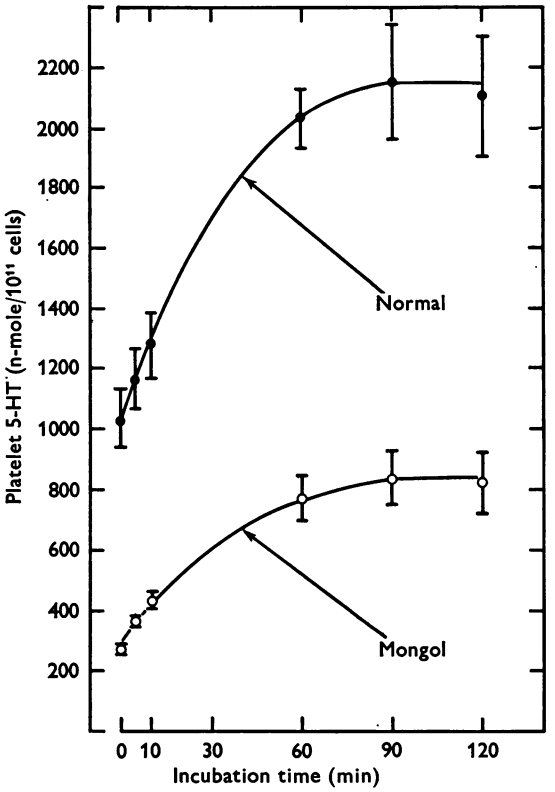


Fig. 1. 5-HT concentrations in normal (●) and mongol (○) platelets before (0 min) and after incubation (10–120 min) with 10^{-5} M-5-HT. Values are the mean ± S.E. of mean of four to twenty-four observations.

binding. Therefore, further experiments were designed to investigate this phenomenon.

Efflux of 5-HT from loaded platelets. One way of assessing the degree of 5-HT retention by platelets was to observe the efflux of amine from 5-HT-loaded cells. The following procedure was adopted. Platelets from normal and mongol children were loaded with 5-HT by incubation with 10^{-5} M [^{14}C]5-HT for 90 min. The cells were then resuspended in plasma without 5-HT. The loss of radioactivity due to resuspension alone was noted, and also the additional loss occurring during the re-incubation at 37° C for up to 2 hr. Determination of the specific activity of platelet 5-HT before and

after 2 hr incubation showed that there was no significant change (Table 3), and therefore indicated that there was no preferential loss of exogenous amine from either platelet type.

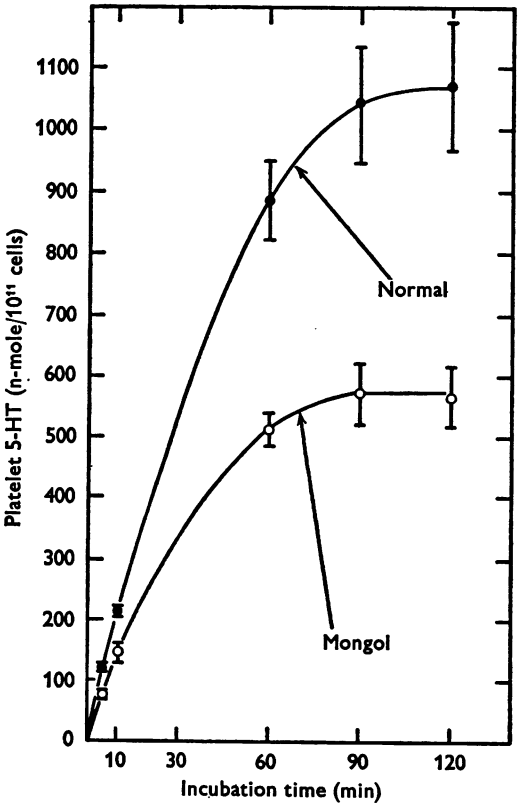


Fig. 2. Net accumulation of 5-HT by normal (●) and mongol (○) platelets incubated with 10^{-5} M-5-HT for 10–120 min. Values are the mean ± S.E. of the mean of four to twenty-four observations.

TABLE 2. Initial rates of uptake of 5-HT by normal and mongol platelets

Plasma 5-HT concentration (n-mole/ml.)	0·05	0·1	1·0	10·0
	n-mole/10^{11} cells.min			
Normal	0·34 ± 0·017	0·74 ± 0·05	7·27 ± 0·47	22·2 ± 1·05
n	13	13	12	8
Mongol	0·36 ± 0·025	0·73 ± 0·049	5·24 ± 0·35	14·4 ± 1·71
n	17	16	17	11

Results were obtained after incubation of platelets in plasma containing [^{14}C]-5-HT, 0·05–10·0 *n*-mole/ml., for 5 min. These values are the mean ± S.E. of the number of observations (*n*) indicated.

The efflux of 5-HT from normal and mongol platelets is shown in Fig. 3. In the case of the normal platelets there was clearly a biphasic pattern of efflux. The initial rate of loss was 30·0 n-mole/10^{11} platelets. hr for the first 70 min. Then the rate declined considerably to only 7·4 n-mole/10^{11} platelets. hr for the remainder of the experiment. Mongol platelets showed a different pattern of 5-HT loss, with little change in rate with time. During the first 15 min the rate was 79·6 n-mole/10^{11} platelets. hr (2·6 times faster than normal) and for the remainder of the experiment the rate was 59·8 n-mole/10^{11} platelets. hr; this was 7·8 times faster than the secondary rate of loss in normal cells.

TABLE 3. Effect of resuspension and 2 hr re-incubation in 5-HT-free plasma on the specific activity of platelet 5-HT

	5-HT specific activity (mc/m-mole)	
	Normal	Mongol
5-HT-loaded platelets	26·7 ± 1·7 (= 100 %)	35·4 ± 1·4 (= 100 %)
Resuspended platelets	97·8 ± 2·1 %	98·3 ± 1·8 %
Re-incubated platelets	96·3 ± 2·1 %	102·3 ± 1·9 %

The values shown, which are the mean ± S.E. of mean of twenty-four normal and twenty-four mongol observations, were obtained as follows: platelets were incubated with 10^{-5} M-[^{14}C]5-HT for 90 min and the specific activity of the platelet 5-HT was measured; these figures are given at the top of the Table, and are assigned values of 100 % for both normal and mongol experiments. Subsequent changes in specific activity of platelet 5-HT are expressed as a percentage (± S.E.) of the original values; resuspension was carried out as described in methods, and platelets were re-incubated after resuspension for 2 hr at 37° C, and then the final specific activity was determined in the platelet pellet obtained after centrifugation.

Berneis, DaPrada & Pletscher (1969) have recently demonstrated micelle formation when concentrated solutions of 5-HT and ATP are mixed *in vitro* in molar ratios of 2:1. In view of this observation and the much earlier work of Born, Ingram & Stacey (1958), who determined the concentration of these substances and suggested that 5-HT was bound to ATP in human platelets, we considered the possibility that the rapid efflux of 5-HT from mongol platelets resulted from a shortage of the essential binding material, ATP.

The experiments described below show this to be the case.

Platelet ATP. The normal platelet ATP content in our experiments with twelve subjects was 3·91 ± 0·08 μ-moles/10^{11} cells; the values are in the range found in earlier work (Born *et al.* 1958; Mills & Thomas, 1969). In thirteen mongols ATP was reduced to 39·6 % (1·55 ± 0·13 μ-mole/10^{11} cells). No change in ATP was noted in platelets which were first saturated with 5-HT by 90 min incubation with 10^{-5} M (Table 4).

The reduction in 5-HT and ATP in mongol platelets was in the molar ratio 1:3. The possible significance of this is considered below.

Relationship between 5-HT and ATP. Table 4 shows the ratio of ATP and 5-HT in unloaded and 5-HT-loaded platelets from normal and mongol children. The values obtained in adults by Born *et al.* (1958) are also included for comparison.

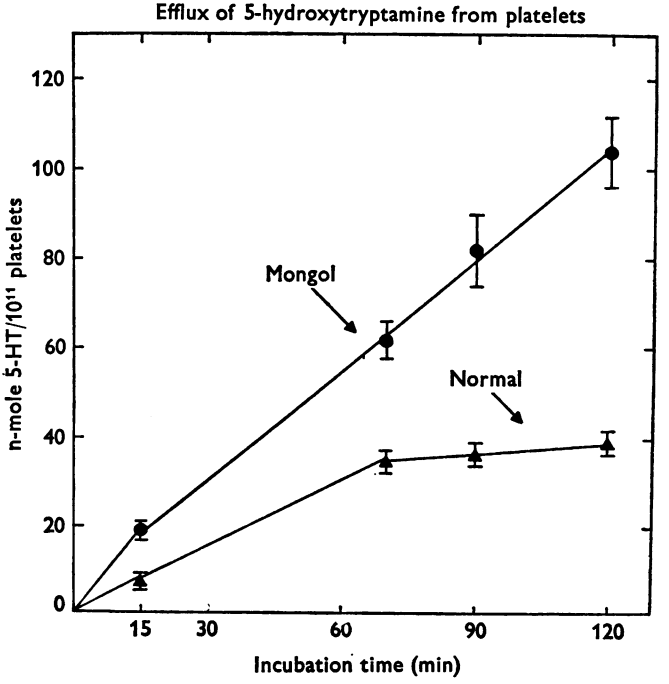


Fig. 3. Loss of total (endogenous + exogenous) 5-HT from platelets loaded with [^{14}C]5-HT by incubation with 10^{-5} M for 90 min as described in Methods. The results are expressed as n-mole 5-HT lost/10^{11} platelets from the loaded platelets after resuspension and re-incubation for 15–120 min in plasma at 37° C. All values are the mean ± S.E. of observations with platelets from twenty-four normal (▲) and twenty-four mongol (●) children.

We find in our experiments with human platelets that the amount of ATP is greater than the amount of 5-HT, whereas DaPrada & Pletscher (1968) found in rabbit platelets that 5-HT exceeded ATP by approximately 2:1. This difference is due to 5-HT rather than ATP, since it may be calculated from DaPrada & Pletscher's results (1968) that platelet ATP in rabbits is 8·8 μ-mole/10^{11} cells, compared with 3·9 μ-mole/10^{11} cells in normal human platelets reported here (Table 4). On the other hand, platelet 5-HT is 15·5 μ-mole/10^{11} cells in rabbits and only

1·06 μ-mole/10^{11} cells in normal children (Table 1). Thus platelet ATP in rabbits is double the human value, whereas 5-HT is 15 times greater. Our results show therefore, at least in man, that not all platelet ATP is involved in 5-HT binding, and agree with the conclusions of Born *et al.* (1958). Now, the ATP:5-HT ratio in rabbit platelets is 0·57:1 (DaPrada & Pletscher, 1968). From the results of Berneis *et al.* (1969) that 2 moles of

TABLE 4. Platelet ATP and ATP: 5-HT molar ratios in normal and mongol platelets before and after loading with 5-HT

	ATP (n-mole/10^{11} cells)	ATP:5-HT	
		Unloaded platelets	Loaded platelets
Normal children	3911 ± 80	3·67 ± 0·3	1·82 ± 0·17
Mongol children	1545 ± 132	5·72 ± 0·49	1·84 ± 0·18
Normal adults*	4340 ± 119	12·8 ± 1·1	3·2 ± 0·30

* Values calculated from the results of Born *et al.* (1958).

Figures are the mean ± S.E. of mean obtained in thirteen normal and twelve experiments. ATP:5-HT ratios obtained with unloaded platelets are based on the endogenous 5-HT content as given in Table 2; ratios for 5-HT loaded platelets are those obtained with platelets which had been incubated with 10^{-5} M-5-HT for 90 min as described in the text and shown in Fig. 1.

TABLE 5. Some possible relationships between ATP and 5-HT in platelets

	Normal	Mongol
Moles ATP required to bind 5-HT in 5-HT-loaded platelets	(*A*) 1072	420
'Excess' ATP	(*B*) 2839	1125
Ratio (*B*)/(*A*)	2·65	2·68

The values are theoretical calculations based on figures for platelet ATP and 5-HT given in Tables 1 and 4 and Fig. 1. The moles of ATP required for binding 5-HT are calculated on the basis that 2 mole 5-HT bind with 1 mole ATP, as has been shown experimentally *in vitro* (Berneis *et al.* 1969) and for rabbit platelets (DaPrada & Pletscher, 1968). The 'excess' ATP is calculated by subtraction of the above values from the figures for the platelet ATP content, as determined experimentally (Table 4).

5-HT are bound with 1 mole of ATP in rabbit platelets, the amount of ATP involved in 5-HT binding may be estimated for normal and mongol platelets. On the basis of calculations with 5-HT loaded cells, it can be seen that there is less 'excess' ATP in mongol than in normal platelets (Table 5).

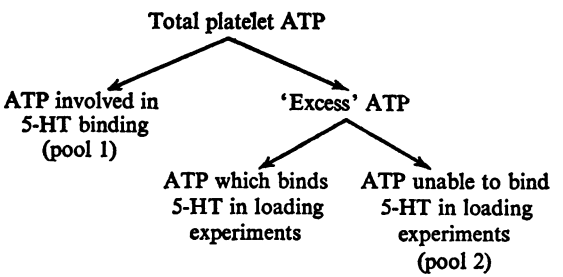
DISCUSSION

In agreement with earlier workers (see Introduction), we find a big reduction in endogenous platelet 5-HT in mongols with trisomy 21, and we think that the results described in this paper enable us to suggest a plausible explanation for this difference.

The reduced 5-HT concentrations found in platelets in Down's syndrome may be due to an abnormal uptake mechanism, plus excessive efflux resulting from shortage of ATP. Regarding uptake, Figs. 1 and 2 show that mongol platelets do not take up and bind 5-HT in the normal way, tissue concentrations being drastically reduced at all times after incubation with exogenous 5-HT *in vitro*. Direct evidence for an abnormality in the uptake mechanism is given in Table 2, which shows that the initial rate of uptake is reduced with plasma 5-HT concentrations of 10^{-6} and 10^{-5} M. It is likely that plasma concentrations of this magnitude may be attained in the circulation in the region of the enterochromaffin cells; from Toh's determinations of the 5-HT content of arterial and portal blood in the dog (1954), it may be calculated that the 5-HT content of plasma in the gastrointestinal tract was in the range 5×10^{-7}–8×10^{-7} M. Thus decreased uptake may be an important factor contributing to the low 5-HT content of mongol platelets.

The other critical factor may be excessive 5-HT efflux as a consequence of the deficiency in ATP for binding 5-HT. It does not appear to involve an abnormality in the actual binding process between ATP and 5-HT since the ratio of ATP:5-HT in loaded cells was the same for both mongol and normal platelets (Table 4). Our results indicate that platelet ATP can be divided into two compartments or pools viz. (1) ATP involved in 5-HT binding and (2) ATP with another function; previously Ireland (1967) and Holmsen, Day & Storm (1969) have produced direct evidence for two ATP pools.

Since the ATP content of normal platelets is not increased when the cells are loaded with 5-HT, and very little of the accumulated amine is subsquently lost (Boullin & O'Brien, 1969; this paper), some of the excess ATP normally present is able to bind 5-HT in loading experiments, as shown below:



The remainder of the 'excess' ATP does not associate with 5-HT in normal platelets saturated with the amine, and presumably has some other function. This is supported by Stacey's finding (1968) that ADP released ATP without releasing 5-HT. As there is less 'excess' ATP in mongol platelets compared to normal (Table 5), it seems that there is a shortage of ATP for 5-HT binding in mongols. The possibility of other abnormalities regarding ATP function is currently under investigation.

Can defects in 5-HT uptake and binding account completely for the low 5-HT content of mongol platelets? There may be at least two other contributory causes: defective synthesis in the enterochromaffin cells in the gastro-intestinal mucosa and increased metabolism by platelet monoamine oxidase. Studies of monoamine oxidase activity in mongolism show that it is impaired rather than increased: urinary 5-HIAA is decreased (Jerome, Lejeune & Turpin, 1960), and we find a decrease in the formation of 5-HT metabolites by mongol platelet monoamine oxidase. These results will be published in a subsequent paper (D. J. Boullin & R. A. O'Brien, unpublished observations). A greater possibility is defective synthesis of 5-HT in the gut. Saxl (1968) reported excess urinary tryptophan, and pathological changes in the enterochromaffin cells. The work of Jerome *et al.* (1960) can also be cited in support of defective synthesis.

Bearing in mind the similarities between platelets and neurones mentioned in the Introduction, the present findings make it worthwhile investigating the relationship between 5-HT and ATP in the mongol brain. Any deficiencies in the synthesis, binding or release of the potential neurohumoral transmitter may be expected to have a profound effect on brain function, and may be involved in the mental abnormalities of Down's syndrome.

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