

An assessment of platelet aggregation induced by 5-hydroxytryptamine

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SYNOPSIS A method of expressing platelet aggregation response after incubation relative to the response before incubation has been used in preference to using direct measurements, and some reasons are given for this choice.

The effect of pre-incubating human platelets with reserpine has been compared with the effect of pre-incubation with 5-hydroxytryptamine (5HT). Reserpine inhibited 5HT-induced aggregation more slowly than 5HT and on this basis their actions could be distinguished.

It was found that the aggregation response of human platelets to 5HT without pre-incubation (R) is inversely proportional to the natural 5HT content of whole blood, and an explanation has been suggested.

A decrease in the aggregation response of human platelets to 5HT without pre-incubation (R) with increasing age has been noted, and is commented upon with reference to the release of amines from blood platelets.

Platelets in plasma obtained from control subjects and incubated with methysergide and from migrainous patients taking methysergide both have reduced aggregation responses to 5HT.

Platelets incubated with clonidine (St155) do not have reduced aggregation responses. A potentiating effect was noted after 20 minutes' incubation, but platelets from migraine patients taking St155 behaved normally.

Tyramine affected the response of platelets to 5HT, giving results similar to those reported for reserpine.

The differences between the aggregation responses to 5HT of platelets from migraine patients and normal control subjects are discussed.

Part I After pre-incubation with 5-hydroxytryptamine and reserpine

The aggregation of platelets *in vitro* has been shown to take place under a number of varying conditions, and 5-hydroxytryptamine (5HT) is one substance capable of inducing aggregation. 5-Hydroxytryptamine is stored in platelets but O'Brien (1964) noted that pre-incubation with 5HT can both potentiate and inhibit 5HT-induced aggregation of human platelets and also that pre-incubation with reserpine for 30 minutes inhibited aggregation; recently Baumgartner and Born (1969) have observed in more detail similar effects using rabbit-platelet-rich plasma. The work reported here studies more closely the effects of the pre-incubation of human plasma with 5HT and reserpine and the influence

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of the natural 5HT content of the blood on platelet aggregation induced by 5HT.

Kloeze (1969) recently introduced a new method of recording platelet aggregation data as the ratio of the change in optical density under experimental conditions (after incubation) and that under baseline conditions (no incubation). A similar ratio, but one using the rate of change of optical density, has been employed in the studies recorded here and the usefulness of both ratios noted.

Method

Samples of human blood were obtained from normal control subjects, using disposable syringes, and

stored in heparin. Siliconized glassware was used except for the diluting pipettes and counting chambers and the test tubes for the measurement of aggregation which were disposable and of non-wettable plastic. The blood was centrifuged within one hour of collection between 800 and 900 rpm for 10 minutes exactly and the resulting plasma divided into 1 ml aliquots and used within four hours of collection. The platelets were counted in the blood and plasma, using Lempert's modification of Kristenson's method. Further samples of heparinized whole blood for 5HT determinations, using the method of Ashcroft, Crawford, Binns, and MacDougall (1964), were kept at -20°C immediately after collection.

Aggregation was followed essentially by the turbidometric methods of Born (1962) and O'Brien (1962) using an EEL platelet aggregation meter. Plasma (1 ml) in a plastic test tube was stirred at a fixed rate (70 of the scale, approximately 2,000 rpm) by a vertical rod. The aggregation of the platelets was accompanied by a decrease in the optical density of the plasma which was recorded automatically on a Honeywell chart recorder. The optical density of distilled water was recorded daily and adjustments made, if necessary, to maintain constant conditions.

The plasma was kept at 37°C in the aggregation meter for one to two minutes before the 5HT was added and the stirrer lowered simultaneously into the plasma. For incubation periods of up to five minutes the incubating agent was added to the plasma in the apparatus, but for longer incubations the plasma was kept in a water bath at 37°C .

Data recorded in the tables and figures give the mean results from 12 different samples of plasma, each from a different subject. Plasma samples from 142 control subjects were used in 180 incubation experiments, some samples being used for more than one incubation time.

Materials

The following solutions were prepared daily at the strengths shown in each 0.1 ml used, stored at 4°C and delivered into 1 ml plasma:

5HT (serotonin creatinine sulphate, B.D.H.)
 5×10^{-8} mol
 Reserpine (2.5 mg, Boots) .. 2×10^{-9} mol

Measurement of Results

Aggregation in general was measured as (a) the maximum rate of aggregation, and (b) the maximum height of aggregation, both being recorded on the chart paper and corrected to a platelet count of 250,000 per cmm.

The following formulae were calculated, whether 5HT or reserpine was used as incubating agent:

- (1) R_i/R where R_i represents the maximum rate of aggregation after incubation; R represents the maximum rate of aggregation before incubation.
- (2) H_i/H where H_i represents the maximum height of aggregation after incubation; H represents the maximum height of aggregation before incubation.

Results

5HT PRE-INCUBATION

In a series of experiments varying the times of pre-incubation with 5HT between 5 sec and 20 min, the aggregation results were plotted as R_i/R and H_i/H , as seen in Figure 1. The ratios are greater than 1 for short incubation times and show that potentiation of the aggregation response has occurred as a result of pre-incubation. For pre-incubation times longer than 30 sec the aggregation response is inhibited and the very small values of R_i/R noted at 5 min were due to an unexpected increase in optical density in some plasmas. Results for aggregation depending on the height recorded were always greater than those based on rate since each unit of incubating or aggregating agent lowers the optical density of the

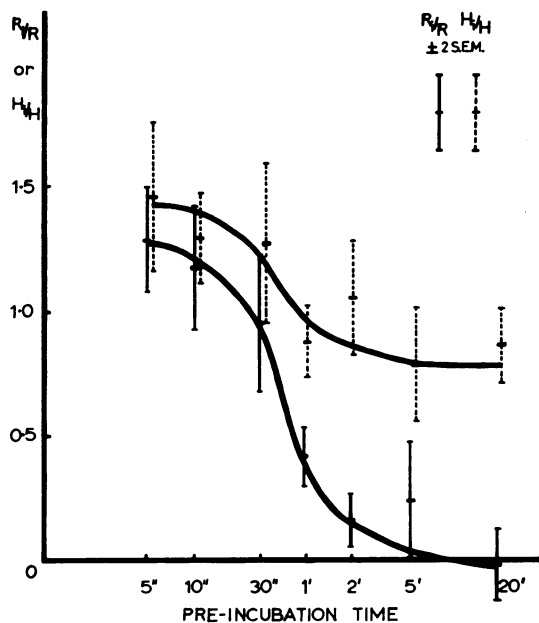


Fig. 1 Aggregation after pre-incubation with 5HT measured relative to aggregation without pre-incubation as R_i/R and H_i/H .

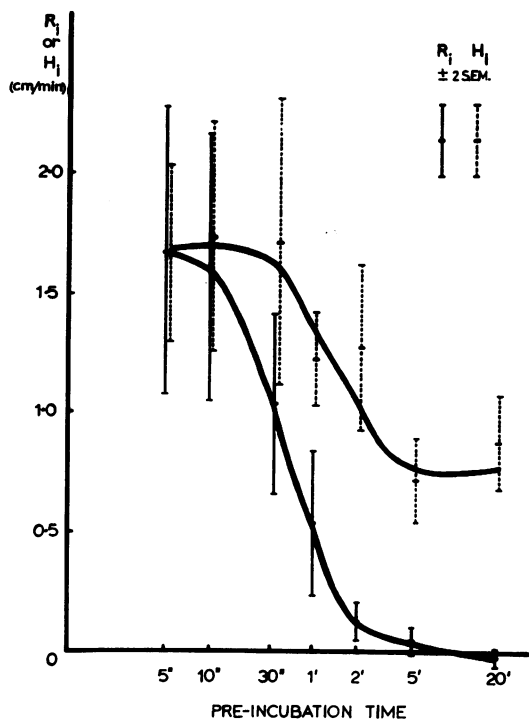


Fig. 2 Aggregation measured after pre-incubation with 5HT as R_1 and H_1 .

plasma before aggregation occurs but rate varies only with aggregation.

When R_1 and H_1 were used to measure aggregation, curves were obtained similar to those for R_1/R and H_1/H as can be seen in Figure 2. The numerical values obtained for aggregation without incubation were in close agreement with those from a preliminary series of experiments, eg, the response to 5HT expressed as R was found to be 1.21 ± 0.16 ($\pm 2 \times$ standard error of mean) for 142 samples of plasma and in the preliminary series had been found to be 1.20 ± 0.36 for 28 samples.

	Incubation	
	Five Seconds	One Minute
R	1.35 ± 1.58	1.19 ± 1.18
R_1	1.68 ± 2.12	0.54 ± 1.02
R_1/R	1.29 ± 0.74	0.42 ± 0.40
H	1.40 ± 1.54	1.49 ± 1.14
H_1	1.67 ± 1.30	1.23 ± 0.70
H_1/H	1.46 ± 1.02	0.88 ± 0.53

Table I Results of 5HT pre-incubation¹

¹Mean result ± 2 standard deviations
For explanation of the symbols see the text.

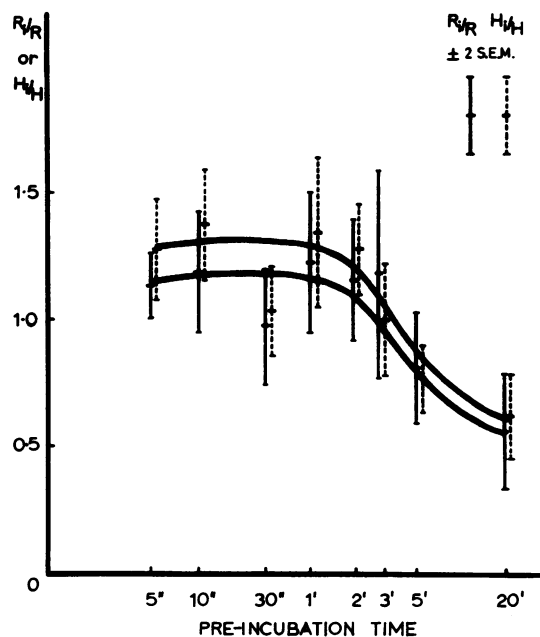


Fig. 3 Aggregation after pre-incubation with reserpine measured relative to aggregation without pre-incubation as R_1/R and H_1/H .

For all the results obtained complete calculations with standard deviations have been made at 5 sec and 1 min incubations, as in Table I, where it can be seen that the ratio method has several advantages. In correcting the aggregation response after incubation for the response before incubation, the standard deviations are less than for other comparable results. Each ratio discriminates thus: if no potentiation or inhibition has occurred, R_1 and R are equal (or H_1 and H are equal), but if potentiation has occurred the ratio is greater than 1, if inhibition is less than 1.

RESERPINE PRE-INCUBATION

The results of pre-incubation with reserpine are seen in Fig. 3, which shows that there is a period of initial potentiation lasting about 3 min using R_1/R or 5 min using H_1/H , rather than 30 sec following 5HT incubation. Even after incubation periods as long as 20 min inhibition is less pronounced than with 5-hydroxytryptamine.

The variance of the results following 5HT and reserpine incubations was analysed statistically and it was found, whether R_1/R or H_1/H was used to measure aggregation, that the incubations gave rise to trends which are significantly different at the level P , less than 0.001.

A study of the curves in Figs. 1 and 3 reveals that individual observations from the two types of incubation could be distinguished if values at 5 sec and 1 min incubations were compared, as in Table II.

Pre-incubation	Five Seconds \pm 2 SEM	One Minute \pm 2 SEM
5HT	1.29 \pm 0.21	0.42 \pm 0.12
Reserpine	1.14 \pm 0.13	1.23 \pm 0.29

Table II Values for R_1/R after 5HT and reserpine pre-incubations¹

¹Each mean result is derived from 12 different plasmas, as described in the method, and is shown here with twice the standard error of the mean.

There was close agreement between results from males and females, as can be seen from Table III, which shows the overall mean values for all the observations measured against each criterion of aggregation. The individual points were drawn on scatter diagrams of aggregation response against age and 5HT content of blood but did not reveal any trend which was masked within the overall mean values.

Criterion of Aggregation	Incubation	Males	Females
R	5HT + reserpine results	1.16 (72)	1.15 (98)
H	5HT + reserpine results	1.42 (72)	1.56 (98)
R_1/R	5HT	0.45 (35)	0.49 (49)
R_1/R	Reserpine	0.94 (43)	0.95 (50)
H_1/H	5HT	1.02 (35)	1.04 (48)
H_1/H	Reserpine	1.09 (42)	0.95 (51)

Table III Means of results from males and females where aggregation was measured under different criteria¹

¹The number of observations contributing to each result is shown in brackets.

A tendency towards a decrease in the rate of aggregation (R) with increasing age was observed as shown in Fig. 4, giving the variation with decades. Results from subjects aged 20 or less, or more than 70, have been ignored in this diagram, since few were available. This affect is less pronounced using aggregation criteria other than R. No change in blood 5HT content with increasing age, nor consistent change in platelet count up to the seventh decade, was noted. No correlation was observed between aggregation after incubation (expressed as R_1/R) and age for 5HT and reserpine incubations.

The 5HT content of whole blood was determined for 105 control subjects and it was seen that the baseline response (R) of the platelets to 5HT appeared inversely proportional to the 5HT content

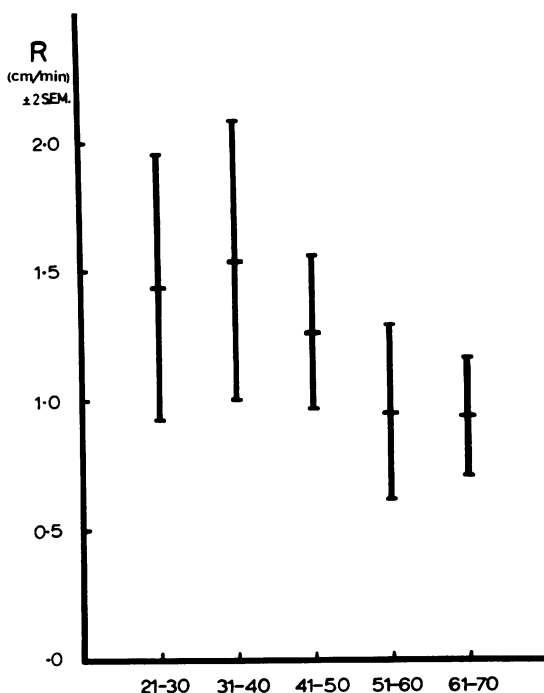


Fig. 4 Aggregation response (R): the number of observations contributing to the mean results for the decades in the order shown is 31, 21, 29, 27, and 22.

corrected to a value for 250,000 platelets per cmm (Fig. 5). This showed a correlation coefficient of -0.57 , indicating a probability of this relationship occurring randomly at a figure less than 0.001. This trend agrees well with results found in the preliminary series of 35 patients where the 5HT content was expressed as weight per millilitre of whole blood (Fig. 6).

Discussion

The results obtained following pre-incubation with 5HT are in agreement with those published elsewhere (O'Brien, 1964; Baumgartner and Born, 1968; Baumgartner, 1969) and support Born's suggestion that the mechanism for the transfer of 5HT into the platelet is involved in 5HT-induced aggregation. It is thought that during 5HT incubation sites on the platelet membrane are filled by 5HT from the external medium, and, as can be seen from the results reported here, for pre-incubation longer than 30 sec, aggregation by 5HT is inhibited. Even longer pre-incubation times lead to greater inhibition and after 5 min and 20 min the degree of aggregation is very small and in some plasmas an increase in

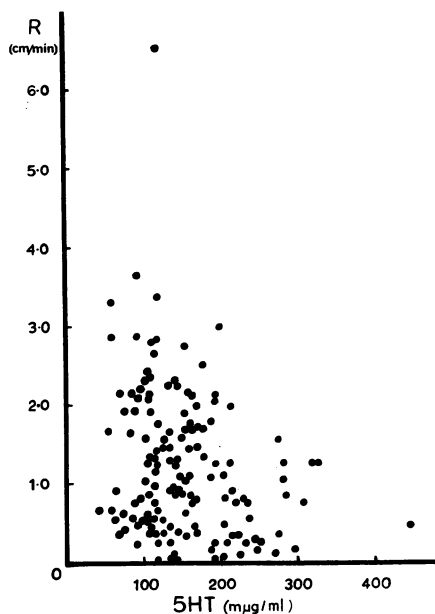


Fig. 5 Aggregation response (R): blood 5HT content (μg) measured per millilitre of blood corrected to a platelet count of 250,000 per cmm.

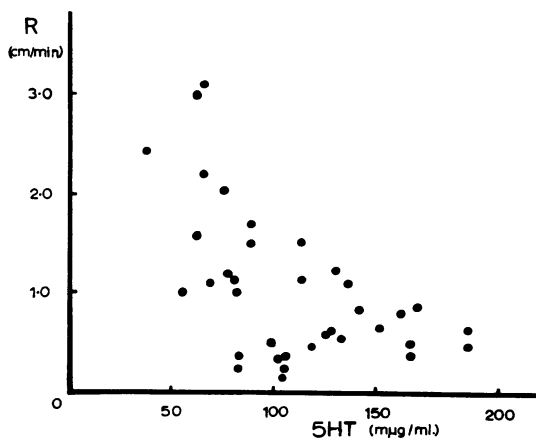


Fig. 6 Aggregation response (R): blood 5HT content (in μg) measured per millilitre of blood (from a preliminary series of experiments).

optical density was observed, which might be explained as disaggregation of loose platelet aggregates formed during pre-incubation.

During pre-incubation with reserpine 5HT is released from within the platelet. Once outside the platelet, the 5HT is presumably free to occupy sites on the platelet membrane and to influence aggregation. Baumgartner (1969) has shown that after pre-incubation with reserpine, aggregation will occur spontaneously and O'Brien (1964) that the pre-incubation with reserpine for 30 min inhibits 5HT-induced aggregation.

However, from the results reported here it can be seen that no inhibition occurs for at least three minutes, presumably this time being required to release a necessary quantity of 5HT from platelet sites. For an addition of reserpine equal to that used here, Pletscher (1968) has shown that the 5HT content of the platelets is lowered by about 22% in 30 minutes. Such an amount released into the plasma is far less than that added as incubating agent and after release from the platelet some 5HT is converted into metabolites and some remains unchanged (Bartholini, Pletscher, and Bruderer, 1964). This may explain why the inhibition was never found to be as pronounced as after incubation with 5-hydroxytryptamine.

The results recorded here have shown that the aggregation response without pre-incubation (R) of human platelets to 5HT is inversely proportional to the natural 5HT content of the whole blood. Nearly all of the 5HT carried in the blood is contained in the platelets (Hardisty and Stacey, 1955) and platelet-free plasma contains only traces of 5HT (Humphrey and Jaques, 1954). It is suggested that under normal, physiological conditions the number of transfer sites for 5HT on the platelet membrane which are occupied is proportional to the 5HT content of the platelets. Thus, a high 5HT content is associated with many occupied sites and the aggregation response would be low and, further, a low 5HT content would imply many vacant sites and a corresponding readiness to aggregate.

It is interesting to speculate on the decrease in aggregation response without pre-incubation (R) noted with increasing age. Curzon, Barrie, and Wilkinson (1969) have suggested that amines are less readily released by agents such as reserpine from the body stores of older rather than younger migraine patients. In the present series of subjects, no variation in blood 5HT content was noted with increasing age, so it might be suggested, also, that the 5HT receptor sites on the membranes of platelets from older patients are less free to participate in aggregation, which may be concomitant with some change in the releasability of amines.

Part II In plasma from control subjects and migraine sufferers in relation to methysergide, St155, and tyramine

The 5HT content of plasma falls during a migraine attack (Curran, Hinterberger, and Lance, 1965; Anthony, Hinterberger, and Lance, 1967). Methysergide is a 5HT antagonist and its usefulness in controlling migraine has been reported by Curran and Lance (1964), Graham (1964), and more recently by Lance, Anthony, and Somerville (1970).

Platelet aggregation induced *in vitro* by 5HT varies inversely with the natural 5HT content of the blood and is affected by pre-incubation with 5HT, as shown in part I of this paper. The influence of methysergide on the 5HT-induced aggregation of platelets has not been studied in detail although O'Brien (1964) showed that pre-incubation of plasma with methysergide for 30 minutes inhibited subsequent aggregation. In the work reported here the 5HT-induced aggregation of platelets in plasma from normal control subjects, pre-incubated with methysergide, has been compared with that of platelets in plasma from migrainous subjects taking methysergide (Deseril).

Clonidine (St155) has recently been used successfully in the treatment of migraine (Wilkinson, 1969) and its effect on the aggregation of platelets by 5HT is also reported here.

Tyramine will induce migraine-like headaches in susceptible persons (Hanington, 1967) and the effects of pre-incubating plasma with tyramine have been compared with earlier results concerning reserpine.

Method

The platelet aggregation tests and whole blood 5HT determinations were carried out as described in part I of this paper.

Blood samples were obtained from three groups of migraine patients who had not suffered headaches for at least three days: (1) 21 female patients (ages 15-57) who had not taken drugs or medication for at least three days; (2) 15 female (ages 18-60) and four male (ages 29-49) patients taking methysergide maleate, from 2-8 mg per day, and no other medication; (3) 13 female patients (ages 36-81) taking St155, from 50-100 μg per day, and no other medication. It has already been shown that in normal control subjects sex does not influence aggregation responses to 5-hydroxytryptamine.

Materials

The following solutions were prepared daily at the strengths shown in each 0.1 ml used, stored at 4°C, and delivered into 1 ml plasma:

5HT	5×10^{-8}
(serotonin creatinine sulphate, B.D.H.)		
Methysergide maleate (Sandoz)	..(1)	8.6×10^{-10} mol
	(2)	4.3×10^{-9} mol
St155 (Boehringer Ingelheim)	..(1)	3.5×10^{-10} mol
	(2)	1.8×10^{-9} mol
Reserpine (Boots)	2×10^{-9} mol
Tyramine hydrochloride (Sigma)	..	3×10^{-7} mol

The amounts of the methysergide maleate (2) and St155 (1) used were in the same ratio as the methysergide maleate (4 mg) and the St155 (50 μg) employed clinically: similarly the amounts of reserpine (2.5 mg) which, when injected intramuscularly, induced migraine-like headaches in migraine sufferers (Kimball and Friedman, 1961) and tyramine (100 mg) which, when taken orally, induces migraine-like headaches in those sufferers with dietary migraine (Hanington, 1967).

Measurement of Results

Aggregation was measured as described in part I.

Results

EXPERIMENTS WITH PLASMA FROM NORMAL CONTROL SUBJECTS

In a series of experiments varying the times of pre-incubation of plasma with methysergide and with St155 between 5 sec and 20 min, the following results were obtained for 5HT-induced aggregation, expressed as R_1/R . The results were compared with those obtained after 5HT pre-incubation, as shown in Table IV.

Methysergide at the stronger concentration is very effective in inhibiting 5HT-induced aggregation but at the lower concentration approximately 50% inhibition is achieved at the incubation times used. St155 in both concentrations does not inhibit aggregation at the incubation times shown but apparently potentiates it after 20 minutes.

Incubation Times	$R_1/R \pm 2$ Standard Errors of Mean				
	5HT ^a		Methysergide		St155
	5×10^{-8} mol	8.6×10^{-10} mol	4.3×10^{-9} mol	3.5×10^{-10} mol	1.8×10^{-8} mol
5 sec	1.29 ± 0.21 (12)		0.54 ± 0.21 (12)		
1 min	0.42 ± 0.12 (12)		0.13 ± 0.11 (12)	0.92 ± 0.28 (12)	1.09 ± 0.22 (6)
5 min	0.24 ± 0.24 (12)	0.52 ± 0.21 (12)	-0.05 ± 0.24 (6)	1.25 ± 0.40 (6)	1.18 ± 0.40 (6)
20 min	-0.023 ± 0.15 (12)	0.51 ± 0.21 (6)		2.53 ± 0.87 (6)	2.04 ± 0.60 (6)

Table IV Effects of pre-incubations with 5HT, methysergide, and St155 on platelet aggregation induced by 5HT¹¹The number of observations contributing to each result is shown in brackets.²See Fig. 1, part I of this paper.

The effect of pre-incubation with tyramine on the subsequent aggregation of platelets by 5HT was compared with the effect of reserpine, and the similarities between the aggregation results are seen in Table V.

Incubation Times (min)	Reserpine ^a	Tyramine
	2×10^{-8} mol	3×10^{-7} mol
1	1.23 ± 0.29 (12)	1.02 ± 0.13 (12)
5	0.81 ± 0.22 (12)	0.64 ± 0.16 (12)
20	0.56 ± 0.23 (12)	0.50 ± 0.22 (12)

Table V Effect of pre-incubation with reserpine and tyramine on platelet aggregation induced by 5HT¹

The number of observations contributing to each result is shown in brackets.

¹ $R_1/R \pm 2$ standard errors of mean²See Fig. 1, part I of this paper.

EXPERIMENTS WITH PLASMA FROM MIGRAINE PATIENTS

The platelet aggregation responses and blood 5HT

levels of migraine patients taking (a) methysergide and (b) St155 were compared with those of normal control subjects and of migraine patients who had been free of headache and medication for at least three days, and are shown in Table VI. The aggregation response without pre-incubation (R) and that after one min pre-incubation with 5HT (R₁) were measured.

It is seen that migraine patients free of headaches and drugs for at least three days gave high values for aggregation responses after pre-incubation with 5HT. Patients on methysergide consistently gave much lower values for R and R₁. The blood 5HT values were slightly higher than those of control subjects. Little change was seen in the platelet aggregation in plasma from patients on St155.

It has previously been shown in part I that the rate of aggregation induced by 5HT without pre-incubation is inversely proportional to the 5HT content of the whole blood for normal control subjects, but no such correlation was apparent for migraine patients free of headache and drugs, or those on methysergide or St155, as shown in Figure 7.

	Control Subjects ¹	Migraine Subjects		
		(I) No Drugs and No Headaches	(II) On Methysergide	(III) On St155
R (cm/min) ± 2 SEM	1.21 ± 0.16 (180)	1.51 ± 0.46 (21)	0.46 ± 0.11 (19)	1.03 ± 0.38 (13)
No incubation				
R ₁ (cm/min) ± 2 SEM	0.54 ± 0.30 (12)	1.34 ± 0.51 (15)	0.29 ± 0.11 (14)	
One min incubation with 5HT				
R ₁ /R One min incubation	0.42 (12)	0.89 (15)	0.64 (14)	
Mean blood 5HT content per ml of whole blood corrected to a platelet count of 250,000 per cmm (mμg)	158 (142)	172 (19)	180 (14)	144 (13)

Table VI Aggregation responses of platelets to 5HT in plasmas from control and migraine subjects²¹See Figs. 2 and 5 in part I of this paper.²The number of observations contributing to each result is shown in brackets.

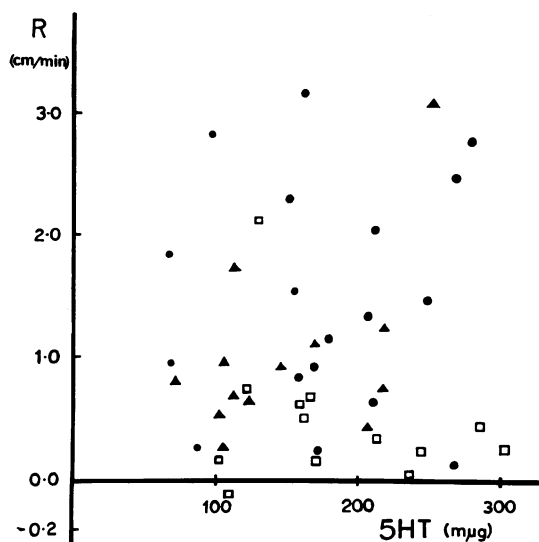


Fig. 7 Aggregation response (R): blood 5HT content (in μg) measured per millilitre of blood corrected to a platelet count of 250,000 per cmm for migraine patients not on drugs and migraine patients taking methysergide and St155.

● = migraine patients not on drugs; □ = migraine patients taking methysergide; ▲ = migraine patients taking St155.

Discussion

The theory accounting for the effect of 5HT on platelet aggregation (Baumgartner and Born, 1968) suggests that transfer sites for 5HT through the platelet membrane are involved in 5HT-induced aggregation. It is thought that during 5HT incubation sites on the platelet membrane are occupied by 5HT and, after a short incubation, aggregation by 5HT is inhibited.

Pre-incubating plasma from normal control subjects with methysergide effectively inhibits 5HT-induced aggregation and the results from migraine patients taking methysergide show a similar pronounced reduction in aggregation response. It is possible that methysergide, a 5HT antagonist, is acting at the level of the platelet membrane preventing the normal aggregation response to 5HT. If methysergide is acting at or near the suggested 5HT sites, it does not seem to interfere with the uptake of 5HT since the mean blood 5HT content of the group of migraine patients taking this drug was very slightly greater than the mean values for control subjects and migraine sufferers not on methysergide, while Curran and Lance (1964) found normal blood 5HT levels in patients taking methysergide.

The treatment of migraine patients with St155 does not seem to affect the behaviour of their blood platelets to 5HT, and further, no evidence was seen of a potentiation of aggregation response similar to that noted after 20 minutes' incubation *in vitro*. It is probable that the action of St155 is quite different, for Zaimis and Hanington (1969) have shown that this substance reduces the responsiveness of blood vessels to either vasodilatation or vasoconstriction.

Pre-incubation with tyramine affects 5HT-induced aggregation, giving results very similar to those after pre-incubation with reserpine. The results with reserpine have been attributed to the release of 5HT from storage sites within the platelet (Baumgartner, 1969); tyramine releases 5HT from platelets (McLean, Nicholson, and Hertler, 1963; Bartholini and Pletscher, 1964) and this is the most likely explanation for the effect of tyramine on 5HT-induced aggregation. Tyramine is also said to release catecholamines from platelets but this would potentiate aggregation (Thomas, 1968; Baumgartner, 1969). It is possible that after ingestion of tyramine by a susceptible migraine sufferer the release of 5HT from platelet stores is an important factor leading to the headache.

The behaviour of platelets from migraine subjects not on drugs differed from that of control subjects in two ways. First, they were readily aggregated by 5HT after one minute's incubation (shown by high R_1 values) and secondly the aggregation response to 5HT before incubation (R) showed no inverse correlation with blood 5HT content, this lack of correlation being noticed also in migraine patients taking drugs. If the aggregation response of the platelets to 5HT is dependent on the state of sites for the transfer of 5HT through the platelet membrane, this might suggest that transfer sites on the membranes of platelets from migraine sufferers differ from those of normal control subjects.

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