The Blood Platelet Release Reaction

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LIST OF ABBREVIATIONS

ATP, ADP and AMP = adenosine tri-, di- and monophosphate.
IMP = Inosine monophosphate.
NEM = N-ethyl maleimide.
EDTA = Ethylenediamino tetraacetate.
NADPH = Nicotine adenine dinucleotide phosphate, reduced form.
TCA = Trichloroacetic acid.

The discovery of Hellem (1960) and Gaarder et al. (1961) that ADP specifically makes blood platelets aggregate and adhere to foreign surfaces has led to a new orientation of research within the field of thromboembolism. Since formation of platelet thrombi prior to coagulation thrombi is the outstanding event, at least in arteries, many laboratories started to explore the mechanism of formation of the hemostatic plug and the platelet thrombus based on these findings (for references see Hellem & Stormorken 1969). These studies disclosed that the platelets themselves contain ADP which can be released as a response to different kinds of stimuli, thereby leading to an increasing accumulation of platelets at the site of injury.

The term 'release reaction' for this event was introduced by Grette (1962) who demonstrated that thrombin in addition to liberating serotonin (Zucker & Borrelli 1955) also caused rapid release of adenine nucleotides, amino acids and protein (fibrinogen) from platelets. Grette also assumed that thrombosthenin (the contractile platelet protein) which he isolated independently of Bettex-Galland & Lüscher (1961), promoted the reaction. Thrombin also releases about 50% of platelet potassium (Buckingham & Maynert 1964) which is inexchangeable with external potassium, in contrast to that retained by the platelets (Zieve, Gamble & Jackson 1964). This indicates compartmentation of this ion, a feature resembling that found for platelet adenine nucleotides (Holmsen 1965 a; Ireland 1967).

Platelets both adhere to (Bounameaux 1959) and are aggregated by (Zucker & Borrelli 1962; Hovig 1963 a) collagen fibres, an aggregation which is secondary to the release of platelet ADP by the fibres (Hovig

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1963 b; Spaet & Cintron, 1963). Spaet & Zucker (1964) showed that collagen also released C^{14}-serotonin taken up *in vitro* prior to release induction, and Holmsen (1965 a) showed that 50% of the endogenous serotonin was released. Spaet & Cintron (1965) and Stormorken & Rapaport (Holmsen 1965 a) further showed that collagen renders platelet factor 3 available. Both collagen and thrombin release a 'permeability factor' (Mustard et al. 1965) and platelet factor 4, the latter also released by ADP and adrenaline (Niewiarowski et al. 1968). Recently, it has been shown that collagen and thrombin induce release of lysosomal enzymes (Mills, Robb & Roberts 1968; Holmsen & Day 1968; Davey & Lüscher 1968). The latter platelet constituents are not released by release inducers such as adrenaline and ADP (Mills, Robb & Roberts 1968).

The present paper attempts to summarize the evidence that the release of all these different platelet constituents by a variety of external stimuli occurs by a common mechanism, *the platelet release reaction.* This process consists in extrusion of material from the platelet granules to the external medium. It clearly differs from cell lysis, general increase in the membrane permeability to the substances released, and a sudden occurrence of tears in the membrane through which intracellular materials escape. In contrast to the latter mechanisms, the platelets seem to have developed the storing and releasing properties of *secretory cells* (Stormorken 1969). The release mechanism can be triggered by a variety of extracellular substances, after which a selected battery of platelet constituents is rapidly secreted without affecting a number of platelet functions, such as aggregation, clot retraction, oxygen consumption, anaerobic glycolysis etc.

Definition of the release reaction

Grette (1962) and Davey & Lüscher (1968) restricted the term 'release reaction' to the sequence of events taking place when platelets are incubated with thrombin. However, the thrombin-induced release reaction resembles that induced by several other agents in so many ways that it justifies the theory of *one* basic release reaction:

1. The substances *released* are (as far as it has been established) located in platelet granules, both the 'usual' α-granules and 'dense' α-granules with 'Bull's eye' as well as 'very dense bodies'.
2. Substances located in either cytoplasm, mitochondria or membranes are *retained* during release.
3. Maximal release is reached within 60 seconds at 37°.
4. The process is dependent on energy derived from both glycolysis and oxidative phosphorylation.
5. After release, the platelet participates almost normally in several of its known functions.
6. Extracellular Ca^{++} promotes the process in many cases.

Experimentally, one sample of platelets (suspended in plasma or an artificial medium) is incubated with a release inducer and another sample with the inducer solvent (control). After 1 min the samples are cooled *rapidly* (i. e. within 1 sec.) to 0° and the cells and extracellular milieu are separated by centrifugation in the cold. A substance is only regarded as being released *when its concentration extracellularly is increased in the experimental sample as compared to the control, and this increase is parallelled by a corresponding intracellular decrease.*

Since little attention has been paid to use of the control sample, many substances have been regarded as released, when they have only appeared extracellularly after unspecific leakage or cell *lysis*. It is our experience that there is a considerable breakdown of platelets during manipulation and incubation, which leads to accumulation of all platelet components in the medium.

The short incubation time allows discrimination between the release reaction and other processes whereby intracellular substances appear in the medium such as reserpine-induced outflux of serotonin (Carlsson, Shore & Brodie 1957) or loss of acid phosphatase and potassium by haematoporphyrin and light (Zieve & Solomon 1966). The latter processes occur slowly and seem for serotonin and potassium to be due to disturbances in active uptake and storage mechanisms. Gaintner, Jackson & Maynert (1962) distinguished the thrombin-induced release of serotonin as 'active release' from 'passive release', caused by reserpine.

Many authors have regarded the amount of a substance *disappearing intracellularly* as the amount which is released. Theoretically this is not correct, as the substance could as well have been converted to some metabolites within the cell without being released. However, for most substances the amount disappearing intracellularly does equal that appearing extracellularly. An important exception is ATP (see 'release energy ATP', p. 9–12).

During the thrombin-platelet interaction, for example, some activities are not released to the extracellular milieu, but rendered available in or on the platelet (i. e. platelet factor 3). However, since these activities have been made *accessible to extracellular reactions* during the release, they also have been included as released platelet constituents.

Release inducers

Apart from thrombin and collagen, a number of chemical and physical stimuli are able to induce the release reaction. Thus, several proteolytic enzymes from conventional and snake venom sources are active such as trypsin (Gaintner et al. 1962), papain (Markwardt et al. 1965), snake venom proteases and pronase, but not chymotrypsin (Davey & Lüscher 1965 and 1967). Further, gamma globulin (Ishizaka & Ishizaka 1962), antigen-antibody complexes (Humphrey & Jaques 1965), viruses (Jerushalmy et al. 1962), bovine pancreatic elastase (Pancreatopeptidase E, E.C.3.4.4.7., Kowalski et al. 1966). Liquoid (Bettex-Galland et al. 1963), endotoxin (Des Prez et al. 1961), polymerizing fibrin (Solum 1966), latex particles (Glynn et al. 1965), particulate fatty acids (Haslam 1964 b), serotonin (Haslam 1967) adrenaline and ADP (MacMillan 1966; Mills, Robb & Roberts 1968) all induce release. Recently, it has been shown that bovine platelet fibrinogen and to a lesser extent, bovine plasma fibrinogen induce release of ADP and ATP from human platelets (Solum 1968).

Compounds released or retained and their intracellular localization

Table I summarizes the present knowledge concerning the intracellular localization of compounds released or made available during the release reaction. The adenine nucleotides released by thrombin, collagen and latex particles are designated 'non-metabolic' since they do not participate in energy metabolism of the platelets (see below, and page 9). All of the other release inducers listed above do release adenine nucleotides, but information is lacking as to whether or

TABLE I
Compounds released and their subcellular localization

Compounds released	Release inducer	References	Subcellular localization	References
Non-metabolic ATP and ADP	Collagen Thrombin Latex particles	Holmsen 1965 a Ireland 1967; Holmsen & Day 1969 Mürer, pers. comm.	Dense α-granules	Holmsen, Day & Storm 1969
Serotonin	Thrombin Collagen ADP Adrenaline	Grette 1962; Buckingham & Maynert 1964; Markwardt et al. 1965 Spaet & Zucker 1964 Holmsen 1965 a Mills, Robb & Roberts 1968	Very dense bodies Dense α-granules	da Prada et al. 1967 Day, Holmsen & Hovig 1969
Potassium (Inexchangeable)	Thrombin "	Buckingham & Maynert 1964 Zieve, Gamble & Jackson 1964	50% in particles 50% in cytoplasm	Buckingham & Maynert 1964
Zinc	Clotting	Foley et al. 1968	—	–
β-glucuronidase	Clotting Thrombin Collagen	Dohrmann & Klesper 1959 Holmsen & Day 1968 Mills, Robb & Roberts 1968	α-granules Vesicles α-granules	Marcus et al. 1966 Siegel & Lüscher 1967 Day, Holmsen & Hovig 1969
β-N-acetylglucosaminidase, β-galactosidase and aryl sulphatase	Thrombin	Holmsen & Day 1969	α-granules	Day, Holmsen & Hovig 1969
Cathepsin	Thrombin	Holmsen & Day 1969	Vesicles α-granules	Siegel & Lüscher 1967 Day, Holmsen & Hovig 1969
Mucopolysaccharides	Thrombin	Ridell & Bier 1965	—	
Fibrinogen	Thrombin Collagen	Grette 1962 Solum & Stormorken 1965	α-granules	Nachman, Marcus & Zucker-Franklin 1967 Solum & Day 1969
Platelet factor 3*	Collagen ADP Thrombin Adrenaline	Spaet & Cintron 1965 Mustard et al. 1964 Hardisty & Hutton 1967	Membraneous structures	Marcus et al. 1966 Day, Stormorken & Holmsen 1968
Platelet factor 4	Collagen Thrombin ADP Adrenaline	Niewiarowski et al. 1968	α-granules	Day, Stormorken & Holmsen 1968

* Not released to the sourroundings, but made available on the surface of platelets.

not they are 'non-metabolic'. However, the parallel release of adenine nucleotides together with serotonin, which is located within 'very dense bodies' or 'dense α-granules', strongly suggests that the nucleotides released in these cases also are non-metabolic and originate from these intracellular compartments.

The intracellular metabolic inaccessibility of the released nucleotides and the inexchangeability of the released potassium is based on the lack of labelling *in vitro* with extracellular radioactive precursors. This is in contrast to that which has been found for serotonin, as C^{14}-serotonin taken up by platelets *in vitro* is readily released by numerous release inducers. However, the adenine nucleotides which becomes labelled during incubation with isotope precursors are located in the cytoplasm, mitochondria or membranes (Table II) whereas serotonin taken up *in vitro* is rapidly incorporated into platelet granules (Minter & Crawford 1967; Davis & White 1968).

It is apparent from Table I that the bulk of compounds *released* are mainly found in α-granules and osmiophilic granules, whereas only small amounts are found in the soluble part. As for serotonin the proportion which is particle bound, varies with the method of homogenization (Day, Holmsen & Hovig 1969). However, it is reasonable to believe that in the intact cell the major part is located in the granules, whereas variable amounts is rendered soluble during homogenization. On the other hand, components *retained* during the release reaction are exclusively located in cytoplasm, membranes or mitochondria (Table II). This therefore strongly indicates that the compounds released are *not freely distributed* within the platelet, but located in specific granules. The findings illustrated in Table I and II clearly indicate that the release reaction is specific, as suggested by Holmsen (1965 a) and further emphasized by Davey & Lüscher (1968) and Holmsen & Day (1969), and not the result of a generalized increase or breakdown of membrane permeability. Strong support for this view is the findings that ATP, ADP and serotonin are released in the same relative proportions as they are present in the subcellular granules (Holmsen, Day & Storm 1969). Further support is that the platelets are still functionally intact as judged by their ability to support clot retraction after release (Zieve, Gamble & Jackson 1964), and that anaerobic metabolism as measured by acid production continues after thrombin treatment (deVreker & deVreker 1965).

It should be noted that acid phosphatase, the most typical lysosomal enzyme and present in higher amounts in platelets than any other acid hydrolase, is *not* released even with 100 units/ml of thrombin for 60 min at 37° (Holmsen & Day 1969). Under these conditions there is a gradual increase of this enzyme extracellularly with a corresponding decrease intracellularly in *absence* of thrombin, but no significant additional increase in the presence of the release inducer. This unspecific release or cell breakdown is probably the reason for the accepted view that platelets release acid phosphatase during clotting (Zucker & Borrelli 1958 and 1959). In the latter process the concentration of thrombin formed far exceeds that necessary for induction of the release reaction.

Intracellular reactions

A possible relationship between energy metabolism and thrombin-induced release of serotonin was first shown by Markwardt, Barthel & Hoffmann (1964 a). Recent studies

TABLE II
Compounds not *released and their subcellular localization*

Compound	Release inducer	References	Subcellular localization	References
Acid Phosphatase	Collagen ADP and adrenaline Thrombin	Mills, Robb & Roberts 1968 Holmsen & Day 1968	α-granules	Marcus et al. 1966 Day, Holmsen & Hovig 1969
Lactate dehydrogenase, pyruvate kinase, adenosine deaminase, adenine phosphoribosyl transferase	Thrombin	Holmsen & Day 1969	Soluble (?)	Holmsen, Day & Pimentel 1969
Adenylate kinase	Collagen, ADP and adrenaline Thrombin	Mills, Robb & Roberts 1968 Holmsen & Day 1969	Soluble (?) Membranes Mitochondria	Holmsen, Day & Pimentel 1969
Hexokinase adenosine kinase	Thrombin	Holmsen & Day 1969	Soluble (?) Membranes Mitochondria	Holmsen, Day & Pimentel 1969
5′-Nucleotidase	Thrombin	Holmsen & Day 1969	Soluble (?) Membranes	Day, Holmsen & Hovig 1969
Cytochrome C oxidase	Thrombin	Holmsen & Day 1968	Mitochondria	Marcus et al. 1966 Day, Holmsen & Hovig 1969
Acid soluble organo-phosphates, metabolic *active*, including ATP and ADP	Collagen Thrombin	Holmsen 1965 a Holmsen & Day 1969	For ATP and ADP: Soluble, membranes and mitochondria	Holmsen, Day & Storm 1969
Amino acids	Thrombin	Buckingham & Maynert 1964	Soluble	Buckingham & Maynert 1964
Exchangeable potassium	Thrombin	Zieve, Gamble & Jackson 1964	—	–
Platelet factor 2	Thrombin ADP	Niewiarowski et al. 1968	—	–
Fibrinogen	ADP adrenaline	Solum & Stormorken 1965	α-granules	Nachman et al. 1967 Solum & Day 1969
Numerous proteins	Thrombin ADP	Davey & Lüscher 1968	—	–
Numerous lipids	Thrombin ADP	Davey & Lüscher 1968	—	–

have revealed that the platelet release reaction is almost completely inhibited when both glycolysis and oxidative phosphorylation are blocked simultaneously. Blocking of one of these processes separately does not inhibit the release reaction, indicating that the reaction requires a continuous supply of ATP, which can be derived from both pathways (Mürer 1968). This is in accordance with the findings of Holmsen (1967), and Ireland (1967) showing that some 'metabolically active' ATP is consumed during the reaction.

Early studies on energy metabolism during clotting of platelet-rich plasma (Born 1958; Löhr, Waller & Gross 1961; Zucker & Borelli 1961) or during interaction between washed platelets and thrombin (Lüscher 1961) had revealed that 50% of platelet ATP disappears rapidly in the platelets or in a system consisting of platelets + suspension medium. This was interpreted as consumption of ATP in an active ATP-requiring process, such as contraction of thrombosthenin. However, with our recent knowledge this may not be correct. The major part (80–90%) of the ATP which disappears intracellularly is probably *released* directly to the external medium (Holmsen, Day & Storm 1969). If the latter is plasma, the released ATP is rapidly broken down by a specific ATP pyrophosphohydrolase (Mills 1966; Holmsen & Holmsen 1969). When a suspension of washed platelets is used, the medium always contain ATP degrading systems from disrupted platelets (Holmsen & Day 1969). Hence, the major part of the 'ATP-consumption' occurs *outside* the platelets by catabolic processes.

Nevertheless, the early studies showed that platelet adenine nucleotides were involved in the thrombin-platelet reaction in some way or other, and the importance of release of ADP for platelet aggregation was soon established (Haslam 1964 b). However, the double function of the adenine nucleotides in the release reaction, both being among the substances released and at the same time providing energy for the process, has been a subject of controversy. Specifically, is ADP *directly* released from preformed platelet ADP or does it originate from ATP by a rapid dephosphorylation? Käser-Glanzmann & Lüscher (1962) and Rossi & Clatanoff (1963) claimed that ATP was the source whereas Haslam (1964 a) provided evidence that ADP was directly released, which also is apparent from the results of Grette (1962). Stronger indications for direct release of ADP have come from studies on platelets containing nucleotides labelled *in vitro* with ^{32}P-orthophosphate, ^{14}C-adenosine or -adenine (Holmsen 1967; Ireland 1967; Holmsen, Day & Storm 1969). When such labelled platelets are exposed to thrombin or collagen *non-radioactive* ADP is released whereas radioactive ADP remains in the cell. At the same time the concentration of the ADP within the cells decreases just as much as the extracellular ADP increases. The concentration of ATP within the cells also decreases to an extent that could explain quantitatively the ADP appearing extracellularly. The ATP disappearing intracellularly becomes *strongly* labelled, but 80–90% can be recovered extracellularly as *poorly* labelled. Furthermore, the ratio between the poorly labelled ATP and ADP released is 0.78. This poorly labelled, 'non-metabolic', pool of adenine nucleotides is located together with serotonin within the osmiophilic α-granules containing 'Bull's eyes' or very dense bodies (Holmsen, Day & Storm 1969), but with almost no lysosomal enzymes (Day, Holmsen & Hovig 1969). The ATP/ADP ratio of these

granular nucleotides is 0.6–1.1, thus strongly indicating that the released ATP and ADP are extruded *directly and unchanged* from the granules (Holmsen, Day & Storm 1969). This is similar to the release of ATP together with catecholamines from the adrenal medulla (Douglas & Poisner 1966).

The 'metabolically active' pool of ATP and ADP becomes labelled by the isotope precursors and do not leave the cell (as nucleotides) during the process. This pool can be subdivided into two functionally different pools, the 'release energy pool', seemingly consisting of ATP only, and the 'basic metabolic pool' which participates in glycolysis and oxidative phosphorylation, and supplies ATP to the 'release energy pool' (Holmsen, Day & Storm 1969). The 'release energy pool' is that ATP which breaks down intracellularly during the release. It shows an apparently different time course of isotope incorporation than the nucleotides remaining in the cells (basic metabolic pool) in that it is slowly, but strongly labelled (Holmsen 1965 b; Holmsen 1967). This 'release energy ATP' has a peculiar mode of breakdown when the cell undergoes the release reaction. Usually, when ATP participates in a process deriving energy from the nucleotide, it is split to ADP which in turn is rephosphorylated to ATP by oxidative phosphorylation and glycolysis. In contrast, the 'release energy ATP' is completely degraded via IMP to inosine and hypoxanthine, none of which can be utilized by the platelet for ATP resynthesis (Holmsen & Rozenberg 1968). This fate of the 'release energy ATP' was first reported by Ireland (1967) using platelets preincubated with C^{14}-adenosine by which this ATP became labelled and could be recovered outside the cells as hypoxanthine after the release reaction. As an intermediate in the breakdown of intracellular ATP to extracellular hypoxanthine he identified IMP inside the cells having maximal concentration one min after thrombin addition. Collagen added to platelet-rich plasma also induces a ^{14}C-ATP-IMP conversion which occurs quantitatively within the first 30–60 sec, and probably accounts for *all* ^{14}C-ATP consumed during the release reaction (Holmsen, Day & Storm 1969). Holmsen & Rozenberg (1968) have shown the presence of 5′-AMP deaminase in platelets besides an IMP specific 5′-nucleotidase and purine riboside phosphorylase. A possible route of ATP – hypoxanthine transformation is thus that the two energy-rich phosphoryl groups of ATP are consumed during the release reaction with the participation of an ATPase and adenylate kinase, the end product AMP deaminated to IMP, which is dephosphorylated by the membrane bound 5′-nucleotidase (Day, Holmsen & Hovig 1969). The inosine formed is rapidly phosphorolyzed to hypoxanthine and ribose-1-phosphate, of which the former probably leaves the cell by mere diffusion whereas the ribose phosphate probably remains in the cell. Shielding of 5′-AMP-deaminase from the adenine nucleotides in platelets by a localization in subcellular structures has been proposed (Holmsen & Rozenberg 1968), but the extreme lability of this platelet enzyme has prevented determination of its exact localization (Holmsen & Pimentel 1968).

The intracellular ATP-IMP conversion starts immediately after addition of release inducer (Ireland 1967; Holmsen, Day & Storm 1969) and stops when the release has terminated, after which the 5′-AMP-deaminase in the platelets is completely inhibited (Holmsen & Day 1969). This indicates that the ATP-IMP conversion is closely linked to the release reaction and perhaps the entire

reaction is controlled by the 5′-AMP-deaminase activity.

The 'release energy ATP' pool shows characteristics of the actomyosin bound ATP of muscle and its binding to the equivalent platelet contractile protein, thrombosthenin, has been suggested (Holmsen, Day & Storm 1969). Binding of adenine nucleotides to purified thrombosthenin has been demonstrated (Haslam 1968 a; Crawford 1968). In this connection the inhibition by NEM of the release reaction (Mürer 1968) is of interest. Haslam (1968 b) has shown that NEM is rapidly taken up by platelets and appreciable amounts of it bind strongly to thrombosthenin in the first few minutes of uptake. Activation of platelet ATPases by thrombin and collagen has been reported (Kowalski et al. 1966) which provides further support for the participation of ATPase in the release reaction.

Evidently, we have some information as to the fate of the AMP part of the 'release energy ATP' pool whereas our knowledge about the two metabolic active phosphoryl groups is scanty. Mürer (1969 b) has shown that P^{32} (probably from ATP) is transferred to TCA insoluble material during thrombin action on washed platelets in the presence of EDTA, a milieu in which aggregation is prevented. However, no specific increase in the radioactivity of the individual phospholipids extracted from the insoluble material was observed. On the contrary, a decrease in the radioactivity of the phosphatidyl inositol fraction could be established. Nishizawa & Haldar (1967) also observed that collagen induced increase in the P^{32} radioactivity of platelet phospholipids, but these experiments were performed with citrated platelet-rich plasma, a system in which platelet aggregation occurred.

The effect of thrombin on platelet glycolysis and respiration has been studied by numerous authors (Bettex-Galland & Lüscher 1960; deVreker & deVreker 1965; Corn 1966 a; Warshaw, Laster & Schulman 1966; Karpatkin 1967; Karpatkin & Siskind 1967; Karpatkin & Langer 1968; Mürer 1969 a). Unfortunately, most of these studies have been performed in the absence of EDTA and the effects observed could as well have been due to ADP-induced aggregation. Apart from aggregation of sticky platelets, the change from a platelet being free to one surrounded by others also might interfere with membrane transport processes which in turn affects glycolysis and respiration. In several of the studies referred to above the effect of both thrombin and ADP on platelet energy metabolism were included. Regarding the ADP experiments as controls for aggregation, additional effects produced by thrombin might be taken to represent those of the release reaction. Both agents produced a sudden increase in lactate production which soon levelled off to the same rate as before stimulation. The effect of thrombin was appreciably greater than that of ADP, suggesting that glycolysis really had been accelerated during, or as a result of, the release reaction. Both Corn (1966 b) and Loder, Hirsh & de Gruchy (1968) found stimulation of lactate production during the collagen action on platelets. This is in accordance with the increase in fructose-1.6-diphosphate radioactivity by treatment of P^{32}-labelled platelets with collagen, an increase far greater than that observed with ADP (Holmsen 1965 b). As fructose-6-phosphate kinase is the main rate limiting enzyme of glycolysis, the accumulation of its product indicates stimulation of glycolysis through lowering of ATP or increase of AMP (the main allosteric effectors of the enzyme). Hence, the increase in the glyco-

lytic flux after addition of a release inducer should be regarded as a *response* to the consumption of 'release energy' ATP in the release reaction, and *not* as has been claimed, that the inducers cause *directly* an elevation of the glycolytic rate.

Both thrombin and latex particles produce a spontaneous burst in platelet oxygen uptake, the time course of which and inhibition by NEM correlates exactly with the release of adenine nucleotides (Mürer 1968). This burst is not connected with increased respiration as it is not influenced by antimycin. It is apparently inhibited by plasma proteins, but this inhibition may as well be due to interference of proteins with the oxygen electrode which has led to conflicting opinions about the significance of the oxygen burst (Hussain & Newcomb 1964; Detwiler 1967; Kitchens & Newcomb 1968). That non-mitochondrial oxygen requiring processes might be involved in the release reaction is of great interest, since leucocyte NADPH oxidases are activitated during phagocytosis (Zatti & Rossi 1965), and platelet phagocytosis might be involved in the release reaction induced by collagen, latex particles or other particulate release inducers.

The role of Ca^{++}

The question about the role of Ca^{++} for the release reaction is confusing. Grette (1962) concluded that extracellular Ca^{++} was necessary, since he could pretreat platelets with thrombin in presence of EDTA without appreciable release, but when excess Ca^{++} was added release occurred immediately. Based on this he inferred that the process consists of two stages, i. e. the action of thrombin for which Ca^{++} is not necessary, and the release process proper for which Ca^{++} is necessary. Grette usually performed his experiments at room temperature, but in some experiments at 37° he showed that Ca^{++} was not necessary. Later investigations (Holmsen & Day 1968; Mürer 1968) have shown that the release from washed platelets may take place in the presence of 0.003 M EDTA at 37°C with thrombin as inducer. The presence of Ca^{++} alone caused some release (or leakage) and addition of Ca^{++} together with thrombin caused a 3–4 fold increase of the release of those substances located in the 'usual' α-granules, lysosomal enzymes and fibrinogen, as compared to release in absence of Ca^{++}. On the other hand, the release of adenine nucleotides and serotonin, located in the 'dense' α-granules, is not affected by addition of Ca^{++}. Extracellular Ca^{++} thus does not seem to be essential for the release reaction, but 'catalyzes' the process as far as release of lysosomal enzymes is concerned.

No one seems to have looked for release of platelet Ca^{++}, its subcellular localization or possible changes of the latter during the release reaction. Its possible release and granular localization would be of great interest, since this ion, similar to the released K^{+}, is inexchangeable with external Ca^{++}, and mainly associated with lipids (Wallach, Surgenor & Steele 1958).

The time course of the release reaction

Careful studies on the velocity of the release reaction have only been performed with the thrombin-induced release of serotonin, adenine nucleotides (Grette 1962; Markwardt & Barthel 1964 b) and lysosomal enzymes (Holmsen & Day 1969). Unfortunately, no time course experiments exist with collagen-induced release in which the released compounds have been measured chemically, but interpretation of aggregation measurements

with the optical method (Born 1962; O'Brien 1962) indicates that when started, the release is of the same order of velocity as with thrombin. The same applies to adrenaline- and ADP-induced release, although time experiments with chemical methods do exist (Mills, Robb & Roberts 1968). Unfortunately, these authors did not give detailed information about the time course of the release reaction, since one minute time intervals were employed. It appears that all four of these release inducers have a lag phase varying from less than a second (thrombin) to up to several minutes (adrenaline and ADP). When started, however, all agents seem to cause release as demonstrated by the thrombin experiments cited above: At 37° the concentration of serotonin and nucleotides increase extracellularly and in parallel within the first 20 sec after thrombin addition and then levels off. After 40 sec no additional release occurs, even with 100 units of thrombin/ml for up to one hour. The extracellular concentration of lysosomal enzymes, however, does not parallel that of serotonin + nucleotides. They increase more slowly and reach their maximal value at about one minute, after which no additional release occurs. The acid hydrolases which have been studied (see Table I) are all released in parallel.

Quantitative considerations

As for the time relationship, the information on 'dose-response' relationship of the thrombin induced release is far more detailed than for any other release inducer. However, all give saturation characteristicts, i. e. the lowest concentration giving maximal release with about 500 000 platelets/μl in PRP is 1–2 μM ADP and 0.5 μM adrenaline (Mills, Robb & Roberts 1968) and 0.5–1 unit thrombin/ml with 0.5–1.0 $\times$ 10^6 washed platelets/ml (Markwardt & Barthel 1964; Jackson, Zieve & Morse 1967; Holmsen, Day & Storm 1969; Holmsen & Day 1969). Quantitation is irrelevant with collagen since the degree of polymerization of the fibrils influences the release.

The maximal amounts released are 70–85% of platelet serotonin, 63% of ADP, 44% ATP, 50% K, but only 20–40% of the lysosomal enzymes (references, see Table I). With the concept that all released substances originate from the subcellular granules, the usual reference to 'total platelet amounts' is irrelevant. Therefore, a release of 60% ADP + ATP (when referred to total platelet ADP + ATP) might as well be 100% of the granule non-metabolic nucleotides. Unfortunately, existing techniques for subcellular fractionation are not yet satisfactory for quantitative measurements, and correlation of released material to what is actually present in the granules is a challenging problem.

Heterogenity of the platelet release reaction (differential release)

It is apparent from Table I that there are two different types of storage-granules (as defined by their localization in sucrose layers of different density on gradient centrifugation) for the substances released. One type contains acid hydrolases, fibrinogen and platelet factor 4 while another type contains serotonin plus adenine nucleotides. The release of the contents of both these granule types is not induced by all release inducers. Thrombin and collagen induce release from both types of granules whereas ADP and adrenaline release only serotonin plus nucleotides or substances from the high-density

particles and 'dense' α-granules (or very dense bodies). This differentiation is also apparent from the time course for the released materials, those located in the dense granules being more rapidly released than those located in the 'usual' α-granules. In addition, the response to extracellular Ca^{++} only affects the slowly released platelet constitutents and not the rapidly released.

Inhibitors

In many studies on inhibitors the rate of platelet aggregation caused by the ADP released from the platelets in plasma has been used as a measure of the release. Since it seems to be a tendency for the inhibitors to act on both aggregation and release, interpretation is, however, often difficult.

Adenosine has been shown (O'Brien 1964) to inhibit the second phase of catecholamine- and thrombin-induced aggregation, indicating inhibition of the release step. Adenosine also inhibits serotonin release by collagen (Valdorf-Hansen & Zucker 1968), and Ireland (1968) has shown that it also inhibits the release of nucleotides, measured chemically.

Phentolamine, an α-blocker, was shown to inhibit the second wave in catecholamine induced platelet aggregation by O'Brien (1963) whereas the β-stimulant isoprenaline was inactive. Mills & Roberts (1967 a) directly measuring the nucleotides released, verified this and in addition showed that the β-receptor blocker propanolol was inactive. It thus seems established that the effect of catecholamines is exerted through the α-receptors.

Chlorpromazine, desmethylimipramine and *amitriptyline* have been shown to inhibit both catecholamine, thrombin and collagen induced release without inhibiting ADP aggregation (Mills & Roberts 1967 b; Mills & Roberts 1967 c). It is not clear whether the effect of these drugs is via their α-blocking effect or through other effects.

Sulphinpyrazone (Anturan) was shown to inhibit collagen induced release by Mustard, Rowsell & Murphy (1966) as is true for the related compound *butazolidine* (Smythe et al. 1965; Evans, Mustard & Packham 1967), and O'Brien (1968 c) showed that together with butazolidine a series of anti-inflammatory drugs inhibited collagen and adrenaline induced release.

Acetylsalicylic acid is of special interest, because it prolongs the bleeding time (Quick 1967; Morris 1967; Weiss & Aledort 1967). Weiss & Aledort showed that acetylsalicylic acid had no effect on the ADP induced aggregation, whereas it decreased collagen induced aggregation by inhibiting the ADP release. The same was found by Evans, Mustard & Packham (1967), and O'Brien (1968 a) found in addition that the secondary wave of adrenaline induced aggregation was inhibited. The inhibitory effect after aspirine ingestion persisted for 4–7 days, and since the ATP and ADP levels of the platelets were not altered (Weiss & Aledort 1968), the inhibition rather affected the release mechanism than the amount of stored nucleotides. Acetylsalicylic acid also inhibits release induced by ADP (Zucker & Peterson 1968) as well as that induced by antigen-antibody complexes, α-globulin coated particles and thrombin (Evans et al. 1968). O'Brien (1968 b) and Weiss & Aledort (1968) found salicylic acid to be nearly without effect, whereas it was inhibitory in the experiments of Evans et al. (1968).

Papaverin was shown to inhibit thrombin and trypsin induced release in rabbit platelets by Markwardt et al. (1967).

Procain, although in high concentrations, inhibits thrombin-induced release (Aledort

& Niemetz 1968); zylocaine also seems to inhibit release (Stormorken, 1968).

Clofibrate (Atromid) was shown to inhibit release by Glynn, Murphy & Mustard (1967).

Serotonin has been shown to inhibit collagen induced release of nucleotides (Holmsen & Storm 1969).

EDTA has been shown to inhibit the release of ADP and serotonin by thrombin and connective tissue (Zucker & Jerushalmy 1965) or purified collagen (Holmsen & Storm 1969) from unwashed platelets in plasma. The chelator did not affect the release of these substances from washed platelets (Holmsen & Day 1968).

The effect of metabolic inhibitors and sulphydrylblockers (NEM) has been described above (page 7–8, 11–12).

Morphological changes during the release reaction

Few studies correlate biochemical evidence of release with morphological findings. Therefore, a review of the latter must be based on indirect interpretation of available data on 'viscous metamorphosis' which has been studied in some detail (for review, see Hovig 1968).

Parmeggiani (1961), Hovig (1962) and Castaldi et al. (1962) stressed the fact that platelets treated in such a way that the release reaction should occur lose their granules while the plasma membrane remains intact. The extensive 'degranulation' observed lead to the suggestion that the platelets were no longer functionally intact. These earlier studies were done with techniques employing clotting of whole blood (Rodman et al. 1962), addition of thrombin (Parmeggiani 1961) and tendon extract (Hovig 1962) containing collagen (Hovig 1963 a) to platelet-rich plasma, and observations were made late after release (more than 30 min) had occurred. Since the release reaction is a rapid process, little relevant information can be deduced from these experiments.

The electron microscopic findings after release vary with the nature of the release inducer. Thus, changes following collagen (Hovig 1962; Rodman & Mason 1967) and thrombin (Rodman, Mason & Brinkhous 1963) are more severe than those with ADP (Rodman, Mason & Brinkhous 1963; Mills, Robb & Roberts 1968; Sixma & Geuze 1968) or adrenaline (Mills, Robb & Roberts 1968). The latter two substances cause pseudopod formation, centralization of the granules, but minimal degranulation (Mills, Robb & Roberts 1968). Addition of thrombin to platelet-rich plasma causes centralization of the organelles and pseudopod formation followed by aggregation (Rodman, Mason & Brinkhous 1963; Rodman &Mason 1967; White 1968 b). Rodman et al. (1963) noted a lag phase lasting several minutes before any changes became apparent. The alterations then proceeded as above through granula disintegration. Hovig (1962) found that small amounts of thrombin cause changes leading to some platelet granule disintegration and some early granule lysis, whereas high doses caused extensive degranulation and membrane disintegration.

Prior washing of the platelets seems to have no effect upon the described sequence of events except that fibrin was not observed and cell rhexis was much slower (Rodman & Mason 1967). When thrombin was used to induce release in the presence of fibrinogen, the resultant fibrin fibrils supported and contained disintegrated platelet masses and platelet cellular debris.

When collagen is added to platelet-rich plasma (Hovig 1962 and 1963 a), the initial centralization of organelles, pseudopod for-

mation and aggregation occur as with thrombin, except that the lag phase is shorter and these changes occur within one minute. Granular disintegration and disruption of plasma membranes may be seen at four minutes (Rodman & Mason 1967). If the reaction to thrombin or collagen is slowed by using EDTA, or using washed platelets, greater delay in the lag period occurs. EDTA itself causes pseudopod formation and swelling of the platelet, but no internal platelet changes (White 1968 a).

The response of platelets in citrated plasma to ADP depends in large part on the amount of ADP used. Rodman & Mason (1967) employed 2 mM (final) which generally resulted in centralization of granules, pseudopod formation, aggregation, but no degranulation within one minute. In contrast to the effect seen with thrombin and collagen, the changes produced by ADP were only transitory in that one hour later most of the platelets had retured to normal. White (1968 b) used ADP concentrations, varying from 0.25–25 mM, which caused disc to sphere transformation of the platelets, followed by centralization and concentration of the organelles by a 'contractile wave' of the microtubules and canalicular system. Finally, the granules lay in membrane to membrane contact without fusing. It would thus seem that in the release reaction induced with ADP, pseudopod formation, platelet swelling, and granular centralization are the prime components.

Platelets aggregated with adrenaline, noradrenaline or serotonin are regarded to undergo similar changes (White 1968 a), and Mills, Robb & Roberts (1968) and Sixma & Geuze (1968) showed experimentally some degree of granular structure loss with ADP and adrenaline.

Other inducers of release such as latex, antigen-antibody complexes or carbon particles (Movat et al. 1965) caused great loss of platelet organelles in addition to phagocytic uptake of particulate material in vesicular structures.

The fate of the granules following release is a subject of much interest. Castaldi et al. (1962) noted release of granules *de toto* during viscous metamorphosis. Vassalli, Simon & Roullier (1964) claimed that some granules became less dense and appeared to be degenerating *in situ,* while others were released into the surrounding interspaces. The same feature was noted by Johnson et al. (1965). Rodman & Mason (1967) were unable to confirm these findings, and Hovig (1968) agrees that if granules are discharged in an intact form from the platelet, it is a rare occurrence, and probably not part of the release reaction. White (1968 c) has provided evidence that the dense bodies are exteriorized before centralization. Libanska (1967) feels that discharge of the α-granule content occurs after centralization into a collecting system without *in situ* lysis.

The electron microscopic investigations have so far not given any clear answer as to what happens to the granules during the release process. The reasons for this might be many. Changes in staining characteristics seem not to have been paid attention to in previous studies. Thus, it is conceivable that following loss of their contents, the granules might change their osmophilia in some way, being no longer recognizable as granules. The degree of granule loss (degranulation) is difficult to ascertain. Because the platelets swell and the granules centralize upon contact with a release inducer, it is obvious that sections through such platelets will cut through areas empty of granular elements, while the cell might have its normal granular content.

The main problems for electronmicroscopists to solve, intracellular granule lysis, fusion of the granules with the plasma membrane or with the canalicular system or extrusion of the complete granule, therefore necessitate further investigation. Important details might have been lost mainly because the morphologists have not realized until now the extreme rapidity of the release reaction which would require better ways for rapid termination of the process.

The mechanism of the release reaction

As numerous and quite different compounds may act as release inducers (see page 5), it is difficult to see how they can share a common mechanism. It should be emphasized, however, that their action is in all probability directly on the platelet membrane and not on the granule membrane. Thus, Markwardt (1967) showed that trypsin and thrombin do not release serotonin and ATP from isolated platelet granules, whereas they do so from intact platelets. Similarly, Bygdeman (1968) has shown that noradrenaline is not taken up by the platelets when it induces release (measured by platelet aggregation).

It is reasonable to divide the release process into three steps:

1. *Induction,* an interaction between the extracellular release inducer and the plasma membrane during which a release 'impulse' is created. The inducer does not penetrate the membrane.
2. *Intracellular transmission,* the transmission of the 'impulse' from the plasma membrane to the α-granules or a system containing these.
3. *Extrusion,* the process triggered by the 'impulse' which leads to specific emptying of the granular content to the extracellular phase.

Induction

As to the proteolytic enzymes inducers which have been studied to some extent, it is reasonable to believe that they act by splitting a target protein in or on the platelet surface, probably producing an active peptide. Morse, Jackson & Conley (1965) suggested that membrane bound fibrinogen was the substrate. However, as the venom of *C. terrificus* causes release without having clotting activity (Markwardt et al. 1966) and other snake venoms are active in clotting fibrinogen, but not in inducing release (Davey & Lüscher 1965 and 1967), platelet fibrinogen is hardly the target protein. Human platelets (Davey & Lüscher 1967) as well as bovine platelets (Lopaciuk & Solum 1969) contain a nonfibrinogen protein showing electrophoretic behaviour similar to the β-globulins, which is a substrate for thrombin. This could be the actual target protein, but further characterization is lacking. It is of interest that trypsin is an active release inducer while chymotrypsin is not, but it is unknown at present whether thrombin and trypsin have the same or different target substrates.

It is known that α-blockers inhibit the release induced by ADP, adrenaline and other biogenic amines, indicating that release induction for these substances is mediated through α-receptors.

The mechanism by which the particulate inducers work is also unknown. It is reasonable to believe that it is in some way coupled with their ability to induce phagocytosis (cfr. the burst in oxygen uptake, page 12). However, we also lack information at present as to how the latter is accomplished. There seems to be some disagreement as to the

role of γ-globulins as co-factors in the particle induced release, at least for latex particles. Glynn et al. (1965) and recently Mueller-Eckhardt & Lüscher (1968) have provided evidence for a definite requirement for coating of the latex particles with γ-globulin in order to make them active as release inducers. In contrast Mürer (1968) demonstrated massive release of nucleotides and burst in oxygen consumption with extensively dialyzed latex particles alone. The discrepancy might consist in presence or absence of Ca^{++} as the experiments of Mürer (1968) were conducted in EDTA-milieu, whereas those of the other authors were done in the presence of Ca^{++}. The size of the latex particles might also be important, Mürer's particles were 0.23 μ in diameter (which gives optimal release) in contrast to 0.8 μ in the experiments of Mueller-Eckhardt & Lüscher (1968).

In spite of the great variation in the type of release inducers, nearly all of them cause one change in the platelet surface: *The platelets become 'sticky'*. That is to say, most release inducers cause a *small* degree of platelet aggregation, which does not seem to be ADP-mediated. It is thus possible that a 'sticky' platelet may act as a release inducer for other platelets.

Intracellular transmission

Grette (1962) assumed that the effect of thrombin was to make Ca^{++} available within the platelet, presumably by making the surface membrane permeable to extracellular Ca^{++}. No studies so far have appeared which shows or even indicates that a rapid influx of extracellular Ca^{++} takes place during the release reaction. Interestingly, Douglas & Poisner (1964) have provided evidence that this is the case for the release reaction in adrenal medulla and posterior lobe. The lack of requirement for extracellular Ca^{++} which is apparent from recent experiments casts some doubt on this mechanism in platelets. However, during intracellular transmission Ca^{++} could be made available at specific loci within the cell from intracellular *platelet* Ca^{++} alone. For example, membrane bound Ca^{++} might be made free and available for structurally bound thrombosthenin, which by contraction both brings the granules to the membrane as well as forcing the granule content out of the cell.

Another candidate for the intracellular transmitter is cyclic 3′.5′-AMP. However, preliminary experiments (French & Stormorken 1969) have revealed that the lipid soluble dibutyryl derivative of cyclic 3′.5′-AMP does not release ADP from platelets in plasma.

The second step in the release might be explained by considering the granules to move freely within the cell and strike the plasma membrane without sticking to it. When the cell is stimulated to release, the electrical charge on the inner side of the membrane is changed in a way that the granules will stick to the membrane on collision. The sticking is then the trigger for membrane fusion and extrusion (Banks 1966).

An attractive theory for intracellular transmission, which covers almost every experimental detail, is *activation of 5′-AMP deaminase* (see page 10–11). Poisner & Douglas (1967) have shown that ATP induces release from *isolated* granules of the adrenal medulla to be itself simultaneously consumed in the process by granule ATPase. Both release and ATPase activity are powerfully inhibited by AMP. One might therefore imagine that in the *intact* resting platelet the cytoplasmic AMP exerts complete inhibition of release in

spite of the presence of extragranular ATP. If 5′-AMP deaminase were activated, AMP would be rapidly removed with resultant loss of ATPase inhibition, the release could start immediately utilizing the available ATP. It is not clear how this activation could proceed, but the extreme lability of the enzyme might indicate that it is under the control of several unknown factors.

Extrusion

At present there is no answer to the question how the contents of the granules are emptied into the extracellular space.

A lysosomal concept (Kowalski et al. 1966) infers that platelet lysosomes are activated and cause release of the specific constituents. However, Holmsen & Day (1968) were not able to demonstrate internal lysosomal activation during thrombin action on washed platelets. It is also difficult to reconcile this with the specificity and the speed of the release reaction.

The possibility that the granules are exteriorized intact has little electron microscopic support, except for the observations by White (1968 c) that the very dense bodies are rapidly extruded through the plasma membrane. Other possibilities are reversed pino/phagocytosis with fusion of the granular membrane with the plasma membrane or with membranes of the canalicular systems. Some electron microscopical evidence for this concept exists (Libanska, 1967). The possibility that the granules are in communication with the surface by tiny channels through which the contents could be expressed to the surface, cannot be excluded at present.

The possible participation of the contractile protein in the release reaction is of interest (Grette 1962). However, only scanty and indirect evidence for this has been provided. The inhibitory effect of NEM and its close binding to thrombosthenin might point in this direction (see page 11). Further, the speed of the reaction would also be easily accounted for if a contractile protein were involved, and likewise the effect of Ca^{++} and the presence of Mg^{++}-Ca^{++} activated ATPase in the granules (French 1969). Although the hypothesis of participation of contractile protein in the release reaction is still speculative, it is of sufficient interest to warrant further investigation.

The physiological and pathological significance of the platelet release reaction

Many of the compounds released from the platelets during the release reaction have been shown to be important for the hemostatic and thrombotic processes. First of all, ADP is released which leads to aggregation of platelets and formation of the primary platelet plug or platelet thrombus. The ATP which is released in parallel with ADP, is rapidly converted in plasma to *adenosine* which inhibits ADP-induced aggregation (Rozenberg & Holmsen 1968 a and b). Platelet factor 3 is made available and is a necessary factor for the intrinsic coagulation mechanism. Although the importance of released platelet factor 4 in hemostasis and thrombosis is still unclear, there are indications that this factor also is of great importance. The release of serotonin might be of importance for haemostasis *via* its vaso-constrictive property. Further, the release of hydrolases and other compounds might be of importance for the initiation of coagulation in the platelet atmosphere.

That the release reaction of platelets really has physiological and pathological significance is evident from the recent description of bleeding disorders caused by a defect release reaction with collagen (Hardisty & Hutton 1967; Hirsh, Castelan & Loder 1967; O'Brien 1967; Caen, Sultan & Larrieu 1968). Also the prolonging effect on bleeding time of acetylsalicylic acid (see above) points in the same direction. Based on the available evidence, therefore, the release reaction has been included as a separate step in the hemostatic and thrombotic mechanism (Hellem & Stormorken 1969).

It should be noted that since the platelet seems to be a true secretory cell (Stormorken 1969) and in addition also has phagocytic abilities, this cell might constitute a simple model for detailed studies of these processes without interference from reactions connected with the function of the nucleus.

CONCLUSIONS

On the basis of available data from the literature end recent developments in our and other laboratories the blood platelet has been established as a secretory cell. The release reaction, as induced by a variety of different external stimuli, follows a basic pattern by which substances stored in subcellular particles are rapidly extruded to the external medium. Many of the extruded substances present in great amounts are of great importance for formation of the platelet plug. Thus, the platelets belong to the *immediate* homeostatic secretory cells, and the release process is a necessary step in the early phase of hemostasis and thrombosis.

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