teins are considered, while having some actions that are parasympathomimetic, may have additional non-cholinergic effects, which involve stimulation of adrenergic alpha- and beta-receptor sites. The precise locus of action of these adrenergic blocking agents, with regard to their alteration of the action of pilocarpine, remains to be explored.

Summary. Pilocarpine is not an equivalent substitute for parasympathetic nerve stimulation of the rat parotid gland when protein composition of the secretion is considered. Levels of amylase for example are appreciably higher with pilocarpine than with auriculo-temporal nerve stimulation. It has been found that Inderal, an agent which blocks adrenergic beta receptors, reduces amylase levels of pilocarpine-evoked saliva to levels observed with auriculo-temporal stimulation; on the other hand, Inderal does not modify amylase levels observed with auriculo-temporal stimulation. It is concluded that pilocarpine while having some actions that are parasympathomimetic has additional effects which involve stimulation of adrenergic receptor sites.

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Received June 24, 1965. P.S.E.B.M., 1965, v120.

Platelet Aggregation and Release of ADP, Serotonin and Histamine Associated with Phagocytosis of Antigen-Antibody Complexes.* (30496)

HENRY Z. MOVAT, J. FRASER MUSTARD, NORTON S. TAICHMAN† AND TSUNEO URIUHARA‡ (Introduced by W. S. Hartroft)

Departments of Pathology and Medicine, and the Blood and Vascular Disease Research Unit, University of Toronto, Toronto, Canada

Platelet aggregation is a well-recognized phenomenon in anaphylaxis, the Arthus reaction, thrombosis and blood coagulation(1,2, 3). *In vitro* studies with rabbit(4), guinea pig(5,6), pig(7) and human(7,8,9) platelet-rich plasma or platelet suspensions demonstrated that addition of antigen-antibody (Ag-Ab) complexes induced platelet aggregation. This platelet aggregation is associated with release of histamine and serotonin (5-hydroxytryptamine, 5-HT), both *in vivo* (10) and *in vitro*(4,11). Reports concerning the role of complement in platelet aggregation associated with release of pharmacologically active substances are not conclusive(12), and until recently the mechanisms initiating platelet aggregation have not been well understood(12).

Recent evidence has shown that platelets can phagocytose latex (polystyrene) particles. This is associated with release of serotonin and ADP from the platelets, which induces platelet aggregation(13,14). The purpose of the present study was to investigate whether platelet aggregation induced by immune complexes involves a similar mechanism.

Materials and methods. The *in vitro* studies were carried out with human or pig native (no anti-coagulant added) or citrated platelet-rich plasma, or with twice washed platelets (prepared from blood taken into EDTA) suspended in Tyrode-gelatine (0.25% gelatine)(13,14).

The Ag-Ab complexes (BSA-anti-BSA or

* Supported by the Ontario Heart Foundation, Medical Research Council of Canada (MT-1309) and Canadian Arthritis and Rheumatism Soc.

† Research Fellow of J. P. Bickell Foundation.

‡ Research Fellow of Medical Research Council of Canada.

TABLE I. *In vitro* Release of 5-HT, Histamine and ADP from Pig Platelets.

Substance added to platelet suspension	Substance released from platelets: Radioactivity of supernatant, cpm/ml: C^{14}-5-HT*	C^{14}-5-HT*	H^3-histamine†	H^3-histamine*	μg ADP/ml supernatant
Buffered-saline	2,300	3,970	3,680	5,440	2.37
Antigen (BSA)		4,000		4,810	2.40
Antibody (anti-BSA)				5,520	2.82
Ag-Ab (equivalence)	22,670		9,210	16,710	10.11
Ag-Ab (×2 Ab excess)			8,840		6.68
BSA (heat aggregated)		19,140			7.13
Latex particles	14,970	18,480	1,360	8,740	5.93
Thrombin (10 units/ml)	11,590	29,110	7,820	11,970	10.20

1 ml labelled platelet suspension (300,000 platelets/mm³) was incubated with agitation for 10 min at 37°C with: * 0.1 ml Ag (0.8 μg-N) or 0.1 ml Ab (4.5 μg-N) or Ag-Ab precipitates (0.8 μg-N and 4.5 μg-N); † 0.1 ml Ag (1.8 μg-N) or 0.1 ml Ab (13.7 μg-N) or Ag-Ab precipitates (1.8 μg-N and 13.7 μg-N).
0.1 ml 5% suspension of Latex (polystyrene) particles.
0.1 ml thrombin (10 units/ml).

ferritin-antiferritin) were prepared either at equivalence, slight Ag excess (× 2½-5 the Ag needed for equivalence) or slight Ab excess (× 2 the Ab needed for equivalence). The final mixture of platelet suspension and immune complex contained between 0.004 and 0.145 mg AB N per ml of suspension. All antisera were heated at 56°C for 30 minutes, absorbed with red cells, dialyzed against saline for 24 hours and centrifuged at 40,000 RPM in an MSE ultracentrifuge for one hour. Such antisera by themselves induced no platelet aggregation or release of serotonin or histamine.

Platelet-rich plasma or platelet suspension (1 ml) containing approx. 300,000 platelets per mm³ was mixed with the Ag-Ab complexes (0.1 ml). Platelet aggregation was recorded with a turbidimetric method(7,13). This consists of a cuvette containing the platelet sample which is continuously stirred. A light beam passes through the cuvette to a photoelectric cell, which is coupled to a recorder. As the platelets aggregate, increasing amounts of light are transmitted, indicated by a deflection of the recorder pen. The platelet aggregates periodically interrupt the light transmission, producing a vertical oscillation in the tracing. The dimensions of the oscillation are proportional to the size of the aggregates. In this fashion a continuous record of platelet aggregation can be made. Inhibition of platelet aggregation was attempted by adding various inhibitors to the platelet-rich plasma or platelet suspension and then adding the Ag-Ab complexes.

The ADP in the supernatant was estimated in the following fashion. After agitation at 37°C for 10 minutes 1 part of a 2% EDTA solution was added to 9 parts of the suspension. (It has been found that EDTA does not interfere with ADP analysis, but prevents conversion of ADP to AMP and adenosine.) Following this the mixture was centrifuged at 7,000 RPM (RCF 5,900) at 4°C. The ADP was determined in the supernatant by an enzymatic method(13).

For the study of serotonin and histamine release *in vitro* platelets were labelled *in vivo* by methods that have been described(13,14), or *in vitro* by adding C^{14}-5-HT or H^3-histamine to EDTA platelet-rich plasma which was then allowed to incubate at room temperature for 10 minutes. Between 0.5 and 2 μc of C^{14}-5-HT or H^3-histamine were used to label the platelets in 30 ml of plasma. A twice washed platelet suspension was then prepared from either the *in vivo* or *in vitro* labelled platelets. In these experiments the total number of radioactive counts in the platelet suspension was always less than 50,000 counts per ml of suspension.

In vivo studies were carried out in rabbits actively immunized with cadmium-free horse ferritin (Nutritional Biochemicals Corp.) or BSA (Pentex, Kankakee, Ill.). Their Ab levels varied between 0.2 mg and 1.2 mg N per ml. They were challenged intravenously with

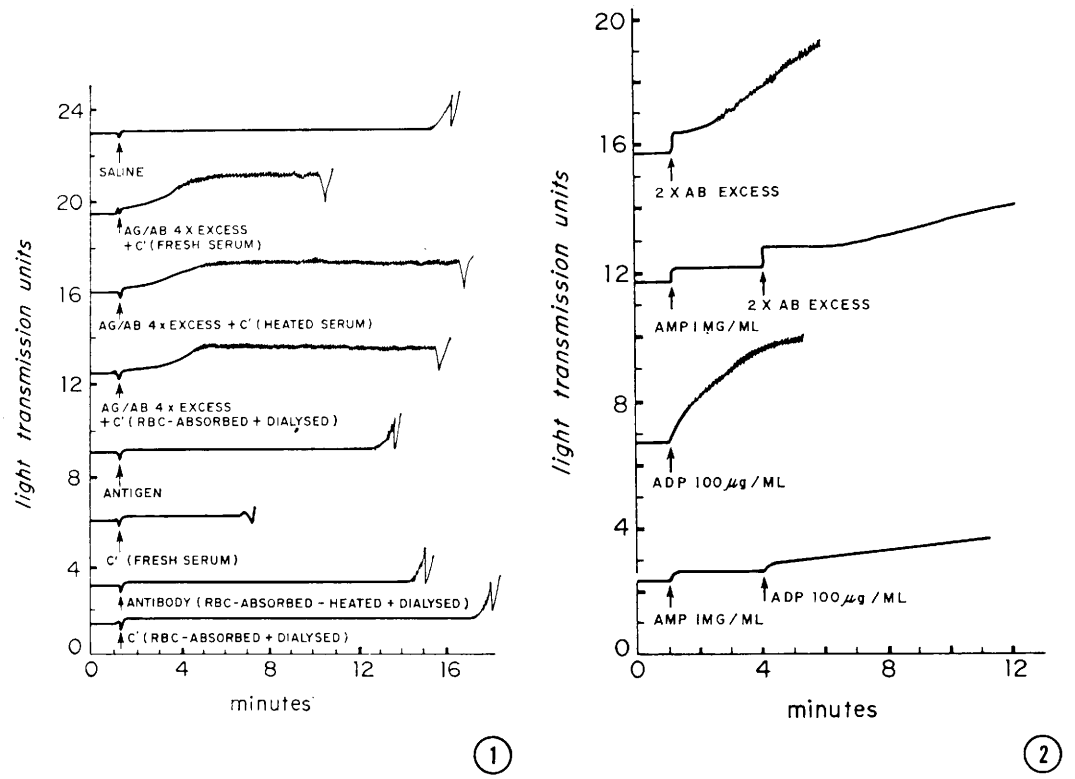

FIG. 1. Turbidimetric recording of platelet aggregation in native platelet-rich plasma (human). *Top tracing:* Addition of saline does not lead to rapid onset of platelet aggregation. At about 15 min, onset of spontaneous platelet aggregation occurs (probably due to formation of traces of thrombin followed by formation of fibrin indicated by sharp downward and then upward deflection). *2nd tracing:* Addition of Ag-Ab complexes in × 4 Ag excess (7.2 µg BSA-N and 13.7 µg anti BSA-N) with fresh guinea pig serum as source of complement causes platelet aggregation and shortens the time required for fibrin formation. *3rd tracing:* Heated guinea pig serum plus Ag-Ab also causes aggregation but fibrin formation is not as rapid. *4th tracing:* When fresh serum is absorbed (30 min at room temp) with human red cells, dialyzed for 16 hr against saline at 4°C and centrifuged at high speed, aggregation and clotting are similar to tracing No. 3. *5th tracing:* Antigen induces no aggregation. *6th tracing:* Fresh guinea pig serum causes no aggregation but enhances clotting. *7th tracing:* Antibody (antiserum) when heated for 30 min at 56°C, absorbed with red cells, dialyzed and centrifuged at high speed causes no platelet aggregation. *8th tracing:* Fresh guinea pig serum when absorbed, dialyzed and centrifuged as in 4th tracing does not accelerate time of onset of platelet aggregation and fibrin formation.

FIG. 2. Effect of immune complexes on platelet aggregation in a suspension of human platelets. *Top tracing:* Aggregation of platelets produced by adding Ag-Ab complexes in × 2 Ab excess (0.9 µg BSA-N and 13.7 µg anti BSA-N). *2nd tracing:* Inhibition by prior addition of AMP of platelet aggregation by the Ag-Ab complexes used in tracing No. 1. *3rd tracing:* ADP-induced aggregation of platelets. *4th tracing:* Inhibition of ADP-platelet aggregation by AMP. Concentrations refer to that in the solution added, not to final concentration (1 part to 20 parts).

the appropriate Ag, using a dose based on the amount of Ag needed for equivalence and the approximate blood volume of the rabbit.

Results. Addition of Ag-Ab complexes to a platelet suspension or platelet-rich plasma *in vitro* produced release of C^{14}-5-HT, H^3-histamine and ADP in the ambient fluid (Table I) and platelet aggregation (Fig. 1, 2 and 3). Electron microscopic examination of these aggregates at different intervals of time showed that by one hour most of the platelets were degranulated. The platelet aggregation appeared to be due to release of ADP since there was a rise in ADP content of the ambient fluid (Table I) and aggregation was blocked by the prior addition of AMP (Fig.

2). Immune complexes in × 4 Ag excess (Fig. 1), at equivalence or in × 2 Ab excess (Fig. 2), caused platelet aggregation. Antigen (BSA) caused no platelet aggregation (Fig. 1) or release of histamine, serotonin or ADP (Table I). Antiserum or fresh serum as a source of complement caused platelet aggregation and clotting (Fig. 1). However, when the antiserum was absorbed with red cells, heated at 56°C for 30 minutes and dialyzed for 16 hours against saline at 4°C, it no longer caused aggregation (Fig. 1). Absorption with red cells and dialysis had a similar effect on fresh serum (complement). Addition of fresh serum as a source of complement was not found to be essential for platelet aggregation (Fig. 3).

Platelet aggregation in citrated platelet-rich plasma or in the platelet suspension could be inhibited by (final concentration in parenthesis): iodoacetate (1×10^{-3} M) and ethylenediamine tetraacetate (0.015 M).

Immunized animals injected intravenously with ferritin developed systemic anaphylaxis. Some died of anaphylaxis within 7 to 15 minutes. Rabbits which did not die were killed one hour after injection of the antigen. All rabbits contained masses of ferritin-antiferritin in their pulmonary vessels, intermingled with aggregated platelets and PMN-leukocytes whether they died spontaneously or were sacrificed. Many of the aggregated platelets contained phagocytosed immune complexes. These were at times within a vacuole which also contained fragments of platelet granules (Fig. 4). Some of the platelets were completely degranulated (Fig. 5). Platelets free of granules were seen both in the animals dying spontaneously in the early phase of anaphylaxis and in those sacrificed one hour after challenge with antigen. Degranulated platelets were usually part of an aggregate, but occasional single degranulated platelets were observed (Fig. 5).

Discussion. The observations with Ag-Ab complexes reported here have been paralleled by studies on aggregation of platelets induced by phagocytosis of latex particles(13,14). As in the studies with Ag-Ab complexes, aggregation of platelets by latex particles could be inhibited by adenosine monophosphate, iodoacetate, 2,4-dinitrophenol and EDTA. Ultrastructural studies showed that some inhibitors (EDTA, iodoacetate and 2,4-dinitrophenol) prevented phagocytosis and aggregation, whereas other inhibitors (AMP and adenosine) prevented platelet aggregation, but not phagocytosis of latex particles. Thus it would seem that phagocytosis by platelets of Ag-Ab complexes is associated with release of ADP from the platelets and the released ADP causes the platelet aggregation. Further, these observations parallel in many respects the action of thrombin and collagen in inducing release of serotonin and ADP from platelets(15,16,17).

The relationship between phagocytosis and release of ADP is uncertain. Of interest in respect to this was the observation that in some platelets there was evidence of fusion of the platelet granules with the phagocytosed immune complexes (Fig. 4) with formation of "digestive vacuoles"(18), indicating that the platelet granules may be involved in the degradation of the phagocytosed immune complexes. We had observed such a process in PMN-leukocytes in the Arthus reaction (3,19) and following *in vitro* incubation of Ag-Ab complexes with PMN-leukocytes(20). Since leukocytes also aggregate during phagocytosis and degranulation(21) it would seem important to examine the relationship of lysosomal enzymes in this process.

The degranulation occurring in PMN-leukocytes *in vitro* was associated with release of a permeability factor into the ambient fluid(21). The activity of this factor could be inhibited by protease inhibitors but not by antihistamines(22). Recent observations in our laboratory indicate that permeability factors are also released from platelets if they aggregate and degranulate following addition of Ag-Ab complexes or other substances such as collagen, latex and thrombin(23). Platelet granules are believed to be lysosomes(24) similar to the granules of PMN-leukocytes (25).

Since neither antihistamines(26) nor 5-HT depletion(27) ameliorate systemic anaphylaxis in the rabbit, and since anaphylaxis in leuko- and thrombocytopenic rabbits is mild (28), platelet degranulation together with

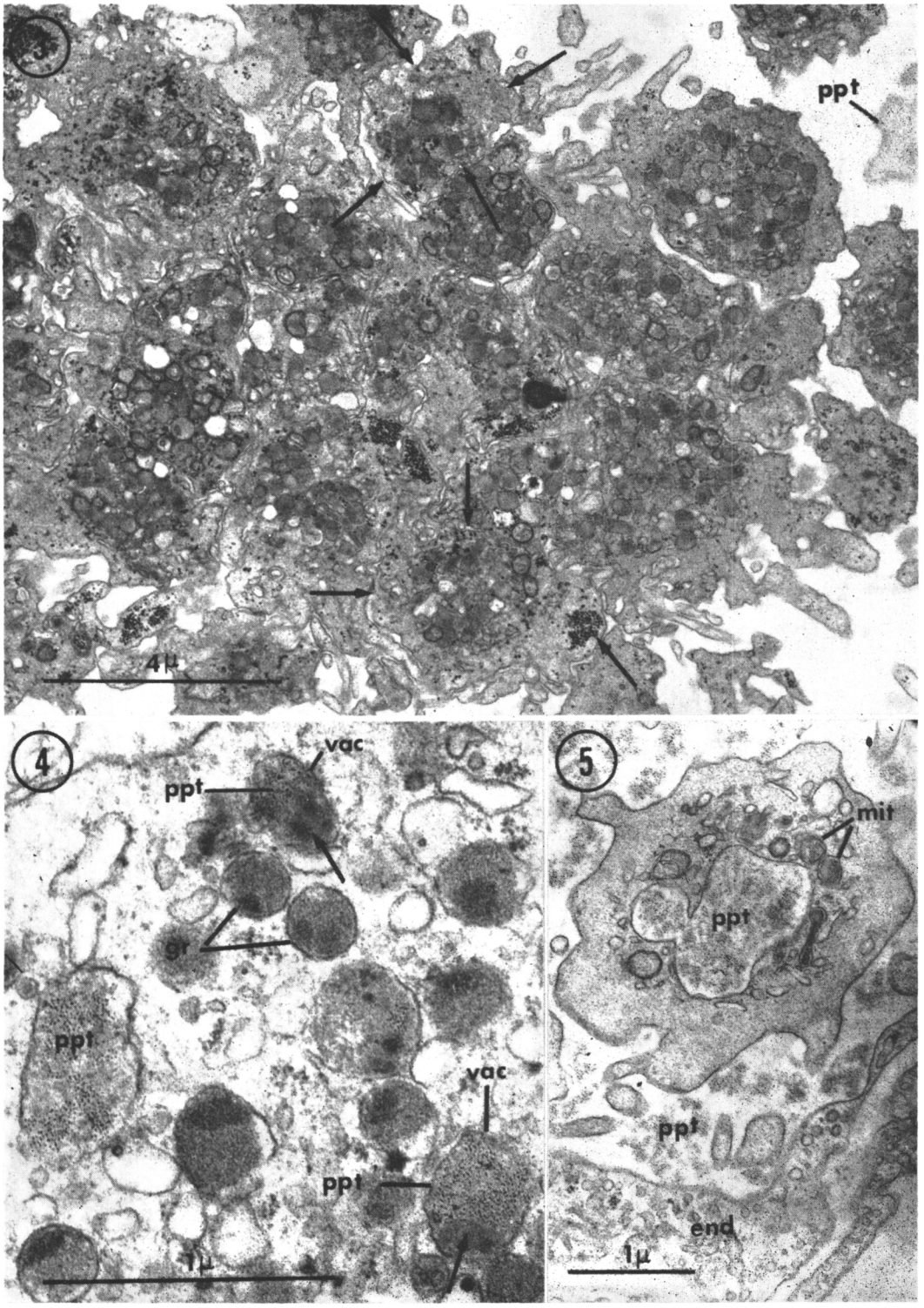

FIG. 3. Aggregation of human platelets formed *in vitro* by adding Ag-Ab complexes to a platelet suspension. Two platelets in the aggregate are outlined by arrows. Immune precipi-

tates (ppt) of BSA-anti BSA are seen in right upper corner. × 9,100.

FIG. 4. High magnification of a platelet in a pulmonary vessel during anaphylaxis of a rabbit. Several granules (gr) are seen in cytoplasm together with phagocytic or digestive vacuoles (vac). These vacuoles contain precipitates (ppt) of ferritin and antiferritin, together with remnants of platelet granules (arrows). × 44,000.

FIG. 5. Platelet in a pulmonary vessel during anaphylaxis. Large vacuole in center of platelet contains precipitates of ferritin-antiferritin (ppt) and similar precipitates are seen free in lumen. The platelet has no granules in plane of sectioning. mit = mitochondria; end = endothelium. × 18,700.

PMN-leukocyte degranulation and release of lysosomal enzymes may play an important role in anaphylaxis in this and perhaps in other species.

Summary and conclusions. Aggregation of human and pig platelets by antigen-antibody complexes is probably induced by release of adenosine diphosphate (ADP) during phagocytosis of the complexes *in vitro*. The *in vitro* aggregation is associated with release of histamine, serotonin and ADP. The aggregation can be inhibited by iodoacetate, EDTA, adenosine monophosphate (AMP) and adenosine. During anaphylaxis in the rabbit induced by intravascular antigen-antibody interaction and precipitation, aggregated platelets in pulmonary vessels contain phagocytosed antigen-antibody complexes. During aggregation *in vitro* and *in vivo* the platelets become degranulated and probably release their lysosomal enzymes. This, together with degranulation of PMN-leukocytes, may be a significant pathogenetic mechanism in anaphylaxis and other hypersensitivity reactions.

The authors wish to express their gratitude to Mr. D. R. L. Macmorine and Mr. A. Sényi for technical assistance.

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Received April 2, 1965. P.S.E.B.M., 1965, v120.