Aggregation of Blood Cells by 5-Hydroxytryptamine (Serotonin)

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A rapid, reproducible method of determining the presence and relative amounts of adhesive and aggregated blood elements in the blood has recently been developed in this laboratory.\(^1\)\(^2\) The method involves measuring the pressure required to force blood at a constant rate through a standardized screen with multiple openings 20 micra square and 20 micra deep. This pressure has been named "screen filtration pressure." During storage of blood\(^1\) and during exsanguination,\(^2\) platelets and leucocytes form aggregates which cause remarkable increases in this pressure. It has also been observed that adenosine diphosphate, a component of platelets, causes platelets suspended in plasma to aggregate,\(^3\) and that monoamine oxidase inhibitors inhibit platelet aggregations.\(^4\) These observations have led to an interest in the composition of the platelets, and to further studies of the effects of certain components of platelets on the suspension stability of blood. One of these constituents, 5-hydroxytryptamine (serotonin), has become particularly interesting in this respect.

Serotonin exerts a strong contractile influence on certain smooth muscles, including the muscular layers of small blood vessels. In this paper, we wish to describe another striking effect of small amounts of 5-hydroxytryptamine—a very significant in vitro and in vivo adhesiveness and aggregation of platelets, leucocytes, and red blood cells resulting in a high screen filtration pressure, and slowing to cessation of circulation in the conjunctival blood vessels.

Methods

Thirty dogs (12 kg to 25 kg), three cats, three rabbits, and blood samples from nine human beings were used. All experimental animals were maintained under pentobarbital anesthesia from beginning to end of the experiments. Three human beings were under surgical anesthesia, the remaining six were not anesthetized. Plastic cannulas were inserted into one or both femoral arteries to the lower end of the abdominal aorta; or glass cannulas, with short rubber tubes attached, were tied into the femoral arteries in various animals. In the dogs, plastic cannulas also were inserted into one femoral vein to the inferior vena cava. Blood samples were taken from these cannulas as follows: 5 ml of blood were withdrawn to remove stagnant blood from the cannula. The sample of blood for analysis was then withdrawn, after which the first 5 ml of blood were reinjected into the dog. Finally, the cannula was flushed with 2 ml of saline and the tip of the cannula washed free of all blood with saline. During most experiments the animals were given an initial dose of 15 mg/kg of heparin, followed by 7.5 mg/kg maintenance doses of heparin every 60 minutes. The other animals received no anticoagulant.

5-Hydroxytryptamine creatinine sulfate (serotonin), 5-methoxytryptamine, 5-methoxy-N-acetyltryptamine (melatonin) and tryptamine were obtained from Cal Biochemical, Los Angeles; 5-hydroxyindole from Nutritional Biochemical, Inc., Cleveland; and 5-hydroxyindole acetic acid from Sigma Chemical Co., St. Louis. 2,3-Dihydro-5-hydroxytryptamine was prepared by a previously described method.\(^5\) The inhibitors of serotonin (\(d\)-lysergic acid diethylamide (LSD-25), 2-bromo-\(d\)-lysergic acid diethylamide (BOL-148), and 1-methyl-\(d\)-lysergic acid (+)-butanolamide bimaleate (UML-491), were generously provided by Mr. Harry Althouse of Sandoz Pharmaceuticals.

The screen filtration assay was performed as described before.\(^1\)\(^2\) Two ml of blood were forced through a screen with multiple pores 20 micra square at a constant rate. The diameter of the area of screen through which the blood passed was 1.8 mm. Thus, approximately 0.08 ml blood passed...
through each square millimeter of screen every second for a period of 10 seconds. The pressure just proximal to the screen was measured by a strain gauge and recorded continuously. The pressure reached at the end of the 10-second period is defined as the screen filtration pressure. Typical screen filtration pressure curves are shown in figure 3 below.

pH measurements were made with a Beckman blood gas analyzer. Erythrocyte sedimentation rates were determined after one hour in Wintrobe tubes which were then spun for 40 minutes at 3000 rev/min to determine the volume of packed red blood cells in the blood. Platelet-free plasma was obtained by centrifuging either plasma or whole blood at 3000 rev/min (2000 × g) for 40 minutes. Platelet-rich plasma was obtained by centrifuging whole blood at 1000 rev/min (250 × g) for 10 minutes. The supernatant platelet-free plasma was then removed by aspiration. Washed red blood cells were obtained by centrifuging and removing the plasma and buffy coat by aspiration. The red blood cells were then resuspended in normal saline. This operation was repeated twice. Red blood cells, relatively free of platelets, were also obtained by centrifuging whole blood at 2000 × g for 20 minutes and then removing by aspiration the lower half of the red blood cell column.

Results

1. IN VITRO OBSERVATIONS

A. Serotonin Effect

In 24 of 30 dogs the addition of 5-hydroxytryptamine creatinine sulfate (5-HT) to heparinized or non-heparinized arterial (or venous) blood resulted in alterations of its screen filtration pressure. This response was dose dependent and biphasic. Addition of from 0.0001 mg to 0.0002 mg of 5-HT/ml of blood caused the screen filtration pressure to decrease. When the concentration was increased the screen filtration pressure also increased, to reach a maximum at concentrations of 0.005 mg to 0.01 mg of 5-HT/ml (fig. 1). The maximum increase in screen filtration pressure (SFP) when 5-HT was added to the blood was usually 300% to 600% of the normal control screen filtration pressure (fig. 3, A). Greater increases in SFP could not be recorded. No effect was noted with creatinine sulfate alone.

Examination of a thick, wet smear of blood exhibiting an increased screen filtration pressure revealed marked aggregation and adhesiveness of platelets and leukocytes. This effect was present when the smears were prepared as soon as a few seconds after the addition of serotonin. The aggregates adhered to the glass slide or coverslip and around them flowed red blood cells, which also exhibited evidence of adhesiveness (fig. 2). Examination of stained smears revealed many polymorphonuclear leukocytes and a few mononuclear leukocytes in the aggregates of platelets. In addition the aggregates often contained red blood cells, and frequently red blood cell masses surrounding the aggregates appeared closely attached to them. The platelets were fused; their morphological individuality was no longer clearly distinguishable but the platelet granules were seen faintly.

Red blood cells, washed free of platelets by physiological saline solution, and resuspended in platelet free plasma, no longer responded to the addition of 5-hydroxytryptamine with an increase in the screen filtration pressure. If small amounts of platelet-rich plasma (0.1
ml/5 ml blood) were added to the suspension, the increase in screen filtration pressure was again present. If instead of platelet-rich plasma obtained by slow centrifugation, platelets which had been washed free of plasma with saline were added, no increase in the screen filtration pressure resulted.

A marked increase in screen filtration pressure was found in blood of two cats and two rabbits to which had been added 0.005 mg of 5-HT/ml. Both heparinized and non-heparinized bloods were tested. In blood from one of the nine human subjects the serotonin reaction also occurred.

B. Absence of Serotonin Effect

In six dogs out of 30, one rabbit out of three, and eight out of nine human beings, the addition of 5-HT to the blood in vitro did not increase the screen filtration pressure (fig. 3, B). When, however, three of the dogs which did not show the in vitro screen filtration pressure effect were exsanguinated sufficiently (200 ml to 400 ml blood removed) to lower the systemic blood pressure by 20 to 30 mm Hg, the screen filtration pressure increased after addition of 5-HT to the blood. Further exsanguination and reduction in mean blood pressure was attended by increase in the screen filtration pressure even without addition of serotonin in vitro and addition of serotonin to a sample of this same blood further increased the screen filtration pressure (fig. 3, C).

C. Factors Influencing the Serotonin Effect

1. Decrease and Disappearance of Serotonin Effect with Time. In three experiments, the increase in SFP was maximal two minutes after the addition of 0.001 mg or 0.005 mg of 5-HT/ml heparinized blood. The effect gradually weakened and 20 minutes later the effect had entirely disappeared (fig. 4). Addition of fresh 5-HT to two of these bloods failed to reactivate the serotonin effect. In a fourth experiment the serotonin effect weak-
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more slowly. The control screen filtration pressure was 28 mm Hg. The screen filtration pressure three minutes after addition of 0.005 mg of 5-HT/ml was 189 mm Hg. Thirty minutes later it was 100, and 70 minutes later 60 mm Hg.

2. Effect of Anticoagulants: Sodium Citrate and Ethylenediaminetetraacetic Acid (EDTA). The screen filtration pressure of arterial blood prevented from clotting by these two agents, was not increased by the addition of 5-HT. Neither did serotonin change the screen filtration pressure when these agents were added to heparinized blood. The subsequent addition of calcium or magnesium to blood containing sodium citrate or EDTA, in amounts sufficient to cause clotting of citrated blood, did not activate the serotonin effect in heparinized bloods. When, however, adenosine diphosphate (ADP) was added along with 5-HT an increase in screen filtration pressure resulted in citrated blood.

3. Effect of Altering Blood pH with Carbon Dioxide and Oxygen. In three experiments carbon dioxide gas was bubbled through freshly drawn arterial blood. The increase of screen filtration pressure upon addition of 0.001 to 0.005 mg of 5-HT/ml blood disappeared when pH had decreased by from 0.05 to 0.1 of a unit from beginning levels of pH 7.35 to 7.5. When, however, oxygen was then bubbled through these same bloods the serotonin effect reappeared when the pH had increased to a level slightly higher than was present originally. It was still present as the blood became more alkaline to pH readings of 7.6 and 7.8, respectively.

In one dog experiment, the serotonin effect both in arterial and in venous blood disappeared after hypoventilation which had been induced by intravenous injections of additional pentobarbital. This was accompanied by a decrease in pH of blood from 7.4 to 7.35. When oxygen was bubbled through these same bloods in vitro the serotonin effect reappeared. The serotonin effect first reappeared after three minutes when the pH was 7.45, and became more marked when the pH had reached 7.55. In venous blood of two human subjects in which the screen filtration pressure did not increase when 5-HT and/or ADP were added, bubbling oxygen through the blood for two minutes restored response to both ADP and 5-HT.

4. Effect of Altered Oxygen Saturation and of Potassium Cyanide. Bubbling nitrogen through arterial blood to rid the blood of its oxygen did not alter the serotonin effect unless the pH was concurrently reduced. Oxygen tensions were not determined, but even though the blood became very dark the serotonin effect persisted if the pH did not fall. The addition of $1 \times 10^{-2}$ molar potassium cyanide to blood completely abolished the serotonin effect.

5. Effect of Filtration Through Pyrex Glass Wool. Passing blood through a three-inch-long column of pyrex glass wool abolished the serotonin effect partially or completely. The effect could be restored by addition of platelet-rich plasma to this filtered blood.

When the entire blood volume of a dog was filtered continuously through a pyrex glass wool filter in an extracorporeal system, the method of filtration is being published separately.

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serotonin effect was also reduced or abolished.

6. Effect of Serotonin-like Substances, Catecholamines, Histamine and Other Compounds. Various catecholamines (l-epinephrine, ephedrine, l-norepinephrine bitartrate, l-metanephrine bitartrate, and nor-metanephrine) added to heparinized blood in concentrations of 1 to 10 μg/ml of blood failed to increase the screen filtration pressure. Substances more nearly related to serotonin were also tested (fig. 5). Of these 5-methoxytryptamine had effects comparable to those of serotonin (figs. 1 and 5).

7. Effect of 5-Hydroxytryptamine Inhibitors. LSD-25, UML-491, and BOL-148 (1 to 5 μg/ml) were added to heparinized blood prior to addition of 5-HT (1 to 5 μg/ml). These substances prevented the rise in screen filtration pressure caused by 5-HT. UML was the most potent inhibitor, being effective in doses of 0.001 mg/ml of blood.

8. Effect of Adenosine Diphosphate (ADP) and Related Substances. Born has reported aggregation of platelets by adenosine diphosphate (ADP) with reversal of this effect by adenosine monophosphate (AMP). Screen filtration tests of these substances revealed that in animals exhibiting no serotonin effect, 0.001 to 0.005 mg/ml of ADP increased the screen filtration pressure by 50% to 100%. Adenosine triphosphate (ATP) had a somewhat lesser effect. When, however, both ADP and 5-HT in doses of 0.001 mg/ml were added to blood, an increase of screen filtration pressure in excess of 600% usually resulted. This combined effect was inhibited by UML (fig.

**FIGURE 5**
Screen filtration pressures in control, heparinized bloods and in others to which serotonin and related substances were added.
Adenosine triphosphate (ATP) and cyclic adenosine monophosphate did not potentiate the effect of serotonin as did ADP.

ADP effectively increased the screen filtration pressure for only one or two minutes. When added to blood the effect was maximal in one minute, and absent three, five, and ten minutes later. When the effect of ADP disappeared, the addition of serotonin no longer increased the screen filtration pressure.

When blood was passed through a glass wool filter it no longer responded to ADP, serotonin, or to a combination of the two.

II. IN VIVO OBSERVATIONS

In three cats, three dogs, and three rabbits (all heparinized) blood circulation was visualized in the conjunctiva (60 to 80 X magnification) with a Leitz binocular microscope. Incident light was used.

In two of the dogs (19 kg and 16 kg) a T-tube was inserted into one common carotid artery and serotonin was injected without stopping the blood flow. After injections of 0.05 mg to 0.1 mg of serotonin, segmentation of the columns of blood in the conjunctival vessels began in about 10 seconds. This became marked in one minute, was accompanied by slight slowing of the flow, and returned to normal in another two to three minutes. With injections of 0.2 mg of serotonin the changes were more dramatic, and red cell aggregation more marked. The circulation generally slowed and, in some areas, stopped. Many aggregates became trapped in small venules and in capillaries. After injections of 1.0 mg of serotonin, aggregation of red blood cells became generalized, and was observed in the opposite eye. Return to normal then required about 10 to 20 minutes. In some instances aggregates ("micro-emboli") could be seen slowly working their way through the blood vessels to the venous channels even a longer time after injections. In smaller venules in which the circulation was not so rapid, masses, light gray in color, were observed. It is presumed that these were composed chiefly of aggregated platelets and leucocytes.

Systemic blood pressure rose by 10 to 30 mm Hg in the dog immediately after intravenous injections of serotonin. This was accompanied by hyperventilation for several minutes. Blood pressure fell to normal or sub-normal levels (60 to 80 mm Hg) for some minutes before returning slowly to normal. When serotonin was injected into the carotid artery, the first response was a sudden fall in blood pressure of 20 to 40 mm Hg 10 seconds after the injection. Pressure then rose slowly to normal.

In the rabbits and cats the circulatory changes just described were studied only after intravenous injections of serotonin. Aggregation of red blood cells and slowing to cessation of circulation were similar to observations in dogs. In these animals the blood pressure was not determined.

The hematocrits slowly decreased after single or multiple intravenous injections of serotonin large enough to cause general aggregation of red blood cells for 30 or more minutes (table 1). This trend was observed 15 minutes after injection and was maximal in about 30 to 120 minutes. In all these experiments injections of serotonin were followed by marked aggregation of blood elements with slowing, or even arrest, of circulation in the blood vessels of the conjunctiva. In most instances red cell aggregates were
observed trapped in the vascular bed. In one case the serotonin response in vitro was absent.

After in vivo aggregation of blood elements had been produced by several injections of serotonin, the serotonin effect previously demonstrated in vitro was no longer demonstrable in freshly drawn blood.

Discussion
The studies just presented indicate that very small amounts of 5-HT added to blood reduced the tendency of blood elements to aggregate. Biologically this could be a factor in reducing peripheral resistance to the flow of blood through the smaller blood vessels including capillaries. The addition of slightly larger amounts of 5-HT, however, increased the aggregation tendency of blood elements. Concentrations of serotonin of this magnitude might result from platelet aggregation, metamorphosis, and release of serotonin which may occur during anaphylaxis and during the reaction to injections of bacterial endotoxins.

It is known that platelets remove added free serotonin from the plasma, and that there are regions of the body, other than platelets, which contain relatively high concentrations of serotonin. Perhaps we should consider the possibility that during the reactions to anaphylaxis and bacterial endotoxin the initial excess of serotonin in the plasma comes not from the platelets, but from these other regions of known high concentration, i.e., gastrointestinal tract, brain, and lungs, and that the platelets change and aggregate when they are unable to remove excess serotonin from the plasma as rapidly as it accumulates. Later, after platelets have aggregated they may become an additional source of plasma serotonin.

Since the screen filtration pressure of blood of some dogs did not increase when serotonin was added in vitro, and since moderate exsanguination of these same dogs resulted in appearance in vitro of the serotonin effect, we assume that the increase in screen filtration pressure depended upon the presence of some other substance. Adenosine diphosphate, observed by Born to cause aggregation of platelets, may be this other substance. In our studies it potentiated the increase in screen filtration pressure produced by serotonin in vitro, and in addition activated the serotonin effect in animals in which this effect was absent. It may be biologically important that ADP no longer caused platelets to aggregate in whole blood three minutes after being added to the blood, whereas serotonin remained active for 10 or more minutes.

Our finding, that the effects of both serotonin and ADP on the screen filtration pressure were absent after blood was filtered through glass wool even though many platelets (usually approximately 75%) still remained in the blood, suggests that all platelets are not the same. One can only speculate as to whether the difference is related to age or to some other cause.

Red blood cells, as well as platelets and leucocytes, were observed to aggregate when serotonin or ADP plus serotonin was added to whole blood, both in vitro and in vivo. This appeared to be due in vitro to adhesiveness.

<table>
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<th>Animal</th>
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<th>Serotonin Injections</th>
<th>Hematocrit</th>
<th>Elapsed time (min)</th>
<th>Per cent reduction hematocrit</th>
<th>Serotonin effect</th>
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of the red blood cells themselves. However, in vivo this could have been more apparent than real, perhaps owing to interruption of the columns of blood in the conjunctival blood vessels by aggregates of platelets. In some but not all experiments, gray masses thought to be aggregated platelets were observed in the smaller blood vessels of the conjunctiva. These appeared too infrequently to be the only cause of the marked segmentation of the columns of red blood cells which were observed. It is also possible that the aggregation was due to interruption or segmentation of the columns of blood by alternating relaxation and contraction of the smaller arteries and arterioles caused by the serotonin. Confirmation of this mechanism by observation of blanching of the conjunctiva and actual narrowing of blood vessels after injection of serotonin was not observed.

Finally, one must consider the possibility that the aggregates of red blood cells had a center of adhesive platelets to which the red blood cells adhered. This mechanism received support from several sources. First, after serotonin had been added to blood, both in vivo and in vitro, there was a tendency for the red blood cells to be mixed with platelets in the buffy coat, suggesting that these elements were adhesive for one another. Second, adhesiveness and aggregation of red blood cells, as indicated by the screen filtration pressure, did not occur in the absence of platelets, nor after filtration of blood through glass wool which removed adhesive and aggregated platelets.

The profound cellular clumping observed in the conjunctiva after injection of serotonin could explain the marked hematocrit reduction which occurred after aggregation had been present for 20 minutes or longer. This could lead to "plasma skimming." Our experiments did not eliminate the possibility, however, that some dilution of the blood by tissue fluids had occurred.

Many cardiovascular phenomena, including the fact that injections of serotonin into the brachial artery were followed by a reduction of circulation through the arm and hand, swelling of these structures, later marked cyanosis of the skin of the hand, and finally by the appearance of petechial hemorrhages, have generally been attributed to spasm of arterioles. Many of these events can be explained by obstruction of these smaller blood vessels by cellular aggregates which form within a few seconds after addition of serotonin to blood. It has been suggested already that part of the increase in pulmonary blood pressure which occurs during anaphylaxis may be due to obstruction of the pulmonary vessels by platelet aggregates.

Summary

Addition of small amounts of 5-hydroxytryptamine to whole blood altered the tendency of the platelets, leucocytes, and red blood cells of dogs, cats, and rabbits to aggregate. The magnitude of this response to 5-hydroxytryptamine was dose dependent and biphasic. Very small doses seemed to decrease the aggregation tendency; larger amounts greatly increased this tendency. This change was determined by measuring the pressure required to force blood at a constant speed through a standardized screen with multiple pores 20 micra square. This pressure was referred to as the "screen filtration pressure." The in vitro serotonin effect was abolished by very slight reductions of pH, by addition of KCN to blood, and by removing adhesive platelets from the blood.

In vivo 5-hydroxytryptamine caused aggregation of blood elements resulting in obstruction of blood flow in many blood vessels of the conjunctiva. This was accompanied by a reduction in hematocrit when multiple injections of 5-hydroxytryptamine were given.

In a few dogs the addition of serotonin to blood in vitro did not increase the screen filtration pressure. In these, adenosine diphosphate (ADP) caused a slight increase in this pressure, and together with serotonin caused a very marked increase in the screen filtration pressure.

Serotonin inhibitors inhibited the effects of 5-hydroxytryptamine, ADP, and combinations of ADP and 5-hydroxytryptamine.
Acknowledgment

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References
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