# THE PROSTAGLANDIN SYNTHESIS PATHWAY OF PLATELETS IN MINK AFFECTED WITH THE CHEDIAK-HIGASHI SYNDROME

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1979

ABSTRACT

THE PROSTAGLANDIN SYNTHESIS PATHWAY OF
PLATELETS IN MINK AFFECTED WITH
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The possibility of a defect in prostaglandin synthesis in Chediak-Higashi mink was studied, and it was concluded that no difference exists between Chediak-Higashi and non-Chediak-Higashi mink as concerns prostaglandin synthesis. Chemiluminescence, platelet aggregations, and malonyldialdehyde assays were utilized to challenge this hypothesis. Significant differences were not observed ($P<0.01$) between the two test groups. It appears that, at least in mink, the primary defect responsible for prolonged bleeding associated with the Chediak-Higashi syndrome is the decreased amount of secretable (storage) pool adenine nucleotides. The possibility that a defect exists in prostaglandin synthesis in other species affected with Chediak-Higashi syndrome is doubtful due to the homology of the disorder between species; however, more research in this area is needed in order to make this conclusion.

TO DEBBIE AND MEGHAN

the most important assets in my life

ACKNOWLEDGEMENTS

Any verbal or written expression of gratitude to Dr. George A. Padgett seems inadequate. As my major professor and chairman of my graduate committee, he supplied continual and concerned support throughout my research; but in my opinion, most importantly he taught me to understand the importance of the educational experience beyond the classroom, which undoubtedly will prove to be a valuable asset throughout my career. For his invaluable advice and insight, I am eternally grateful.

Special thanks are deserving to the other members of my committee also. Dr. Thomas Bell, Dr. Robert Bull, Ms. Kathy Colando, and Mrs. Martha Thomas provided continual support, which especially with Dr. Bell was at times more psychological than technical. Kathy and Martha, who I guess would be considered as dual academic advisors, first helped me adjust to my return, after five years, to the academic environment, which was totally unlike that I had ever experienced before.

Ms. Irene Brett was very helpful by providing technical advice and assistance whenever it was needed, and a well deserved expression of thanks is extended to her.

Although I have dedicated this thesis to my wife and daughter, I am especially grateful for the patience and understanding that my wife, Debbie, showed during difficult times. She deserves as much of the credit as I for the accomplishments made during my graduate program.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine diphosphate |
| ATP | Adenosine triphosphate |
| BHT | Butylated hydroxytoluene |
| c-AMP | Cyclic-adenosine monophosphate |
| C-HS | Chediak-Higashi syndrome |
| CL | Chemiluminescence |
| cpm | Counts per minute |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| 5-HT | 5-Hydroxytryptamine |
| HBSS | Hank's balanced salt solution |
| LASS | Labile aggregation stimulating substance |
| MDA | Malonyldialdehyde |
| $PGD_2$ | Prostaglandin $D_2$ |
| $PGE_1$ | Prostaglandin $E_1$ |
| $PGE_2$ | Prostaglandin $E_2$ |
| $PGF_{2\alpha}$ | Prostaglandin $F_{2\alpha}$ |
| $PGG_2$ | Prostaglandin $G_2$ |
| $PGH_2$ | Prostaglandin $H_2$ |
| $PGI_2$ | Prostacyclin |
| PPP | Platelet poor plasma |
| PRP | Platelet rich plasma |

| | |
|---|---|
| TBA | Thio-barbituric acid |
| $TxA_2$ | Thromboxane $A_2$ |

# INTRODUCTION

The Chediak-Higashi syndrome (C-HS) is a genetic disease that is known to occur in 6 different species: man,[1] mink,[2,3] cattle,[3] cats,[4] mice,[5] and killer whales.[6] The syndrome is inherited as an autosomal recessive trait[7] and has several striking defects which affect all species in much the same way. One of the readily observed defects is the formation of abnormally large intracytoplasmic granules in neutrophils and other cells which normally produce such structures.[8,9] Affected individuals of all species display a partial oculocutaneous albinism which can be seen in the hair, skin, and eyes[10] due to the presence of giant melanin granules in melanocytes.[11] In every species affected with C-HS, there are reports of increased susceptibility to infection.[12] All species show a marked increase in frequency and severity of infections when exposed to agents that cause either minor infections or no infection at all in individuals not affected with C-HS.[13,14,15] Lastly, another defect in C-HS individuals is an abnormally prolonged bleeding time. Prothrombin times, partial thromboplastin times, coagulation factor assays, and platelet counts all are within the normal ranges.[16] This then indicates a defect in the platelet itself.[17] Several investigators subsequently reported a storage pool defect characterized by decreased stores of serotonin (5-HT) and non-metabolic adenine nucleotides,[17,18,19] which are stored in

the dense granules of the platelet.[20] It is the release of the storage pool adenine nucleotides, namely adenosine diphosphate (ADP), that is responsible for the second wave of aggregation that is observed during platelet aggregation induced with epinephrine and ADP.[21,22]

It is well accepted that the cause of the prolonged bleeding time in C-HS individuals is the reduced amount of "storage pool" constituents. However, the possibility that there may be a defect in the arachidonic acid to prostaglandin pathway, which recently has evolved as "the other mechanism" of platelet aggregation, has not been studied in C-HS. The pathway has been elucidated and the interactions of by-products of the pathway have been studied in depth.[23,24,25] Several intermediates and by-products of the pathway are known to be potent inducers of aggregation.

The purpose of this research project was to compare the prostaglandin pathway of C-HS affected mink to non-C-HS mink. The methods used to study the prostaglandin pathway activity and viability were chemiluminescence, platelet aggregations, and measurement of a stable by-product of the pathway, malonyldialdehyde (MDA). After correlating the results, a useful comparison was made and evaluated in order to determine if any defect existed in the prostaglandin pathway of C-HS mink.

# LITERATURE REVIEW

## General Aspects of the Chediak-Higashi Syndrome

The first published reports of the Chediak-Higashi syndrome were those of Bequez-Cesar (1943),[1] in which he observed abnormal granules in the leukocytes of children from the same family. In addition to the abnormal granule formation, he also described partial albinism, and an increased susceptibility to infection, in these C-HS children. Several years later, Chediak (1952)[26] and Higashi (1954)[27] reported on the same syndrome, and in 1954 Sato[28] named the condition the Chediak-Higashi syndrome. In the years to follow, several investigators described the occurrence of C-HS in other species. Leader et al. (1963)[2] reported finding abnormally large intracytoplasmic granules in mink leukocytes. Padgett et al. (1964)[3] were the first to report the occurrence of the Chediak-Higashi syndrome in mink and cattle, with particular reference to a familial (autosomal recessive) occurrence in these species. Abnormal granules in the leukocytes of the beige mouse were reported by Lutzner et al. (1967)[5] and Taylor and Farrell (1973)[6] reported the same finding in killer whales. Kramer et al. (1974)[4] found abnormal melanin and leukocyte granules in blue smoke colored Persian cats and compared their results to those reported in other species affected with C-HS.

Reviews have been published[7,8] that compare each species as to clinical and morphological findings. There seem to be 4 characteristics

common to all species affected with C-HS, with only minor variations between species. The first of these characteristics is the development of abnormally large intracytoplasmic granules in cells that normally produce these structures. The two most commonly studied cells exhibiting this defect are neutrophils and melanocytes. Electron microscopic and biochemical studies have determined that the large granules in neutrophils are aberrant primary granules, some of which contain various acid hydrolases. Normally, proteins synthesized by polyribosomes on the endoplasmic reticulum move through the endoplasmic reticulum to the Golgi complex, where they are concentrated, surrounded by a membrane, and ultimately released into the cytoplasm as primary granules. Any theory that explains the abnormal formation must then encompass all cells that produce granules and not be specific for one cell type, i.e., in all cells the formation of the granules is Golgi mediated.[7] Lutzner et al. (1965)[29] suggested that the Golgi involvement could be one of the following: a defect in the transfer of material from endoplasmic reticulum to Golgi bodies, a defect in the process of granule assembly, or a defect in granule membrane formation, any of which could be caused by a mutant gene.

Another feature of C-HS is a partial oculocutaneous albinism secondary to fusion of melanocytic granules. Melanocytes are cells that are responsible for skin pigmentation. In C-HS, the pigmentary dilution is caused by an abnormal fusion of melanocyte granules into large melanin-containing structures with the albinism occurring because of an abnormal melanosome distribution. This defect can be seen in the hair, skin, and eyes of affected individuals of all species.[10,11]

A third characteristic common to the Chediak-Higashi syndrome is the increased susceptibility to infection. Affected individuals of all species seem to have more frequent and severe infections than non-C-HS individuals. In all species there have been reports of severe pyogenic infections in C-HS individuals, caused by agents that also affected non-C-HS members of each species. The non-C-HS individuals, however, were not stricken as frequently or as severely as their counterparts. Chediak-Higashi syndrome cattle exhibited greatly increased bacterial infection rate, while non-affected cattle exposed to the same infectious agents often had no infection at all.[12] In mink, the findings are similar. Chediak-Higashi syndrome mink can exhibit an almost chronic periodontitis and seem to be more susceptible to pyogenic abscesses (personal observation). In addition, in mink there is a particular susceptibility to a disease of viral etiology (i.e., Aleutian disease). All mink may contract Aleutian disease; however, in C-HS mink the disease follows a more severe and rapid course with the life expectancy of C-HS mink being much shorter than non-C-HS mink.[12,13] There are several reports that indicate that neutrophil dysfunction may be the primary cause of the increased susceptibility to infection.[31,32,33,34] It appears that the defect lies in impaired intracellular killing of bacteria by neutrophils in C-HS and not in normal individuals.[15] Several investigators have implicated an abnormal microtubular assembly to account for defective chemotaxis, which is also present in C-HS individuals,[35,36] and one researcher has reported a correction of the defective chemotaxis by administering ascorbate, which is said to lower the intracellular level of cyclic-adenosine monophosphate (c-AMP).[37]

Lastly, a defect common to all species affected with C-HS is an abnormally prolonged bleeding time and hemorrhagic tendency which was recognized relatively early in the study of the disease. Several investigators attempted to determine the cause of this hemorrhagic disorder, employing many different methods. Prothrombin times, partial thromboplastin times, platelet counts, and clotting factor assays were done, and all results were found to be within normal limits.[16] However, the template bleeding times were in some cases as much as 10 times normal.[17] These results suggested an intrinsic platelet defect in C-HS. Platelet aggregations of C-HS individuals revealed a marked reduction in the extent and rate of aggregation in response to collagen and an absence of secondary aggregation in response to critical concentrations of ADP and epinephrine.[17] Biochemical analysis of platelet-rich plasma (PRP) yielded data showing a marked decrease in adenine nucleotides (ATP and ADP), an increase in the ATP/ADP ratio, and a reduction in the amount of stored serotonin as well as total serotonin uptake.[17,19] Several investigators have reported that serotonin and the nonmetabolic adenine nucleotides ATP and ADP are stored in the same granules (the dense granules) of the platelet.[18,20] All of the above-mentioned studies suggest a "storage pool" disorder which leads us to a discussion of platelet anatomy and function.

## Platelet Anatomy

In the peripheral circulation of normal individuals, the platelet is disc-shaped and is about 2.5 μm in diameter. One of the most remarkable characteristics of platelets is the capability to change shape in response to a number of stimuli.[38] The shape change involves

an increase in surface area with little increase in volume. The changes can be seen microscopically by the formation of pseudopodia as it transforms into a "spiny sphere."[39,40] This change of shape appears to be the first response of platelets to almost any stimulus,[41] including exposure to cold; but more importantly it is achieved by the addition of various aggregating agents which will be discussed later.

The outer layer of the platelet is a relatively thick glycoprotein coat which may play a role in platelet adhesion. Inside this coat is a trilaminar platelet membrane containing platelet specific proteins.[42] Two types of granules have been described in the platelet cytosol: primary (alpha) granules and dense bodies. The primary granules are the most numerous and have been shown to contain various acid hydrolases. The dense bodies are less numerous and contain adenine nucleotides, serotonin, and calcium. The adenine nucleotides in the dense bodies are ADP and ATP, which are metabolically inert and are considered to be "storage pool" constituents.[40,42] Platelets are capable of carrying out the glycolytic, the tricarboxylic acid cycle, and the hexose monophosphate shunt reactions for the production of ATP needed in energy requiring reactions.[39] One of the energy consuming reactions that utilizes the metabolic ATP is shape change, which was previously mentioned, and is mediated by the action of the microtubular system.

## Platelet Production

Normal bone marrow contains giant multinucleated cells, cells called megakaryocytes, which arise from multipotential stem cells.[39] The nucleus of the megakaryocyte divides without division of the

cytoplasm, and in a mature megakaryocyte the nucleus is polyploidy.[39,43] The cytoplasm increases in volume during this process and is basophilic, but becomes acidophilic as the megakaryocyte matures.[43,44] The mature megakaryocyte cytoplasm, by invaginations of the plasma membrane, forms demarcation membranes encapsulating cytoplasm.[43,44] The platelets are then separated from the megakaryocyte and, when the cytoplasm is depleted, the remaining nucleus is promptly disposed of by macrophages.[43,44] The regulation of megakaryocyte proliferation and, ultimately, of platelet production is very strictly controlled, and there is recent evidence for the existence of thrombopoietin, a substance similar to erythropoietin, that regulates platelet production on a hormonal level.[45,46] Normally, platelet production is approximately equal to platelet destruction, with a normal platelet count being between 150,000 and 450,000, with the mean of about 250,000/$mm^3$ in the peripheral circulation. The life span of the platelet is about 10 days.[44]

## Platelet Aggregating Agents

As a prelude to discussing platelet function, it would probably be helpful to review some of the agents that cause visible changes in the platelet, i.e., platelet aggregation. Platelet aggregation can be induced *in vitro* in normal individuals by numerous compounds. Among these are low molecular weight molecules (ADP, epinephrine, serotonin), proteolytic enzymes (thrombin, trypsin, snake venom), particulate matter (collagen, fatty acids, latex particles, endotoxin, viruses, antigen-antibody complexes), strongly positive charged ions (polylysine), and an antibiotic (Ristocetin).[39] The most commonly used aggregation inducers, for the assessment of a bleeding diathesis that

is platelet related, are ADP, epinephrine, and collagen. Some of the other agents are useful if measurement of released products is desired but may not provide insight into aggregation problems.

## Platelet Function

Normally, platelets circulate in the peripheral blood in a discoid shape for approximately 10 days, which is their normal life span. However, when vascular trauma occurs which exposes certain tissues in vessel walls to circulating platelets, a rapid chain of events takes place that stops the bleeding and allows for healing of the wound. There are several reactions that occur in platelets that allow them to arrest bleeding by "plugging" the hole from which bleeding is occurring. The series of events that takes place in the order that they occur is as follows: adhesion to exposed tissue and subsequent shape change, release of granule contents, and aggregation caused by released products.[42] Each of these reactions will be discussed further in subsequent pages.

Trauma to a blood vessel that disrupts the endothelium exposes subendothelial layers to which the platelets can adhere. Of all the components of the subendothelial layers, platelets have the greatest affinity for collagen.[42,47] The affinity for collagen is very specific and dependent on the proper quaternary structure in order to elicit the required reactions. The helical portion of the collagen molecule is necessary to initiate the platelet response.[48] Platelets moving in the blood stream recognize the exposed collagen and immediately adhere to it. The exact mechanism of adhesion is unknown at present. The next reaction to occur is shape change. As previously mentioned, shape change is the first reaction of the platelet to most

stimuli and is a sign of platelet stimulation. A small and reversible amount of aggregation directly to collagen fibers takes place and lays the groundwork for the following reactions necessary for the formation of a stable hemostatic plug.

The release mechanism is actually a complex set of reactions which causes the expulsion of the platelet granules. There are actually two release reactions. Release I is the release of dense body contents, i.e., ADP and serotonin,[49] and Release II is the release of the alpha granule contents.[50] Release I can be induced by ADP and epinephrine,[48] and Release II is induced by thrombin or collagen[50] and occurs later than Release I. After adhesion of platelets to collagen, the platelet is stimulated by an unknown mechanism to release its granule contents into the surrounding medium. One of the constituents of the dense bodies, ADP, has been shown to be a potent inducer of platelet aggregation.[40,42,47,51] The released ADP then causes other platelets which are flowing by to aggregate, and the process continues until the platelet plug is formed. The formation of the platelet plug is called primary hemostasis and without release is reversible, and the aggregation of platelets by released ADP is irreversible second wave aggregation. Controls on the mechanism of primary hemostasis will be discussed later.

## Prostaglandin Synthesis

Another mechanism which occurs simultaneously with and causes release was not discussed in the preceding section because of its complexity, but should not be thought of as separate. This mechanism is the production of prostaglandins from arachidonic acid. The investigation of the conversion of arachidonic acid into biologically

active compounds is relatively young, with most of the research starting in the early 1970s. In 1973 a biochemical intermediate in prostaglandin synthesis, labile aggregation stimulating substance (LASS), was isolated and proven to act as a physiologic intercellular messenger that promoted platelet aggregation and release.[52,53] The relationship between prostaglandin synthesis, the platelet release reaction, and second wave aggregation was recognized fairly early. It has been known for some time that the platelet release reaction is associated with second wave aggregation (aggregation caused by the release of endogenous ADP from platelets). Several investigators blocked prostaglandin production with the use of aspirin, and found that this also blocked second wave aggregation.[24,54] It was then suggested that, because of their potent aggregating abilities, the prostaglandin endoperoxides, prostaglandin $G_2$ ($PGG_2$) and prostaglandin $H_2$ ($PGH_2$), played an important role in platelet aggregation[23,55] and are the first products that are biologically active in the prostaglandin pathway.[56,57] These endoperoxide intermediates, however, are very short-lived and degrade to more stable and measurable end-products. Figure 1 is a representation of the prostaglandin pathway of active platelets, showing the ultimate fate of arachidonate and the endoperoxides.

The arachidonic acid to prostaglandin pathway was first elucidated in sheep vesicular glands[58,59] and later found to be true for platelets. Platelet membranes contain phospholipids which are made up of fatty acids; the most important, as far as the platelet is concerned, is arachidonic acid. When platelets are exposed to collagen and thrombin, a specific phospholipase (phospholipase $A_2$) is activated[60] which cleaves arachidonic acid from the membrane phosphatides,

Figure 1. Possible fates of arachidonic acid released from membrane phospholipids. Cyclooxygenase is the key enzyme for platelet aggregation and is irreversibly acetylated by aspirin. Prostaglandin $G_2$ ($PGG_2$), $PGH_2$ and thromboxane $A_2$ are potent inducers of aggregation; $PGE_2$ acts synergistically with biological aggregating agents to potentiate aggregation; and $PGI_2$ is a potent inhibitor of platelet aggregation by stimulating adenylate cyclase.

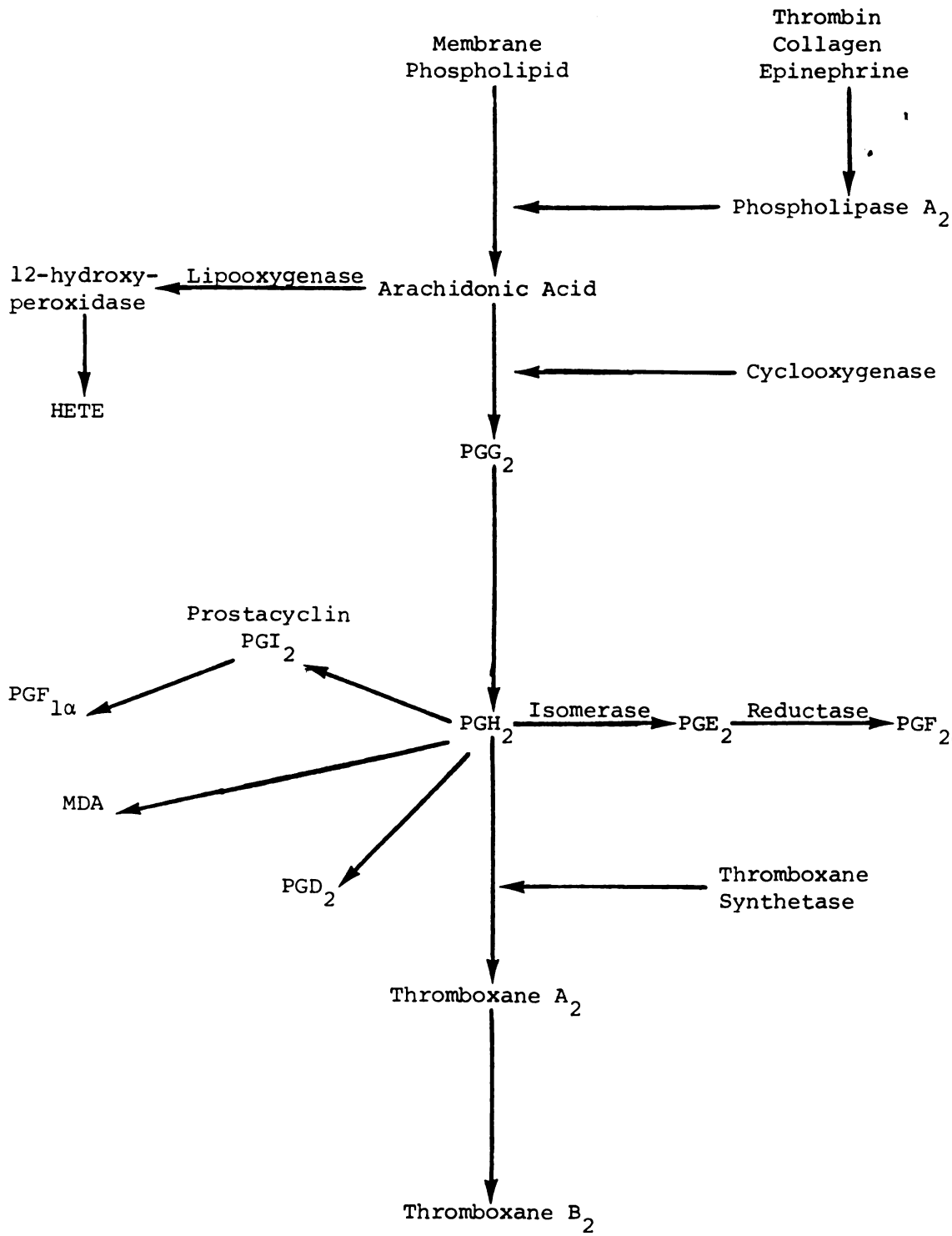

Membrane
Phospholipid
Thrombin
Collagen
Epinephrine
Phospholipase A2
12-hydroxy-
peroxidase
Lipooxygenase
Arachidonic Acid
HETE
Cyclooxygenase
PGG2
Prostacyclin
PGI2
PGF1α
PGH2
Isomerase
PGE2
Reductase
PGF2α
MDA
PGD2
Thromboxane
Synthetase
Thromboxane A2
Thromboxane B2

Figure 1

phosphatidylcholine and phosphatidylinositol.[42,61,62] The available arachidonic acid then is converted, through the prostaglandin endoperoxides, to prostaglandin $E_2$ and $F_{2\alpha}$ ($PGE_2$ and $PGF_{2\alpha}$) by the action of cyclooxygenase, prostaglandin synthetase, and other enzymes.[61,63,64] The end-products of the pathway, $PGE_2$ and $PGF_{2\alpha}$, have been shown to act synergistically with aggregation inducers, but in low concentrations are not actually aggregation inducers per se.[55]

It has been shown that the formation of prostaglandin endoperoxides is associated with the release reaction of platelets, namely second wave aggregation, but the exact association is unclear. Most investigators believe that endoperoxide formation precedes and is the cause of release of platelet granules. A recent article, however, draws conclusions that are perhaps slightly divergent. Charo et al. (1977)[65] suggest that there may be two different methods of inducing release, one being aggregation dependent and the other being aggregation independent. Their data show that release and second wave aggregation are parallel events and that one does not necessarily cause the other. Because of these data, the theory that second wave aggregation is caused by released ADP, etc., may in fact be incorrect. Two other articles suggest the possibility that the released substances, adenine nucleotides and serotonin, may act as cofactors for prostaglandin synthesis.[22,25] In one article by Willis and Weiss,[22] storage pool deficient platelets were shown to have defective prostaglandin production. It should be obvious, then that there is much more research needed in this area.

## Control of Prostaglandin Production and Aggregation

In any biological process, the control of that process is tantamount in preventing overstimulation or overreaction of that process, and platelet function is not an exception. Normally the response to vascular injury is rapid and complete in attaining hemostasis, and the control of the mechanisms involved is taken for granted unless thrombotic episodes occur. Numerous drugs, to be discussed later, have been used in an attempt to augment the biologic control, but we will now consider some of the physiological control mechanisms.

Until 1971, not much was known about the blockade of prostaglandin synthesis. At that time it was proposed that variations in the oxygenation system of prostaglandin synthesis could be a rate-limiting step.[66] In 1974, a previously considered physiologically inert prostaglandin, $PGD_2$, was found to be very portent as an inhibitor of platelet aggregation.[67] In fact, it was found to be more than twice as potent as $PGE_1$ as an inhibitor of platelet aggregation *in vitro*. Prostaglandin $E_1$, which is a derivative of the metabolism of dihomo-$\gamma$-linolenic acid,[68] was shown to be an inhibitor of platelet aggregation.[69,70] Holmsen (1977)[63] reported a positive feedback mechanism that could account for the rapid hemostatic reaction of platelets to vessel trauma. He stated that exogenous substances, the platelet membrane, and released substances act synergistically to potentiate platelet aggregation and hemostasis. He made no mention, however, of a negative feedback system.

Recently a series of articles appeared that stress the importance of prostacyclin, $PGI_2$.[68,71,72] Prostacyclin was first characterized by Moncada et al. (1976) to be derived from $PGH_2$ and, among other properties, is an inhibitor of platelet aggregation. In platelets,

the major metabolite of prostaglandin synthesis is thromboxane $A_2$ ($TxA_2$),[68] a potent inducer of platelet aggregation.[73] It is suggested that a delicate balance exists between $TxA_2$ and $PGI_2$ production, and it is this balance that regulates platelet aggregation.[68] Prostacyclin production is confined mainly to vascular tissue.[68,71] $PGH_2$ produced by the platelet might migrate to vessel walls and be converted to $PGI_2$.[71] Thromboxane $A_2$ and $PGI_2$ have opposite effects on c-AMP levels, which may be the mechanism by which regulation of aggregation occurs ($TxA_2$ decreases c-AMP levels and $PGI_2$ increases c-AMP levels).[68,71] The process of regulation of thrombosis is interesting and intriguing and is now a frequent topic of discussion; however, exact mechanisms of regulation will require more research before they are generally accepted.

## Measurement of Prostaglandin Production

In order to ascertain the activity and viability of any enzymatic pathway, measurement of intermediates, end-products, or by-products of that pathway is necessary. Measurement of intermediates of the prostaglandin pathway, however, is particularly difficult due to the short half-life of the endoperoxides, $PGG_2$ and $PGH_2$.[74] Equally difficult to quantify are the end-product prostaglandins, $PGE_2$ and $PGF_{2\alpha}$. Measurements of $PGE_2$ and $PGF_{2\alpha}$ in the past have been done by thin-layer chromatography[22] or radioimmunoassay[25] procedures, which are not inherently difficult but, due to the small and sometimes variable amounts of these prostaglandins that are produced, give results that are oftentimes equally variable.[74] An often used and more reliable method for assessing prostaglandin synthesis is measurement of a stable by-product of the PG pathway, malonyldialdehyde (MDA).[75]

Malonyldialdehyde is produced in quantities directly proportional to the amount of prostaglandins that are produced,[64] and is biochemically stable and measurable by direct methods.[74,75,76] The method involves reacting an acid extract containing MDA with thiobarbituric acid, and then reading the absorbance at 532 nm on a spectrophotometer.

## Chemiluminescence

A recently published method being employed to assess the activity of the arachidonic acid to prostaglandin pathway is chemiluminescence (CL).[77,78] Chemiluminescence was initially employed with human neutrophils[79,80,81] and later with monocytes[81,82] to measure phagocytic activity. There is some disagreement as to the cause of the chemiluminescence. Some investigators suggest that is is caused by a decay of a singlet oxygen from an excited state to a more relaxed state, or perhaps by electronically excited compounds formed in secondary reactions between a singlet oxygen and other molecules. For instance, carbon-carbon double bonds could react with singlet oxygen to form dioxetanes which, in turn, cleave to form aldehydes and ketones.[80] Nelson et al. (1978)[82] suggests that, since CL can be reduced by the addition of superoxide dismutase, a possible source of CL is the non-enzymatic conversion of superoxide (a product of oxidative metabolism) to singlet oxygen, which then relaxes to a ground state. This might explain the reports that the decay of singlet oxygen is not the major emitter of CL.[79,80] It is agreed, though, that an electronically excited intermediate, which by the emission of photons measurable as CL, relaxes to a more steady state with the amount of CL being proportionate to the number of excited species produced. Chemiluminescence has also been demonstrated in

platelet suspensions stimulated by the addition of arachidonic acid. It has been used as a measure of prostaglandin pathway enzyme activity, and the results correlate well with MDA production.[78] This could be a useful tool in determining whether any differences in platelet aggregation exist between normal controls and abnormal subjects caused by defects in prostaglandin production. For the purposes of this project, CL was used to compare PG production between non-C-HS mink and C-HS mink. Most investigators feel that the bleeding defect in C-HS individuals is caused by the decrease in "storage pool" adenine nucleotides; however, no work has been done to date that specifically evaluates the prostaglandin pathway in these subjects.

## Drug Effects on Platelets

Another prime area of interest in the study of platelet function is the evaluation of drugs as anti-thrombotic agents, considering that a major medical problem for our present population is occlusive vascular diseases such as atherosclerosis. There has been increasing recognition of the platelet's role in thrombus formation, especially in arteries; therefore, there is an active search for true platelet suppressive agents.[83,84,85] Several drugs have been tried in clinical situations with varying results; e.g., aspirin, sulfinpyrazone, dipyridamole, clofibrate, and others (non-clinical)[86] show some potential as anti-thrombotic agents.[84,85]

There are several ways in which platelet aggregation could be affected by drugs: 1) direct inhibition of release, 2) blockage of enzyme pathways, or 3) creating an increase in a cellular constituent that inhibits some part of platelet function are possible mechanisms

to be considered. An example of the latter is supported by reports indicating that drugs that cause an increase in c-AMP cause an inhibition of platelet aggregation.[87,88,89] Increases in c-AMP may occur by either stimulating adenyl cyclase or inhibiting phosphodiesterase, enzymes which regulate c-AMP levels.[90] Cyclic-AMP has been shown to be a potent inhibitor of platelet aggregation.[87]

Another approach to inhibiting platelet aggregation is to block the prostaglandin pathway. Perhaps the most ideal method of achieving blockade is by inhibition of one of the enzymes in the pathway; however, the blockade must occur before the production of endoperoxides, since $PGH_2$ can be utilized in the production of vasoactive compounds.

There are two important steps in the pathway that occur before endoperoxide production: the mobilization of arachidonic acid by phospholipase $A_2$ and the oxidative transformation of arachidonic acid by cyclooxygenase. Blocking arachidonic acid mobilization might have deleterious effects *in vivo*, since it is an essential fatty acid; however, if the action of platelet cyclooxygenase could be blocked, the physiologic effects would probably be minimal.

The inhibition of platelet cyclooxygenase is the mode of action of aspirin and other non-steroidal anti-inflammatory agents,[61,91] whereas clofibrate appears to inhibit arachidonic acid release from phospholipids.[61] Aspirin inhibition of aggregation occurs due to an irreversible acetylation of cyclooxygenase.[85,91] Due to the irreversible inhibition of platelet cyclooxygenase, inhibition of aggregation remains even after the drug has been completely excreted. It is this fact that makes aspirin very attractive as a possible anti-thrombotic agent. In this study, aspirin was employed to

determine if the quantity or activity of platelet cyclooxygenase differs in C-HS mink as compared to non-C-HS mink.

# MATERIALS AND METHODS

## Animals

The mink utilized for this project were C-HS and non-C-HS males and females ranging in age from 6 months to 1 1/2 years. Some were obtained from a commercial breeder (Phil Clemmons Fur Farm, Barryton, Michigan) and maintained at the mink research facility at Michigan State University. Specimen collection and some testing were done on location at the farm. All mink were determined to be free of Aleutian disease prior to testing, since the presence of the disease could interfere with the results. When the identity of C-HS mink was questioned, a peripheral blood smear was made and examined for the presence of abnormal neutrophil granules.

## Blood and Platelet Collection

Blood was collected directly from the heart of the mink while under ether anesthesia. The blood was drawn through a 21-gauge, 1 1/2 inch needle into a 10 cc syringe containing 3.8% trisodium citrate solution with the ratio of citrate to blood being 0.8 ml:9.20 ml. Several ratios, including 1 part citrate to 9 parts blood, were tested for citrate inhibition, and the 0.8:9.20 ratio was found to be optimum for mink. The specimens were transferred to 15 ml plastic centrifuge tubes and centrifuged for 10 min at 200 g to obtain platelet rich plasma (PRP). If the specimen was to be used for platelet aggregations, 0.5 ml aliquots of PRP were transferred to

siliconized glass aggregation tubes, and the remaining blood specimen was recentrifuged at maximum rpm to obtain platelet poor plasma (PPP). Each mink specimen yielded from 1.5 to 2.0 ml PRP, which allowed 3 to 4 aggregation tracings on each mink. Platelet counts on the PRP were done using the unopette method (Becton-Dickinson and Co., Rutherford, New Jersey).

Aliquots for chemiluminescence were collected and PRP prepared as previously described. Washed platelet suspensions were then prepared by adding 1 part 10% EDTA (0.343 M, pH 7.4) to 9 parts PRP, centrifuging at 600 g for 10 min at 4°C, discarding the plasma, and resuspending the platelets in 1 ml Hank's balanced salt solution (HBSS). Platelet counts were done on the resuspended solution and adjustments to platelet suspensions were made to equalize the counts to 0.8-1.2 x $10^9$ platelets/ml.

## Platelet Aggregations

Platelet aggregations were done on a Payton Dual Channel Aggregometer, with a Houston Instruments dual pen recorder. The aggregometer tracing range was adjusted to 90% of the possible range using PPP as 100% transmittance and PRP as approximately 5% transmittance to allow maximal pen movement to show shape change. Platelet rich plasma was continuously stirred at 900 rpm by a metal rod in the aggregation tube, allowing maximum platelet-platelet contact, and was simultaneously incubated at 37°C during the entire procedure. Aggregation was induced by adding 0.030 ml collagen (0.05 mg/ml as determined by the Bio-Rad protein assay) or 0.050 ml arachidonic acid (8 μM) to the PRP. Incubation at 37°C occurred for 2 min prior to and 4 min after the addition of the aggregating agent to allow for maximum

aggregation. The plasma was immediately centrifuged in an Eppendorf microcentrifuge for 2 min and the supernatant fluid frozen for later malonyldialdehyde measurements.

## Collagen Preparation

Collagen was prepared using Sigma bovine collagen (Sigma Chemical Co., St. Louis, Missouri). One gram of collagen was added to 100 ml of 83.5 mM acetic acid. The collagen-acetic acid mixture was mixed using a tissue homogenizer to break up the collagen fibers and make an even suspension of collagen in the acetic acid. This homogenation was done for 30 sec while keeping the mixture as close to 0°C as possible by means of an ice water bath. After achieving an even suspension of collagen, the volume was adjusted to 1 liter with 16 mM acetic acid. Approximately 1 ml aliquots were pipetted into small vials and frozen for later use. The assayed value of collagen concentration was determined to be 0.05 mg/ml. When needed for platelet aggregation, a vial was thawed, mixed vigorously on a vortex mixer to insure an even suspension of collagen, and kept in an ice bath during use.

## Arachidonic Acid Preparation

Arachidonic acid (>99% pure, Sigma Chemical Co., St. Louis, Missouri, 50 mg vials) was made up as a solution of the sodium salt by the addition of 0.164 ml 1 N NaOH and then adding Tris buffer (0.015 M, pH 8.5) to a final volume of 1 ml. After the addition of Tris buffer, the vial was shaken vigorously for approximately 1 min to allow portions of the fatty acid that had crystallized to go into solution. The final concentration of fatty acid was calculated to be 0.16 mM/ml. The solution was kept frozen until immediately

before use and was then refrigerated and protected from exposure to light during use to prevent autooxidation. Excess arachidonate was refrozen and used later with no degeneration in effectiveness observed.

## Chemiluminescence Measurements

All scintillation vials for this procedure were dark adapted for at least 1 hr prior to testing, and were not exposed to light until after testing in order to prevent elevated CL results. Three milliliters phosphate-buffered saline (pH 7.4) was added to the 1 ml washed platelet suspension in the scintillation vials under red illumination. After obtaining a background count which was later subtracted from the final results, 50 µl (8 µM) arachidonic acid was added and the solution hand-agitated vigorously for 2 sec. The specimens were then counted for 2 min at 0.1 min intervals. Counting was accomplished on a β scintillation counter (Model LS-9000, Beckman Instruments, Inc., Fullerton, California) at room temperature with the counter adjusted out of coincidence and the window opened wide to count the entire measurable light spectrum emitted. The results were plotted as counts per minute (cpm) vs. time.

## Malonylaldehyde Assay

The measurement of MDA was accomplished by reacting an acidified protein-free filtrate of the reacted platelet suspension with thiobarbituric acid (TBA) in the following manner. The sample was decanted into an equal volume of 20% trichloroacetic acid in 6 N HCl, mixed by gentle inversion, and allowed to stand at room temperature for 10 min to allow complete protein precipitation. The acid solution was centrifuged at 1500 g for 15 min or in a serofuge for 5 min.

The supernatant was then filtered through glass wool to remove any remaining protein precipitate. Thiobarbituric acid was prepared by making a 0.12 M solution of TBA in Tris buffer (0.26 M) and the pH adjusted to 7.0 with 0.1 N HCl. Just prior to using, 0.1 ml 1% butylated hydroxytoluene (BHT) in absolute ethanol was added to 10 ml TBA solution to make a final concentration of 0.01% BHT. This solution was prepared fresh daily. Standards were prepared by hydrolyzing 16.7 µl malonaldehyde tetramethyl acetal MDA/TMA with 3 or 4 drops concentrated HCl and adjusting the volume to 10 ml with distilled $H_2O$ to make a final concentration of 10 µM/ml. A 10 nM/ml standard solution was prepared by adjusting 0.010 ml of 10 µM/ml standard solution to 10 ml with water. A 5 mM/ml and 2.5 mM/ml standard were prepared from the 10 mM/ml solution and all standards were run with the unknowns.

The reactant solutions were prepared by adding 0.2 vol TBA reagent to 1.0 vol of acid extract and incubating at 60°C for 30 min. The specimens were cooled slowly to room temperature and the absorbance of the standards plotted vs. a blank at 532 nm was recorded. The absorbance of the standards plotted vs. the known concentrations on standard graph paper yielded a straight line, indicating linearity at least to 10 nM/ml concentration. Varying concentrations of standard were tested and found to be linear to 40 nM/ml; however, standard concentrations above 10 nM/ml were not needed in this study since the specimens fell in the 2.5-10 nM/ml range (TBA, MDA/TMA, and BHT were procured from Eastman Organic Chemicals, Rochester, New York).

## Platelet Particulate Fraction

A platelet particulate fraction was prepared and run as a control with all CL assays to make certain that all reagents and equipment were working properly. The particulate fraction was prepared by obtaining human PRP as described previously and centrifuging the PRP at 600 g for 10 min after the addition of 10% EDTA (1 vol EDTA to 9 vol PRP). The supernatant was discarded, the platelet button resuspended in HBSS, and the preceding step repeated. After the second centrifugation, the supernatant was discarded and the platelet button resuspended in half the original volume of PRP with HBSS. The platelets were fractured by freezing, thawing, and sonicating for 10 min, followed by centrifugation at 13,000 g for 10 min at 0°C. Keeping the solution at 0°C, the supernatant was pipetted in 2 ml volumes into small vials, quick frozen in a dry ice and acetone bath, and kept frozen until use. On the days that CL assays were done, a vial of particulate fraction was thawed and assayed along with the mink suspensions with consistent results.

## Aspirin Preparation and Injection

Powdered aspirin (Sigma Chemical Co., St. Louis, Missouri) was prepared as a 30 mM solution in 0.9% NaCl. The solution was made immediately before use and was not stored for extended periods of time, since aspirin in this type solution degrades very quickly. A 5 ml aliquot of the aspirin solution was administered to the mink by intraperitoneal injection and blood specimens obtained 1 hr after injection. The PRP was then tested for platelet aggregation by collagen and arachidonic acid and the specimens for MDA assays frozen and analyzed later.

## Statistical Analysis

The results were analyzed by the use of the Student's t-test (unpaired) and in all cases are expressed as means $\pm$ standard deviation. Differences with $P<0.01$ were considered to be significant.

## RESULTS

The results of chemiluminescence are depicted in Figure 2, with the platelet counts adjusted to range from 0.8-1.2 x $10^9$ platelets/ml. The mean peak value 0.2 min after arachidonic acid injection was 74,000 $\pm$ 15,000 cpm for the C-HS mink and 71,000 $\pm$ 21,000 cpm for the non-C-HS mink. The other interval counts for both groups of mink were approximately equal for the remainder of the assay, with the ranges of counts for both groups overlapping. A human curve was also plotted as a control, as was the platelet particulate fraction.

As a more quantitative measure of the chemiluminescent response, malonyldialdehyde assays were done on the scintillation vial contents after CL, and Figure 3 graphically represents the results of the MDA assays. The mean MDA value for non-C-HS mink was 8.2 $\pm$ 2.8 ng/ml, and for C-HS mink it was 8.6 $\pm$ 2.6 ng/ml. The MDA results for platelet particulate fraction (not shown) were 15.5 $\pm$ 1.5 ng/ml. The reason for the major difference in MDA results between the mink specimens and the particulate fraction is hypothesized to be due to a higher concentration of platelets used to prepare the particulate fraction. No platelet counts were done on the particulate fraction during preparation since its use was only to provide consistent day-to-day results.

In Figure 4, the MDA results after correction for platelet counts to 0.3 x $10^9$/ml are shown. Each mink PRP was aggregated with

Figure 2. Chemiluminescence response plotted as cpm x $10^3$ vs. time. Human platelets and C-HS mink platelets both had a mean peak chemiluminescent response at 74,000 cpm and decayed as shown. Non-C-HS mink platelets had a mean peak chemiluminscent response at 71,000 cpm and a platelet particulate fraction mean peak was at 48,000 cpm. (n = 5 for the human curve; n = 8 for C-HS, non-C-HS, and particulate fraction curves)

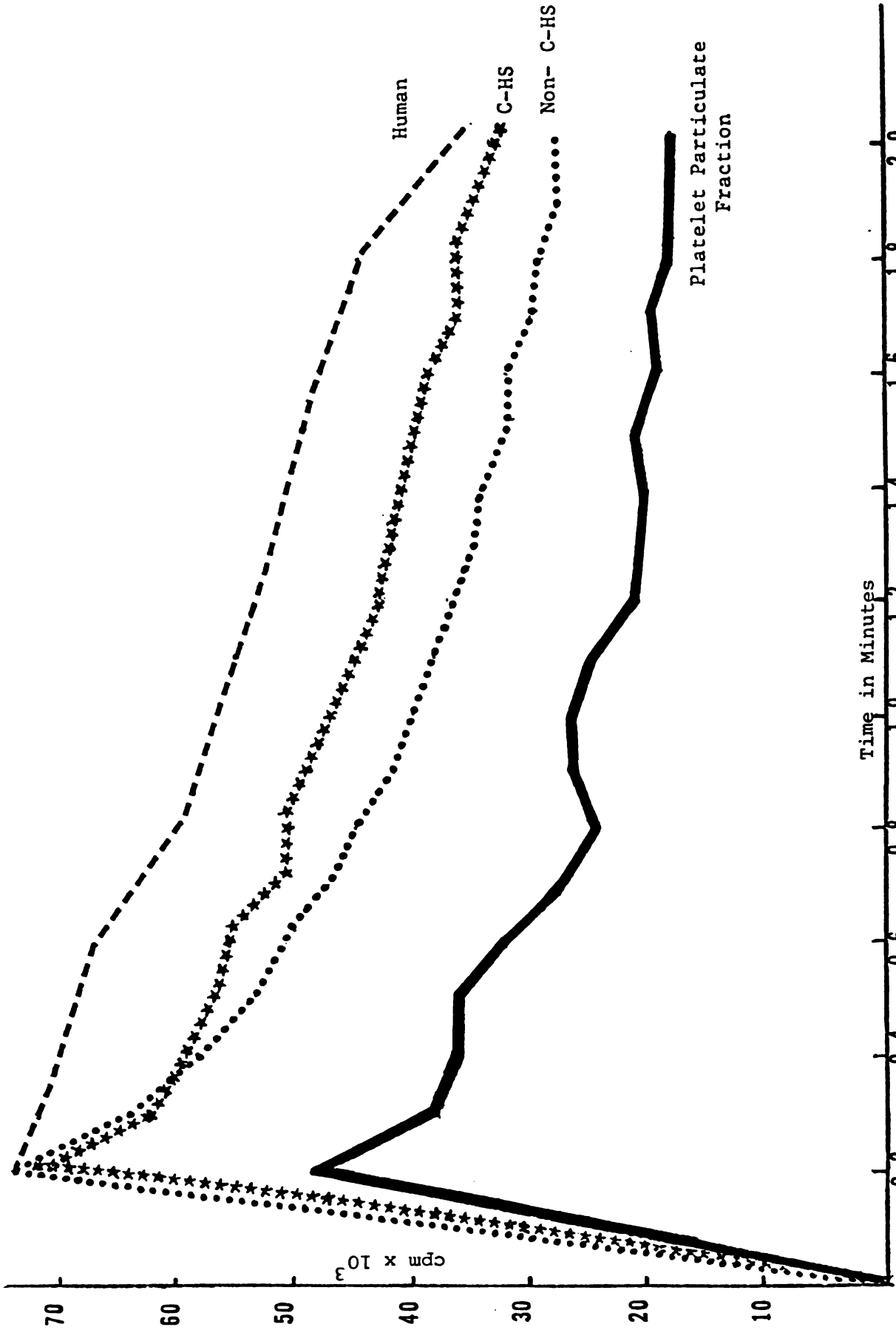

Human
C-HS
Non- C-HS
Platelet Particulate Fraction
cpm x $10^3$
70
60
50
40
30
20
10
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Time in Minutes

Figure 2

Figure 3. Mean malonyldialdehyde assay results produced by non-C-HS and C-HS mink platelets during chemiluminescence induced with arachidonic acid. The mean values were 8.2 ± 2.8 ng/ml for non-C-HS mink and 8.6 ± 2.6 ng/ml for C-HS mink. Platelet counts for both groups were corrected to range from 0.8 to 1.2 x $10^9$ platelets/vial analyzed for chemiluminescent response. (n = 8)

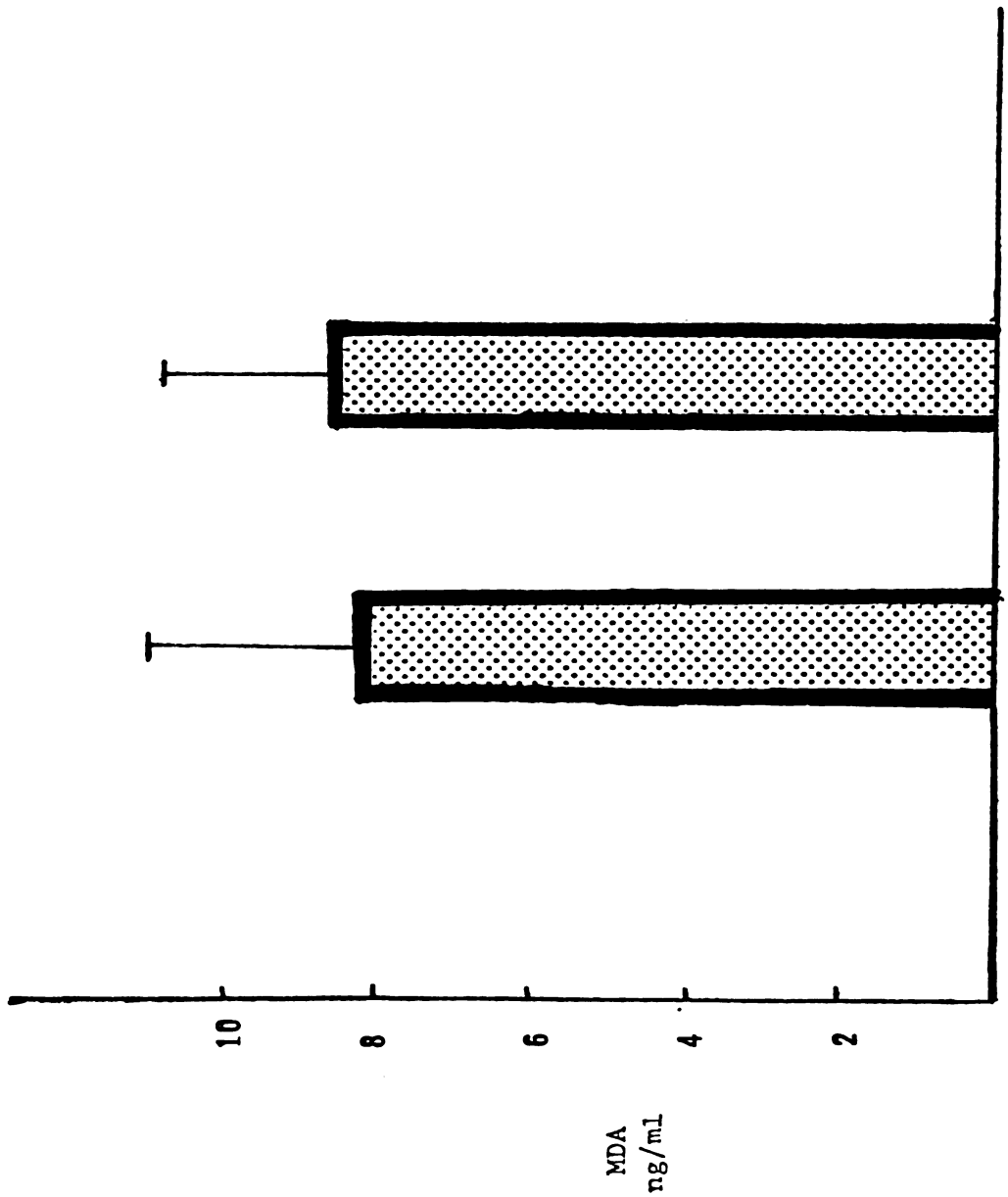



Figure 3

Figure 4. Mean malonyldialdehyde assay results produced by non-C-HS and C-HS mink platelets during platelet aggregation induced by arachidonic acid and collagen . The mean values were 6.8 ± 1.1 and 2.5 ± 0.9 ng/ml for non-C-HS mink and 6.9 ± 0.8 and 3.1 ± 0.9 ng/ml for C-HS mink induced by arachidonic acid and collagen, respectively. (n = 8)

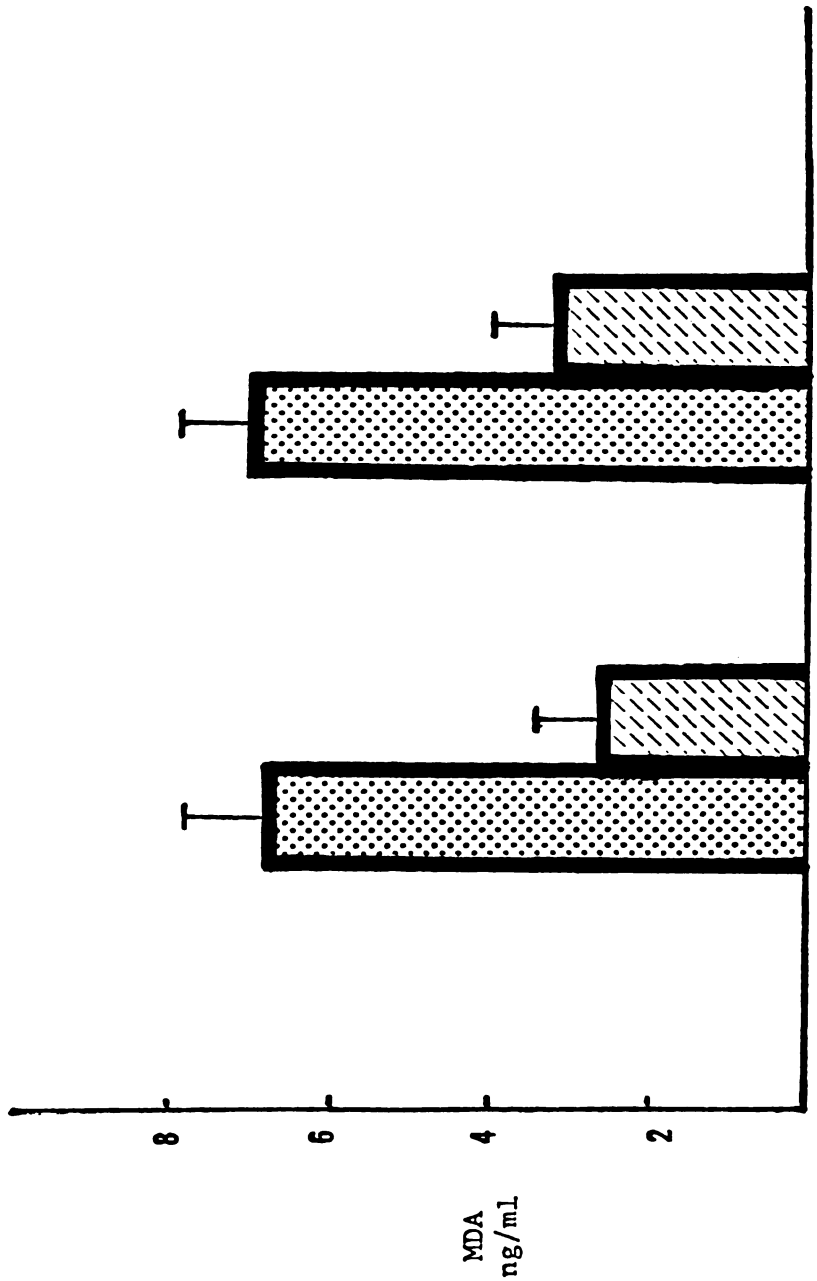
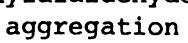
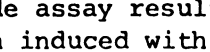



Figure 4

arachidonic acid and collagen and then assayed for MDA. The non-C-HS mink values were 6.8 ± 1.1 and 2.5 ± 0.9 ng/ml, and the C-HS mink results were 6.9 ± 0.8 and 3.1 ± 0.9 ng/ml using arachidonic acid and collagen, respectively, as inducing agents.

The MDA assay results after aspirin injection and platelet aggregation are illustrated in Figure 5. The mean values for non-C-HS mink were 5.1 ± 1.0 and 2.3 ± 0.6 ng/ml and, for C-HS mink, 4.4 ± 0.9 and 1.7 ± 0.4 ng/ml using arachidonic acid and collagen, respectively, as aggregating agents and correcting platelet counts as previously described.

Figures 6 and 7 represent typical aggregation curves of non-C-HS and C-HS mink in response to arachidonic acid and collagen used as inducing agents before (Figure 6) and after (Figure 7) the injection of aspirin. A marked decrease in the extent and rate of aggregation is seen after aspirin injection.

Figure 5. Mean malonyldialdehyde assay results produced by non-C-HS and C-HS mink platelets during platelet aggregation induced with arachidonic acid  and collagen  1 hr after aspirin injection. The mean values were 5.1 ± 1.0 and 2.3 ± 0.6 ng/ml for non-C-HS mink and 4.4 ± 0.9 and 1.7 ± 0.4 ng/ml for C-HS mink induced by arachidonic acid and collagen, respectively. (n = 8)

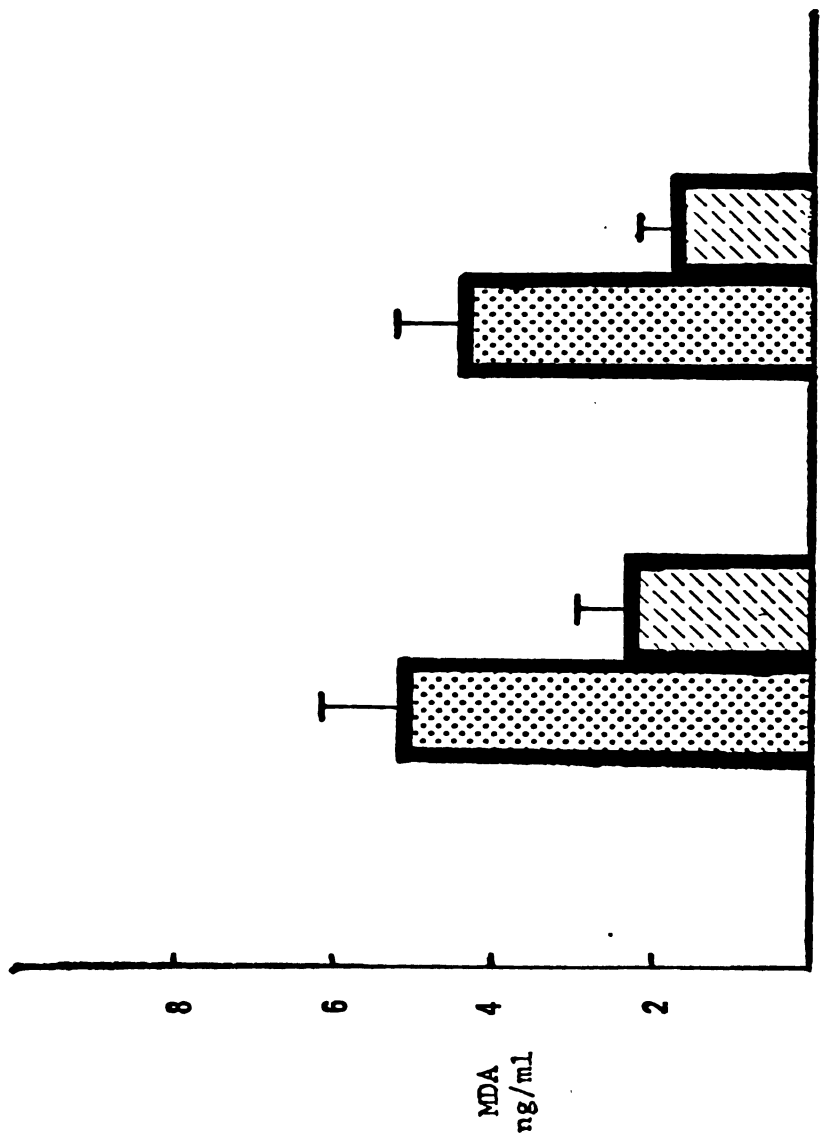
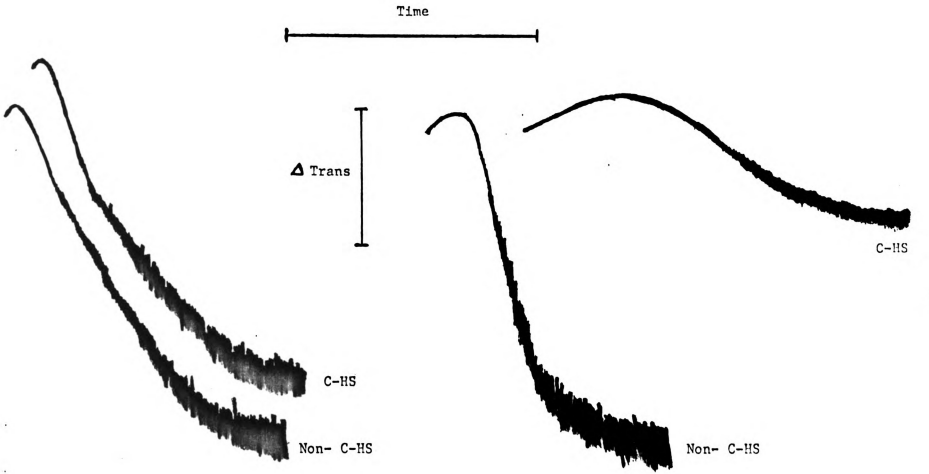



Figure 5

Figure 6. Platelet aggregation curves for non-C-HS and C-HS mink in response to arachidonic acid (8 μM) and collagen (0.05 ng/ml).

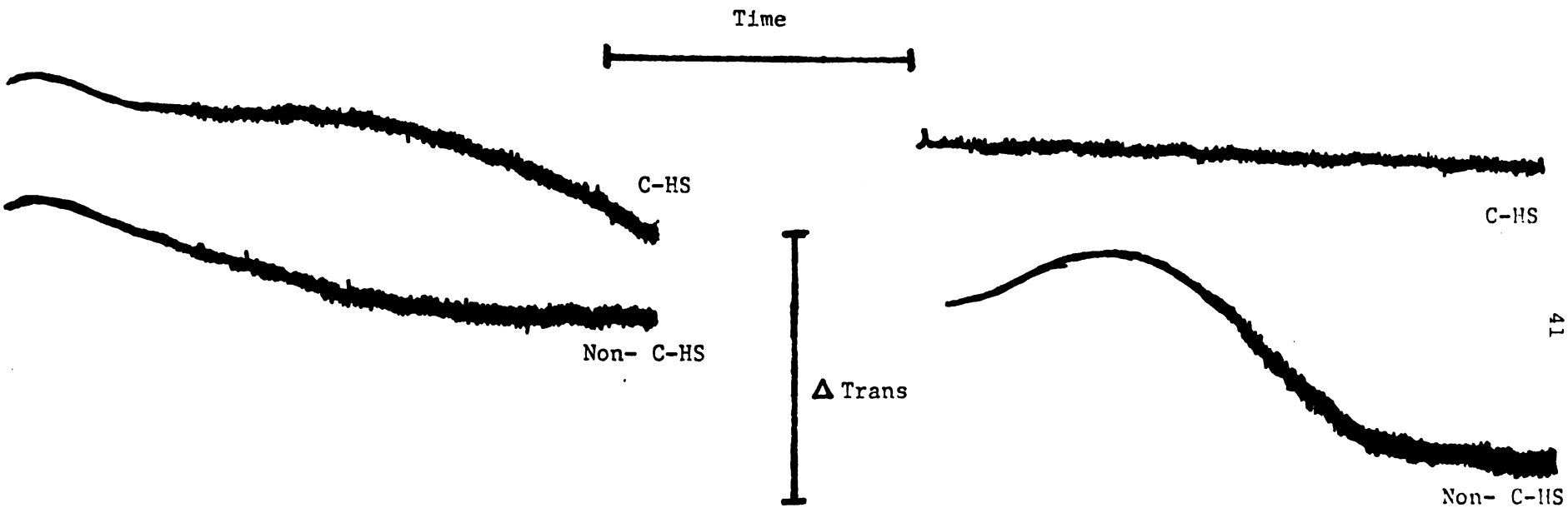



Arachidonic Acid Aggregation

Collagen Aggregation

Figure 6

Figure 7. Platelet aggregation curves for non-C-HS and C-HS mink in response to arachidonic acid (8 μM) and collagen (0.05 ng/ml) 1 hr after aspirin injection.




Arachidonic Acid Aggregation

Collagen Aggregation

Figure 7

# DISCUSSION

It has been shown in the past that C-HS affected individuals of all species studied have an abnormally prolonged bleeding time. The prolongation of bleeding times is related to the platelet count and/or platelet function.[92] Similar findings are also observed to a lesser extent in patients who have ingested aspirin. *In vitro* studies illustrate defective platelet aggregation, which is directly related to primary hemostasis, and in C-HS was determined to be caused by decreased amounts of "storage pool" ADP released by platelets during aggregation with collagen. Collagen normally causes a release of storage pool ADP during the platelet release reaction of aggregation and the released ADP causes aggregation. Like ADP, the proper concentration of epinephrine first has a direct effect on platelets in initiating aggregation and is followed by a second wave of aggregation (biphasic response) caused by released ADP. It is this second wave of aggregation which is absent in C-HS individuals. In summary, a decreased or absent pool of releasable ADP characterizes itself functionally as a reduction in response to collagen and a loss of second wave aggregation in response to ADP and epinephrine. These defective responses are seen both in storage pool disease (C-HS) and in patients who have ingested aspirin. Interestingly, non-primate species, except for cats, do not exhibit a biphasic response to ADP and epinephrine even with normal amounts of storage pool adenine

nucleotides present. Therefore, a storage pool deficiency is somewhat more difficult to characterize in these species.

Failure to produce a secondary wave of aggregation may also be due to a defect in prostaglandin synthesis. Certain myeloproliferative disorders exhibit decreased prostaglandin synthesis,[64] suggesting that defects in platelet aggregation previously attributed to storage pool deficiencies could also have underlying defects in prostaglandin synthesis. In this study, using C-HS mink, the possibility of prostaglandin synthesis defects in addition to storage pool deficiencies was explored.

Assays measuring the chemiluminescent response of non-C-HS and C-HS mink platelets found no significant differences between the two groups ($P<0.01$). Peak CL values were, for all practical purposes, the same. The rates of relaxation to a more steady state depicted by the decrease in counts per minute over time were also basically equal. Malonyldialdehyde values after chemiluminescence were nearly equal for both groups ($P<0.01$) and no significant differences were detected.

Another approach, the measurement of MDA after platelet aggregation, was also undertaken using 2 different aggregating agents, arachidonic acid and collagen. As with the chemiluminescence results, no significant differences were detected between non-C-HS and C-HS mink ($P<0.01$) with either aggregating agent.

Measurement of MDA after aspirin injection and platelet aggregations revealed no significant differences between the two test groups ($P<0.01$). However, there were significant differences between the non-aspirin treated and aspirin treated values. Theoretically, treatment of platelets with aspirin would completely stop MDA and prostaglandin production. Since this was an *in vivo* injection of

aspirin, however, problems in the concentration injected and/or absorbance from the peritoneal cavity could prevent all platelets from being exposed to aspirin and the non-exposed platelets could account for the MDA that was produced. The one hour delay between injection of aspirin and specimen collection was possibly not adequate for complete absorption of the drug.

The minor difference between C-HS and non-C-HS mink after aspirin treatment ($P<0.01$), although not significant, might be supported by two different, relatively recent studies. First, it has been shown that the effects of drugs both *in vivo* and *in vitro* are influenced by the concentration of red cells present in PRP.[93] It is difficult to obtain PRP completely void of red cells, and it is entirely possible that differences in red cell concentrations in PRP contributed to the minor difference observed. Secondly, the concentration of divalent cations, especially calcium, is important in platelet aggregation and the inhibitory effects aspirin and other drugs have on platelet aggregation are enhanced as the calcium concentration decreases.[94] Any one or combinations of the above possibilities could account for the minor observed differences in MDA production between the test groups after aspirin injection.

Considering the previously presented data, the author concludes that no difference exists between C-HS and non-C-HS mink as concerns the prostaglandin synthesis pathway. Similar studies in other species could reveal a difference and are deserving of further study. However, due to the great degree of homology of the disease between species, the existence of a significant difference is doubtful.

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# VITA

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The author was born in Houlton, Maine, on November 22, 1949, and as the son of a dairy farmer, he grew up in the small town of Sherman, Maine. His elementary and secondary education was attained in Sherman and culminated with graduation from Katohdin High School in 1968. He then entered the United States Air Force Academy located near Colorado Springs, Colorado. Graduation from the service school occurred on June 7, 1972, when he received a Bachelor of Science degree in Life Sciences and a commission in the United States Air Force. From the academy he was assigned to Wilford Hall USAF Medical Center in San Antonio, Texas, to attend a 12-month clinical laboratory internship. Upon completion of the internship, he tested for and received a registry as Medical Technologist from the American Society of Clinical Pathologists. His next assignment was to USAF Hospital Loring, Loring AFB, Maine, as Director of Laboratory Services, which began in August 1973. In the fall of 1976, the author received a scholarship for the Air Force Institute of Technology (AFIT) to obtain an advanced degree beginning the subsequent school year. The author chose Michigan State University as his first choice of schools and matriculated in September 1977. Upon completion of his degree requirements, Captain Patterson will be assigned as Chief of Hematology and Coagulation at Keesler AFB Medical Center, Keesler AFB, Biloxi, Mississippi.

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