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MAYO, JOSEPH RAYMOND

A STUDY ON THE MECHANISM OF TRIMETOQUINOL AS AN INHIBITOR OF HUMAN PLATELET AGGREGATION

The Ohio State University

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PH.D. 1980
A STUDY ON THE MECHANISM OF
TRIMETOQUINOL AS AN INHIBITOR
OF HUMAN PLATELET AGGREGATION

DISSertation

Presented in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy in the Graduate School of
The Ohio State University

By

Joseph R. Mayo, B.A.

* * * * *

The Ohio State University
1980

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To my wife, Debbe, through her love, understanding, encouragement and sacrifice, all of this has been possible.

To my parents, whose love, encouragement and support is unwavering.
ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to:

Dr. Dennis R. Feller

for his direction, for his encouragement and support, for his friendship, and for giving me the freedom to pursue my research independently.

Dr. Norman J. Uretsky

for his encouragement and support, for his friendship, and for our many fruitful discussions.

Drs. Allan M. Burkman and Duane D. Miller

for their suggestions, discussions and guidance throughout this undertaking.

The American Red Cross, Columbus, Ohio and Riverside Methodist Hospital for their generous donations of stored human platelet rich plasma.
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Major Field: Pharmacology

Biochemical pharmacology; hepatic drug oxidations, mechanisms of thrombosis and hemostasis.

Drug metabolism as related to the toxicity of metabolites.

Mechanisms of prostaglandins as stimulators of platelet aggregation and thrombosis.
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Physiology and general properties of platelets

Blood platelets, in common with red cells and white cells, are formed elements of the blood. These thrombocytes are formed within the cytoplasm of granular megakaryocytes in bone marrow and are released into the circulation when the megakaryocytes expire. Like red cells, platelets have no nucleus. They do not contain DNA and may or may not contain small quantities of RNA (Marcus, 1978). Electron microscopy reveals a cytoplasmic matrix with an endoplasmic reticulum-like network of membranes, Golgi apparatus, mitochondria, microvesicles, lysosomes and highly refractile granules.

The various granules within the platelet contain a variety of constituents with known biological activities. Dense granules contain serotonin, ADP, calcium and antiplasmin (Helmsen, Day and Stormorken, 1969) (Feinman and Detwiler, 1974). Alpha granules contain beta-thromboglobulin, platelet factor 4, cell growth factor and fibrinogen (MacIntyre, 1979). Granules with a reported lysosomal function contain acid hydrolases and also platelet factor 3 (Owen, 1979).

Platelets are thought to have two basic physiological functions. The first important platelet function is to somehow maintain the
integrity of the vasculature. In general, blood vessel fragility and permeability can be correlated with the quantity of platelets in the circulation (only viable platelets circulate). An abrupt reduction in the platelet count may be accompanied by hemorrhage into the skin and mucous membranes. This defect can be rapidly corrected by infusion of platelet concentrates (Marcus, 1978).

A second important platelet function which has been widely studied is their contribution to hemostasis via the intrinsic coagulation system. There are several processes which collectively contribute to hemostasis. The properties of adhesion, aggregation and the synthesis, storage and release of biologically active substances from platelets are probably the most important. These events are interrelated and share the general property of being highly efficient mechanisms that require only a few stimulated platelets for activity. All of these processes and their roles in hemostasis will be discussed in the ensuing description of platelet aggregation and the endogenous roles of arachidonic acid and its metabolites in the aggregation response.

Primary and secondary aggregation

The initial response of platelets to interruption of continuity of vascular surfaces may be termed primary hemostasis. The process can be arbitrarily subdivided into phases of adhesion and primary aggregation. During adhesion, platelets contact and adhere to vascular tissues below the endothelium (primarily collagen). Such adhesion is a property unique to platelets and may be defective in disorders such
as von Willebrand's disease and the Bernard-Soulier syndrome (Hardisty, 1977). One of the major consequences of adhesion is that the platelet membrane develops new biochemical properties. For example, the stimulated plasma membrane develops the capacity to catalyze interactions between proteins of the coagulation system which eventually results in thrombin formation (Walsh, 1974).

Morphologically, the platelets become spherical and extend pseudopodia; this is termed shape change. The stimulated platelet membrane also transmits a signal directed intracellularly which results in a primary release reaction (Holmsen, 1975). This event is characterized by secretion of components previously stored in platelet dense granules, especially calcium, serotonin and various nucleotides. Morphologically, the platelets that have undergone shape change interdigitate with one another, recruit their fellows as a result of the primary release reaction and aggregate. One of the most important substances released during the primary release reaction is ADP. This nucleotide can cause the aggregation of platelets by a direct effect on the plasma membrane. The mechanism is not well understood but the two main hypotheses are (1) ADP may reduce the ability of the platelets to repel one another via a normally present negative surface charge and (2) an ATPase may reside in or near the platelet membrane and ADP may inhibit this enzyme and thereby promote aggregation (Owen, 1979).

Thrombin or collagen can induce further secondary release where other organelles discharge their contents via channels in the open canicular-dense tubular system. The substances which appear in the
plasma are those especially associated with lysosomes and alpha granules (Holmsen, 1975).

The release reactions have been linked to secretory processes in other cell types (endocrine, neuronal, leukocytes). However, in many secretory cells release is associated with increased levels of cyclic AMP (cAMP), but in platelets aggregation and secretion are accompanied by a fall in cAMP (Marcus, 1978). Clearly, the endogenous levels of cAMP within the platelet are very important insofar as they dramatically influence aggregation. This is supported by evidence that the organism controls normal platelet hemostasis by balancing the opposite effects prostacyclin and thromboxane A₂ have on cAMP levels (see subsequent sections for details).

Primary aggregation merges into secondary aggregation sometime after primary release and before secondary release. For primary aggregation, the aggregates may be dispersed, but with the onset of secondary release (and even sometime before) the aggregates are fixed (Holmsen, 1969). Secondary aggregation culminates in the evolution of thrombin. Thrombin in turn can promote aggregation (Marcus, 1978) as well as act on its natural substrate fibrinogen to form a fibrin monomer which polymerizes into insoluble fibrin strands held together by hydrogen bonds. The fibrin strands reinforce the primary aggregation plug converting it to a consolidated mass of stimulated platelets. This mass is reinforced further by activated factor XIII (activated by thrombin, calcium) which forms covalent amide bonds between fibrin polymers (Owen, 1979).
The formation of prostaglandins from arachidonic acid

In 1934 von Euler described an endogenous substance with vaso-depressor and smooth-muscle stimulating activity which was called "prostaglandin" (von Euler, 1935). The activity was subsequently discovered to be due to several different acidic lipids. In years to follow, two independent groups (Bergstrom, Danielson and Samuelsson, 1964) (Van Dorp et al., 1964) demonstrated that prostaglandins are biosynthesized from polyunsaturated fatty acids. Arachidonic acid (eicosatetraenoic acid) is the precursor of all bisenoic prostaglandins, which are those that are the most relevant to platelets and to this text. Sources of arachidonic acid (A.A.) can include membrane phospholipids, or diet where the phospholipid is transported in blood largely bound to albumin and is eventually incorporated as a component of cell membranes throughout the body (Ramwell et al., 1977).

Arachidonic acid may be released from platelets and other cell membranes by the action of phospholipase A₂. This enzyme may be activated by chemical stimuli or simple mechanical stimulation in the case of platelets or lung tissues (Salzman, Lindon and Rodvien, 1976) (Piper and Vane, 1971). Prior to about 1973 it was believed that the only metabolic products of A.A. that possessed substantial biological activity were PGE₂ and PGF₂α. However, it is now recognized that there are several intermediates in A.A. metabolism which are biologically active and also are critically important to the scheme of hemostasis (Figure 1). The synthesis and biological activity of the prostaglandin endoperoxides (PGG₂ and PGH₂), thromboxane A₂ (TxA₂)
and prostacyclin (PG\textsubscript{12}) are particularly crucial to the scheme of hemostasis and will be discussed separately. Other products of A.A. metabolism are formed from lipoxygenase enzymes. These enzymes attack certain double bonds in the A.A. structure and form unstable compounds called hydroperoxyarachidonic acids. There is little information on the properties of these substances and therefore these will not be discussed here or elsewhere in this text (Moncada and Vane, 1979c).

Formation of the cyclic endoperoxides involves the enzyme prostaglandin endoperoxide synthase (EC 1.14.99.1) (hereafter referred to as cyclo-oxygenase). This enzyme transforms A.A. into the unstable cyclic endoperoxide PG\textsubscript{2} (15-hydroperoxy-9α,11α-peroxidoprosta-5,13-dienoic acid). PG\textsubscript{2} is in turn converted by the same cyclo-oxygenase enzyme into the more polar endoperoxide PGH\textsubscript{2} (15-hydroxy-9α,11α-peroxidoprosta-5,13-dienoic acid). The existence of the cyclic prostaglandin endoperoxides was first postulated by Samuelsson (1965) while studying the formation of PGE\textsubscript{1}. PG\textsubscript{2} and PGH\textsubscript{2} were first "detected" as unknown components of a material called rabbit aorta contracting substance (RCS) by Piper and Vane in 1969. Finally, Hamberg and Samuelsson (1973) confirmed the formation of PGH\textsubscript{2}, a 15-hydroxy cyclic endoperoxide of A.A. Now 15-hydroxy and 15-hydroperoxy endoperoxides (PGH\textsubscript{2} and PG\textsubscript{2} respectively) are recognized as initial metabolic products of A.A. in platelets (Fig. 1). The PG\textsubscript{2} and PGH\textsubscript{2} endoperoxides are unstable in aqueous media (T\textsubscript{1/2} is 5min at 37°) decomposing to the stable
FIG. 1. THE METABOLISM OF ARACHIDONIC ACID
(taken from Moncada and Vane, 1979c)
prostaglandins $E_2$, $D_2$ and $F_{2\alpha}$. Their presence and activity during platelet aggregation was initially hypothesized by Vargaftig and Zirinis (1973) and later reported by Willis et al. (1974). The cyclic endoperoxides were strong inducers of platelet aggregation and their involvement in the aggregation process explained why cyclooxygenase inhibitors (such as aspirin) (see following section for details) could inhibit the second phase of aggregation in vitro. Previously, it had not been possible to explain why inhibitors of prostaglandin biosynthesis could inhibit platelet aggregation since $PGE_2$ and $PGF_{2\alpha}$ have no pro-aggregatory activity).

Agents which inhibit the cyclo-oxygenase enzyme are often called aspirin-like inhibitors. Aspirin is now recognized to acetylate the active site of the cyclo-oxygenase enzyme (Samuelsson, 1978) thereby preventing the formation of the cyclic endoperoxides and all of their metabolites. Indomethacin also inhibits the cyclo-oxygenase enzyme and produces similar effects in platelets. Over the last few years, evidence has supported the concept that the analgesic, antipyretic and anti-inflammatory actions of the aspirin-like drugs are mediated by inhibition of the cyclo-oxygenase enzyme (Moncada and Vane, 1979c). As a consequence, use of these drugs has been suggested for clinical conditions including Bartter's syndrome and the refractory diarrhea that accompanies some cases of medullary carcinoma of the thyroid and Crohn's disease. Aspirin or aspirin-like drugs are also being tested in the prevention of premature labor and abortion, the closure of a patent ductus arteriosus, the prevention of second
heart attacks and in other forms of antithrombotic therapy (Moncada and Vane, 1979b).

Hamberg and Samuelsson (1974) also identified and named TxA$_2$ as a component of rabbit aorta contracting substance (TxA$_2$ was initially called PHD, but the current accepted notation is TxA$_2$) (TxA$_2$; 7-\{3-(3-hydroxy-1,5-octadienyl)-2,6-dioxabicyclo [3.1.1] hept-4-yl\}-\{1S-\{1\alpha,3d(1E,3R*,52),4\beta(2),5\alpha\}5-heptenoic acid\}). TxA$_2$ is formed by the action of prostaglandin endoperoxide: thromboxane A isomerase on PGH$_2$. (hereafter called thromboxane synthetase). TxA$_2$ is highly unstable in aqueous media with a half-life of 30 sec at 37° and is an extremely potent inducer of platelet aggregation. The ability of a particular organ or cell to synthesize TxA$_2$ from PGH$_2$ depends on the activity of thromboxane synthetase and therefore varies with each system. In platelets as in lung there is a high conversion of the cyclic endoperoxides to TxA$_2$, but in vascular tissues the cyclic endoperoxides are primarily converted to prostacyclin. The relevance of cyclic endoperoxide conversion to predominantly TxA$_2$ or prostacyclin will be discussed later.

Thromboxane synthetase inhibitors are less clearly defined than cyclo-oxygenase inhibitors because the formation of TxA$_2$ has not been easily measured until the recent use of RIA techniques (Gorman, 1980). Gorman (1980) has suggested that agents which are suspected inhibitors of thromboxane synthetase should be subjected to more rigorous testing before any statements are made as to their activity.
to avoid confusion that currently exists in the area. Two agents
which are accepted thromboxane synthetase inhibitors are imidazole,
and azo analog I (9,11-azoprosta-5,13-dienoic acid) (Gorman, 1980).
These agents are currently not used clinically but find their
greatest use as experimental tools.

In 1976, Moncada and associates found that the prostaglandin
cyclic endoperoxide PGH₂ was transformed by a microsomal enzyme
(prostaglandin endoperoxide I isomerase) (hereafter called prostacyclin
synthetase) from blood vessels into an unstable material that was
a vasodilator and a potent inhibitor of platelet aggregation. This
material was identified as an intermediate in the formation of
6-oxo-PGF₁₀ and labeled prostacyclin (prostacyclin was originally
abbreviated as PGX but current accepted nomenclature is PGI₂)(Fig 1).
Formation of PGI₂ in vivo depends on the presence of prostacyclin
synthetase. This enzyme and consequently PGI₂ formation occurs in
vascular tissues from all species investigated and is the main
metabolic product of A.A. in human vascular tissue (Moncada, Higgs
and Vane, 1977). Prostacyclin synthetase is most highly concentrated
in the intimal surface of the vasculature and progressively decreases
in activity towards the adventitial surface (Moncada, Higgs
and Vane, 1977)(This activity gradient is important in hemostasis and
will be discussed later). PGI₂ is the most potent endogenous
inhibitor of platelet aggregation yet discovered (Moncada and Vane,
1979c). It is also a potent vasodilator and is being implicated as
a mediator in the regulation of blood pressure, the release of renin
from the renal cortex as well as other processes (Moncada and Vane, 1979c). PGI\textsubscript{2} is unstable with a half-life of 2-3 min in blood (37°) and forms 6-oxo-PGF\textsubscript{1\alpha} upon decomposition. Since it is not inactivated by the pulmonary circulation, PGI\textsubscript{2} is equipotent as a vasodilator when given intraarterially or intravenously in animal models (Dusting, Moncada and Vane, 1977; 1978).

The role of prostaglandin cyclic endoperoxides, TxA\textsubscript{2} and PGI\textsubscript{2} in aggregation and hemostasis

Hamberg et al. (1974) demonstrated that the cyclic endoperoxides induced the release of platelet constituents and caused rapid aggregation. Their data showed that the extent and reversibility of the aggregation was concentration dependent with low concentrations (< 10 ng/ml) giving small, reversible aggregation, whereas higher concentrations (> 50 ng/ml) elicited a maximal, irreversible aggregation. The aggregation response to the cyclic endoperoxides was not sensitive to aspirin. Salzman (1977) extended these studies and found that PGG\textsubscript{2}, at low doses, produced aggregation without secretion. At somewhat higher doses, there was irreversible aggregation with secretion that was sensitive to indomethacin, and with even higher doses, the same effects occurred but were insensitive to indomethacin. The indomethacin sensitive doses were attributed to an "autocatalytic action of exogenous PGG\textsubscript{2} on cyclo-oxygenation of endogenous A.A.".
Hamberg et al. (1975) proposed that TxA$_2$ and not PGG$_2$ or PGH$_2$ was the arachidonate metabolite that mediated aggregation and release. Their position was based upon experiments which showed that the aggregating effects of A.A. declined with a half-life similar to TxA$_2$ and not the cyclic endoperoxides. Clearly, TxA$_2$ is a more potent inducer of aggregation than the endoperoxides, however, whether TxA$_2$ is obligatory for aggregation is unclear (Hamberg et al., 1975). The ability of PGH$_2$ to cause rapid aggregation and release with little conversion (<1%) into TxB$_2$ suggests that the endoperoxides have a direct action on platelets to induce aggregation (Smith, Ingerman and Silver, 1975). The question has been extensively studied using thromboxane synthetase inhibitors and synthetic PGH$_2$ analogues. Briefly, studies using imidazole gave conflicting results which may be explained by the ability of imidazole to enhance PGH$_2$-induced aggregation in washed platelets and to block the aggregation in platelet rich plasma (PRP) (Fitzpatrick and Gorman, 1978). Another thromboxane synthetase inhibitor, azo analogue I inhibited both TxA$_2$ synthesis and aggregation due to PGH$_2$ in PRP or washed platelets (Fitzpatrick and Gorman, 1978). However, these results are difficult to interpret since this azo analogue may also be a receptor antagonist to PGH$_2$ as well as a thromboxane synthetase inhibitor (based on structural similarity to the cyclic endoperoxides). The synthetic PGH$_2$ analogues U44069 [(15S)-hydroxy-9α,11α-(epoxymethano)prosta-5Z,13E-dienoic acid] and U46619 [(15S)-hydroxy-11α,9α-(epoxymethano)prosta-5Z,13E-dienoic acid] are not substrates for the
synthetase enzyme and are also pro-aggregating and releasing agents suggesting that cyclic endoperoxides can be active on their own (Smith, Ingerman and Silver, 1977). However, this view is also questionable since the cyclic endoperoxide analogues may act more as TxA\(_2\) mimetics than endoperoxide stimuli (Coleman et al., 1980; Smith, Ingerman and Silver, 1977). These questions are certainly not clear but based on all the current data a reasonable hypothesis is that the PGG\(_2\) and PGH\(_2\) endoperoxides can exert direct pharmacological effects when their metabolism to TxA\(_2\) is inhibited. Normally, however, the endoperoxides probably elicit a response through conversion to TxA\(_2\). The question of whether the cyclic endoperoxides can interact at a TxA\(_2\) "receptor" site or at distinct sites is unclear.

The mechanism through which the endoperoxides or TxA\(_2\) cause aggregation is not firmly established. The currently accepted hypothesis is one where TxA\(_2\) acts to mobilize calcium from platelet intracellular stores. The mobilized calcium is then available to catalyze the process of shape change, release and aggregation (Gerrard et al., 1978b; Gorman et al., 1978; Haslam et al., 1978; Moncada and Vane, 1979c; White, 1979). The decrease in cAMP in platelets seen after stimulation by aggregating agents would be explained by an inhibitory effect of calcium on adenylate cyclase. The reverse would also be true where elevated cAMP levels would inhibit calcium mobilization and also inhibit the cyclo-oxygenase
enzyme. This scheme is supported by evidence which suggests that
$\text{TxA}_2$ can act as an ionophore (Gerrard et al., 1976) and also by
experiments using TMB-8 (8-N,N-diethylamino-octyl-3,4,5-trimethoxy-
benzoate) (an intracellular calcium antagonist) in which increasing
concentrations of TMB-8 were able to inhibit secretion and $\text{PGH}_2$-
induced cAMP accumulation in a dose dependent fashion (Gorman, 1979).
The important role cAMP plays in the regulation of platelet function
is difficult to study in vitro. The reason is that when in situ,
platelets are thought to have a normal physiological stimulation of
their adenylate cyclase by PGI$_2$, whereas in vitro platelets would
have very low basal levels of cAMP and any change from this level
would be difficult to detect. This fact explains why the mechanism
of PGI$_2$ is difficult to determine in in vitro studies and underscores
the role PGI$_2$ plays as a hemostatic element in situ.

The interplay between platelets and PGI$_2$ is exemplified by the
current theory that the vessel wall can not only synthesize PGI$_2$
from its own precursors, but also from prostaglandin endoperoxides
released by platelets (Moncada and Vane, 1979c). Although this view
is not supported by all investigators (Needleman, Wyche and Raz,
1979) this author believes that the major evidence supports this
position. To summarize, the enzyme which forms PGI$_2$ is most
concentrated in endothelial cells and in fact most apparent in the
intimal surface (Moncada et al., 1977). Clearly, the generation of
PGI$_2$ is an active mechanism through which the vessel wall may be
protected from platelet deposition. When there is vessel damage,
and adventitial layers become exposed, (low in PGI₂ synthetase) platelets will adhere to that site and may form large thrombi depending on the extent of injury. When the injury is minor, the small thrombi that form will break away from the vessel wall and be washed away. In order for the development of thrombosis to occur, there must be a physical detachment of the endothelium such as might occur with severe damage. This regulating gradient of enzyme activity is not only true for PGI₂ but also occurs for the enzymes which synthesize TxA₂ which increase from the subendothelium to the adventitia (Moncada et al., 1977). In this way, these two opposing synthetic pathways cause the endothelial lining to be antiaggregatory and the outer layers of the vessel wall thrombogenic.

An additional control over aggregation is apparent with the ability of PGI₂ to inhibit aggregation at much lower concentrations than those needed to inhibit adhesion (Higgs et al., 1978). This suggests that PGI₂ will allow platelets to adhere to vascular tissue while at the same time limiting thrombus formation. One might envision that platelets, after adhering to a site where PGI₂ synthetase was present, would feed the enzyme with cyclic endoperoxides, thus producing more PGI₂ and therefore preventing other platelets from attaching and aggregating thus limiting the cells to a monolayer. There are also suggestions that formed blood elements such as white cells which produce endoperoxides may feed the vessel enzymes to produce PGI₂. A reciprocal modulation may also be present here since
PGI₂ may modulate white cell behavior and thereby control migration during inflammation (Weksler, Knapp and Jaffe, 1977).

Prostacyclin inhibits platelet aggregation by stimulating adenylate cyclase, causing an increase in cAMP levels in the platelet (Tateson, Moncada and Vane, 1977). Although PGE₁ and PGD₂ both cause cAMP levels to increase, PGI₂ seems to act on the same receptor as PGE₁ while PGD₂ acts on another. This is supported by species differences in activity (Williams and Downing, 1977) and by the observation that a specific antagonist (N-0164) can inhibit aggregation induced by PGD₂ but not by PGI₂ or PGE₁ (Whittle, Moncada and Vane, 1978) (Eakins et al., 1976). The final suggestion is that the PGE₁ receptor in platelets may also be identical to the PGI₂ receptor.

All of the effects that PGI₂ has in platelets, its ability to enhance calcium sequestration (Kazer-Glanzman et al., 1977), its inhibition of phospholipase and cyclo-oxygenase enzymes (Malmsten et al., 1976) are all manifest through cAMP (Minkes et al., 1977). Since PGI₂ can inhibit several steps in the A.A. cascade, it exerts a powerful overall control of platelet function in vivo. The fact that PGI₂ can also increase cAMP in other cells (Gorman et al., 1979) and the fact that TXA₂ has effects on a variety of cells suggests the PGI₂/TXA₂ system may have a broad significance in cell regulation.
Quantitation of platelet function

Platelet function tests are an important tool in the study of platelets. The tests may be done either in vitro or in vivo and each has certain advantages over the other in the type of information that may be learned.

Tests used in vitro include quantitation of adhesiveness and aggregation. As a measure of platelet adhesion, a platelet count is done before and after the passage of citrated whole blood through a known volume of glass beads of uniform size. The difference between counts is taken as a measure of adhesiveness (Owen, 1979). For aggregation, the method that is commonly used is a nephelometric method first suggested by Born (1962). Citrated PRP is pipetted into a cuvette and placed in an aggregometer where it is stirred at a constant rate and kept at a constant temperature. Light of known wavelength is passed through the tube and a baseline transmission is adjusted. A stimulating agent is added (with or without previous inhibitor introduction) which causes the platelets to aggregate and increase light transmission. The changes in transmission are recorded automatically by a recorder and a tracing is produced. Two common interpretations of the tracings are (1) the maximum increase in light transmission and (2) the slope of the curve indicating the ratio between the vertical distance (maximum deflection) and the horizontal distance (elapsed time). Each method of interpretation is accurate and the method of choice is dependent on experimental design (Owen, 1979).
Methods for quantitation of platelet function in vivo include haemostatic platelet plug formation and thrombus formation. Thrombus formation measures the time required for the formation of a haemostatic plug, which is effectively the bleeding time. A micropuncture wound is made in a small vein in a mesenteric vessel (usually mouse). The appearance of a platelet plug may be visualized and the corresponding time for stoppage of bleeding recorded. Several bleeding times obtained from multiple sites are taken for each animal (Owen, 1979). Thrombus formation may be measured by the use of an extracorporeal shunt between the left carotid artery and the left jugular vein (as an example). A silk thread is introduced into the shunt and the thrombus that forms on the silk may then be removed, dried and weighed. Comparisons are made between sizes of thrombi before and after drug treatment of the animal. Many other methods are also used some of which include: A.A.-induced pulmonary embolism, platelet mural thrombus in the hamster cheek pouch and endotoxin-induced aggregation (Owen, 1979).

As a way of summary, it is important to recognize that experiments done in vitro may be difficult to extrapolate to in vivo conditions. Some of the reasons for the limitations may be summarized as follows:

1) Most in vitro tests use anticoagulants, the presence of which may affect the results obtained.

2) Rates of flow and shear in experimental studies very often do not correspond to the "normal" rates in human circulation.

3) Platelets in PRP do not interact in the same manner as platelets flowing in whole blood.
4) Species differences exist with regard to platelet surface interactions.

5) Isolated platelets are deprived of the normal interaction with PGI2 from the vessel wall and therefore basal cAMP levels are changed.

6) Animal models of thrombosis often do not accurately reflect the clinical disorders they are selected to mimic.

( Owen, 1979)

Pharmacology of tetrahydropapaveroline and trimetoquinol

Shortly after the initial synthesis of tetrahydropapaveroline (THP) in 1909 by Pyman, Laidlaw tested the alkaloid and discovered that THP lowered blood pressure and increased heart rate; he had indirect evidence for increased force of contraction of the heart and also found that THP relaxed uterus (Laidlaw, 1910) (see Fig 2 for structure). In the 1960's confirmation of the beta-agonist activity appeared (Holtz et al., 1964; Santì et al., 1967) including a demonstration of lipolytic activity in vivo and in vitro. In 1967, Japanese workers became interested in THP and analogues and found that the tetrahydroisoquinoline analogue trimetoquinol (TMQ;[1-(3',4',5'-trimethoxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline]) (Fig 2) was a more potent bronchodilator than isoproterenol (Iwasawa and Kiyomoto, 1967). Further research showed that the beta-stimulant properties of tetrahydroisoquinolines were stereoselective (Kiyomoto et al., 1970) and also that TMQ was less potent on guinea pig heart preparations than isoproterenol (Kiyomoto et al., 1969; Brittain et al., 1970; Farmer et al., 1970). Later, Shonk et al.
(1971) examined the activity of the TMQ isomers using lipolysis as an index of activity. Their results revealed that the S(-)-TMQ isomer was at least 2.9 log units more potent than the R(+) isomer. Buckner and Abel (1974) extended the work on TMQ by showing isomeric potency differences to be 1.61 and 1.56 log units for isolated atria and tracheal preparations respectively. Feller et al. (1975) also failed to find any difference in isomeric-activity ratios (IAR) in isolated atria and trachea.

This similarity in IAR (negative log molar $ED_{50}(-)$ isomer minus negative log molar $ED_{50}(+)$ isomer) suggests that TMQ is a non-selective beta-adrenoceptor stimulant based on the criteria suggested by Patil (1969). He suggested the use of stereoisomers of agonists to categorize receptor types. This is based on the premise that stereoisomers of a given agonist are treated in an identical fashion by all physiological processes that are not stereoselective themselves. Patil (1969) has used that property to describe a relationship between IAR and tissue receptors which states that if the IAR of a given compound are the same in different tissues, then the compound is equiactive at both receptors and the receptor types in the tissues may be assumed to be identical.

In addition to beta-stimulant activity, TMQ also has some blocking activity against alpha-adrenoceptors. Although there was no activity on guinea pig aortic preparations, the S(-)-isomer was able to inhibit norepinephrine-induced aortic contractions.
(Miller et al., 1975). The pA₂ value for S(-)-TMQ was 4.7 but the activity was not stereoselective since the pA₂ value of the R(+) isomer was 4.8.

Other reported properties of THP and tetrahydroisoquinolines include inhibition of COMT activity in vitro. This activity was demonstrated in a variety of tissues including rat liver mitochondria, rat brain and brainstem as well as intact neurons (adrenergic nerve plexus of isolated mouse atrium) (Hattori et al., 1969; Yamanaka, 1971; Katz and Cohen, 1976). Cohen et al. (1972) have also shown that 6,7-dihydroxy-tetrahydroisoquinoline can be taken up into peripheral sympathetic neurons of the rat iris. Likewise, many other reports have appeared which indicate that THP and tetrahydroisoquinolines can be taken up and stored in nerve terminals as well as cause the release of biogenic amines (especially dopamine) from brain slices and synaptosomes (reader is referred to Deitrich and Erwin, 1980 for a comprehensive review).

One of the most interesting properties of TMQ was shown in 1976 by Shtacher et al. when they demonstrated that TMQ could inhibit platelet aggregation. What was particularly interesting was that the inhibition of collagen, second-wave ADP and second-wave epinephrine induced aggregation was unaffected by propranolol, practolol or phentolamine and was independent of platelet adenylate cyclase levels and cAMP or cGMP phosphodiesterase activities. Dalton et al. (1976) described an additional unique property of TMQ. Their work used the stereoisomers of TMQ and they reported that the stereoselectivity
Fig 2. STRUCTURES OF TRIMETOQUINOL AND TETRAHYDROPAPAVEROLINE

♦ Indicates an assymetric center
for inhibition of collagen, ADP and epinephrine-induced aggregation was reversed relative to the selectivity for beta-adrenoceptor stimulation. This reversal of activities for stereoisomers of a biologically active compound is highly unusual and this author is aware of only one other example. (-)-Isoproterenol is more active than (+)-isoproterenol when tested for heart rate, blood pressure and tracheal relaxation; but (+)-isoproterenol is more potent than the (-)-form in lowering the intraocular pressure of the rabbit eye (Seidehamel et al., 1975).

The most recent work with TMQ centers on its properties apart from beta-stimulation. MacIntyre and Willis (1978) and Mayo et al. (1979) have described the ability of TMQ to inhibit prostaglandin endoperoxide-induced aggregation. Mayo et al. (1979) have shown that R(+)-TMQ is significantly more effective than S(-)-TMQ as an inhibitor of aggregation induced by A.A., collagen and the stable PGH₂ analogues U44069 and U46619 and have also indicated that inhibition of the U46619 analogue is competitive. MacIntyre and Willis (1978) reported single doses of racemic-TMQ that could inhibit aggregation induced by U44069, U46619 as well as PGG₂,PGH₂ and TxA₂.

Bennett and Sanger (1979) have shown that racemic-TMQ can selectively antagonize longitudinal muscle contractions of rat isolated gastric fundus to thromboxane B₂ as well as U44069 and U46619. Hanna et al. (1980) report that racemic-TMQ can relax bovine pulmonary vein independent of adrenergic influence. This author believes that a variety of non-adrenergic mediated activities will be
described for TMQ in the future and these may possibly be explained
by the mechanisms for TMQ detailed in this text (that is, related to an
inhibition of intracellular calcium movement).

Statement of the problem

Trimetoquinol [1-(3',4',5'-trimethoxybenzyl)-6,7-dihydroxy-
1,2,3,4-tetrahydroisoquinoline; TMQ] represents a successful effort
to alter the physiological response a compound will elicit through
the designed manipulation of its structure. This compound is a
modification of tetrahydropapaveroline (THP), a stimulant of
beta-adrenoceptors (Holtz et al., 1964; Santi et al., 1967).
Japanese workers, in 1967, found that TMQ was a more potent broncho-
dilator than isoproterenol (Iwasawa and Kiyomoto, 1967) and that
its activities were stereoselective (Kiyomoto et al., 1970). The
S(-)-isomer of TMQ is currently used as a therapeutic regimen for
bronchial dilation in Japan.

Apart from the ability to stimulate beta-adrenoceptors, TMQ
was shown ten years later to inhibit platelet aggregation induced
by collagen, epinephrine and ADP (Shtacher et al., 1976). Since
the antiaggregatory activity was unchanged in the presence of phentol-
amine or propranolol and practolol and was independent of cAMP levels,
or cAMP and cGMP phosphodiesterase activities, adrenergic mechanisms
were not implicated. Also, when TMQ was given to rats by oral
administration, formation of PGF$_{2\alpha}$ in serum was unchanged indicating
that TMQ was not a cyclo-oxygenase inhibitor in vivo (Shtacher et al.,
A noteworthy discovery was made by Dalton et al. (1976) when they reported that the isomeric activities of TMQ were reversed for inhibition of aggregation as compared to beta-adrenoceptor stimulation. This suggested further that the mechanism was independent of beta-receptor/adenylate cyclase coupled activation.

Since little was known about the mechanism of TMQ action as an antiaggregatory agent, our approach was to examine TMQ as an inhibitor of aggregation induced by agents which were known intermediates in the A.A. cascade of platelet aggregation. The systematic use of these stimulants would help us to localize the pharmacological site of action of TMQ in the metabolic scheme (Fig 1).

It is proposed to use human blood as the source of platelets since considerable differences are often observed using animal platelets (Moncada and Vane, 1974). It is also proposed to use PRP as the source of platelets rather than platelets which have been washed and resuspended in buffer. It is the opinion of this author that since recognizable differences exist between the activity of enzymes in PRP versus washed platelets (Fitzpatrick and Gorman, 1979), that the cofactors, proteins and lipids present in the plasma may help to more closely approximate in vivo conditions than platelets suspended in a buffer solution.

To support the studies done which measured aggregation, biochemical studies were proposed to monitor the release of serotonin from platelets as a consequence of the early stages of aggregation.
The examination of this event with and without the presence of TMQ would help to more precisely define what biochemical events TMQ was inhibiting during platelet aggregation. These studies are favorably marked by the precise manner in which platelets take up, store and release serotonin (Bhargava et al., 1979).

Based on initial studies, it is proposed that any effect TMQ has on calcium movement in platelets should be identified. The hypothesis (based on current evidence) is that platelets contain calcium storage organelles which bind calcium and then release it under the influence of a pro-aggregating stimulus (Gerrard et al., 1976). The discharged calcium would diffuse out of the cell but it would also activate any calcium-sensitive intracellular process (Detwiler and Feinman, 1973) including secretion and phospholipase activation (Robblee and Shepro, 1976). Since calcium is a proposed central determinant of platelet aggregation, any alteration in its normal movement would affect the release reaction and aggregation. The model implies that intracellular calcium is the source of activation for platelets unlike most other secretory cells which depend on extracellular calcium. This premise is strongly supported by the demonstration of relatively large platelet intracellular storage pools for calcium and by the observation that extracellular calcium is not required for platelet activity (Robblee and Shepro, 1976).

It is proposed to use human intracellular platelet membranes to observe calcium uptake and release. This author believes that small
changes in intracellular calcium that may occur during aggregation might be undetected in whole platelets given the present methodology. The work done by Robblee et al. (1973), isolating and identifying viable platelet intracellular membranes provided the methodological background for the proposed experiments. The rationale is to use the stereoisomers of TMQ as a tool in order to compare and contrast the effects of TMQ as an inhibitor of aggregation with the effects of TMQ on calcium movement from platelet membranes. Since little evidence on the mechanism of action of TMQ was available at the onset of these studies, they represent an initial probe into the properties of TMQ as an antiaggregatory agent.

The significance of these studies lies in the nature of TMQ as an inhibitor of platelet aggregation. Preliminary studies done by Shtacher et al. (1976), Dalton et al. (1976), MacIntyre and Willis (1978) and Mayo et al. (1979) indicate that TMQ is capable of inhibiting platelet aggregation at some point after TxA₂ action. A reasonable hypothesis is that TMQ is a receptor-level antagonist of TxA₂.

This level of prostaglandin antagonism may have important clinical applications when one considers the scheme of A.A. metabolism in platelets and blood vessel walls (Fig 3). The human platelet can synthesize only the pro-aggregatory and vasoconstrictive TxA₂, while the vessel wall can synthesize only the vasodilator and antiaggregatory PGI₂ (Gorman, 1980). Under normal circumstances, the balance between these two prostaglandins controls human platelet aggregation through
FIG 3. THE INTERACTION BETWEEN THROMBOXANE A$_2$
AND PROSTACYCLIN IN VIVO
(taken from Gorman, 1980)
a reciprocal regulation of platelet cAMP and calcium levels (White, 1979). In certain pathological states, PGI₂ synthesis may be impaired (atherosclerosis) and TxA₂ would become the dominating force inducing thrombotic episodes and vasoconstriction (Gorman, 1980). In theory, a cyclo-oxygenase inhibitor would not be desirable since the synthesis of PGI₂ would also be impaired. However, a thromboxane synthetase inhibitor or a TxA₂ receptor-level antagonist would be most useful since the metabolic pathway for PGI₂ formation would be unimpaired by these agents (see Fig 1). The greatest therapeutic effect may in fact be a combination of the two agents to achieve inhibition at the receptor level and at the enzyme level together. Since TMQ may inhibit aggregation at the level of the TxA₂ receptor, its mechanism of action is of interest.

Also, TMQ appears structurally unrelated to TxA₂ (see Figs 1 & 2). To date, only the prostaglandin analogues 13-APA (13-azaprostanolic acid; LeBreton et al., 1979) and 9,11-EIP (9α,11α-epoxyiminoprost-5,13-dienoic acid; Fitzpatrick et al., 1978) have been reported to act at the receptor-level for TxA₂ antagonism. TMQ therefore may represent a novel chemical entity.
CHAPTER II
MATERIALS AND METHODS

Chemicals

Adenosine-5'-triphosphate, and albumin (bovine fraction V) were purchased from Sigma Chemical Co., St. Louis, MO. A23187 was obtained from Eli Lilly and Co., Indianapolis, IN. [5-\(^{14}\)C]-Serotonin was purchased from Amersham Corp., Arlington Heights, IL, and diluted with 0.05 M potassium phosphate buffer. \(^{45}\)Ca]-as chloride in water was obtained from New England Nuclear Corp., Boston, MA. Stock solutions of indomethacin (Merck and Co., Inc., Rahway, N.J.), arachidonic acid (Nuchek Corp., Elysian, MN.), prostaglandin E\(_1\) (Upjohn, Kalamazoo, MI.) and the epoxymethano prostaglandin endoperoxide analogues U44069 [(15S)-hydroxy-9\(\alpha\),11\(\alpha\)-(epoxymethano)prosta-5Z,13E-dienoic acid] and U46619 [(15S)-hydroxy-11\(\alpha\),9\(\alpha\)-(epoxymethano)prosta-5Z,13E-dienoic acid] (Upjohn, Kalamazoo, MI) (courtesy of Dr. John Pike) were prepared in absolute ethanol and working solutions were diluted with phosphate buffer. Collagen and ADP were obtained from Chrono-log Corporation (Haverton, PA.) as aqueous solutions. Racemic-Trimetoquinol (TMQ) was synthesized in our laboratories as reported by Miller et al., (1975). The stereoisomers of TMQ were gifts from Tanabe Seiyako (Tokyo, Japan). All compounds were freshly prepared in 0.05 M potassium phosphate buffer, pH 7.4 containing 0.05% metabisulfite.
Collection of blood and preparation of platelet rich plasma

Human blood was taken by venipuncture from volunteers who had fasted overnight and reported being free of aspirin containing medication for at least 14 days. A human subjects protocol form is appended. The whole blood was combined and mixed with 3.8% trisodium citrate (9:1, v/v). Platelet rich plasma (PRP) was then prepared by centrifugation at 200g for 10 min at room temperature and used within 2 hr of isolation (International Model HN centrifuge). Platelet poor plasma (PPP) was obtained by centrifuging PRP for 10 min at 4,000g. The platelet concentration of PRP was consistently between 300,000 and 400,000/cmm, as determined using a Neubauer counting chamber (Spencer, Inc., Buffalo, NY).

Dog blood was taken by venipuncture from mixed-breed animals and combined with 3.8% trisodium citrate as previously described. PRP was prepared by centrifugation at 67g for 10 min in an International Model K centrifuge (International Equipment Co., Needham Hts., Mass.)(International type 240 rotor).

Aggregation

Platelet aggregation was monitored at 37° by nephelometry in a Chrono-log aggregometer (Model 330; Haverton, PA) with constant stirring at 1100 rev/min. PRP (0.5 ml) was incubated for 3 min at 37° prior to the initiation of aggregation. This time period also served as the incubation interval for modulators of the system.
All inhibitors or stimulators of platelet aggregation were added to PRP as aliquots ranging from 5 μl to 20 μl in volume. In all experiments (unless indicated otherwise) the minimal concentration of aggregating agent that produced irreversible aggregation was used. The light transmission through PPP was used to determine a maximum aggregation response.

**Serotonin release**

[5-^{14}C]-Serotonin was incubated with PRP for 25 min prior to aggregation studies (0.05 μCi/ml PRP). Aliquots of PRP were removed and platelets caused to aggregate by various agents in the presence or absence of TMQ. Control samples received aliquots of solvent in place of an aggregating agent. The samples were immediately centrifuged at 10,000g for 30 sec (Beckman microfuge B, Beckman Instruments, Inc., Fullerton, CA). Aliquots of the supernatant were transferred to scintillation vials and following the addition of an emulsion-type scintillation solution (Thrift-Solv, Kew Scientific, Columbus, OH) were examined for radioactivity. $^{14}$C was measured on a Beckman (LS 345) scintillation counter using external standardization to monitor the extent of quench. Counting efficiencies of not less than 90% were obtained. The amount of $[^{14}C]$-serotonin released was calculated by subtracting the activity contained in the control samples from the total activity contained in each drug modified sample. Maximal release was defined as the radioactivity present in the supernatant after chemically-inducing irreversible aggregation.
without modifiers present. The concentration of $^{14}$C-serotonin after maximal aggregation was always at least 4 times greater than control values (average maximal aggregation = 9000 dpm).

**Thromboxane A$_2$ generation**

Thromboxane A$_2$ (TxA$_2$) was generated by a modification of the method reported by Chignard and Vergaftig (1977). In this procedure, the unusual property of dog platelets to synthesize TxA$_2$ in the presence of exogenously added arachidonic acid (A.A.) without aggregating themselves is exploited. Dog platelets were isolated as previously described. Aliquots of dog PRP (0.5 ml) were incubated with 0.2 mM arachidonic acid with constant stirring at room temperature for 2 min. This interval was determined to be optimal for maximal TxA$_2$ production by Chignard and Vergaftig (1977) and also in our own experiments using aggregation of human PRP as an indicator of activity. After the incubation interval, 0.2 ml of dog PRP were immediately transferred to a cuvette containing 0.5 ml human PRP. The human PRP was incubated with 0.5 mM indomethacin for 2 min prior to the addition of dog PRP to prevent interference by A.A. The aggregation response of human PRP as a result of stimulation by TxA$_2$ was monitored as previously described.

**Isolation of intracellular membranes**

The method used to isolate platelet intracellular membranes represents a modification of the method reported by Robblee, Shepro
and Belamarich (1973). Human platelet-rich plasma was obtained from The American Red Cross or local hospital blood banks. All steps were carried out at 0-4°C unless otherwise stated and all glassware was previously siliconized. The platelet-rich plasma was transferred to plastic round bottom test tubes (12 ml capacity) and centrifuged at 150g in a refrigerated Sorval RC-2B centrifuge for 3 min. This procedure removed red blood cells (RBC's) from the plasma. The supernatant was transferred to glass conical test tubes and centrifuged at 1800g for 20 min. The supernatant was discarded and the pellet was carefully resuspended in citrated saline (1/10 vol of 3.8% trisodium citrate). The suspension was centrifuged at 150g for 3 min to remove remaining RBC's and the resulting supernatant was centrifuged at 1800g for 20 min. The pellet was resuspended in citrated saline as before and the procedure repeated. The final supernatant was discarded and the pellet was resuspended in 8 ml of freshly prepared homogenizing solution (homogenizing solution contained 30 mM KCl, 5 mM MgCl₂, 50 mM potassium oxalate and 20 mM TRIS-HCl pH 7.0). The platelet suspension was subjected to sonication in a glass vessel using a Sonic 300 dismembrator (Artek systems Corp., Farmingdale, N.Y.). The period of sonication was 1.5 min accomplished in 30 sec intervals spaced by 30 sec rest periods. The disrupted platelets were centrifuged at 14,000g for 10 min in a Beckman model L ultracentrifuge (type 40 rotor). The supernatant was recovered and re-centrifuged at 40,000g for 60 min. The 40,000g pellet was resuspended in a minimal volume of homogenizing solution. The
proteins. Protein concentration was adjusted to 0.2 mg/ml using the method of Lowry et al. (1951) with bovine serum albumin (fraction V) as a standard.

**Calcium uptake by platelet subcellular fractions**

The isolated membranes were induced to accumulate $^{45}$Ca by using a modification of the method reported by Robblee, Shepro and Belamarich (1973). Reaction mixtures contained 2 mM ATP, 100 mM KCl, 5 mM MgCl$_2$, 8.1 μM CaCl$_2$ of which 0.063 nmols was $^{45}$Ca (0.126 μCi/ml) in a final volume of from 20-30 ml 20 mM TRIS-HCl pH 7.0. Unless otherwise stated in the figure legends, complete reaction mixtures contained 5 mM K oxalate which was introduced with the platelet fraction (from the homogenizing medium). Because the activity of the preparations is reported to deteriorate rapidly at 37° and also has been shown to be suppressed at 4° (Robblee, Shepro and Belamarich, 1973), all reactions were carried out at ambient temperature (22-24°). The reaction was initiated by the addition of from 2-3 ml of the membrane suspension to a final protein concentration of 0.2 mg/ml. This mixture was constantly stirred (200 rev/min) throughout the incubation period. The reaction mixture was assayed after various time intervals by removing aliquots (0.5 or 1.0 ml), transferring them to a Millipore Filtration Manifold (# 3025)(Millipore Corp., Bedford, MA) where the membranes were trapped by filtration under negative pressure (Millipore type HA 0.45 μm filters). Any calcium which was not tightly associated with the membranes was removed by
washing the immobilized membranes four times successively with 10 ml portions of ice cold 0.9% saline. Additional washing was shown not to remove any additional calcium. The membranes were allowed to air dry before placing them in an emulsion-type scintillation cocktail (Thrift-Solv) and dissolving them by vigorous shaking in a capped scintillation vial. $^{45}$Ca was assayed by liquid scintillation spectrometry using the entire window (0-1000) of a Beckman LS 8100 scintillation counter where efficiencies of not less than 92% were obtained. Control samples demonstrated that the filter retained less than 0.005% of the total added $^{45}$Ca in the absence of membranes.

Calcium mobilization

The membrane continually accumulated calcium over time until a steady state level was reached sometime between 60 and 70 min. At given times during the equilibrium period, aliquots (0.5 or 1.0 ml) were transferred to small plastic vials (2 ml capacity) which were continuously shaken at 120 oscillations/min at ambient temperature (22-24°) in a Dubnoff Metabolic Incubator (Precision Scientific, Chicago, IL.). Vials were divided into control and treated groups and after receiving 20 μl additions of trimetoquinol or vehicle alone were incubated for 3 min. The epoxymethano PGH$_2$ analogues U44069, U46619, the cation ionophore A23187 or vehicle alone were then added (20 μl vol) to appropriate vessels and allowed to incubate for 3 min unless otherwise stated. After this interval, the contents of the vials were filtered on a Millipore Filtration Manifold
followed by successive washings with 10 ml of ice-cold 0.9% saline as previously described. The filters were air dried, dissolved by shaking in scintillation cocktail and assayed as before.

Calculations

The potency ratios for the TMQ stereoisomers were calculated as the negative log molar IC\textsubscript{50} R(+) - isomer minus the negative log molar IC\textsubscript{50} S(-) - isomer. (IC\textsubscript{50} = inhibitory concentration-50)

Statistical Analysis

Where appropriate, statistical comparisons of independent sample means were made using the Student-t-test at the 95% level of confidence (Sokal and Rohlf, 1969).

Comparisons between several independent sample means were made using a one-way analysis of variance and by examining 95% confidence intervals for overlapping limits (Sokal and Rohlf, 1969).
CHAPTER III
RESULTS

Platelet aggregation

Preliminary studies of aggregation in platelet rich plasma (PRP) preparations examined concentration thresholds for induction of maximal aggregation by arachidonic acid (A.A.), ADP, collagen, U44069, U46619, A23187 and thromboxane A₂ (TXA₂). In these experiments, the minimal concentration of inducer that caused the platelets to aggregate was identified and used in subsequent experiments. Levels of inducer concentration used in these studies were: A.A. (1 mM), ADP(5 µM), collagen(0.12 mg/ml), U44069(10 µM), U46619(2 µM) and A23187(5 µM).

The initial studies with trimetoquinol (TMQ) began by examining its inhibitory effects on A.A.-induced platelet aggregation. Platelets were caused to aggregate by the introduction of A.A.(1 mM) in the presence or absence of TMQ. Each of the TMQ stereoisomers caused a dose-dependent inhibition of A.A.-induced aggregation as seen in Figure 4. R(+) -TMQ was calculated to be 11.7 times more potent than the S(-)-isomer and the mean IC₅₀ values for the R(+) and S(-)-isomers were 4.1 µM and 47.6 µM respectively.

Collagen (0.12 mg/ml)-induced aggregation differed from A.A. in that five min was required in order to develop maximal aggregation as compared to about three min for A.A. (Fig 5). An explanation
Figure 4. Dose dependent inhibition of human platelet aggregation by the stereoisomers of trimetoquinol. Aggregation was induced by the addition of 1 mM arachidonic acid as described in Materials and Methods. Each point is the mean ± S.E.M. of 3 determinations. Inhibitory concentration-50 values together with the 95% confidence limits are 4.1 μM (2.5-6.6 μM) and 47.6 μM (43.7-51.3 μM) for the R(+) and S(-)-isomers respectively. The potency ratio is 11.7 (9.4-14.8).
INHIBITION OF ARACHIDONIC ACID (1 mM)-INDUCED AGGREGATION
BY THE TRIMETOQUINOL ISOMERS IN HUMAN PLATELETS

Fig. 4
Figure 5. Representative tracings of aggregation induced by collagen (0.12 mg/ml) and arachidonic acid (1 mM) in the presence and absence of the stereoisomers of trimetoquinol. Tracings were obtained as described in Materials and Methods. Both tracings indicate that the R(+) isomer is more potent as an inhibitor of platelet aggregation than the S(-) isomer. The calculated potency ratios and the 95% confidence limits for the trimetoquinol isomers are 12.3 (9.9-15.1) for collagen and 11.7 (9.4-14.8) for arachidonic acid-induced aggregation. Each tracing represents the pen response to each drug concentration and the individual responses are superimposed for visual comparison.
INHIBITORY EFFECTS OF THE TRIMETOQUINOL ISOMERS ON COLLAGEN AND ARACHIDONIC ACID-INDUCED AGGREGATION IN HUMAN PLATELETS

Fig. 5
is that collagen must induce the liberation of A.A. from platelet phospholipids in the membrane which is then further metabolized to physiologically active products. By contrast, A.A. which is introduced directly into the system becomes an immediate substrate of platelet enzymes forming physiologically active prostaglandin metabolites. Inhibition of collagen-induced aggregation by TMQ however, was similar to inhibition of A.A. (Fig 5) (Table 1) as demonstrated by nearly identical IC<sub>50</sub> values of 4.2 μM and 50.8 μM for the R(+) - and S(-)-isomers. The corresponding calculated potency ratio of 12.3 was also comparable to the potency ratio obtained for the inhibition of A.A.-induced aggregation by the TMQ isomers (see Fig 4).

The cyclic prostaglandin endoperoxides are the first intermediates formed in the platelet from A.A. metabolism (see Fig 1). Since the TMQ-isomers were inhibitors of comparable potency against A.A. and collagen-induced aggregation, it was desirable to examine platelet aggregation stimulated by the cyclic prostaglandin endoperoxides. However, PGG<sub>2</sub> and PGH<sub>2</sub> are very unstable and are easily converted to the products PGE<sub>2</sub>, PGD<sub>2</sub> and PGF<sub>2α</sub> which makes their biosynthetic preparation difficult (Moncada and Vane, 1979c). These endoperoxides have recently become available from commercial sources (Ran Biochemicals, Israel); however their cost is prohibitive. The synthesis and availability of cyclic prostaglandin endoperoxide analogues (Bundy, 1975) which are very stable to spontaneous degradation make these attractive to use as experimental replacements for the natural endoperoxides. These analogues are structurally
different from naturally occurring \( \text{PGH}_2 \) in the substitution of a methyl group for oxygen in the 9-position for analogue U46619 and the 11-position for analogue U44069 (Fig 6). In preliminary experiments, it was found that analogue U44069 was a less potent stimulator of maximal platelet aggregation than U46619. The minimal concentration of U44069 that was required to induce maximal aggregation was five-fold greater than the minimal concentration required for U46619 (10 \( \mu \text{M} \) vs 2 \( \mu \text{M} \)). These results are in agreement with the reported observations of others (Malmsten, 1975; MacIntyre et al., 1978).

As shown in Figure 7, TMQ was an effective, dose-dependent inhibitor of aggregation induced by both of these stable endoperoxide analogues. Inhibition of U44069-induced aggregation was characterized by IC\(_{50}\) values of 1.42 \( \mu \text{M} \) and 56.2 \( \mu \text{M} \) for the R(+)– and S(−)-isomers respectively and a calculated potency ratio of 39.2 (Fig 7). TMQ was found to be a more effective inhibitor of U46619-induced aggregation since IC\(_{50}\) values of 0.14 \( \mu \text{M} \) and 11.2 \( \mu \text{M} \) were observed for the R(+)– and S(−)-isomers respectively. The potency ratio was calculated to be 82.9 (Fig 8). These data are in agreement with the observations of MacIntyre and Willis (1978) who examined racemic-TMQ as an inhibitor of the prostaglandin cyclic endoperoxides (both natural and the synthetic analogues) and reported IC\(_{50}\) values ranging from 0.28 \( \mu \text{M} \) for both U44069 and U46619 to 1.4 \( \mu \text{M} \) for \( \text{PGH}_2 \).

Although the synthetic endoperoxide analogues are structurally like \( \text{PGH}_2 \), reports have appeared indicating that these analogues produced physiological effects more like that of \( \text{TXA}_2 \) than \( \text{PGH}_2 \).
STABLE PROSTAGLANDIN ANALOGS OF PGH₂

(15S)-hydroxy-9α,11α-(epoxy methano)prosta-5Z,13E-dienoic acid

(15S)-hydroxy-11α,9α-(epoxy methano)prosta-5Z,13E-dienoic acid

PGH₂
Figure 7. Dose dependent inhibition of human platelet aggregation by the stereoisomers of trimetoquinol. Aggregation was induced by the addition of 10 μM U44069 as described in Materials and Methods. Each point is the mean ± S.E.M. of 3 determinations. Inhibitory concentration-50 values together with the 95% confidence limits are 1.4 μM (0.68-3.3 μM) and 56.2 μM (39.8-80.2 μM) for the R(+) and S(-)-isomers respectively. The calculated potency ratio is 39.2 (23.4-65.6).
INHIBITION OF U44069 (10 \( \mu \text{M} \))-INDUCED AGGREGATION BY THE TRIMETOQUINOL ISOMERS IN HUMAN PLATELETS

Fig. 7
Figure 8. Dose dependent inhibition of human platelet aggregation by the stereoisomers of trimetoquinol. Aggregation was induced by the addition of 2 μM U46619 as described in Materials and Methods. Each point is the mean ± S.E.M. of 3 determinations. Inhibitory concentration-50 values together with the 95% confidence limits are 0.14 μM (0.02-0.91 μM) and 11.2 μM (2.6-48.9 μM) for the R(+) and S(−)-isomers respectively. The calculated potency ratio for the trimetoquinol isomers was 82.9 (53.7-129).
INHIBITION OF U46619 (2μM)-INDUCED AGGREGATION BY THE TRIMETOQUINOL ISOMERS IN HUMAN PLATELETS

N=3

82.9*  
(53.7-129)

* Potency Ratio

Fig. 8
Since the data indicated that TMQ was a potent inhibitor of U46619 and U44069, the implication was that TMQ may also inhibit the action of TxA$_2$.

Studies with TxA$_2$ are marked by the same problems of availability and stability as previously discussed for PGG$_2$ and PGH$_2$. A stable analogue of TxA$_2$ has recently been described (carbocyclic TxA$_2$; [2(2),3-(1E,3R*)-3-(3-hydroxyyl(1-octenyl)-bicyclo(3.1.1)hept-2-yl-5 heptenoic acid]) but this analogue is an inhibitor of platelet aggregation and is therefore not useful as a TxA$_2$-mimetic in platelet systems (Lefer et al., 1980). The lack of a stable analogue of TxA$_2$ which acts as a mimic on platelets makes biosynthetic preparation of TxA$_2$ a necessity. Representative tracings of aggregation induced by U46619 and TxA$_2$ in the presence and absence of TMQ are shown in Figure 9. As can be seen, differences are noted in the extent of maximal aggregation by U46619 and TxA$_2$ and are attributable to the technical problems associated with the generation of TxA$_2$.

The isomers of TMQ were found to potently and stereoselectively inhibit the aggregation response to TxA$_2$. The IC$_{50}$ values for the R(+) and S(-)-isomers of TMQ were 0.64 µM and 23.2 µM respectively. The potency ratio for the TMQ-isomers was calculated to be 36.0 (Fig 10).

The ability to stereoselectively antagonize TxA$_2$ activity is in agreement with MacIntyre and Willis (1978) who reported the ability of racemic-TMQ to inhibit TxA$_2$-induced aggregation and also Bennett
Figure 9. Representative tracings of platelet aggregation induced by U46619 (2 μM) and thromboxane A\(_2\) (TXA\(_2\)) in the presence and absence of the stereoisomers of trimetoquinol. Tracings were obtained as described in Materials and Methods. Both tracings indicate that the R(+) isomer is more potent as an inhibitor of aggregation than the S(-) isomer. Each tracing represents the pen response to each drug concentration and the individual responses are superimposed for visual comparison.
INHIBITORY EFFECTS OF THE TRIMETOQUINOL ISOMERS ON U46619 AND TxA2 - INDUCED AGGREGATION IN HUMAN PLATELETS

Fig. 9
Figure 10. Dose dependent inhibition of human platelet aggregation by the stereoisomers of trimetoquinol. Aggregation was induced by the addition of thromboxane A\(_2\) as described in Materials and Methods. Each point is the mean ± S.E.M. of 4 determinations. Inhibitory concentration-50 values together with the 95% confidence limits are 0.64 \(\mu\)M (0.39-1.07 \(\mu\)M) and 23.2 \(\mu\)M (17.4-30.9 \(\mu\)M) for the R(+) and S(-)-isomers respectively. The calculated potency ratio for the trimetoquinol isomers was 36.0 (19.1-67.6).
INHIBITION OF THROMBOXANE A₂-INDUCED AGGREGATION BY THE TRIMETOQUINOL ISOMERS IN HUMAN PLATELETS

![Graph showing percent inhibition of thromboxane A₂-induced aggregation by R(+)- and S(-)-trimetoquinol isomers. The graph includes data points and error bars, with a potency ratio labeled as 36.0 (19.1-67.6).]

Fig. 10
and Sanger (1979) who reported on the ability of racemic-TMQ to inhibit U46619- and TxB$_2$-induced contraction of rat isolated gastric fundus.

The IC$_{50}$ values of each TMQ isomer together with the calculated potency ratios for the inhibition of A.A.-, collagen-, U44069-, U46619- and TxA$_2$-induced aggregation are shown in Table 1. As can be seen, the data appear to fall into two groups based on the similarity of IC$_{50}$ values and calculated potency ratios. In the first group, collagen and A.A. have similar IC$_{50}$ values for R(+) and S(-)-TMQ as well as having similar calculated potency ratios (based on the overlap of the 95% confidence limits). These values (as a group) are different however than the IC$_{50}$ values and the calculated potency ratios (as a group) for inhibition of U44069-, U46619- and TxA$_2$-induced aggregation. Within the latter group, all of the values for R(+)TMQ are similar (based on confidence interval overlap). The calculated potency ratios for inhibition of U44069-, U46619- and TxA$_2$-induced aggregation were similar and ranged from 46 to 83. The observed difference between potency ratio values in the latter group is related to differing IC$_{50}$ values of S(-)-TMQ. IC$_{50}$ values for S(-)-TMQ against U44069- and U46619-induced aggregation are not significantly different. However, the S(-)IC$_{50}$ value for TxA$_2$ is about 2-fold larger than the S(-)IC$_{50}$ for U46619 and about 2-fold smaller than the S(-)IC$_{50}$ value for U44069.

In addition to the A.A. cascade there are other mechanisms through which platelets may aggregate. One avenue distinct from the
<table>
<thead>
<tr>
<th>Aggregating Agent</th>
<th>IC_{50} R(+) Isomer</th>
<th>IC_{50} S(-) Isomer</th>
<th>Potency Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic Acid (10^{-3}M)</td>
<td>4.1 (2.5-6.6)</td>
<td>47.6 (43.7-51.3)</td>
<td>11.7 (9.4-14.8)</td>
</tr>
<tr>
<td>Collagen (0.12 mg/ml)</td>
<td>4.2 (2.4-7.2)</td>
<td>50.8 (35.4-72.4)</td>
<td>12.3 (9.9-15.1)</td>
</tr>
<tr>
<td>U44069 (10^{-5}M)</td>
<td>1.4 (0.7-3.1)</td>
<td>56.2 (34.8-80.2)</td>
<td>39.2 (23.4-65.6)</td>
</tr>
<tr>
<td>U46619 (2 X 10^{-6}M)</td>
<td>0.14 (0.02-0.9)</td>
<td>11.2 (2.6-48.9)</td>
<td>82.9 (53.7-129)</td>
</tr>
<tr>
<td>TxA2 ***</td>
<td>0.64 (0.4-1.1)</td>
<td>23.2 (17.4-30.9)</td>
<td>36.0 (19.1-67.6)</td>
</tr>
</tbody>
</table>

*The minimal concentration of each agent which produced irreversible aggregation was used.

**Inhibitory Concentration-50 (IC_{50}); values are the mean of N = 4; 95% confidence interval shown in parenthesis.

***Thromboxane A_{2} (TxA_{2}) was generated as indicated in Materials and Methods.

\dagger IC_{50} for R(+)–trimetoquinol/IC_{50} for S(-)–trimetoquinol; 95% confidence intervals shown in parenthesis.

\ddagger IC_{50} for racemic-trimetoquinol was 4.0 (2.1-6.9) (\mu M; 95% confidence interval).
A.A.-prostaglandin scheme is the interaction of ADP with the platelet membrane (see Introduction for details). When ADP was used as a stimulator of platelets, maximal aggregation was apparent at 5 μM ADP and racemic-TMQ was not an effective inhibitor of the ADP-induced aggregation since only a 17% inhibition was apparent at 10⁻⁴ M TMQ (Figure 11). For comparison purposes, racemic-TMQ is shown in the figure to inhibit A.A.-induced aggregation in the same potency range as previously shown for the R(+)–TMQ isomer alone. The fact that TMQ was shown to be an effective inhibitor of aggregating agents which act through a prostaglandin-dependent pathway and an ineffective inhibitor of ADP-induced aggregation suggested that TMQ was a specific inhibitor of prostaglandin-induced aggregation.

In preliminary experiments, the inhibition of U46619-induced aggregation by R(+)–TMQ was reversed by the further addition of higher concentrations of U46619. Figure 12 shows that the control aggregatory response to 2 μM 46619 was reduced to about 8% of control in the presence of 1 μM R(+)–TMQ. This inhibition by TMQ was completely reversed by the addition of a higher (10 μM) concentration of U46619. In a similar manner, the 100% inhibition of platelet aggregation by 5 μM TMQ was also reversed and returned to 100% of control (0% inhibition) after the addition of 10 μM U46619. Finally, even in the presence of 50 μM TMQ, the addition of U46619 up to 100 μM returned the response to about 60% of control aggregation from 0% aggregation. The ability to overcome TMQ inhibition of platelet aggregation by increasing the concentration of U46619 suggested that a competitive-like inhibition was operative.
Figure 11. Representative tracings of platelet aggregation induced by U46619 (2 μM), arachidonic acid (1 mM) and adenosine diphosphate (ADP; 5 μM) in the presence or absence of racemic-trimetoquinol or the R(+)-trimetoquinol isomer. Tracings were obtained as described in the Materials and Methods. The tracings indicate that trimetoquinol is a potent inhibitor of aggregation induced by U46619 or arachidonic acid but not an effective inhibitor of aggregation stimulated by ADP. Each tracing represents the pen response to each drug concentration and the individual responses are superimposed for visual comparison.
INHIBITORY EFFECTS OF TRIMETOQUINOL ON U46619, ARACHIDONIC ACID AND ADP INDUCED AGGREGATION IN HUMAN PLATELETS

Fig. 11
Figure 12. The reversal of R(+)−trimetoquinol inhibition of U46619-induced aggregation in human platelets by the addition of higher concentrations of U46619. Aggregation was induced by the addition of varying concentrations of U46619 (as indicated) and monitored as described in Materials and Methods. Each value is the mean of duplicate determinations.
REVERSAL OF R(+)-TRIMETOQUINOL INHIBITION OF U46619-INDUCED AGGREGATION IN HUMAN PLATELETS

PERCENT MAXIMAL AGGREGATION

R(+)-TRIMETOQUINOL
Conc; µM: 0 1 1 5 5 50 50 50

U46619
Conc; µM: 2 2 10 2 10 2 20 100

Fig. 12
Figure 13. Estimation of the inhibitory constant (Ki) of racemic-
trimetoquinol against U46619-induced aggregation in human platelets.
Data were plotted according to the method of Dixon (1953).
Experiment A estimated an apparent Ki value of 0.25 μM; experiment B,
0.09 μM and experiment C, 0.06 μM. The mean Ki value for trimetoquinol
for these determinations was 0.13 μM ± 0.06 (S.E.M.) (n=3).
ESTIMATION OF THE INHIBITORY POTENCY (Ki value) OF RACEMIC -TRIMETOQUINOL AGAINST U46619-INDUCED AGGREGATION IN HUMAN PLATELETS

\[ \text{Ki} = 0.13 \pm 0.06 (\mu M \pm \text{S.E.M.}) \]

**Fig. 13**
Subsequent experiments were designed in order to monitor aggregation induced by two separate concentrations of U46619 in the presence of increasing concentrations of TMQ (Fig 13). In experiment A, a control aggregatory response was elicited by the addition of 1 \( \mu \text{M} \) U46619. This control response was modified in subsequent replications by the presence of increasing concentrations of racemic-TMQ. The procedure was repeated again for a control response to 1.5 \( \mu \text{M} \) U46619 and the data were plotted according to the method of Dixon (1953). The intersection of the lines above the abscissa suggested that a competitive inhibition was operative, with the point of intersection being an estimate of an apparent inhibitory constant (\( K_i \)). Experiment A revealed an inhibitory constant for racemic-TMQ of 0.25 \( \mu \text{M} \). The entire procedure was repeated in experiment B except for the use of 1.5 \( \mu \text{M} \) and 2.0 \( \mu \text{M} \) concentrations of U46619. Experiment C used 1 \( \mu \text{M} \) and 1.5 \( \mu \text{M} \) concentrations as before. Experiments B and C revealed apparent \( K_i \) values of 0.09 \( \mu \text{M} \) and 0.06 \( \mu \text{M} \) respectively. An approximate \( K_i \) value for racemic-TMQ was calculated as the mean ± S.E.M. of the three experimental \( K_i \) values and was found to be 0.13 \( \mu \text{M} ± 0.06 \) (S.E.M.). The identification of an apparent \( K_i \) value for racemic-TMQ strongly suggested that the inhibition of U46619-induced aggregation was competitive.

**Release of serotonin from platelet stores**

One of the consequences of prostaglandin-stimulation of platelets besides aggregation is the platelet granular secretion reaction i.e. the release of serotonin and other materials from storage vesicles.
(see Introduction for details). Since TMQ was shown in previous experiments to inhibit prostaglandin-mediated aggregation (and not ADP-induced aggregation) it was of particular interest to determine whether TMQ could also inhibit the release of serotonin since that process is known to be mediated through a prostaglandin-dependent pathway (Moncada and Vane, 1979c). Platelets were pre-incubated with $[^{14}\text{C}]-\text{serotonin}$ as described previously and caused to aggregate and release serotonin by the addition of 2 $\mu$M U46619. Racemic-TMQ was shown to dose-dependently inhibit both aggregation and the secretion of $[^{14}\text{C}]-\text{serotonin}$(Fig 14). The concentrations of racemic-TMQ required for the inhibition of aggregation and release were nearly identical. The $IC_{50}$ value for the inhibition of the release of serotonin was 1.29 $\mu$M with a 95% confidence interval of 0.56–2.95 $\mu$M. The $IC_{50}$ value for the inhibition of U46619-induced aggregation was 1.49 $\mu$M with a 95% confidence interval of 0.58–3.8 $\mu$M. Similar experiments were done using the stereoisomers of TMQ and as before, each isomer produced a stereoselective, dose-dependent inhibition of both aggregation and serotonin release (Fig 15). Once again, the inhibition of aggregation for each isomer was nearly identical to the inhibition of serotonin release.

Experiments were also done using ADP as the inducer of aggregation. Even though racemic-TMQ was not an effective inhibitor of ADP-induced platelet aggregation, (see Fig 11) the release of serotonin by ADP was inhibited maximally by 0.01, 0.1 and 1.1 mM racemic-TMQ (Table 2).
Figure 14. Inhibition of U46619-induced (2 µM) aggregation and serotonin release by racemic-trimetoquinol in human platelet rich plasma. Aggregation and serotonin release were induced by the addition of U46619 as described in Materials and Methods. Racemic-Trimetoquinol dose-dependently inhibited both aggregation and the release of serotonin. The IC$_{50}$ value together with the 95% confidence limits for the inhibition of aggregation was 1.29 µM (0.56–2.95 µM). The IC$_{50}$ value for inhibition of serotonin release was 1.49 µM (0.58–3.8 µM). Each point is the mean ± S.E.M. of 3 determinations.
Fig. 14

PERCENT INHIBITION vs. LOG MOLAR CONCENTRATION

- Aggregation
- Serotonin Release

N=3
Figure 15. Inhibition of U46619-induced (2 μM) aggregation and serotonin release by the stereoisomers of trimetoquinol in human platelet rich plasma. Aggregation and serotonin release were induced by the addition of U46619 as described in Materials and Methods. Both the R(+)- and S(-)-trimetoquinol isomers dose-dependently inhibited aggregation and the release of serotonin from platelet stores in a nearly identical fashion. The incubation of R(+)–trimetoquinol in the absence of U46619 and platelet aggregation did not increase serotonin release above control samples. Also, the incubation of racemic-trimetoquinol (10⁻⁴M) alone did not cause lysis of the platelet membrane when compared to controls. A lactate dehydrogenase enzyme assay was used as an indicator of lysis (personal communication with Stephen S. Navran, 1980).
INHIBITION OF U46619-INDUCED (2 µM) AGGREGATION AND
SEROTONIN RELEASE BY THE ISOMERS OF TRIMETOQUINOL
IN HUMAN PLATELETS

Fig. 15
TABLE 2. INHIBITION OF ADP INDUCED SEROTONIN RELEASE BY RACEMIC-TRIMETOQUINOL

<table>
<thead>
<tr>
<th>TMQ (mM)</th>
<th>Percent inhibition of aggregation*</th>
<th>Percent inhibition of serotonin release**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>9.2 ± 0.4</td>
<td>100</td>
</tr>
<tr>
<td>1.0</td>
<td>17.0 ± 0.6</td>
<td>100</td>
</tr>
</tbody>
</table>

* Human platelets were caused to aggregate by the addition of 5 μM ADP

** See text for description of method.

* Results are mean values ± S.E.M. from 3 experiments.

Calcium accumulation and release

Current theories on the biochemical events which trigger platelet aggregation suggest that calcium is the final mediator in the prostaglandin-dependent pathway which leads to the release reaction and aggregation (Gerrard et al., 1978b; Haslam et al., 1978; Moncada and Vane, 1979; White, 1979). Experiments were initiated to determine whether TMQ could influence intracellular calcium movement in the platelet since the data suggested that it was a potent inhibitor of prostaglandin-mediated events in the platelet. For these experiments, intracellular platelet membranes were isolated by a modification of
the method reported by Robblee et al. (1973). These investigators described the biochemical properties of an isolated subcellular preparation of platelet membranes. It was shown that a 40,000g platelet subcellular fraction accumulated calcium in the presence of ATP and oxalate and reached a steady-state level of accumulation after 40-60 minutes. The calcium uptake process required ATP and magnesium, was enhanced by oxalate and was accompanied by the release of inorganic phosphate. Calcium accumulation and phosphate release were found to be inhibited by salyrgan (10 µM) and ADP (1 mM) but not by ouabain (0.1 mM). Electron microscopic histochemistry using lead nitrate showed lead precipitates to be localized primarily at the inner surface of membrane vesicles suggesting that an ATPase was present at that site. They postulated that the observed calcium uptake activity was an in vitro manifestation of a calcium pump in the intact platelet.

The experimental design was to examine the prostaglandin-stimulated mobilization of calcium from a similar preparation of isolated platelet membranes. In preliminary studies, the subcellular membrane fraction accumulated calcium in the presence of ATP and oxalate to a steady-state level of 24.5 nmol/mg platelet protein which was reached after 60 min of incubation at room temperature (23°) (Fig 16). Both the rate of accumulation and the steady-state level reached were found to be unaffected in the presence of 10^{-4}M racemic-TMQ. After steady-state levels of calcium accumulation were reached, the addition of U46619 to the preparations caused a specific release and loss of calcium from the membranes (see Materials and
Methods for details). Preliminary experiments indicated that 0.42 nmoles calcium/mg protein (± 0.02 S.E.M., N=3) were mobilized after a 10 sec incubation interval with U46619 and that the maximal level of release (1.2 nmol calcium/mg protein) was achieved between 2 and 3 min.

On the basis of these findings, an interval of 3 min was selected in subsequent experiments. Additional experiments showed that the mobilization of calcium was dose-dependent and reached a maximal level at 2 µM U46619 (Fig 17). This represented the loss of 5.0% (± 0.5 S.E.M.) of the total bound calcium. This percentage loss is in agreement with Owen et al. (1980) who reported the mobilization of about 4% of the total bound calcium from intact platelets after the addition of 0.5 µM U46619. As a comparison, the addition of 2 µM A23187 to the preparations caused a dramatic reduction in the accumulated calcium (> 8 nmol calcium/mg protein lost) within the same time interval (Fig 17).

The U44069 analogue was also incubated with the membranes but unlike U46619 only mobilized 0.56 nmol calcium/mg protein (± 0.7 S.E.M.) (2.3% of the total calcium) at a concentration of 50 µM. Lower concentrations were found not to cause any significant mobilization when compared to controls. The fact that the U44069 analogue was a less potent stimulator of calcium mobilization than the U46619 analogue is in agreement with the order of potency observed for stimulation of platelet aggregation. Figure 18 shows that the U46619 analogue is about 5-fold more potent than the U44069 analogue as a
Figure 16. Accumulation of calcium versus the duration of incubation time. Calcium uptake is expressed as nmoles calcium accumulated / mg platelet protein and also as a percentage of the total calcium added to the incubation mixture. Each value is the mean ± S.E.M. of 4 determinations.
CALCIUM UPTAKE (n moles/mg protein)

CALCIUM UPTAKE (per cent of total calcium)

MINUTES

0  10  20  30

0  20  40  60  80  100 110

Fig. 16
Figure 17. Mobilization of calcium from a human platelet subcellular membrane fraction after incubation with U46619 or A23187. Incubation mixtures and methods are described in Materials and Methods. Amount of calcium mobilized is expressed as nmoles calcium removed/ mg protein and as a percentage of the total calcium bound to the membrane fraction. Each value represents the mean ± S.E.M. of 4 determinations. *Value is significantly different from control incubations, p < 0.05. +No corrections were made for endogenous calcium present.
stimulator of aggregation. Also the U46619 analogue is about 40-fold more potent than the U44069 analogue as an inducer of calcium mobilization from isolated platelet subcellular membranes. It is also noteworthy that the dose of U46619 which induces half-maximal aggregation is nearly identical to the dose which causes half-maximal calcium mobilization (see Fig 18).

All of these data were used to design experiments where TMQ was examined as an inhibitor of calcium mobilization. In these experiments, the R(+) or S(-)-TMQ isomer was pre-incubated with the membranes for 3 min prior to the addition of 2 µM U46619. Both isomers were found to inhibit calcium mobilization in a dose-dependent fashion compared to controls (Fig 19). The IC$_{50}$ values for the R(+) and S(-)-isomers were 0.197 µM and 2.39 µM respectively. The calculated potency ratio was 12.1. These data show that TMQ can stereoselectively inhibit calcium mobilization stimulated by U46619.

In other experiments, membranes were pre-incubated with racemic-TMQ before the addition of A23187 (2 µM). At the highest concentration of racemic-TMQ ($10^{-4}$ M), calcium mobilization was inhibited by only 23%. This suggests that TMQ is not an effective inhibitor of calcium mobilization stimulated by the ionophore A23187 but that it is effective at inhibiting U46619-induced mobilization (Fig 20). These data are in agreement with the relative inability of racemic-TMQ to inhibit A23187 (5 µM)-induced platelet aggregation (Fig 20). In those experiments the rate of maximal aggregation (increase in light transmission/ time) of human platelet rich plasma induced by A23187
Figure 18. Direct comparison of the stimulation of human platelet aggregation (in platelet rich plasma) and calcium mobilization (from isolated platelet subcellular membranes) by the PGH$_2$ analogues U46619 and U44069. Incubation mixtures and methods are described in Materials and Methods. Results are expressed as a percentage of the maximal aggregation response (closed and open circles) and as a percentage of the total bound calcium that was released (closed and open squares) after stimulation by U46619 or U44069. Each value represents the mean ± the range of values of 3–5 determinations. No corrections were made for endogenous calcium present.
STIMULATION OF HUMAN PLATELET AGGREGATION IN PRP AND CALCIUM MOBILIZATION FROM ISOLATED SUBCELLULAR MEMBRANES BY U46619 AND U44069

![Graph showing stimulation of human platelet aggregation and calcium mobilization by U46619 and U44069.](Fig. 18)
Figure 19. Inhibition of U46619 (2 μM)-induced calcium mobilization from a platelet subcellular membrane fraction by the stereoisomers of trimetoquinol. Incubation mixtures and methods are described in Materials and Methods. Results are expressed as a percentage of inhibition of calcium removal from the membrane fraction. Zero percent inhibition corresponds to 5.0 nmoles calcium mobilized/mg protein ± 0.5 S.E.M. No corrections were made for endogenous calcium present. Each value represents the mean ± S.E.M. of 4 determinations. The calculated potency ratio for the trimetoquinol isomers together with the 95% confidence limits was 12.1 (3.1-47.8).
INHIBITION OF U46619 (2 μM)-INDUCED MOBILIZATION OF CALCIUM FROM ISOLATED PLATELET MEMBRANES BY THE STEREOISOMERS OF TRIMETOQUINOL

Fig. 19
Figure 20. Inhibition of A23187-induced platelet aggregation and calcium mobilization by racemic-trimetoquinol. Incubation mixtures and methods are described in Materials and Methods. Data on aggregation are expressed as a percentage reduction of the initial slope change at various trimetoquinol doses relative to the control (or maximal slope change) (closed circles). Data on calcium mobilization are expressed as a percentage of the maximal calcium removed from a platelet membrane fraction (open circles). Zero percent inhibition corresponds to about 8.5 nmol calcium mobilized/mg protein. No corrections were made for endogenous calcium present. Each value represents the mean ± the range of values of 2-4 determinations.
INHIBITION OF A23187-INDUCED PLATELET AGGREGATION AND CALCIUM MOBILIZATION BY RACEMIC-TRIMETOQUINOL

Fig. 20
(5 \mu M) was reduced by 47.8\% and 16.0\% of control in the presence of $10^{-4}$ M and $10^{-5}$ M \textit{racemic}-TMQ respectively. Although the rate of aggregation was reduced, the maximal aggregatory response was unaffected.
CHAPTER IV
DISCUSSION

This investigation has been concerned with the process of platelet aggregation and the in vitro modification of that process by the beta-receptor stimulant trimetoquinol (TMQ; Inolin$^R$). Inolin$^R$ is the S(-)-TMQ stereoisomer and is used clinically as a bronchodilator in Japan (Yamamura and Kishimoto, 1968). Early work in 1976 (Shtacher et al., 1976) demonstrated that TMQ could inhibit platelet aggregation induced by collagen, epinephrine and ADP. These authors clearly showed that this activity was independent of cyclic AMP levels or cyclic AMP and cyclic GMP phosphodiesterase activities. Furthermore, the antiaggregatory effects of TMQ were not blocked by the presence of phentolamine or propranolol and practolol. Because of these findings, alpha or beta-adrenoceptors were not implicated in the inhibitory process. Dalton et al. (1976) extended these findings and reported that the potency ratio for the TMQ isomers was reversed for inhibition of aggregation as compared to beta-adrenoceptor stimulation. This further suggested that the mechanism was independent of beta-receptor/adenylate cyclase coupled activation.

To identify possible mechanisms of antiaggregatory action for TMQ, studies were done where TMQ was examined as an inhibitor of human platelet aggregation induced by various pro-aggregatory
compounds which are known intermediates in the arachidonic acid (A.A.)
cascade of human platelets (Fig 21). Collagen and A.A. were used in
initial experiments because they are agents which play roles early in
the metabolic conversion of A.A. to active prostaglandin intermediates
and products (Samuelsson et al., 1978; Moncada and Vane, 1979c;
Mustard et al., 1980). TMQ was found to stereoselectively inhibit
human platelet aggregation stimulated by these agents (Figs 4 and 5).
As can be seen, R(+)-TMQ was a more potent inhibitor of platelet
aggregation than the S(−)-isomer. The calculated potency ratios
for the TMQ isomers were found to be similar for inhibition of
collagen-(12.3) and A.A.-induced (11.7) aggregation suggesting that
TMQ was not a phospholipase A₂ inhibitor and that the site of
inhibition was an event subsequent to the formation and action of A.A.
(Fig 21).

Sequentially, the next active intermediates formed in the
metabolism of A.A. within the platelet are the prostaglandin endo-
peroxides PGG₂ and PGH₂ (Hamberg and Samuelsson, 1973). Subsequent
studies were directed toward the ability of TMQ to block endoperoxide-
induced aggregation. Both PGG₂ and PGH₂ are extremely labile.
However, two stable, synthetic PGH₂ analogues (U44069 and U46619)
(Fig 6) (Bundy, 1975) were available as investigational agents. The
greater stability of these analogues made them attractive substitutes
for the natural endoperoxides as stimulators of platelet aggregation.
When aggregation was induced using U44069 (10 μM) the TMQ isomers were
found to be unequal and effective inhibitors of aggregation. The
Modified from Gorman et al. (Biochim. Biophys. Acta, 1975)

Fig. 21
R(+) isomer was a more potent inhibitor and was found to be 39.2 times more active than S(-)-TMQ (Fig 7). In a similar fashion, U46619 (2 μM)-induced aggregation was stereoselectively inhibited by the TMQ isomers and the potency ratio was calculated to be 82.9 (Fig 8). These ratios are certainly of comparable magnitude based on their respective 95% confidence limits (see Table 1) and suggest that TMQ is inhibiting each of these agents at a similar site (Patil, 1969).

The demonstration of a dose-dependent, stereoselective inhibition of U44069- and U46619-induced platelet aggregation (Mayo et al., 1979) significantly characterized the inhibitory nature of TMQ and extended the preliminary and limited observations of MacIntyre et al. (1978). These workers reported that selected doses of racemic-TMQ could inhibit platelet aggregation induced by U44069, U46619, PGG\(_2\) and PGH\(_2\). Bennett and Sanger (1979) using a different approach demonstrated that the inhibitory action of TMQ was also apparent on other tissues such as the longitudinal muscle from rat gastric fundus where racemic-TMQ was shown to inhibit thromboxane B\(_2\) (TXB\(_2\))- U44069- and U46619-induced contractions. The ability to inhibit both the natural and the synthetic endoperoxides in platelet systems and TXB\(_2\) in gastric fundus suggested that the site of TMQ inhibition was still further along the A.A. cascade (Fig 21). This interpretation was strengthened by a recent report indicating that the PGH\(_2\) analogues produced physiological effects more like that of TxA\(_2\) than PGH\(_2\). Coleman et al. (1980) used bioassay techniques on aortic, saphenous vein, lung, ileum, iris sphincter muscle and fundus preparations to study the comparative
actions of U46619 and thromboxane A₂ (TxA₂). Both U46619 and TxA₂ caused the contraction of aortic, saphenous vein and lung preparations only while PGH₂ contracted all preparations suggesting that U46619 and TxA₂ produced similar physiological effects which are different than those produced by PGH₂. These findings implied that TMQ may be an inhibitor of TxA₂ action in platelets.

Studies using TxA₂ are complicated by the instability of the thromboxane molecule and therefore a biosynthetic technique was used to generate TxA₂ (Chignard and Vargaftig, 1977). This technique does not provide for the introduction of known quantities of TxA₂ into platelet systems and therefore different aliquots of the biosynthetic media may be added to platelets until an aggregatory response is recorded. In these experiments, TMQ was found to be a dose-dependent, stereoselective inhibitor of TxA₂-induced aggregation (Fig 10). The inhibition occurred over the same concentration range as observed for inhibition of U46619- and U44069-induced aggregation (Figs 7 and 8) and the potency ratio for the TMQ isomers against TxA₂-induced aggregation was calculated to be 36.0.

Examination of the 95% confidence intervals of each of the potency ratios for inhibition of U44069-, U46619- and TxA₂-induced aggregation revealed that these ratios are of comparable magnitude (Table 1). The similarity in potency ratios for the TMQ isomers may indicate that a similar receptor site is involved in the stimulation of platelet aggregation by U44069, U46619 and TxA₂ (Patil, 1969). This hypothesis is supported by Coleman et al. (1980)
and Smith et al. (1977) who report that U46619 and TxA2 produce identical physiological effects in many different tissues including platelets. For purposes of clarification, U46619 will be considered a PGH2/TxA2 mimetic in all discussions after this point.

The fact that TMQ was a stereoselective inhibitor of all aggregating agents which act through the platelet A.A. cascade suggested that TMQ may inhibit a site sensitive to TxA2 or both TxA2 and PGH2 stimulation. This hypothesis was tested in experiments using ADP as an inducing agent of platelet aggregation. ADP is known to stimulate primary platelet aggregation through mechanisms which are independent of prostaglandin influence (Owen, 1979) and therefore if TMQ is an antagonist of TxA2 or of both TxA2 and PGH2 sensitive sites then it should have little effect on ADP-induced aggregation. That is in fact what was observed when a high dose (10^-4 M) of racemic-TMQ was unable to inhibit ADP-induced aggregation by more than 17% (Fig 11). This inability to potently inhibit ADP-induced aggregation was also observed by MacIntyre et al. (1978) and suggested further that TMQ was a specific antagonist of TxA2 and/or both TxA2 and PGH2 sensitive sites within the platelet. It should be noted that the early work of Shtacher et al. (1976) suggested that TMQ was a potent inhibitor of ADP-induced platelet aggregation. Although these results appear contradictory, they are not. Shtacher et al. (1976) dissociated the primary and secondary phases of ADP-induced aggregation and measured the ability of racemic-TMQ to inhibit the secondary phase only. Since the secondary phase of ADP-induced aggregation is
mediated through prostaglandins (unlike the primary phase) (Owen, 1979) their observed inhibition of this phase is consistent with the results of Mayo et al. (1979) and MacIntyre et al. (1978). The latter investigators measured both phases of aggregation induced by ADP and therefore were only able to inhibit a portion of the total aggregatory response i.e. the portion mediated by prostaglandins.

Further experiments showed that R(+)–TMQ exerted a competitive-like inhibitory action on U46619-induced aggregation. In these studies, the complete inhibition by TMQ of U46619-induced aggregation was reversed by increasing the concentration of U46619. The inhibition was reversed such that a maximal aggregation response was achieved even in the presence of a highly effective dose of R(+)–TMQ (5 μM). Since the maximum velocity is unchanged in the presence of a competitive inhibitor in enzymatic reactions, (Dixon and Webb, 1964) it may be suggested that TMQ was acting as a competitive inhibitor for U46619-induced aggregation. These preliminary studies were amplified by using the graphical method of Dixon (1954) to estimate an apparent inhibitory constant (Ki) value for racemic-TMQ (Fig 13). By calculation, the Ki value for TMQ was found to be 0.13 μM ± 0.06 (S.E.M.). This value is nearly identical to the IC_{50} value of R(+)–TMQ obtained for the inhibition of U46619-induced aggregation. The Ki value is also of comparable magnitude to the IC_{50} of R(+)–TMQ for the inhibition of TxA_{2}-induced aggregation (Table 1). These similarities provide support for TMQ as being a competitive inhibitor of U46619-induced aggregation.
The platelet granular secretion reaction, i.e., the release of serotonin and other materials from platelet storage vesicles is known to be mediated through a prostaglandin dependent pathway (Moncada and Vane, 1979c). An inhibitor of TxA$_2$ and/or PGH$_2$ would be expected therefore to inhibit the secretion reaction stimulated by these agents. When aggregation and the release of serotonin was measured simultaneously in platelets, racemic-TMQ was found to inhibit U46619-induced aggregation and serotonin release in a nearly identical fashion (Fig 14). These results were supported by additional studies using the TMQ-isomers. In these experiments, each isomer caused a stereoselective, dose-dependent inhibition of both aggregation and serotonin release (Fig 15) indicating that TMQ was a stereoselective inhibitor of the release reaction. Also even though TMQ was not an effective inhibitor of ADP-induced aggregation, racemic-TMQ was shown to completely inhibit the release reaction after platelet stimulation with ADP (Table 2). The release reaction is known to be dependent on TxA$_2$ and/or PGH$_2$ stimulation (Moncada and Vane, 1979c) and therefore these data are consistent with the premise that TMQ is an inhibitor of TxA$_2$ or both TxA$_2$ and PGH$_2$ action.

The studies which monitored calcium movement from isolated platelet membrane stores were designed as a model of TxA$_2$ and/or PGH$_2$ action. The goal was to find an isolated platelet membrane preparation which would mobilize calcium upon stimulation by U46619, This mobilization of calcium in vitro was to be used as a model of the calcium movement that is hypothesized to occur in an intact platelet
after TxA₂ stimulation (Gerrard et al., 1978b; Haslam et al., 1978; White, 1979).

The addition of U46619 to isolated platelet membranes which were pre-loaded with ⁴⁵Ca and in a steady-state of accumulation caused the mobilization and loss of calcium from those preparations. This mobilization of calcium by U46619 was found to be dose-dependent, (Fig 17) reaching a maximum loss of 5.0% of the total bound calcium. The U46619 analogue was found to be a more potent stimulator of calcium mobilization than the U44069 analogue which parallels the potency differences observed for platelet aggregation (Fig 18) Malmsten, 1975; MacIntyre et al., 1978; Mayo et al., 1979). However, A23187 was able to cause the release of about 36% of the total bound calcium as compared to 5% for U46619 (Fig 17). This potency difference is consistent with the biochemical properties of A23187 as a calcium ionophore.

Preincubation of the membranes with the TMQ isomers before the addition of U46619 resulted in a stereoselective, dose-dependent inhibition of U46619-induced calcium mobilization (Fig 19). This inhibition was found to occur over the same dose-range as the stereoselective inhibition of U46619-induced platelet aggregation (Fig 8). The calculated potency ratio for the TMQ-isomers was 12.1. This calculated potency ratio is similar to the potency ratio observed for the inhibition of TxA₂-induced aggregation and is of comparable magnitude to the calculated potency ratios for U44069- and U46619-induced aggregation (see Table 1). These similarities suggest that a
relationship exists between the ability of TMQ to inhibit U46619-induced calcium mobilization from isolated platelet subcellular membranes and platelet aggregation in platelet rich plasma.

The ability of U46619 to evoke calcium mobilization has also recently been reported by Owen and LeBreton (1980) who used $^{45}$Ca labeled whole platelets and demonstrated that U46619 stimulation directly caused the mobilization of approximately 4% of the total bound calcium from intracellular sites. Their observed 4% mobilization of calcium by U46619 is in agreement with this report of 5% (Fig 17). Gerrard et al. (1978a) have also used an isolated intracellular platelet membrane preparation and have shown that the accumulated calcium within the membranes can be released by the addition of A.A. or PGG$_2$ and PGH$_2$. These investigators found that the A.A.-induced release of calcium could be blocked by the presence of aspirin. To summarize, U46619 had the same relative effects on calcium mobilization in whole platelets as in platelet subcellular membranes and TMQ stereoselectively inhibited that calcium mobilization in platelet membrane preparations in the same dose range as it inhibits U46619-induced aggregation. These data suggest that the ability of U46619 to mobilize calcium may be in part a mechanism whereby it stimulates platelets to aggregate. Furthermore, the data also suggest that the ability of TMQ to inhibit prostaglandin-stimulated calcium mobilization from isolated membranes may be a mechanism for its action as an antiaggregatory agent.
In other experiments, racemic-TMQ (10^{-4} M) was shown to only slightly inhibit (by 23%) the mobilization of calcium by A23187. This inability to effectively inhibit A23187-induced calcium mobilization parallels the relative inability of racemic-TMQ to inhibit A23187-induced platelet aggregation (Fig 20) and further suggests a relationship between inhibition of calcium mobilization and inhibition of platelet aggregation by TMQ.

These suppositions are in agreement with a working hypothesis of platelet aggregation proposed by White (1979) and supported by several others including Gerrard et al. (1978b), Haslam et al. (1978), Gorman et al. (1978), and Moncada and Vane (1979c). The hypothesis is based on the current concept that the movement of calcium from platelet membranous storage sites to the platelet cytoplasm is the final common pathway for activation of platelets by pro-aggregatory agents. The maintenance of an inactive state would therefore require systems to keep calcium out of the cytoplasm or suppress activities that cause the translocation of calcium. The former would be accomplished by appropriate levels of cyclic AMP which would stimulate a calcium extrusion pump (ATPase) and the inactivity of prostaglandin synthesis in the unstimulated platelet for the latter. Agents that stimulate platelets would interact with specific receptors on the plasma membrane or at an intracellular site. The interaction would result in a signal causing small amounts of calcium to move into the cytoplasm or to the inner face of the membranes of the dense tubular system. The small amount of calcium would activate a nearby
phospholipase (phospholipase A₂) which would cleave A.A. from membrane phospholipids and initiate the A.A. cascade resulting in the production of the cyclic endoperoxides and TxA₂ (Fig 21). The membrane signal would also stimulate the interaction of actinin with its binding protein resulting in pseudopod formation. This would be the stage of platelet shape change which is reversible in the presence of inhibitors such as aspirin or other agents which inhibit the cyclo-oxygenase enzyme.

An uninhibited liberation of A.A. would result in TxA₂ formation. One group suggests, based on indirect evidence, that TxA₂ can transport calcium from the dense tubular system storage sites to the cytoplasm where it stimulates activation of the contractile system and results in the release reaction (Gerrard et al., 1978b). The calcium may be released from TxA₂ by hydrolysis or in exchange for magnesium. Some of the TxA₂ may be able to re-cycle back to the dense tubular system before it spontaneously degrades to the more inactive product TxB₂. On the basis of my findings, I propose that TxA₂ may interact at a specific site within the dense tubular system to cause the release of calcium from storage sites within that organelle. In this way TxA₂ would not be a carrier of calcium but rather a stimulator of mobilization. A calcium binding protein such as calmodulin may play a significant role in this scheme by acting as the "gate" for calcium release.

This author would like to suggest as a working hypothesis for future studies that TMQ can interfere with the ability of TxA₂ and/or both TxA₂ and PGH₂ to mobilize calcium from the dense tubular system.
by interacting at the site where TxA₂ combines with calcium/calcium "gate" thereby interfering with the release of calcium from the membranous system. This concept of TMQ will certainly be modified as new information is learned about its properties and also about the biochemical events of platelet aggregation but presently it may serve as a working model for the inhibitory activity of TMQ in human platelet systems.
1. The effects of trimetoquinol as an inhibitor of platelet aggregation, the platelet release reaction and calcium mobilization from platelet stores were studied in either fresh human platelets obtained from healthy volunteers or in stored platelet rich plasma preparations.

2. A series of experiments were designed to evaluate the inhibition by trimetoquinol of platelet aggregation induced by intermediates of the platelet arachidonic acid cascade including collagen, arachidonic acid, prostaglandin H\textsubscript{2} analogues (U44069, U46619), and thromboxane A\textsubscript{2}.

3. Trimetoquinol was a potent and stereoselective inhibitor (R(+)\text{-}isomer > S(-)-isomer) of aggregation induced by all of these agents. Inhibitory concentration-50 (IC\textsubscript{50}) values for the R(+)\text{-}isomer were similar for inhibition of collagen- and arachidonic acid-induced aggregation (4.2 \text{ \mu M} and 4.1 \text{ \mu M} respectively). The R(+)\text{-}isomer IC\textsubscript{50} values for inhibition of U44069-, U46619- and thromboxane A\textsubscript{2}-induced aggregation were also similar and found to be 1.4 \text{ \mu M}, 0.14 \text{ \mu M}, and 0.64 \text{ \mu M} respectively. Calculated potency ratios against aggregation induced by collagen, arachidonic acid, U44069, U46619 and thromboxane A\textsubscript{2} were 12.3, 11.7, 39.2, 82.9 and 36.0 respectively.
4. In other experiments, platelets were stimulated with U46619 and
the platelet granular release reaction was studied concomitantly with
aggregation. The release of $^{14}$C-serotonin from platelet stores and
its subsequent assay in platelet rich plasma was used as an indicator
of the release reaction. Racemic-Trimetoquinol was found to inhibit
both the release of serotonin and the aggregation of platelets in a
nearly identical, dose-dependent fashion. Additional experiments
confirmed that the trimetoquinol stereoisomers also inhibited the
release of serotonin in a nearly identical, stereoselective, dose-
dependent fashion as obtained for the inhibition of aggregation.
These results suggested that an event obligatory for both aggregation
and the release of serotonin was inhibited by trimetoquinol.

5. Studies with ADP revealed that racemic-trimetoquinol was not a
potent inhibitor of aggregation induced by that agent since only a
17% inhibition was apparent using $10^{-4} \text{M}$ trimetoquinol. Despite
the inability to inhibit aggregation, racemic-trimetoquinol was a
potent inhibitor of the ADP-induced release of serotonin from platelet
stores. These results are consistent with the accepted view that the
release of serotonin from platelet stores is dependent on prosta-
glandin influence whereas ADP-induced primary aggregation occurs
through mechanisms independent of prostaglandin formation and action.
6. The nature of the inhibition of U46619-induced aggregation was studied by selecting several different stimulating concentrations of U46619 and then measuring the inhibition of the aggregatory responses by the presence of increasing concentrations of racemic-trimetoquinol. After plotting the data according to Dixon (1953), apparent inhibitory constants (Ki) were estimated for each of three separate experiments. The calculated Ki value for trimetoquinol was 0.13 μM ± 0.06 (S.E.M.). The Ki value was nearly identical to the R(+)-trimetoquinol IC₅₀ value for the inhibition of U46619-induced aggregation. It was also of comparable magnitude to the IC₅₀ value of R(+)-trimetoquinol for the inhibition of TxA₂-induced aggregation.

7. Experiments done with ⁴⁵Ca revealed that a subcellular fraction of platelet membranes could actively accumulate calcium in the presence of ATP and oxalate up to a steady-state level. The addition of U46619 to these preparations caused a dose-dependent mobilization and loss of calcium from the membranes up to a maximum amount of 1.25 nmoles/mg platelet protein. The U46619 analogue was a more potent stimulator of calcium mobilization than the U44069 analogue. Also, doses of the U46619 analogue which produced 50% of the maximal response were nearly identical for the stimulation of aggregation and calcium mobilization, whereas the U44069 analogue was a more potent inducer of aggregation than calcium mobilization.
8. The isomers of trimetoquinol were found to stereoselectively inhibit the U46619-induced mobilization of calcium in the same potency range as observed for the inhibition of U46619-induced aggregation. IC\textsubscript{50} values were 0.197 \mu M and 2.39 \mu M for the R(+) and S(-) isomers respectively and the calculated potency ratio was 12.1. This potency ratio is similar to the calculated potency ratio observed for the inhibition of TxA\textsubscript{2}-induced aggregation and is of comparable magnitude to the calculated potency ratios for U44069- and U46619-induced platelet aggregation.

9. In other experiments, racemic-trimetoquinol (10^{-4} M) was shown to only slightly inhibit (by 23%) the mobilization of calcium by A23187. This inability to effectively inhibit A23187-induced calcium mobilization was found to parallel the relative inability of racemic-trimetoquinol to inhibit A23187-induced platelet aggregation.

10. In conclusion, the results of these studies suggest that trimetoquinol is a stereoselective inhibitor of human platelet aggregation mediated by pro-aggregatory prostaglandins. Furthermore it appears that trimetoquinol is a competitive inhibitor of PGH\textsubscript{2}-analogue/ thromboxane A\textsubscript{2}-induced aggregation.

11. Studies with isolated human platelet intracellular membranes revealed that the PGH\textsubscript{2}-analogues, U46619 and U44069, could stimulate the mobilization of calcium from membrane stores. This observation
supports current theories of prostaglandin-induced calcium mobilization as being an important mediator in the platelet aggregatory response. Finally, the ability of trimetoquinol to stereoselectively inhibit U46619-induced calcium mobilization in a platelet subcellular membrane preparation suggests that this type of inhibition may be an explanation for the inhibitory activity seen in human platelet rich plasma preparations after stimulation with pro-aggregatory prostaglandins.
APPENDIX

Human Protocol and Consent Form
CONSENT TO SPECIAL TREATMENT OR PROCEDURE

I, ______________, hereby authorize or direct Drs. Blanchine, Feller and/or Witlak or associates or assistants of his or her choosing, to perform the following treatment or procedure and such additional services as they may deem reasonably necessary in its performance (describe in general terms)

Draw 50ml (1.7oz) of blood from the arm vein. ______________
upon ______________ (myself or name of subject)
The experimental portion of the treatment or procedure is: Platelets in your blood drawn from your arm will be tested to see if they are changed biochemically by trimetoquinol and related drugs.
This is done as part of an investigation entitled: Effect of Trimetoquinol Analogues in Human Platelets, in vitro.

1. Purpose of the procedure or treatment: To remove 50ml of blood from your arm.

2. Possible appropriate alternative methods of treatment: NONE.

3. Discomforts and risks reasonably to be expected: Pain and bruising at the blood drawing site. There is the possibility of fainting while having your blood drawn. There will be one (1) venipuncture with 50ml (approx. 1/5 Cup) of blood drawn from your arm.

4. Benefits which may be expected: NONE.

5. Likely results of the experimental treatment or procedure: To test the effects of trimetoquinol and related derivatives on platelet function. _________________________________________________

I hereby acknowledge that I have had a full opportunity to ask any questions regarding the procedure described above and that all questions have been answered by Drs. J.R. Blanchine/D. Feller and/or Witlak to my full satisfaction. He/She has explained the risks described above and I understand them, and he/she has also offered to explain all possible risks or complications.

I understand that any further inquiries I may make concerning the procedure described above will be answered, and I understand that I am free to withdraw my consent and participation in this project at any time after notifying the project director without prejudicing my future care. No guarantee has been given to me concerning this treatment or procedure.

In the unlikely event of physical injury resulting from my participation in this study, I understand that immediate medical treatment is available at University Hospital of The Ohio State University. I also understand that the costs of such treatment will be at my expense and that financial compensation is not available.

I have read and fully understand the consent form. I have signed it freely and voluntarily and understand a copy is available upon request.

Signed: ___________________________ (Subject)

Witness: ___________________________

Witness: ___________________________ (Person Authorized to Consent for Subject - If Required)

I certify that I have personally completed all blanks in this form and explained them to the subject or his/her representative before requesting the subject or his/her representative to sign it.

Signed: ___________________________ (Signature of the project director or authorized representative)

Document: PA-028A (1/79)
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