THE INHIBITORY ACTION OF MYOCHRYSINE, A GOLD BASED, ANTI-RHEUMATIC DRUG, ON PLATELET ACTIVATION

By

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for my two favourite men

Bob and Michael

THE ACTION OF MYOCHRYSINE ON PLATELETS

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ABSTRACT

Rheumatoid arthritis is a chronic, systemic inflammatory disease. It can be treated with the gold(I) based drug, Myochrysine. Much research has been done in the past, in an attempt to elucidate the mechanism of action of this drug. To date, the mechanism remains a mystery. It is known however that Myochrysine inhibits the action of inflammatory cells. Myochrysine has been shown to inhibit phagocytosis, chemotaxis and the respiratory burst in mononuclear phagocytes and polymorphonuclear cells; T and B cell proliferation; interleukin 2 secretion from T cells; interleukin 2 receptor expression on T cells; as well as thrombin stimulated platelet aggregation and release.

All of these inflammatory cell functions are mediated by a biochemical pathway involving receptor-G-protein mediated phospholipase C activation which results in Ca^{2+} mobilization and protein kinase C activation. It is proposed that Myochrysine acts to relieve rheumatoid arthritis by interfering with some point(s) in this biochemical pathway in inflammatory cells. Through the use of the platelet as a model system, it has been shown that Myochrysine inhibits the collagen, ADP, and U46619 induced platelet aggregation and serotonin release; that Myochrysine inhibits collagen induced myosin light chain and pleckstrin phosphorylation; that the inhibitory action is fast and reversible; that the inhibitory effect is not dependent on the presence of albumin; that the inhibitory effect does not involve cyclic nucleotide increases and that Myochrysine does not inhibit TPA induced pleckstrin phosphorylation or A23187 induced myosin light chain phosphorylation. More importantly, it is shown that Myochrysine inhibits platelet activation in a manner very similar to inhibition of platelet activation by other sulfhydryl reacting

agents, including cell impermeant compounds. The data presented are consistent with the action of Myochrysine being at a membrane surface sulfhydryl group, most probably at a common structurally important sulfhydryl group within platelet receptors involved in the activation of the protein kinase C/Ca^{2+} mobilization pathway.

Data are also presented which indicate that both the gold(I) moiety and the thiomalate ligand of Myochrysine can inhibit platelet function.

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ABBREVIATIONS

Au	Gold
Au(I)	Gold, +1 Oxidation State
ACD	Acid Citrate Dextrose
ADP	Adenosine Diphosphate
cAMP	Cyclic Adenosine Monophosphate
cGMP	Cyclic Guanosine Monophosphate
Con A	Concanavalin A
CPDS	6,6'-Dithiodinicotinic Acid
DAG	Diacylglycerol
DPM	Disintegrations per Minute
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethyleneglycolbis-(β-aminoethylether)N,N,N',N'-tetraacetic acid
ESSE	5,5'-dithiobis-2-nitrobenzoic Acid
fMLP	N-Formylmethionylleucylphenylalanine
G-Protein	GTP-Binding Protein
Gs	GTP-Binding Protein Linked to Adenylate Cyclase in a Stimulatory Manner
GTP	Guanosine Triphosphate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HLA	Human Leukocyte Antigen
HLA-DR	Human Leukocyte Antigen - D-related Region
IFN-γ	Gamma Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M

ABBREVIATIONS Continued

IL-1	Interleukin 1
IL-2	Interleukin 2
IP_3	1,4,5-Inositol Triphosphate
LPS	Lipopolysaccharide
MLR	Mixed Lymphocyte Reaction
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-Ethylmaleimide
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
P20	Myosin Light Chain, 20 Kilodalton Platelet Protein
P40	Pleckstrin, 40 Kilodalton Platelet Protein
PAGE	Polyacrylamide Gel Electrophoresis
PHA	Phytohemagglutinin
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PK-C	Protein Kinase C
PL-C	Phospholipase C
PMNLs	Polymorphonuclear Leukocytes
PWM	Pokeweed Mitogen
RA	Rheumatoid Arthritis
RBC	Red Blood Cell
SDS	Sodium dodecyl sulphate
SNP	Sodium nitroprusside
TCA	Trichloroacetic Acid
TEMED	N,N,N'N'-Tetramethylethylenediamine
Ti-CD3	Antigen receptor on T lymphocytes
TMA	Thiomalic Acid
TPA	12-O-Tetradecanoylphorbol-13-acetate
TRIS	Tris(hydroxymethyl)aminomethane

ABBREVIATIONS Continued

TXA_2	Thromboxane A_2
U44619	9,11-Dideoxy-11α,9α-Epoxymethano-Prostaglandin-$F_{2\alpha}$

TABLE OF CONTENTS

TABLE OF CONTENTS

continued

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 RHEUMATOID ARTHRITIS

Chronic, systemic, inflammatory are all adjectives describing the debilitating disease, rheumatoid arthritis (RA).(1,2,3) RA is a disease which combines genetic and immunological factors to result in inflammation of synovial joints.(4) RA, which has a world wide distribution, occurs with a prevalence of approximately 1% and affects 2-3 times more women than men.(1,2,5) This disease can begin at any age, but occurs most commonly in the mid fifty age group.(1)

Clinically, the presentation and course of RA is highly varied.(1,2,3) Most often, the onset of the disease is insidious but RA can have an acute onset of action which is seen in about 20% of patients.(1) RA initially presents with generalized aching, weakness, stiffness, fatigue, weight loss, anorexia and low grade fever.(1,2) As the disease process progresses, pain and stiffness in the affected joints becomes predominant.(1,3) The classic signs of inflammation; pain, redness, warmth, swelling and loss of function, can all be seen in these joints.(1,2) Any synovial joint can be affected but the more common joints include the small joints of the hands and feet, the wrist and the knee.(1) Long term outcome is varied and ranges from a very short term illness with no resulting disability to a long term disease resulting in destruction of joints, deformity, disability and a significant systemic involvement of other organ systems.(1,2,5,6) Spontaneous remission is

seen in approximately 10-30% of patients.(1,2,3)

The cause of RA is unknown.(3,4,5,7,8) Many hypotheses suggest that some exogenous antigenic material enters the joint and initiates the inflammatory process.(4,7) Possibilities for this exogenous antigen include viruses such as Epstein-Barr virus (EBV), hidden in macrophages or lymphocytes and non-biodegradable products of bacteria.(4) Whatever the cause, it still remains that an inflammatory response is initiated and maintained.

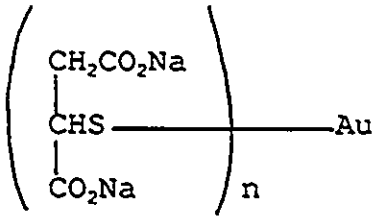
Damage to the synovial micro-vasculature endothelium is one of the first histological changes observed in RA.(4,9) This is followed by mild synovial cell proliferation.(9) In early stages of the disease there is an influx of polymorphonuclear leukocytes (PMNLs) into the joint fluid.(9) As the disease progresses, small lymphocytes, plasma cells and macrophages become the predominate infiltrating, inflammatory cell types in the subsynovial lining while PMNLs predominate in the synovial joint effusion.(1,5,9) The synovial lining becomes hypertrophied.(9) The pathology of RA in the affected joint results from a complex interaction between the inflammatory cells (lymphocytes, PMNLs and macrophage), resident synovial cells, chondrocytes and soluble mediators released from these cells such as interleukins, immunoglobulins and hydrolytic enzymes.(1,5,8,9) One of the results of this interaction is inflammation which resembles a delayed type hypersensitivity reaction.(5,7) Another result is degradation of proteoglycans and collagen which make up the cartilage of the joint.(1,5,9)

Generally, treatment of RA begins conservatively with patient education, rest and exercise.(1) Usually, the first drug which is given is aspirin or some other non-steroidal anti-inflammatory drug (NSAID).(2,5) These drugs alleviate the inflammatory symptoms of RA but do not affect disease progression.(2,5) Corticosteroids which are anti-

inflammatory are also used in the treatment of RA (2,5), but because of potential serious side effects, use of corticosteroids is limited.(2,5) As the disease continues to become more active and becomes unresponsive to NSAIDs, slow-acting, remission inducing drugs are used.(5) These include gold-containing drugs, D-penicillamine and anti-malarials.(3,5) These drugs generally have a slow onset of action and have been shown to slow progression of the disease.(5) For severe disease unresponsive to NSAID or remission inducing agents, immuno-suppressive drugs such as cyclophosphamide or methotrexate may be used.(2,5) Some immunosuppressive drugs have been reported to induce remission of RA but have potentially serious side effects.(2,5)

1.2 MYOCHRYSINE

FIGURE 1.2.1 STRUCTURE OF MYOCHRYSINE



Myochrysine or gold sodium aurothiomalate (see Figure 1.2.1) is a gold based drug used in the second-line treatment of RA. The use of gold for the treatment of RA began in the late 1920's when Dr. J. Forrestier used gold thiopropanol sodium sulphonate to treat 15 individuals with chronic rheumatoid arthritis.(10,11) He used this drug based on the findings in 1890 by Koch, that gold cyanide inhibited the growth of tubercle bacilli and on the belief at that time that RA and

tuberculosis may be caused by the same organism.(10,11,12) Dr. Forrestier showed through several clinical trials with large numbers of patients, that gold was indeed effective in the treatment of RA in approximately 70-80% of the cases.(11) Since that time several clinical trials have shown the beneficial effects of Myochrysine with regards to RA.(11,13-17) It was found from these studies that Myochrysine provided benefit for RA patients in 50-80% of the cases, that it took several months of continual therapy to see the beneficial effects of gold therapy, that gold therapy must be maintained to continue providing benefit, that Myochrysine appeared to slow radiological progression of the disease and that Myochrysine had a fairly high level of toxicity.(10,11,13-18) Myochrysine is used to treat patients with RA who show a lack of benefit from more conventional types of therapy such as rest, exercise, and NSAIDs.(11,12,19)

A significant number of adverse effects are associated with the clinical use of Myochrysine.(12,19) Major side effects of the drug are muco-cutaneous, renal and hematological.(12,19,20) Muco-cutaneous reactions occur in 15-30% of RA patients on Myochrysine.(11,20) The appearance, severity and duration of gold-induced rash is highly variable(12,19). Renal side effects include proteinuria and nephrotic syndrome.(12,19,20) Proteinuria can occur in up to 26% of patients taking Myochrysine and the more serious nephrotic syndrome, only occurs in 0.2-2.6% of patients.(12) Hematologic disorders caused by Myochrysine include eosinophilia, leukopenia, thrombocytopenia, anemia, pancytopenia and aplastic anemia.(12,19) These complications are more rare but can be very serious and in some cases life threatening.(12,19) A side effect of gold therapy, which is unique to Myochrysine, is a nitritoid reaction.(11) This causes the patient to feel weak, light-headed, flush, faint and can cause nausea and vomiting.(11,12,19)

Pharmacokinetics and distribution of Myochrysine have been studied in animals and man. The drug is given by intramuscular injection.(12,19) An early study by Lawrence using ^{199}Au showed that by 15 minutes after injection, gold could be detected in the hands and feet.(21) Several studies have shown that Myochrysine dissociates once it enters the body into thiomalate and protein bound gold. (22-26) Approximately 95% of the dissociated gold binds to albumin.(27-30) Cottrill *et al.*, using ^{199}Au and ^{35}S labelled Myochrysine in rats(22) and Jellum *et al.*, using ^{199}Au and ^{14}C labelled Myochrysine in mice(23) have shown dissociation of gold from its thiomalate ligand. It is interesting to note that Cottrill found only 0.9% ^{35}S remaining in the rats by 48 hours(22) while Jellum found that at 20 hours, 40% of the ^{14}C label was still within the mice.(23) These differences may reflect changes between species but more likely reflect differences in metabolism between sulfur and carbon. Dissociation of Myochrysine has also been shown in man.(24,25,26) Free thiomalate and thiomalate disulfide have been found in human serum and urine.(24,25,26)

After a single dose of 50 mg Myochrysine, serum gold concentration peaks at 4-8 µg/mL within 2-6 hours and then declines over the next week to about 3 µg/mL.(28,31,32) The half life for gold clearance from serum is 5.2 - 5.5 days.(31.33) Weekly administration of Myochrysine results in an initial peak of serum concentration after injection and then a decline over the next week.(32) The decline does not reach base line so that over time there is a steady increase in serum concentration until 5-10 weeks when a plateau in serum concentration is reached.(28,32) Plateau levels range from 0.52 - 7.76 µg/mL.(15,16,17,34,35)

Excretion of Myochrysine occurs via urine and feces with 70% of gold being found in urine and 30% being found in feces.(27,28,31,32) The amount found in feces is highly variable and appears to be dependent on stool mass.(27,31) Excretion kinetics follow the same general pattern as

serum kinetics.(27) The highest concentrations of gold are found in the first 24 hours post injection.(27,32,36) Levels of gold in the urine then decline over the next week.(27,32,36)

Tissue distribution of gold has been studied in mice, rats and man.(21,22,23,37,383) Gold is widely distributed throughout the body and has been found in virtually every organ investigated.(21,37) It has been found that gold tends to concentrate in reticuloendothelial tissues.(27,28,37) Highest concentrations of gold are found in lymph nodes, adrenal glands, liver, kidney, bone marrow and spleen.(27,28,31,39) Gold has also been found to accumulate in inflamed joints of RA patients with very little gold being found in uninvolved joints.(21,27,38) Tissue concentrations of gold range from 1-200 µg/mL.(31)

A prevailing theme seen with Myochrysine is that there appears to be a large variation in responses between individuals. Measurements of blood gold levels, rate of excretion, amount of gold in urine and feces, amount of gold in individual tissue samples all show large variations between individuals.(34,37,38,40) Variation is also seen in the clinical response to gold and the development of toxicity. It is important to note that several studies have shown that there is no correlation between serum gold concentration and either efficacy or toxicity of Myochrysine.(21,35,36,40) This lack of correlation was shown in a controlled, double-blind, multi-centre, randomized trial.(17) Auranofin, Myochrysine and placebo were tested in an attempt to find a correlation between whole blood gold concentration and clinical efficacy.(17) None was found.(17)

Although the kinetics and distribution of gold are fairly well known for Myochrysine, there are several things about the drug which are still not known. One of the major unknowns is the identity of the pharmacologically active component of Myochrysine. Myochrysine is a

gold(I) based drug. Gold in the +1 oxidation state usually has two ligands attached in a linear fashion, therefore Myochrysine must be polymeric (see Figure 1.2.1).(41,42) Whether it is a hexamer or pentamer is still not known with certainty. Isab and Sadler, suggest the drug exists as a hexamer(43), while Al-Sa'ady *et al.*, give evidence that Myochrysine is likely a pentamer.(44) Unfortunately, crystals of the drug have proven difficult to produce, so that an X-ray structure determination of Myochrysine has not been undertaken.(42)

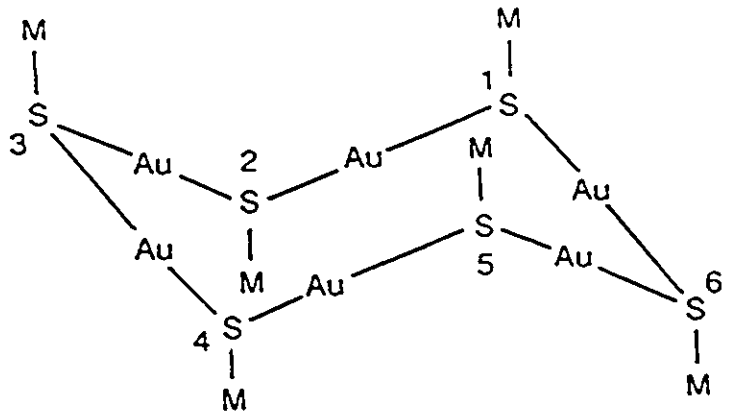
Another issue which complicates the identification of the active component is that Myochrysine is chiral.(41) Several combinations of chiral forms of the drug exist in equilibrium (see Figure 1.2.2). The exact combinations of the drug depends on the number of units in the polymer.(41) Figure 1.2.2 shows these combinations assuming the Myochrysine polymer has six units. Whether "all d" or "all l" or a combination of "d" and "l" Myochrysine is responsible for clinical efficacy is not known.

Other complicating factors include the fact that each batch of Myochrysine contains contaminants such as glycerol, water, chlorocresol and excess thiomalate and that the composition of the drug and its contaminants varies from batch to batch.(42,45)

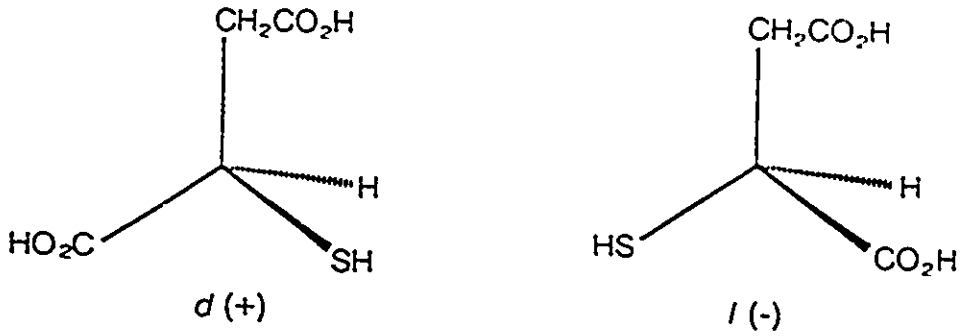
Myochrysine solid, when dissolved in water and sterilized for patient use, undergoes several changes.(45,46,47) Upon dissolution, two short lived species of unknown chemical identity are formed.(45,46) Their presence can be followed by colour changes and changes in platelet reactivity.(45,46,47) Sterilization also causes the production of another unknown component which is yellow and may be responsible for the nitritoid reaction caused by Myochrysine.(11,45)

FIGURE 1.2.2 THE POLYMERIC AND CHIRAL NATURE OF MYOCHRYSINE

(A)



(B)



(C)

MOLECULE	RATIO IN MIXTURE
6d(+)	1
5d(+),1l(-)	6
4d(+),2l(-)	15
3d(+),3l(-)	20
2d(+),4l(-)	15
1d(+),5l(-)	6
6l(-)	1

Figure taken from W.F. Kean, C.J.L. Lock, H.E. Howard-Lock (1991) Lancet 338: 1565-1568.

(A) Numbers in hexamer denote sulphur groups about which the thiomalate ligand (M) is chiral.

(B) Shown are d(+) and l(-) configurations that may occur at these sulfur groups.

(C) Different chirality combinations and the ratios with which they occur.

1.3 MECHANISM OF ACTION OF MYOCHRYSINE

The definitive mechanism(s) of action of Myochrysine in rheumatoid arthritis is(are) not known. Myochrysine is known however to inhibit many inflammatory cell functions.

1.3.1 EFFECT OF MYOCHRYSINE ON LYMPHOCYTES

Myochrysine has been shown to affect lymphocytes in a variety of ways. *In vivo*, Myochrysine decreases circulating IgM, IgG and IgA in RA patients.(48,49,50) This may result from an inhibition of immunoglobulin production or from an inhibition of lymphocyte proliferation, both of which have been shown to occur.(50,51) *In vitro*, 0.25 - 25 x 10^{-5}M Myochrysine irreversibly inhibited, in a dose dependent manner, production of IgM, IgG and IgA from pokeweed mitogen (PWM) and EBV stimulated human mononuclear cells.(50,51) Incubation of Myochrysine with either macrophage or B cells resulted in inhibition of the antibody response.(50,51)

Inhibition by Myochrysine of lymphocyte proliferation, as measured by ^{3}H-thymidine uptake in stimulated cells, has been shown to occur in many studies which used a variety of stimulators such as PWM, phytohemagglutinin (PHA), concanavalin A (ConA), *mycoplasma arthritidis* T cell mitogen, streptokinase/streptodornase and interleukins 1 and 2.(50,52-58) Myochrysine has also been shown to inhibit mixed lymphocyte reactions (MLR).(53,54,55,59) One study has suggested that the inhibitory action of Myochrysine on lymphocyte proliferation was the result of inhibition of monocyte accessory function rather than inhibition of the lymphocyte response.(53)

An effect of Myochrysine on thymocytes has also been shown. It

has been found that concentrations of Myochrysine greater than 2.5 µg/mL had a suppressive effect on ConA and PHA induced interleukin-2 (IL-2) production and IL-2 receptor expression in thymocytes of mice and in human peripheral blood mononuclear cells.(56)

1.3.2 EFFECT OF MYOCHRYSINE ON MONONUCLEAR PHAGOCYTES

Several functions of mononuclear phagocytes including chemotaxis, phagocytosis, and the respiratory burst are inhibited by Myochrysine. Myochrysine has been shown to inhibit chemotaxis of human monocytes towards lymphocyte derived chemotactic factor (60) and towards the chemotactic agent N-formylmethionylleucylphenylalanine (fMLP).(61) Concentrations of up to 100 µg/mL Myochrysine have been shown to inhibit Fc receptor mediated phagocytosis of sheep red blood cells (RBC) (62,63) and engulfment of *Candida albicans* by human mononuclear blood cells is inhibited by 10-100 µg/mL Myochrysine.(64) Jessop *et al.*, showed that, *in vivo*, phagocytic activity of macrophage and PMNLs was decreased in RA patients receiving Myochrysine compared to RA patients not on gold therapy.(65)

A respiratory burst as measured by superoxide generation and chemiluminescence occurs upon activation of phagocytosis in polymorphonuclear and peripheral blood mononuclear cells.(66) Myochrysine, 5-25 µg/mL, was shown to inhibit zymosan and calcium ionophore induced chemiluminescence in peripheral blood mononuclear cells and in adherent cells from both controls and RA patients.(67) Superoxide anion generation in rat lung macrophage was also inhibited by Myochrysine.(68)

Interleukin-1 (IL-1) production by human mononuclear phagocytes was also inhibited by Myochrysine.(56,57) However, in one study using

rabbits, concentrations of Mycchrysine which inhibited IL-1 production, killed most of the monocytes.(69)

Differentiation of monocytes to macrophage is marked by synthesis of the second complement component, gamma-interferon (IFN-γ) induced human leukocyte antigen - D-related region (HLA-DR) expression, loss of peroxidase activity and phorbol myristate acetate induced lysis of chicken erythrocytes.(70,71,72) Myochrysine at concentrations of 12.5 - 50 µg/mL, inhibited all these functions.(70,71,72) From these results, it can be inferred that Myochrysine inhibited the maturation of monocytes.(70,71,72)

1.3.3 EFFECT OF MYOCHRYSINE ON POLYMORPHONUCLEAR CELLS

Myochrysine has been shown to inhibit chemotaxis and phagocytosis of PMNLs. PMNLs isolated from RA patients treated with Myochrysine, 1 - 1000 µg/mL showed a decreased casein induced chemotaxis, compared to PMNLs from controls.(73) *In vivo* administration of Myochrysine to rats inhibited random migration and fMLP induced chemotaxis of PMNLs, while *in vitro* addition of Myochrysine inhibited only chemotaxis of the rat PMNLs.(74)

In vivo administration of Myochrysine to rats also results in an inhibition of phagocytosis.(75) This inhibitory effect of Myochrysine on phagocytosis by PMN is also seen in RA patients treated with Myochrysine.(65) *In vitro*, up to 100 µg/mL Myochrysine showed only mild suppression of phagocytosis of *Candida albicans* by human PMNLs.(76)

The effect of Myochrysine on the respiratory burst by PMNLs is less evident. In several studies, up to 100 µg/mL Myochrysine failed to inhibit chemiluminescence and superoxide anion generation.(76-79) Other studies, though, show that Myochrysine can inhibit these functions. In a study by Davis and Johnston, Myochrysine up to 100 µg/mL was shown to

inhibit PMA and fMLP induced superoxide generation in human PMNLs.(80) Roisman *et al.* have also shown that 20 µg/mL Myochrysine shows modest inhibition of superoxide anion generation in human PMNLs.(81)

Lysosomal enzyme release is another phagocytic cell function which may be inhibited by Myochrysine. Carevic showed though that 10^{-5} - 10^{-7}M Myochrysine had no significant effect on release of lysosomal enzymes.(82) On the other hand, 20-40 µg/ Au/mL Myochrysine was shown to decrease release of β-glucuronidase, lysozyme and acid phosphatase from human PMNLs by 20-35%.(83,84) Also, 1 mM Myochrysine significantly decreased release of these enzymes from zymosan stimulated rat leukocytes.(85)

1.3.4 EFFECT OF MYOCHRYSINE ON LYSOSOMAL ENZYMES

In addition to inhibiting lysosomal enzyme release, Myochrysine has been shown to inhibit the action of these enzymes. Turkall *et al.* showed that *in vitro*, Myochrysine could inhibit the activity of β-glucuronidase and acid phosphatase.(68) Myochrysine, 5 x 10^{-3}M, inhibited β-glucuronidase and acid phosphatase activity in extracts from guinea pig peritoneal macrophage.(86) Activity of these enzymes was also inhibited by incubation of intact cells with Myochrysine.(86) Myochrysine also has been shown to inhibit human lysosomal elastase.(87)

1.3.5 EFFECT OF MYOCHRYSINE ON PLATELETS

Myochrysine has been shown to inhibit platelet function. At concentrations of 1.3 - 6.5 x 10^{-5}M, Myochrysine has been shown to prolong the time of platelet shape change and the time required to attain maximum aggregation in thrombin stimulated platelets.(88) 1.3-2.6 x 10^{-4}M Myochrysine added to thrombin stimulated platelets inhibited aggregation

completely but did not inhibit platelet shape change.(88)

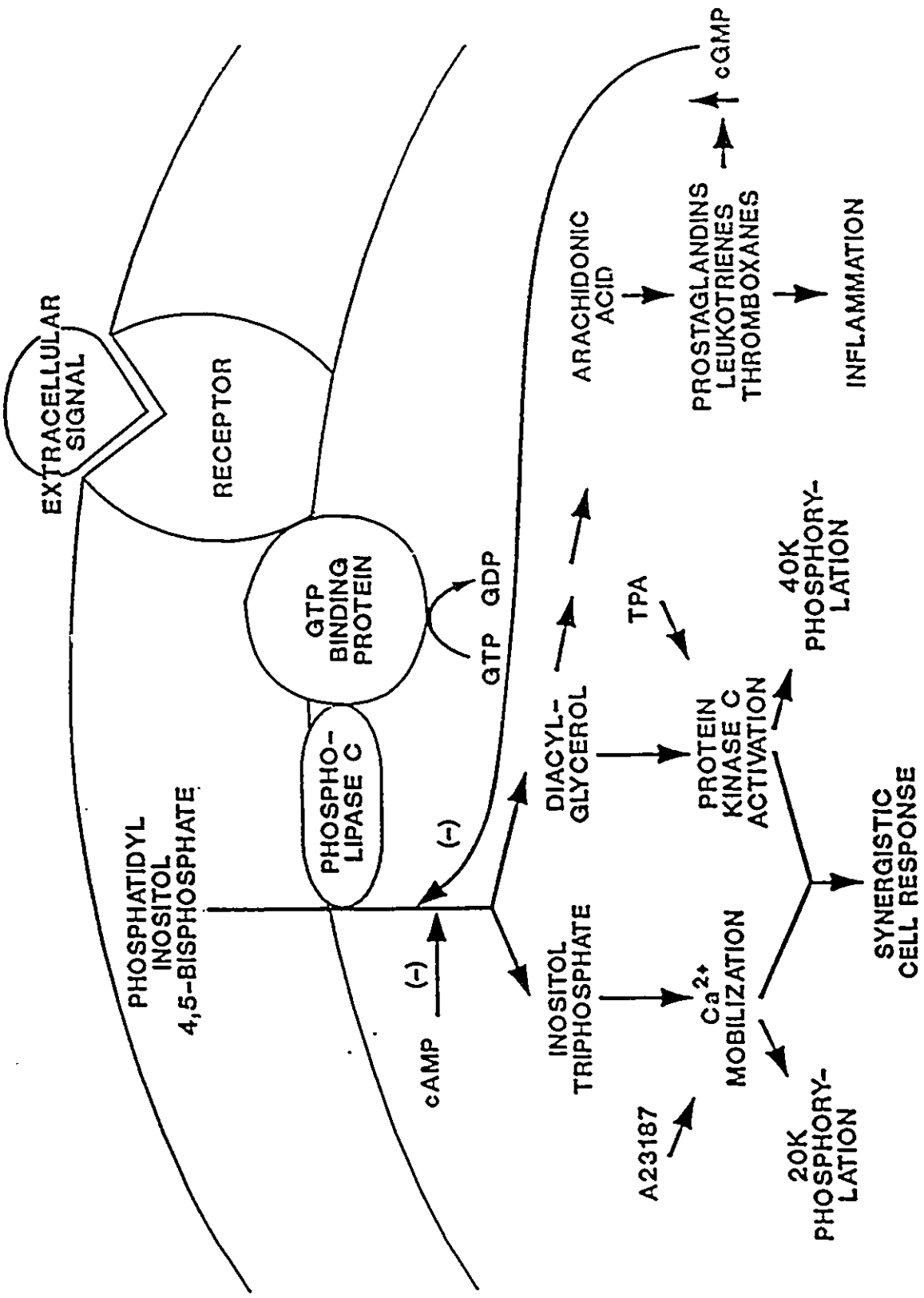
1.3.6 OTHER EFFECTS OF MYOCHRYSINE

Other effects of Myochrysine which may have importance in its effect on RA are shown in two studies. The first, by Koch *et al.*, investigated the action of Myochrysine on angiogenesis, an important feature in the resolution of inflammation.(89) It was shown that Myochrysine was capable of inhibiting the production of macrophage-derived angiogenic activity.(89) The second study by Kawakami *et al.* investigates the action of Myochrysine on endothelial cells.(90) Endothelial cells line small blood vessels and are thought to play a role in the inflammatory process seen in RA.(90) Kawakami *et al.* have shown that 1-20 µg/mL Myochrysine inhibited proliferation of endothelial cells and that 15-20 µg/mL inhibited IFN-γ induced HLA-DR expression in these cells.(90)

1.4 BIOCHEMISTRY OF INFLAMMATION

There is a biochemical pathway which is universal to the activation of all inflammatory cells. This pathway involves concurrent activation of protein kinase C (PK-C) and Ca^{2+} mobilization (see Figure 1.4.1). An extracellular signal which among others can be thrombin, collagen and mitogen, binds to its specific cell receptor. The receptor then interacts with a GTP-binding protein (G-protein) which is responsible for coupling the receptor to PL-C.(91,92) The identity of the G-protein is as yet unknown with certainty.(91,92) PL-C is then activated by the interaction with this G-protein.(91,92) PL-C cleaves phosphatidyl inositol 4,5 bisphosphate (PIP_2) into 1,4,5-inositol triphosphate (IP_3) and diacylglycerol (DAG).(91,92,93)

FIGURE 1.4.1 PROTEIN KINASE C/Ca^{2+} PATHWAY



DAG released from PIP_2 is responsible for the activation of protein kinase C.(91,92,93) Upon activation, translocation of PK-C from cytosol to membrane occurs.(91) DAG increases the affinity of PK-C for phospholipid (phosphatidylserine) and Ca^{2+} which are required for the enzyme's activity.(93) DAG as well as activating PK-C, is involved in the regeneration of phosphatidyl inositols and in the formation of arachidonic acid.(91,92) Arachidonic acid can also be released by the action of the Ca^{2+} dependent phospholipase A_2 on other phospholipids in the cell membrane.(91,93) Arachidonic acid once released can be metabolized into prostaglandins and leukotrienes which are mediators of inflammation.(91)

Arachidonic acid metabolites are also thought to be activators of guanylate cyclase.(91,93) The increase in cyclic guanosine monophosphate (cGMP), is believed to act as a negative feedback inhibitor of this pathway.(93) PK-C is also believed to be a negative feedback inhibitor of this activation pathway.(91) The exact mechanism of feedback inhibition is not known, but PK-C is capable of phosphorylating many receptors causing a decrease in affinity of these receptors for their agonist.(91) PK-C has also been shown to phosphorylate PL-C and G-proteins *in vitro*.(91,92) Cyclic adenosine monophosphate (cAMP), which can be formed by activation of a different set of cell receptors, also inhibits this pathway, possibly by preventing signal dependent phospholipid breakdown.(92,93)

Both Ca^{2+} and PK-C can mediate cell activation by phosphorylation of specific target proteins.(91) Ca^{2+} acts through a Ca^{2+}-calmodulin dependent protein kinase.(91) Ca^{2+} ionophores such as A23187 or ionomycin cause a rise in intracellular Ca^{2+} without the activation of PK-C (93,94) and phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) or synthetic DAG (1-oleoyl-2-acetylglycerol) directly activate PK-C without a concomitant rise in intracellular Ca^{2+}.(93,94,95) Through the use of

these two agents, it has been shown that Ca^{2+} and PK-C act synergistically to produce a variety of cellular responses.(91,93,94,95)

1.4.1 ACTIVATION IN THE LYMPHOCYTE

In T lymphocytes, the pathway involving PIP_2 hydrolysis, PK-C activation and Ca^{2+} mobilization is responsible for cell proliferation, IL-2 secretion and IL-2 receptor expression.(93,96,97,98) The link between the pathway and these cellular events has been shown in many studies. Agents which interact with the Ti-CD3 receptor complex (antigen receptor) have been shown to activate PL-C (99), to increase IP_3 (100,101) and to increase intracellular Ca^{2+}.(101) PK-C has also been implicated in this activation.(97,102,103,104) TPA, only in combination with calcium ionophores, causes lymphocyte proliferation, IL-2 secretion and IL-2 receptor expression.(105) This emphasizes the fact that PK-C and Ca^{2+} act synergistically to give a maximal cell response.

In B lymphocytes, signals which cross-link cell surface immunoglobulin receptors (antigen receptor) cause hydrolysis of PIP_2 (106-112) and translocation of PK-C from the cytosol to the plasma membrane.(107,113) Activation of B cells results in proliferation and differentiation into antibody secreting cells.(114) A combination of synthetic DAG and Ca^{2+} has been shown to induce growth of B cells.(115) Cross-linking of HLA class II receptors is associated with PIP_2 hydrolysis, increased intracellular Ca^{2+} and proliferation in B cells.(110) Mitogenic lectins which cause B cell proliferation are also believed to involve PK-C activation.(116)

1.4.2 ACTIVATION IN THE MONONUCLEAR PHAGOCYTE

Macrophage activation has also been shown to be mediated by the pathway involving PK-C activation and Ca^{2+} mobilization. Mitogens such as lipopolysaccharide (LPS) which cause macrophage proliferation have been shown to result in PIP_2 hydrolysis.(117) Oxygen radical release during the respiratory burst in macrophages correlates with translocation of PK-C to the plasma membrane (106) and PK-C has also been implicated in production of IL-1.(118) IFN-γ regulates macrophage differentiation (monitored by Fc receptor expression), antigen presentation, phagocytosis and cytokine production.(119) The combination of ionomycin and TPA can also induce Fc receptor expression in macrophage.(119)

1.4.3 ACTIVATION IN THE POLYMORHONUCLEAR CELLS

There are several inflammatory processes which can be activated in neutrophils including chemotaxis, adherence, respiratory burst, lysosomal enzyme and granule release and phagocytosis.(120,121,122) The respiratory burst results in activation of NADPH oxidase and release of O_2^- and H_2O_2.(123,124,125) Both PK-C and Ca^{2+} have been implicated in these processes. A combination of both TPA and ionomycin were required to produce the same degree of neutrophil adherence as fMLP which acts through binding of a specific receptor (126) and the action of fMLP has been shown to depend on PIP_2 hydrolysis.(121) The respiratory burst in neutrophils has been shown in several studies to require PK-C.(120,124,125,127-131) Activators of PK-C activate NADPH-oxidase.(124,128,129) Translocation of PK-C to the membrane fraction correlates with oxidase activation.(120,127) Inhibitors of PK-C inhibit the respiratory burst (125,131) and NADPH oxidase can be phosphorylated *in vitro* by PK-C.(130) The release of

granules and lysosomal enzymes can both be induced by the combination of synthetic DAG and A23187.(93,132) Phagocytosis may also be associated with enhanced PIP_2 hydrolysis.(122) Receptor activation leading to phagocytosis results in increased levels of DAG and IP_3.(122)

1.4.4 ACTIVATION IN THE PLATELET

Thrombin (133-136), collagen (133,136), ADP (136,137,138), arachidonic acid (136,139), thromboxane A_2 (U46619) (139,140) and platelet activating factor (136,141) have all been shown to exert their effects through receptor mediated PIP_2 hydrolysis by PL-C. Receptor-PL-C coupling has been shown to be mediated by a G-protein.(142-145) The identity is not known with certainty but αi has been implicated in this action.(146,147)

PIP_2 hydrolysis results in the formation of IP_3 and DAG.(136) Intracellular Ca^{2+} is increased through the action of IP_3 and from extracellular sources as well.(148) DAG activates PK-C.(136) Ca^{2+}, through a Ca^{2+}-calmodulin dependent kinase, phosphorylates a 20 kilodalton protein, known to be myosin light chain (P20).(149,150) This can be mimicked by the action of A23187.(151) PK-C has been shown to phosphorylate a 40 kilodalton protein which has been named pleckstrin (P40).(152) The function of this protein is not known but it is thought to be involved in secretion.(152) The actions of PK-C can be directly initiated by phorbol esters such as 12-O-tetradecanoyl-13-acetate.(151,153)

Arachidonic acid formation from the action of diacylglycerol lipase on DAG (154,155) and arachidonic acid liberation from the action of

phospholipase A_2 on membrane phospholipids (154,155,156) also occurs in activated platelets. Phospholipase A_2 is activated from the increase in intracellular Ca^{2+} (157) and possibly through the action of PK-C.(158)

Both Ca^{2+} and PK-C are important in secretion of platelet granules.(153,159,160) The pathway involving PK-C activation and Ca^{2+} may also be involved in aggregation but primary aggregation can be induced by epinephrine through a mechanism not involving PIP_2 hydrolysis.(136) Secondary aggregation however is most likely dependent on processes initiated by PIP_2 hydrolysis.(136)

The role of this pathway in shape change is controversial. On one hand, all agonists which induce PIP_2 hydrolysis induce shape change (136) and phosphorylation of myosin light chain appears to correlate with shape change.(161,162) However, through the use of a TXA_2 antagonist, two TXA_2 receptor types have been found.(163) The first receptor type was low affinity and was associated with shape change and an increase in platelet Ca^{2+}.(163) The second type was high affinity and resulted in PL-C activation.(163) Also, inhibitors of PK-C do not affect platelet shape change(163).

A Na^+/H^+ exchanger is also activated during platelet activation.(164) The steps involved in this activation and the role of the exchanger in platelet activation are still under debate.(165-169)

Platelet cGMP has been shown to be increased by platelet activators.(170,171) The increase in this cyclic nucleotide is thought to act as a negative feedback on the activation pathway, possibly through inhibition of PL-C.(171) PK-C is also thought to be a negative feedback inhibitor (172) with potential actions on the G-protein and/or PL-C.(173) Another possibility is that PK-C exerts its effects by phosphorylating inositol-P3-5'-phosphomonoesterase rendering it active.(174) This would result in an increase in IP_3 breakdown.(174)

Each platelet activator, although initially acting through the same mechanism, differs in its overall action. Collagen activation appears to be dependent on TXA_2 and ADP released after the initial activation by collagen fibres.(175,176) Thrombin depends on these mediators but also has a third unknown activation mechanism.(176)

1.5 PROPOSAL

It can be clearly seen that there is a great overlap between the inflammatory cell responses which are inhibited by Myochrysine and those which are mediated by the PK-C/Ca^{2+} pathway (see Table 1.5.1).

TABLE 1.5.1: COMPARISON OF INFLAMMATORY CELL PROCESSES MEDIATED BY PK-C/Ca^{2+} PATHWAY AND PROCESSED INHIBITED BY MYOCHRYSINE

Inflammatory Cell Function	Inhibition by Myochrysine	Mediated by PK-C/Ca^{2+} Pathway
T LYMPHOCYTE		
proliferation	YES	YES
IL-2 secretion	YES	YES
IL-2 receptor expression	YES	YES
B LYMPHOCYTE		
proliferation	YES	YES
antibody production	YES	?
MONONUCLEAR PHAGOCYTE		
chemotaxis	YES	?
respiratory burst	YES	YES
phagocytosis	YES	?
IL-1 production	YES	YES
IFNγ induced differentiation	YES	YES
POLYMORPHONUCLEAR CELL		
chemotaxis	YES	YES
phagocytosis	YES	YES
respiratory burst	YES	YES
lysosomal enzyme release	YES	YES
adherence	?	YES
PLATELETS		
aggregation	YES	?
release	?	YES

It is proposed that Myochrysine acts to relieve rheumatoid arthritis by interfering with some point(s) in the biochemical pathway in inflammatory cells involving PIP$_2$ hydrolysis, PK-C activation and Ca^{2+}.

The action of Myochrysine on this pathway is investigated here by use of human and rabbit platelets as a model system. The reason for choosing the platelet is that the platelet is the most extensively studied system with regards to this pathway. Each part of the pathway is fairly well worked out. Knowing that Ca^{2+} directly mediates phosphorylation of myosin light chain and that PK-C directly phosphorylates pleckstrin allows for the activity of each arm of the pathway to be monitored.

The platelet is not entirely an obscure model since platelets do have a role in the inflammatory process. In autoimmune diseases such as RA, endothelial injury, caused by the deposition of immune complexes, resulting in exposure of underlying collagen is common.(177) The exposed collagen can then activate platelets to release alpha and dense granule contents.(178) Platelet factor 4, released from alpha granules and serotonin released from dense granules, contribute to the inflammatory process.(179) Endersen has shown in patients with RA, that peripheral platelets as well as platelets in the synovial fluid have been activated.(179)

Platelets have also been shown to interact with neutrophils in a manner which affects the inflammatory response.(180) Platelets have been shown to increase adherence and cytolytic ability of neutrophils.(180) Platelet factor 4 is believed to be responsible for this interaction.(180)

A role for platelets in the inflammatory response is also suggested by the ability of platelets to accumulate in vessels adjacent to inflammation and their ability to interact with bacteria, viruses, complement and antigen-antibody complexes.(181) In addition, activation of platelets leads to the formation of arachidonic acid with subsequent

metabolism to known mediators of inflammation.(181)

In this report, the action of Myochrysine on human and rabbit platelet activation was investigated. Initial studies were performed with human platelets but because of a large inter- and intra- person variation, subsequent studies were performed with rabbit platelets.

The effect of Myochrysine on platelet shape change, aggregation and serotonin release was investigated and the implications of these effects with regards to the PK-C/Ca^{2+} pathway discussed. Further studies investigating the action of Myochrysine on platelet protein phosphorylation and cyclic nucleotide levels were also performed.

In addition, the action of Myochrysine compared with other sulfhydryl reacting compounds was investigated. An attempt at determining the active component of the drug, that is, gold versus thiomalate, was also made through chiral drug studies and investigation of gold compounds containing non-sulfhydryl reacting ligands.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 PLATELET ISOLATION BUFFERS

Acid Citrate Dextrose (ACD)
- 85 mM citric acid
- 67 mM trisodium citrate
- 0.1 M dextrose

Tyrodes Buffer
- 0.14 M NaCl
- 2.7 mM KCl
- 12 mM $NaHCC_3$
- 1 mM NaH_2PO_4
- 1 mM $MgCl_2$
- 2 mM $CaCl_2$
- 5 mM glucose

Phosphate-Free Tyrodes Buffer
- 0.14 M NaCl
- 2.7 mM KCl
- 12 mM $NaHCO_3$
- 5 mM HEPES
- 1 mM $MgCl_2$
- 2 mM $CaCl_2$
- 5 mM glucose

Tyrodes Albumin Buffer

Same as Tyrodes with the addition of 0.35% albumin.

Tyrodes Gelatin Buffer

Gelatin (225 Bloom, Sigma) at 0.875% + 1.5 mM EDTA (ethylenediaminetetraacetic acid) was first dialysed against deionized, distilled water overnight at room temperature to remove contaminating Ca^{2+}. The dialysed gelatin was added to Tyrodes buffer to give a final concentration of 0.35% gelatin.

Calcium-Free Tyrodes

Same as Tyrodes with the omission of $CaCl_2$.

2% EGTA

EGTA [ethylene-glycol-bis-(β-aminoethyl ether) N,N,N',N' tetraacetic acid] (3.8 g) was dissolved in 80 mL deionized, distilled H_2O by dropwise addition of 10-20 N NaOH, final pH 7. Isotonic saline (9.0 mL, 0.9% final) was added to 10 mL 3.8% EGTA to make 2.0% solution which is isotonic.

2.1.2 ELECTROPHORESIS BUFFERS

30-0.8% Acrylamide Solution
30% acrylamide
0.8% N,N'methylene-bis-acrylamide
Made up in distilled, deionized water.
Filtered and stored in dark at 4°C.

Separating Gel Buffer
1.5 M Tris (tris(hydroxymethyl)aminomethane), pH 8.8

Stacking Gel Buffer
0.5 M Tris, pH 6.8

Reservoir Buffer (10X)
0.25 M Tris
1.9 M Glycine
1% SDS (sodium dodecyl sulphate)
Diluted 10X before use.

3.75% Stacking Gel (for 2 gels)
23.4 mL deionized, distilled H_2O
10.0 mL stacking gel buffer
5.0 mL 30-0.8% acrylamide solution
0.2 mL 20% SDS
0.2 mL 10% glycerol
Just before pouring gel, 20 µL TEMED (N,N,N',N'-tetramethyl-ethylene-diamine) and 1.2 mL 10% ammonium persulfate were added.

10% Separating Gel (for 2 gels)
39.0 mL deionized, distilled H_2O
25.0 mL separating gel buffer
33.3 mL 30-0.8% acrylamide solution
0.5 mL 20% SDS
1.0 mL 10% glycerol
Just before pouring gel, 25 µL TEMED and 0.75 mL 10% ammonium persulfate were added.

SDS-PAGE Sample Buffer
1.25 mL stacking gel buffer
1.5 mL 20% SDS
1.0 mL 100% glycerol
0.5 mL 1% bromophenol blue
5.25 mL deionized, distilled H_2O
0.5 mL mercaptoethanol
Divided into 200 µL aliquots and stored at -20°C.

Stain Solution
1 g Coomassie Blue
455 mL methanol
455 mL deionized, distilled H_2O
90 mL acetic acid, glacial

Destain Solution
455 mL methanol
455 mL deionized, distilled H_2O
90 mL acetic acid, glacial

2.1.3. OTHER REAGENTS

Apyrase, U46619, aspirin and acid soluble collagen were kindly supplied by Dr. R.L. Kinlough-Rathbone, McMaster University, Hamilton, Ontario. Apyrase had an activity of 2.38 x 10^{-6} moles min^{-1} mg^{-1} ADPase activity and 3.33 x 10^{-6} moles min^{-1} mg^{-1} ATPase activity.

Acid soluble collagen (Type I, Bovine Achilles Tendon) was prepared as 0.25% collagen in 0.522M acetic acid. The concentration of collagen, ADP and U46619 used in each experiment was the minimum concentration required to induce full aggregation. This varied from experiment to experiment. In experiments in which U46619 was used to stimulate platelets, the platelets were treated with 0.5 μM aspirin for 1 minute before addition of U46619.

Myochrysine was a gift from Rhone Poulenc Pharma, Montreal.

Imipramine, ADP, thiomalic acid, 5,5'-dithiobis-2-nitrobenzoic acid (ESSE), 6,6'-dithiodinicotinic acid (CPDS) and D-Penicillamine were purchased from Sigma Chemical Company, St. Louis.

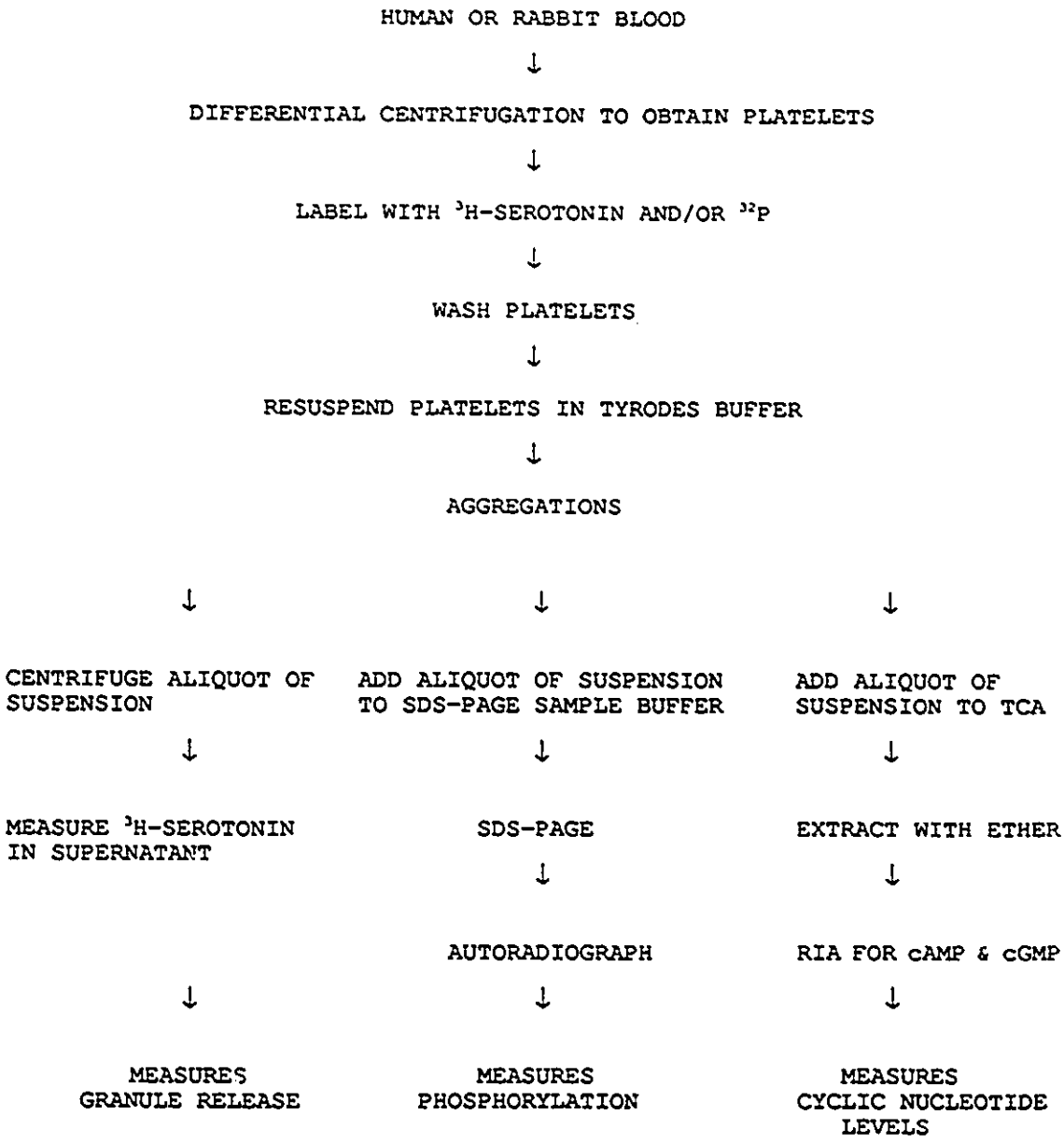
Bovine Serum Albumin, Fraction V was purchased from Boehringer Mannheim Canada Ltd, Laval.

Chloro[tris(2-pyridyl)phosphine]gold(I) and chloro(t-butyl isocyanide)gold(I) were prepared in the laboratory of Dr. C.J.L. Lock by Daren LeBlanc and Katherine Warren.

All glassware used for platelet experiments was siliconized with surfasil siliconizing fluid (Terochem, Markham).

2.2 METHODS

FIGURE 2.2.1 OUTLINE OF METHODS USED



2.2.1 Human Platelet Isolation

Washed human platelets were prepared by the method of Mustard *et al.*(182) Venous blood (129 mL) from healthy human volunteers was taken into 21 mL of ACD anti-coagulant. The citrated blood was then centrifuged at 1200 g at 37°C for 3 minutes. The upper phase, platelet rich plasma, was collected and centrifuged at 1200 g at 37°C for 15 minutes. The supernatant, platelet poor plasma, was discarded and the platelet pellet was resuspended in 10 mL Tyrodes-albumin buffer containing 50 units mL^{-1} heparin and 0.1 mL apyrase. The platelet suspension was labelled with 20 µCi of ^{3}H-serotonin (23.4 Ci $mmol^{-1}$ 5-[1,2-^{3}H]-hydroxytryptamine, Creatinine sulphate, Dupont, Boston, MA) and/or 0.5 mCi ^{32}P (Phosphorous-32 in 0.02 N HCl, 8500-9120 Ci/$mmole^{-1}$, NEN Research Products) for 1 hour at 37°C. The labelled platelet suspension was centrifuged at 1200 g at 37°C for 10 minutes. The supernatant was discarded and the platelets were resuspended in 10 mL Tyrodes-albumin buffer with 0.1 mL apyrase. This platelet suspension was washed once more and was finally resuspended in Tyrodes-albumin buffer at a concentration of 5 x 10^{5} platelets mm^{-3}. Apyrase (1 µL mL^{-1}) and imipramine (final conc. 10^{-6}M) were added and the washed platelets were kept at 37°C until needed.

2.2.2 Rabbit Platelet Isolation

Washed platelets for determination of aggregation and serotonin release were prepared from New Zealand White male rabbits by the method of Ardlie *et al.*(183) Blood was withdrawn by cannulation from rabbits anaesthetized with 1 - 1.5 mL 6.5% sodium pentabarbitol. The blood (100-150 mL) was collected into 60 mL syringes containing ACD at a ratio of 7:1 (blood:ACD). The citrated blood was then centrifuged at 1200 g at room

temperature for 3 minutes. The upper phase, platelet rich plasma, was collected and centrifuged at 1200 g at room temperature for 15 minutes. The supernatant, platelet poor plasma, was discarded and the platelet pellet was resuspended in 10 mL calcium free, Tyrodes-albumin buffer containing 0.01% EGTA. The platelet suspension was labelled with 20 µCi of ^{3}H-serotonin (23.4 Ci mmol^{-1} 5-[1,2-^{3}H]-hydroxytryptamine, Creatinine sulphate, Dupont, Boston, MA) for 30 minutes. The labelled platelet suspension was centrifuged at 1200 g at room temperature for 10 minutes. The supernatant was discarded and the platelets were resuspended in 10 mL calcium free, Tyrodes-albumin buffer. This platelet suspension was washed once more and was finally resuspended in Tyrodes-albumin buffer at a concentration of 5 x 10^{5} platelets mm^{-3}. For some experiments, platelets were isolated and washed with calcium-free, Tyrodes-gelatin buffer and Tyrodes-gelatin buffer. Apyrase (1 µL mL^{-1}) and imipramine (final conc. 10^{-5}M) were added and the washed platelets were kept at 37°C until needed.

Platelets to be used for cyclic nucleotide determinations were prepared as above except that they were not labelled with ^{3}H-serotonin.

Platelets to be used for phosphorylation determinations were prepared as follows. Blood was withdrawn by cannulation from rabbits anaesthetized with 1 - 1.5 mL 6.5% sodium pentabarbitol. The blood (100-150 mL) was collected into 60 mL syringes containing ACD at a ratio of 7:1 (blood:ACD). The citrated blood was then centrifuged at 1200 g at room temperature for 3 minutes. The upper phase, platelet rich plasma, was collected and centrifuged at 1200 g at room temperature for 15 minutes. The supernatant, platelet poor plasma, was discarded and the platelet pellet was resuspended in 10 mL calcium free, phosphate free, Tyrodes-albumin buffer containing 0.01% EGTA (Suspension A). For experiments examining the effect of Myochrysine on collagen and TPA induced phosphorylation, suspension A was labelled with 0.5 mCi ^{32}P (Phosphorous-32

in 0.02 N HCl, 8500-9120 Ci/mmole^{-1}, NEN Research Products) for 1 hour at room temperature. The labelled platelet suspension was centrifuged at 1200 g at room temperature for 10 minutes. The supernatant was discarded and the platelets were resuspended in 10 mL calcium free Tyrodes-albumin buffer. This platelet suspension was washed once more and was finally resuspended in Tyrodes-albumin buffer at a concentration of 5 x 10^5 platelets mm^{-3}. Apyrase (1 µL mL^{-1}) was added and the washed platelets were kept at 37°C until needed.

For experiments determining the effect of Myochrysine on A23187 induced phosphorylation, suspension A was centrifuged at 1200 g at room temperature for 10 minutes. The supernatant was discarded and the platelets were resuspended in calcium free, phosphate free, Tyrodes-albumin buffer, containing 0.01% EGTA, at a concentration of 5,000,000 platelets mm^{-3}. This platelet suspension was labelled with 0.75 mCi ^{32}P per 5 x 10^8 platelets for 1 hour at room temperature. After 1 hour, the platelet suspension was adjusted to 10 mL with calcium free, phosphate free, Tyrodes-albumin buffer, containing 0.01% EGTA, and centrifuged at 1200 g at room temperature for 10 minutes. The supernatant was discarded and the platelet pellet was resuspended in 10 mL calcium free, Tyrodes-albumin buffer. Aspirin (5.5 x 10^{-2}M) was added to the platelet suspension and incubated for 5 minutes at room temperature. The platelet suspension was washed one more time and finally resuspended in Tyrodes-albumin buffer at a concentration of 500,000 platelets mm^{-3}. Apyrase (1 µL mL^{-1}) was added and the washed platelets were kept at 37°C until needed.

2.2.3 Platelet Aggregation

Light transmission of 1 mL samples of platelet suspension were measured on a Payton Aggregation Module (Payton Associates, Scarborough,

Ontario) and recorded on a Linear 1200 recorder (Canlab Scientific Products, Mississauga, Ontario). Platelet shape change and aggregation were recorded after interaction with test compounds.

2.2.4 ^{3}H-Serotonin Release

After the platelets had been in the aggregometer for the 3 minutes the platelet suspension was added to a microcentrifuge tube and centrifuged for 30 seconds in an Eppendorf microcentrifuge. To measure ^{3}H levels, the supernatant (25 µL) was added to 10 mL of aqueous counting scintillant and counted in a scintillation counter (Phillips PW 4700 Liquid Scintillation Counter). The unstimulated, uncentrifuged platelet suspension (25 µL) was used to determine the total counts; 25 µL of unstimulated, centrifuged platelet suspension supernatant was used to determine the background counts.

$$\% \text{ release} = \frac{\text{Sample DPM} - \text{background DPM}}{\text{total DPM}} \times 100$$

2.2.5 Determination of Myosin Light Chain and Pleckstrin Phosphorylation

At the end of the aggregation reaction, 200 µL of the platelet suspension was added to 200 µL of SDS-PAGE sample buffer. The samples were boiled for 3 minutes. Each sample (75 µL) was applied to a 10% SDS-polyacrylamide gels. A current of 60 mA was applied to each gel for 2 hours. At the end of 2 hours, the gels were placed in staining solution for 1 hour and then in destaining solution until protein bands were visible. The destained gels were wrapped in Saran wrap and autoradiographed for 2-4 days at -70°C with the use of an intensifying screen. The film was developed. The intensity of the bands were then measured with use of either a Joyce-Loebl densitometer or a Biorad

Densitometer, Model 620, Video Densitometer. For the Joyce-Loebl densitometer, the heights of the peaks corresponding to the protein bands gave a measure of the amount of phosphorylation. For the Biorad densitometer, the area of the peaks corresponding to the protein bands were calculated with use of Biorad 1-D Analyst Data Software, Version 2.01.

For experiments in which the effect of Myochrysine on A23187 induced phosphorylation was determined, autoradiographs of the gels were used to identify the P20 and P40 bands. The P20 and P40 bands were cut out from the gel and added to 10 mL aqueous counting scintillant and counted for ^{32}P in a scintillation counter (Phillips PW 4700 Liquid Scintillation Counter).

2.2.6 Preincubation and Washing Experiment

1 mL samples of platelet suspension were placed in a series of cuvettes. Some had Myochrysine added and some were washed according to the conditions summarized in Table 2.2.1

TABLE 2.2.1 CONDITIONS FOR THE PREINCUBATION EXPERIMENTS

Treatment	Cuvettes							
	A0[i]	B0	B15	C15	D15	B30	C30	D30
Myochrysine[ii]	---	x	x	x	---	x	x	---
Washing[iii]	---	--	---	x	x	---	x	x

i. The letters represent the sample and the numbers represent the time of preincubation in minutes.
ii. Myochrysine was added at time zero in samples marked x.
iii. Samples marked with x were washed. Washing involved centrifuging the sample, discarding the supernatant and resuspending the pellet in fresh Tyrodes.

At the end of each procedure, the samples were tested for the ability of the platelets to aggregate in response to collagen. At the end of a three minute aggregation with collagen, samples were centrifuged and supernatants were tested for ^{3}H-serotonin release. Each concentration of Myochrysine was tested at least 3 times. These experiments were performed with platelets isolated in Tyrodes-albumin buffer and with platelets isolated in Tyrodes-gelatin buffer.

2.2.7 Cyclic Nucleotide Determination

At the end of the aggregation, the reaction was terminated by adding 500 µL of ice cold 30% trichloroacetic acid (TCA) to the platelet suspension. The sample was then centrifuged in the microcentrifuge for 30 seconds. 600 µL of supernatant was transferred to each of 2 microcentrifuge tubes, one for cGMP analysis and one for cAMP analysis. 11,000 dpm (480 pmol) ^{3}H-cGMP ([8-^{3}H]-Guanosine 3',5'-cyclic phosphate, ammonium salt, 21 Ci mmol^{-1} Amersham International) was added to each of the cGMP sample tubes and 11,000 dpm (290 pmol) ^{3}H-cAMP ([2,8-^{3}H]-Adenosine 3'5'-cyclic phosphate, ammonium salt, 34 Ci mmol^{-1} Amersham International) was added to each of the cAMP sample tubes to determine recovery. The tubes were mixed and stored at -20°C until extractions could be done.

Each sample was extracted 3 times with 5 volumes (3 mL) H_2O saturated ether. The ether layer was discarded. Samples were kept at -20°C until lyophilized.

Samples were lyophilized and resuspended in 600 µL sodium acetate buffer (0.05 M, pH 6.2) a half day before samples were analyzed with the RIA. Samples were kept at 4°C during this time.

Samples were analyzed with either a cAMP RIA kit or cGMP RIA kit from Cedarlane Laboratories, Hornby, Ontario. Kit instructions were

followed. Samples were first acetylated with acetic anhydride according to kit instructions. Acetylation of samples increases the sensitivity of the assays. After samples were acetylated, [^{125}I]-succinyl-cGMP-methylester or [^{125}I]-cAMP-methylester and rabbit anti-cGMP or anti-cAMP anti-sera was added to each sample depending on which cyclic nucleotide was being measured. The samples were incubated overnight at 4°C. Goat, anti-rabbit, IgG was then added. After a 20 minute incubation at room temperature, samples were centrifuged at 1000 g, 4°C for 15 minutes to separate bound from unbound cyclic nucleotide. The supernatant (unbound cyclic nucleotide) was discarded and the pellet (bound cyclic nucleotide) was counted in a gamma counter. Since this is a competition assay between sample cyclic nucleotide and tracer cyclic nucleotide, a higher sample cyclic nucleotide content in the reaction tube resulted in lower counts. Standards were run for each assay and a standard curve (% B/Bo vs Log pmol ml^{-1} standard) was plotted. Sample concentrations were read from the standard curve and corrected for recovery.

CHAPTER 3

THE EFFECT OF MYOCHRYSINE ON HUMAN PLATELET ACTIVATION

The effect of Myochrysine on collagen induced aggregation and serotonin release from platelets isolated from human volunteers was investigated. Aggregation and serotonin release are both measures of platelet activation and are believed to result from receptor-coupled protein kinase C activation and Ca^{2+} mobilization.(136) Human platelets were isolated and aggregation and serotonin release was measured as per pp. 26-29, Chapter 2. The effect of Myochrysine on human platelet activation was tested in 10 caucasian volunteers; 7 female and 3 male.

3.1 RESULTS

Results are shown in Tables 1,2 and 3 and in Figures 3.1.1 - 3.1.6. Table 1 shows that Myochrysine by itself, caused a certain degree of platelet activation in some individuals. This activation occurred at 6.3×10^{-4}M Myochrysine and greater. The amount of aggregation caused by Myochrysine ranged from platelet shape change to 88% aggregation. The amount of serotonin release varied from 4% to 59.4%.

Table 2 shows that Myochrysine can inhibit collagen induced activation in some individuals. Both collagen induced aggregation and serotonin release were inhibited by Myochrysine. The concentration of Myochrysine required to cause inhibition of these processes differs for each individual and for an individual between different experiments. The minimum concentration of Myochrysine required to inhibit collagen induced

aggregation and serotonin release ranged from 0.13-12.7 x 10^{-4}M. From this table, it can also be noted that, in general, the minimum concentration of Myochrysine required to inhibit collagen induced serotonin release is less than that required to inhibit collagen induced aggregation. In some cases, serotonin release was inhibited while aggregation was not.

The effect of Myochrysine on collagen induced aggregation and release can be seen more clearly in Figures 3.1.1 and 3.1.2 respectively. The effect of Myochrysine on these processes is shown graphically. These figures emphasize the variation within and between different individuals. A point, which is not indicated by these graphs, but is worthy of mention, is that even when Myochrysine inhibited collagen induced aggregation by 100%, the drug was not able to inhibit collagen induced platelet shape change.

A point which is indicated by these graphs is that, in those individuals in which high concentrations of Myochrysine causes platelet activation, there is an apparent loss of inhibition by the drug with regards to collagen activation. In fact, the activation by Myochrysine seems additive with activation caused by collagen. This can be very clearly seen in Figure 3.1.2, subject 3.

Figure 3.1.3 and 3.1.4 show the variation within one particular individual. This individual was tested 5 times and each time a different response was seen. Not all individuals show this variation. Subject 2 seen in Figures 3.1.1 and 3.1.2 was tested 3 times and each time the same response was found.

Table 3 shows the ID_{50} (dose of Myochrysine required to cause 50% inhibition) for the action of Myochrysine on collagen induced aggregation and serotonin release in human platelets. Only those individuals for which Myochrysine was shown to have a clear dose-response effect are included in this table. The ID_{50} for the action of Myochrysine on collagen

induced aggregation ranged from 0.9 - 5.6 x 10^{-4}M and for collagen induced release ranged from 0.25 - 6.6 x 10^{-4}M. Figures 3.1.5 and 3.1.6 show the dose-response data graphically. The last block in each of Figures 3.1.5 and 3.1.6 combines the different dose-response curves into one plot. From these figures, it can be seen that in those cases in which there is a clear dose-response effect, the effect is very similar between different individuals. This is more clearly seen with regards to collagen induced aggregation.

TABLE 3.1.1: The Effect of Myochrysine on Human Platelet Aggregation and Serotonin Release

Subject #	Aggregation		Serotonin Release		Conc. Range Tested (x 10^{-4} M)
	Conc. Myochrysine (x 10^{-4} M)	% Maximum Aggregation	Conc. Myochrysine (x 10^{-4} M)	% Serotonin Release	
1A	25.4	40.4	25.4	17	2.5-32
1B	12.7	S.C.	12.7	7.5	0.25-12.7
1C	---	---	---	---	0.06-0.25
1D	---	---	6.3	8.8	1.3-6.3
1E	2.5	S.C.	6.3	15.1	0.25-12.7
2A	25.4	S.0	25.4	3.75	2.5-25.4
2B	---	---	---	---	0.25-12.7
2C	---	---	6.3	7	0.25-6.3
3	---	---	25.4	13	12.7-32
4	---	---	---	---	0.25-12.7
5A	---	---	---	---	0.25-6.3
5B	---	---	2.5	6.1	0.13-2.5
5C	---	---	---	---	0.06-0.25
6A	---	---	---	---	0.13-2.5
6B	---	---	---	---	0.13-2.5
7	---	---	---	---	0.25-2.5
8	---	---	---	---	0.13-2.5
9A	6.3	S.C.	---	---	0.25-6.3
9B	6.3	S.C.	N.D.	N.D.	0.25-6.3
10A	4.4	S.C.	---	---	0.25-6.3
10B	6.3	S.C.	---	---	0.25-6.3

Note: 1) Conc. Myochrysine represents the minimum concentration of Myochrysine to cause a platelet response.
2) S.C. = Shape change
3) N.D. = Not Determined
4) --- = No reaction caused by Myochrysine

TABLE 3.1.2: The Effect of Myochrysine on Collagen Induced Human Platelet Aggregation and Serotonin Release

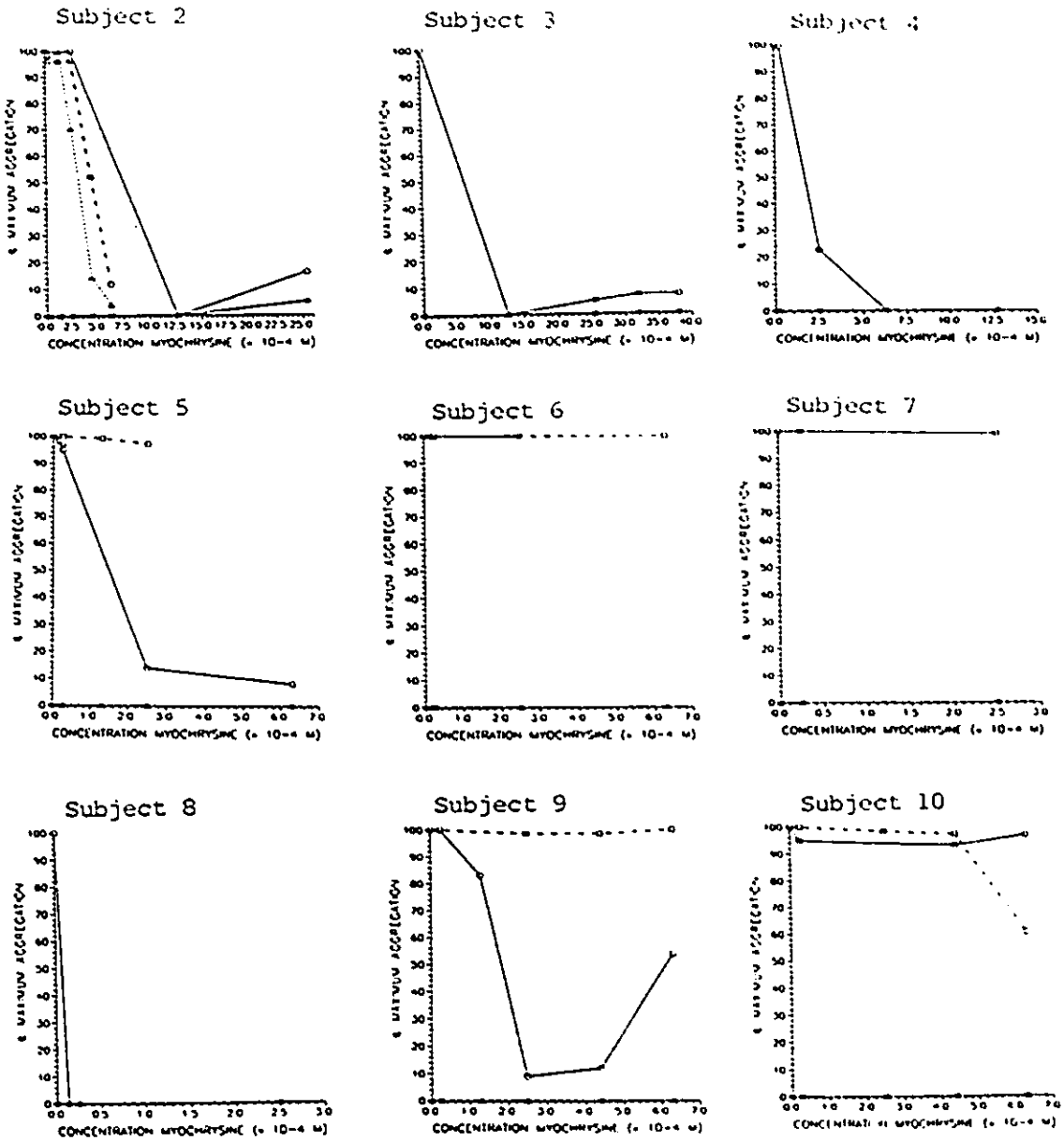
Subject #	Aggregation		Serotonin Release		Conc. Range Tested (x 10^{-4}M)
	Conc. Myochrysine (x 10^{-4} M)	% Inhibition	Conc. Myochrysine (x 10^{-4} M)	% Inhibition	
1A	---	---	---	---	2.5-32
1B	2.5	48.3	2.5	44.4	0.25-12.7
1C	0.13	82.2	0.13	72.6	0.06-0.25
1D	---	---	1.3	27.5	1.3-6.3
1E	---	---	---	---	0.25-12.7
2A	12.7	100.0	12.7	83.9	2.5-25.4
2B	4.4	48	1.3	17.7	0.25-12.7
2C	2.5	29.6	1.3	39.1	0.25-6.3
3	12.7	100	12.7	54.2	12.7-32
4	2.5	77.3	0.25	33.8	0.25-12.7
5A	2.5	86	0.25	68	0.25-6.3
5B	---	---	1.3	34	0.13-2.5
5C	---	---	---	---	0.06-0.25
6A	---	---	2.5	28.2	0.13-2.5
6B	---	---	2.5	32.8	0.13-6.3
7	---	---	---	---	0.25-2.5
8	1.3	100	1.3	76.5	0.13-2.5
9A	1.3	17	1.3	60.1	0.25-6.3
9B	---	---	N.D.	N.D.	0.25-6.3
10A	---	---	0.25	19.8	0.25-6.3
10B	6.3	39.3	0.25	26.8	0.25-6.3

Note: 1) Conc. Myochrysine represents the minimum concentration of Myochrysine required to inhibit collagen induced aggregation or serotonin release.
2) N.D. = Not Determined
3) --- = No inhibition of aggregation or serotonin release occurred at the concentrations tested.

TABLE 3.1.3: ID_{50} for the Action of Myochrysine on Collagen Induced Aggregation and Serotonin Release in Human Platelets

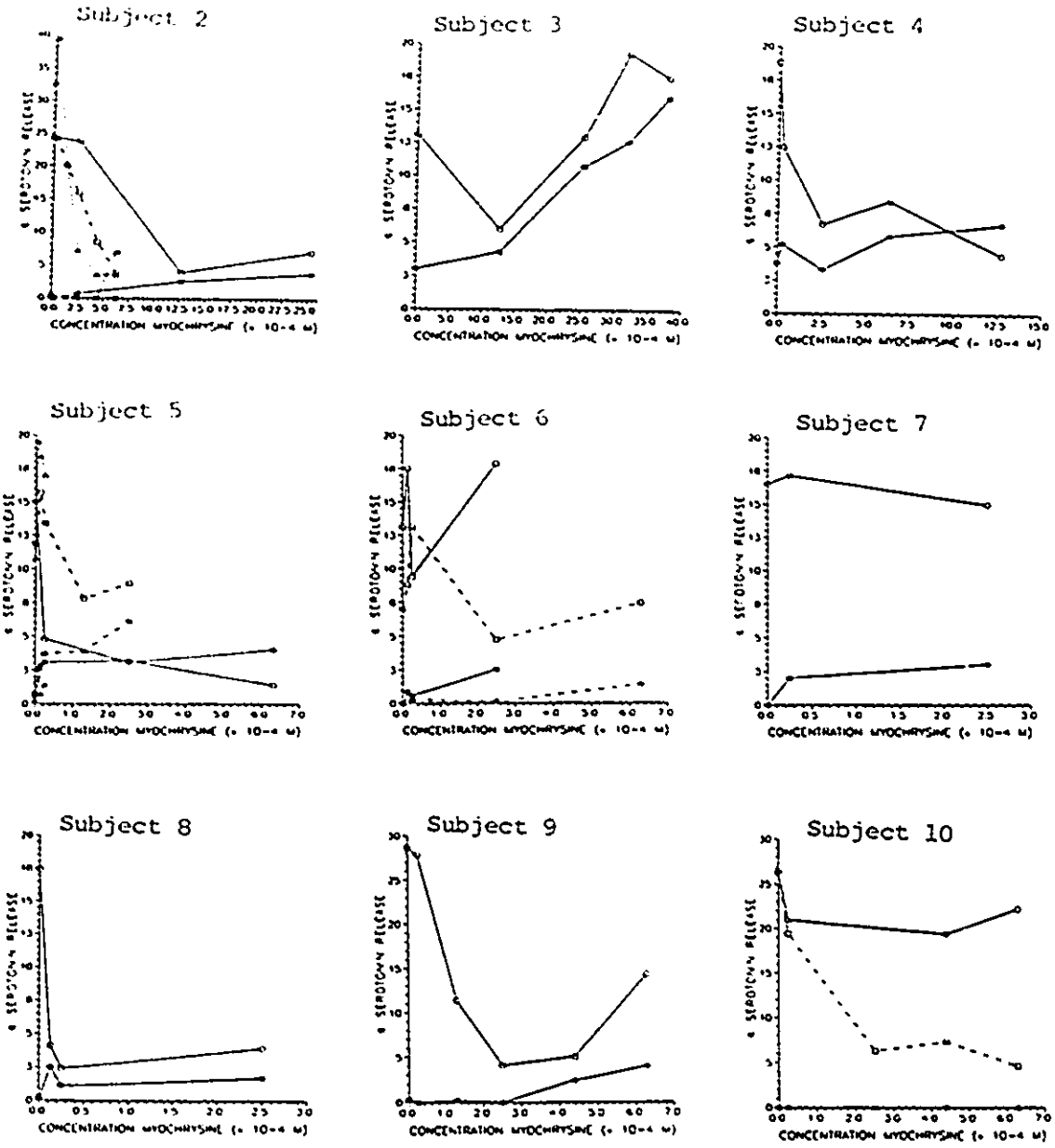
Subject #	ID_{50} for Aggregation ($\times 10^{-4}$ M)	ID_{50} for Serotonin Release ($\times 10^{-4}$ M)
2A	5.6	6.6
2B	4.5	3.4
2C	3	3
4	1.1	0.8
5A	0.9	0.25
9A	1.8	1

FIGURE 3.1.1 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED PLATELET AGGREGATION IN DIFFERENT INDIVIDUALS



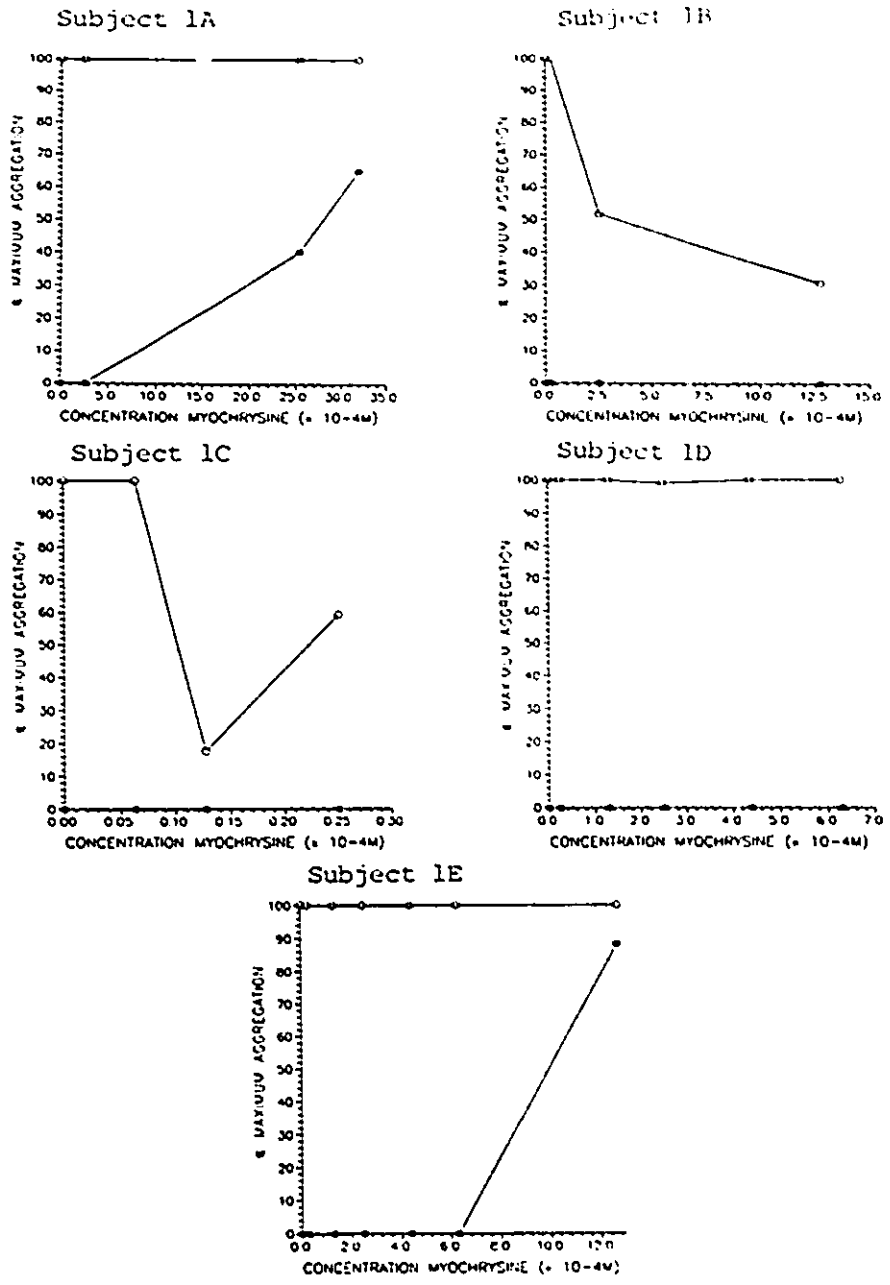
LEGEND: Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen. Different symbols represent different experiments.

FIGURE 3.1.2 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED PLATELET SEROTONIN RELEASE IN DIFFERENT INDIVIDUALS



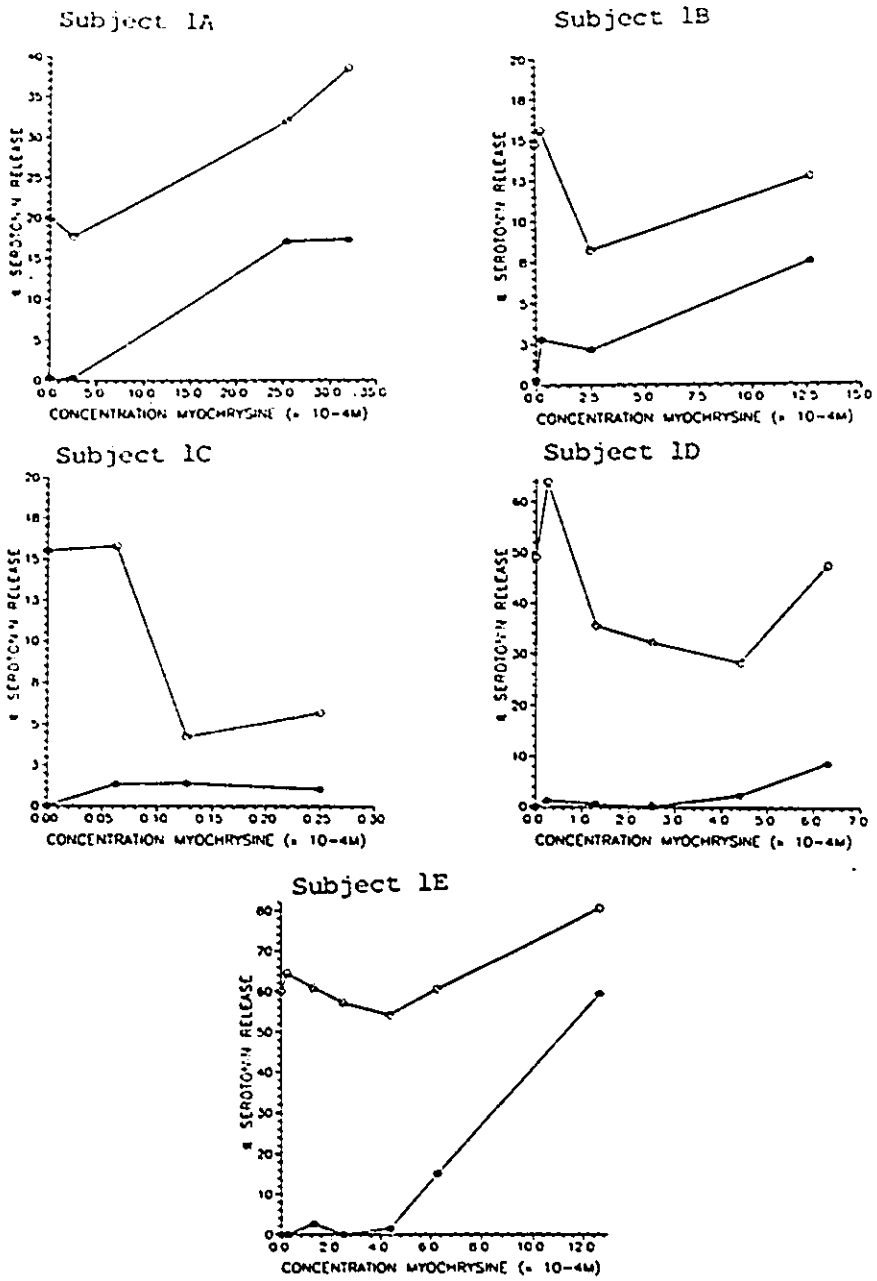
LEGEND: Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen. Different symbols represent different experiments.

FIGURE 3.1.3 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED PLATELET AGGREGATION WITHIN ONE INDIVIDUAL



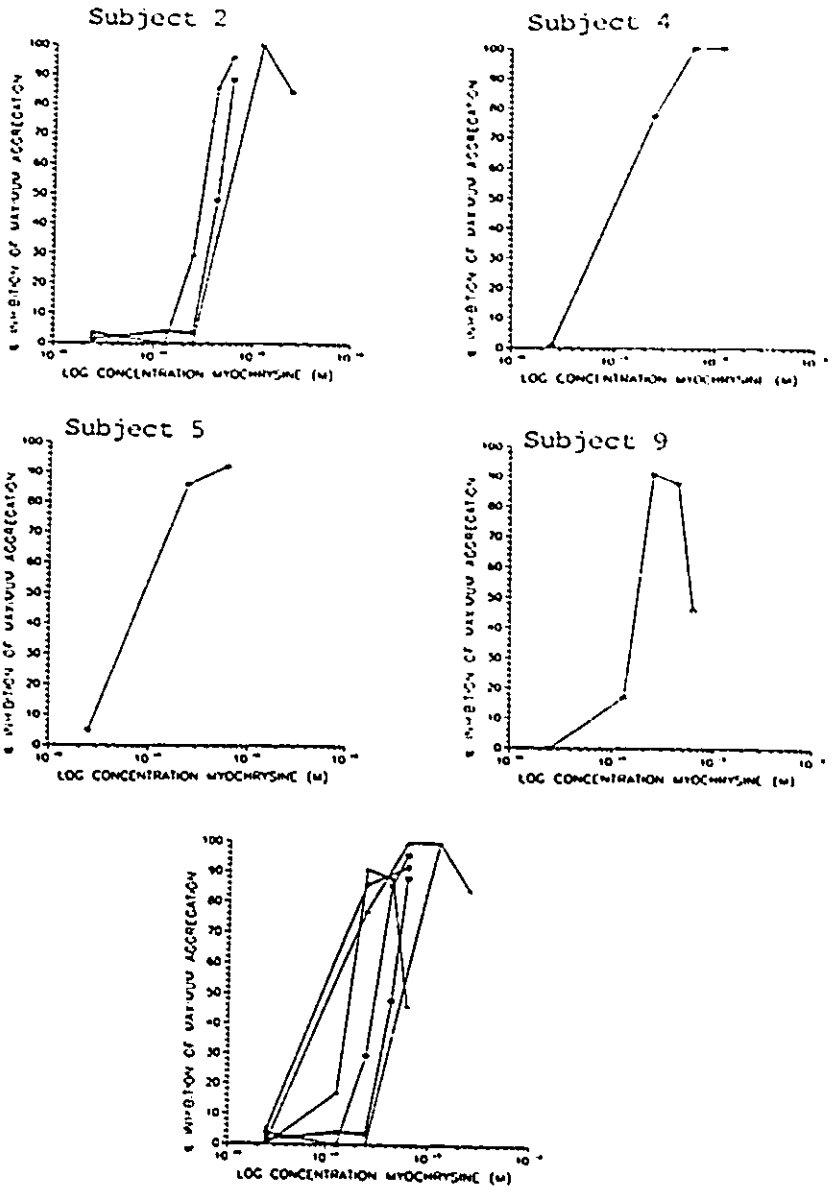
LEGEND: Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen.

FIGURE 3.1.4 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED PLATELET SEROTONIN RELEASE WITHIN ONE INDIVIDUAL



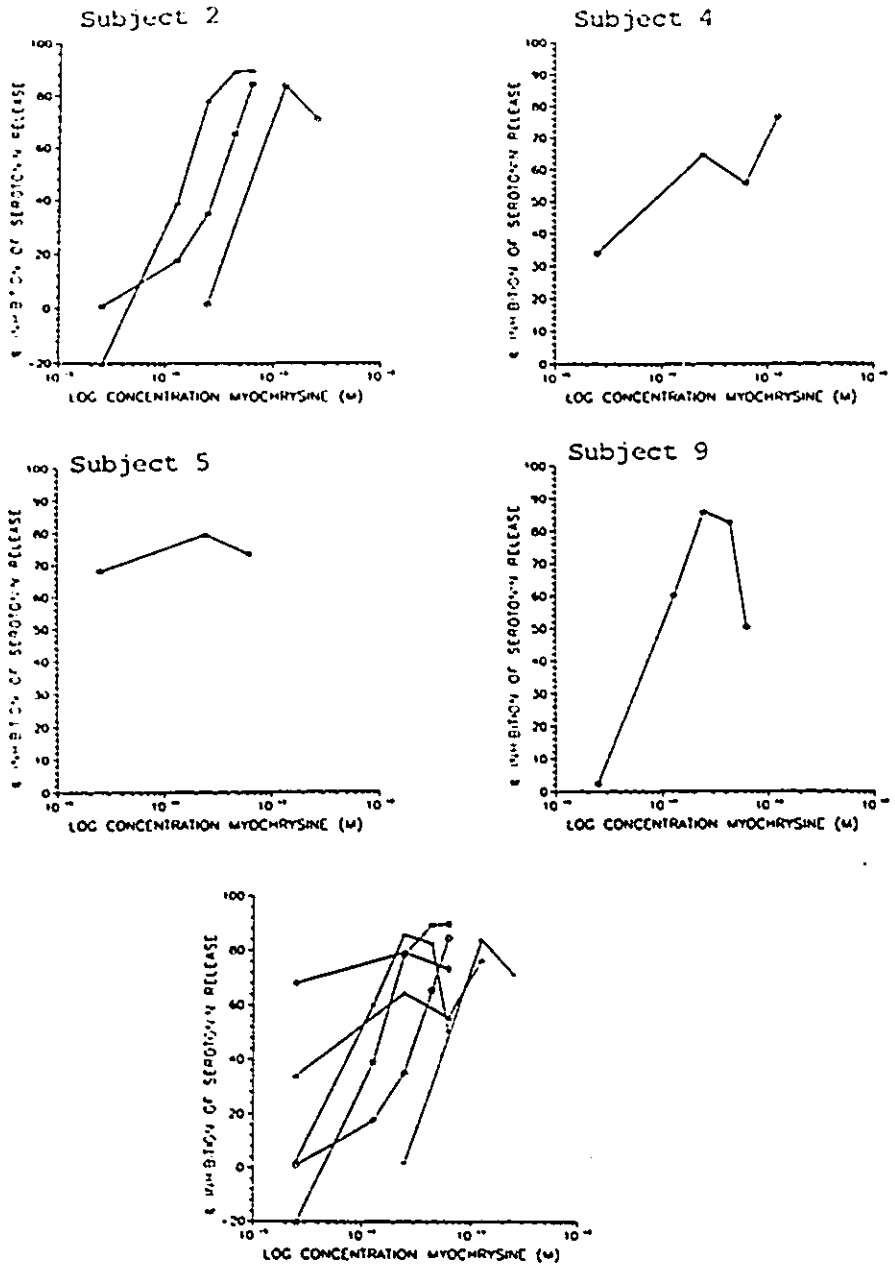
LEGEND: Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen.

FIGURE 3.1.5 LOG DOSE-RESPONSE CURVES FOR THE ACTION OF MYOCHRYSINE ON COLLAGEN INDUCED AGGREGATION



LEGEND: Open symbols represent Myochrysine added together with collagen. Different symbols represent different experiments. Bottom graph is a combination of the top four graphs.

FIGURE 3.1.6 LOG DOSE-RESPONSE CURVES FOR THE ACTION OF MYOCHRYSINE ON COLLAGEN INDUCED SEROTONIN RELEASE



LEGEND: Open symbols represent Myochrysine added together with collagen. Different symbols represent different experiments. Bottom graph is a combination of the top four graphs.

3.2 DISCUSSION

Three major points can be concluded from the data presented. The first is that Myochrysine can cause platelet activation at concentrations greater than 6.3×10^{-4}M in some individuals. Secondly, Myochrysine at concentrations of $0.13 - 32 \times 10^{-4}$M, can inhibit collagen induced aggregation and serotonin release in human platelets in a dose-response manner. Thirdly, the action of Myochrysine in different individuals is highly variable.

The fact that Myochrysine can cause stimulation of responses at higher concentrations is an uncommon finding. There are very few reports of stimulation of cells by Myochrysine. Kean *et al.*, report that dissolution of Myochrysine powder in water at a concentration of 12.7×10^{-4}M produces a short lived component that causes human platelet aggregation and that commercial Myochrysine at this same concentration also caused platelet aggregation in the individuals they tested.(46) Anderson *et al.*, showed that Myochrysine caused stimulation of random migration in human neutrophils.(184) It has also been reported that, Myochrysine (1-100 µg/mL), stimulated proliferation in unstimulated lymphocytes taken from RA patients.(185) Other reports of Myochrysine being stimulatory, state that stimulation occurs at very low concentrations. In one report by Blistein-Willinger and Streller, concentrations of $0.8 - 2.5 \times 10^{-6}$M increased the proliferation of thymocytes in response to ConA and PHA and also increased IL-1 production in the murine macrophage line P388D.(56) At concentrations of 6.3×10^{-6}M or greater, Myochrysine inhibited these processes.(56) A similar stimulatory effect by low concentrations of Myochrysine can be seen in Figure 3.1.2 for subjects 2, 5 and 6. Myochrysine at a concentration of $0.063 - 0.25 \times 10^{-4}$M caused an increase in collagen induced serotonin release over the control. At higher

concentrations, Myochrysine inhibited collagen induced serotonin release in these individuals.

The reason for the stimulatory effect of low concentrations of Myochrysine is not known but it may be related to a finding by Parente *et al.*, that Myochrysine can induce the translocation of PK-C from the cytosol to the membrane fraction of human neutrophils.(186) This act of translocation of PK-C is associated with stimulation of cells.(91)

Both platelet aggregation and serotonin release are believed to be mediated by a receptor coupled process which results in the breakdown of PIP_2 and subsequent Ca^{2+} mobilization and PK-C activation.(135) The fact that Myochrysine has been shown to inhibit aggregation and release gives evidence that the drug is inhibiting the PK-C/Ca^{2+} pathway at some point. The site of inhibition may be direct, such as at the receptor level, or may be indirect. Both cAMP and cGMP are known to inhibit this pathway in platelets.(136) One way in which Myochrysine may be causing inhibition of collagen induced platelet activation is to increase levels of these nucleotides within the platelet.

The variation in response between individuals was not surprising. Variation in response, is recognized for several activities of Myochrysine. There is variation in clinical response to the drug, both with regards to efficacy and toxicity, (10,13-18) and there is variation in response in a variety of studies that have been done to test the action of Myochrysine on inflammatory cells.(54,60,65,76,80,187,188,189) Both Davis and Harth have commented in two separate studies that there is a wide variation between individuals in their response to Myochrysine.(76,187) Other studies, although it is not commented on, also show a wide variation in response between individuals.(54,60,65,80,187,188) In one study, standard deviations ranged from 20-130% of the mean for the response of peripheral blood

mononuclear cells from controls and RA patients to Myochrysine induced inhibition of chemotaxis, Fc and C3b receptor expression and cAMP production.(60)

There are several potential sources of variation in humans including overall fitness, health, diet and genetics. Health status has been shown to affect platelet function in one study which indicated that platelets from hyper-lipidemics are in a proaggregant state.(190) It was shown also that an increase in dietary fish oil normalized the function of their platelets.(190) Dietary fish oil consumption has also been shown to significantly decrease the response of platelets to collagen and platelet activating factor.(191) High dietary salt intake is another factor which affects platelet function *in vitro*.(192) It has also been demonstrated that cigarette smoking can affect platelet function by impairing metabolic platelet capacity.(193)

Genetics may also play a role in determining the variability in response between individuals. There is a known genetic component to RA and to the formation of toxicity to Myochrysine.(8,194) A correlation has been shown between individuals bearing HLA-DR4 antigens and those who have RA and between individuals bearing HLA-DR3 and those who form toxicity to Myochrysine.(194,195) Although no association with HLA type has been found for the efficacy of Myochrysine, it is possible that a more subtle relationship exists.(194)

CHAPTER 4

THE EFFECT OF MYOCHRYSINE ON RABBIT PLATELET ACTIVATION

In the experiments with human platelets, it was shown that there was considerable variability in terms of aggregation and serotonin release following stimulation with collagen. In order to utilize a system with minimal variability, rabbit platelets were chosen for the remaining experiments. Myochrysine action on collagen induced platelet activation was investigated with use of rabbit platelets isolated with Tyrodes-albumin buffer and rabbit platelets isolated with Tyrodes-gelatin buffer. The effect of Myochrysine was also tested on U46619 (a thromboxane A_2 mimetic) and ADP induced platelet activation. In addition, the effect of preincubating platelets with Myochrysine was investigated.

4.1 RESULTS

4.1.1 THE EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED RABBIT PLATELET ACTIVATION

Myochrysine, at concentrations of 0.25 - 12.7 x 10^{-4}M, caused a dose dependent inhibition of collagen induced aggregation and serotonin release in rabbit platelets isolated in Tyrodes-albumin buffer (see Figure 4.4.1). The ID_{50} for the action of Myochrysine on collagen induced aggregation was 5.35 ± 3.19 x 10^{-4}M, n = 17. The ID_{50} for the action of Myochrysine on collagen induced serotonin release was 4.33 ± 2.28 x 10^{-4}M, n = 14. Typical log dose versus response curves are shown in Figure

4.1.2. Unlike the results found with human platelets, Myochrysine, up to 12.7 x 10^{-4}M, did not cause any degree of platelet activation in any of the rabbits tested.

Myochrysine did not inhibit collagen induced shape change at any of the concentrations tested (data not shown in graphs). Myochrysine did not inhibit platelet shape change even when a collagen concentration was used which only caused platelet shape change (see Figure 4.1.3). The inability of Myochrysine to inhibit platelet shape change was also seen in the experiments using human platelets. (Chapter 3)

Myochrysine, at concentrations of 0.25 - 12.7 x 10^{-4}M, inhibited collagen induced aggregation and serotonin release in a dose dependent manner in rabbit platelets isolated with a Tyrodes-gelatin buffer (see Figure 4.1.4). The ID_{50} was 1.57 ± 1.12 x 10^{-4}M, n = 3, for Myochrysine action on collagen induced aggregation and was 2.78 ± 2.31 x 10^{-4}M, n = 2, for Myochrysine action on collagen induced serotonin release.

4.1.2 THE EFFECT OF MYOCHRYSINE ON ADP AND U46619 INDUCED RABBIT PLATELET ACTIVATION

The effect of Myochrysine was tested on platelet activation by other platelet agonists. It was found that at concentrations of 0.25 - 12.7 x 10^{-4}M, Myochrysine inhibited platelet aggregation and serotonin release induced by U44619 and platelet aggregation induced by ADP in a dose dependent manner (see Figures 4.1.5 and 4.1.6). The concentrations of ADP used to stimulate platelets in these latter experiments were too low to cause platelet serotonin release. The ID_{50} for Myochrysine action on U46619 induced aggregation was 9.7 ± 2.47 x 10^{-4}M, n = 3. The ID_{50} for Myochrysine action on U46619 induced serotonin release ranged from 1.2 x 10^{-4}M to greater than 12.7 x 10^{-4}M, n=3. The ID_{50} for Myochrysine action on

ADP induced aggregation was 4.9 ± 2.43 x 10^{-4}M, n = 4.

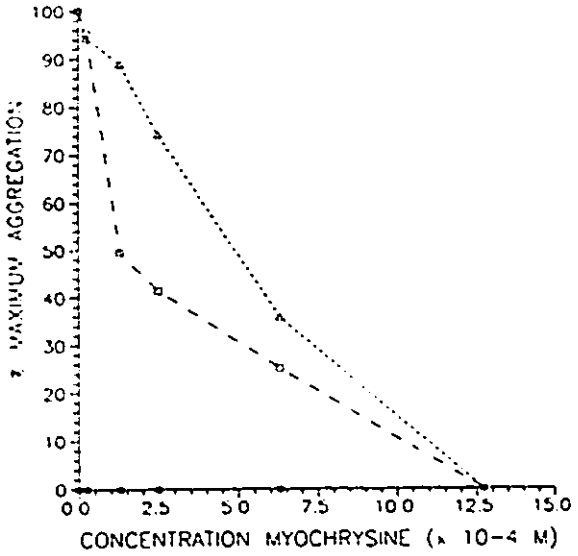
4.1.3 THE EFFECT OF PREINCUBATION OF RABBIT PLATELETS WITH MYOCHRYSINE

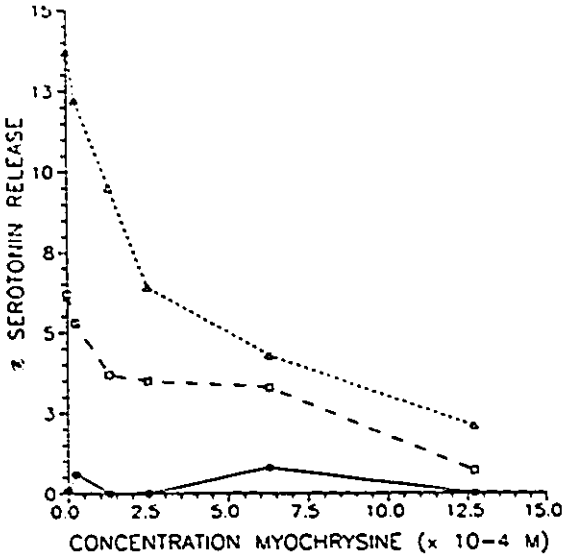
The effect of preincubation of platelets with Myochrysine for 15 and 30 minutes either in the Tyrodes-albumin buffer system or Tyrodes-gelatin buffer system was examined. Platelets were washed after this incubation, to determine whether the effect of the drug was permanent (see Figure 4.1.7). Similar results were found for both buffer systems. Myochrysine concentrations of 0.25 - 1.3 x 10^{-4}M showed little or no inhibition of collagen activation at time 0, 15 or 30 minutes. Myochrysine concentrations of 2.5 - 6.3 X 10^{-4} M showed inhibition of collagen induced aggregation at time 0, but as the time increased to 15 and 30 minutes the degree of inhibition decreased (Figure 4.1.7). A Myochrysine concentration of 12.7 x 10^{-4} M inhibited collagen induced aggregation completely at time zero and this inhibition was maintained for the 15 and 30 minute incubation times. In all cases in which Myochrysine inhibited collagen induced aggregation, the effect could be removed by washing. In approximately 30% of the experiments with Tyrodes-albumin buffer and 40% of the experiments with Tyrodes-gelatin buffer, the act of washing and resuspending the platelets resulted in the platelets being refractory to collagen stimulation. Myochrysine prevented the platelets from becoming refractory.

The effect of Myochrysine on collagen induced ^{3}H-serotonin release in these preincubation experiments followed the same pattern as the effect of Myochrysine on collagen induced aggregation. Some differences were seen however between the two buffer systems. In the Tyrodes-gelatin system, release induced by collagen at time zero was on average half that seen in the Tyrodes-albumin system (6.3 ± 2.7%,n=20 compared to 13.1 ±

6.1%,n=22, respectively). For the Tyrodes-albumin buffer system, washing resulted in a drastic decrease in release induced by collagen. The presence of Myochrysine in the platelet suspension during washing prevented this decrease. In the Tyrodes-gelatin system, washing decreased collagen induced serotonin release. In this case, however, Myochrysine did not appear to give any protection.

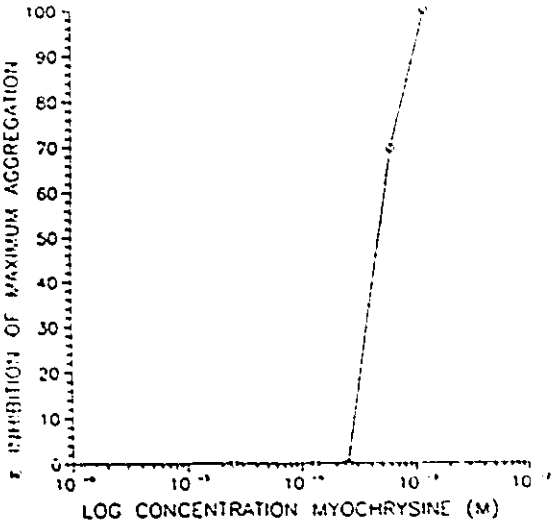
FIGURE 4.1.1 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED RABBIT PLATELET AGGREGATION AND SEROTONIN RELEASE

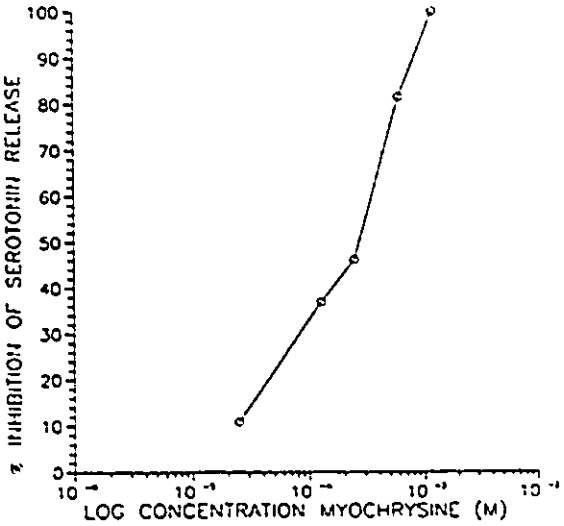




LEGEND: Closed circles represent Myochrysine alone. Open triangles represent Myochrysine added together with 7.5 µL collagen and open squares represent Myochrysine added together with 5 µL collagen.

FIGURE 4.1.2 LOG DOSE-RESPONSE CURVE FOR THE ACTION OF MYOCHRYSINE ON COLLAGEN INDUCED AGGREGATION AND SEROTONIN RELEASE





LEGEND: Open circles represent Myochrysine added together with collagen.

FIGURE 4.1.3 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED RABBIT PLATELET SHAPE CHANGE

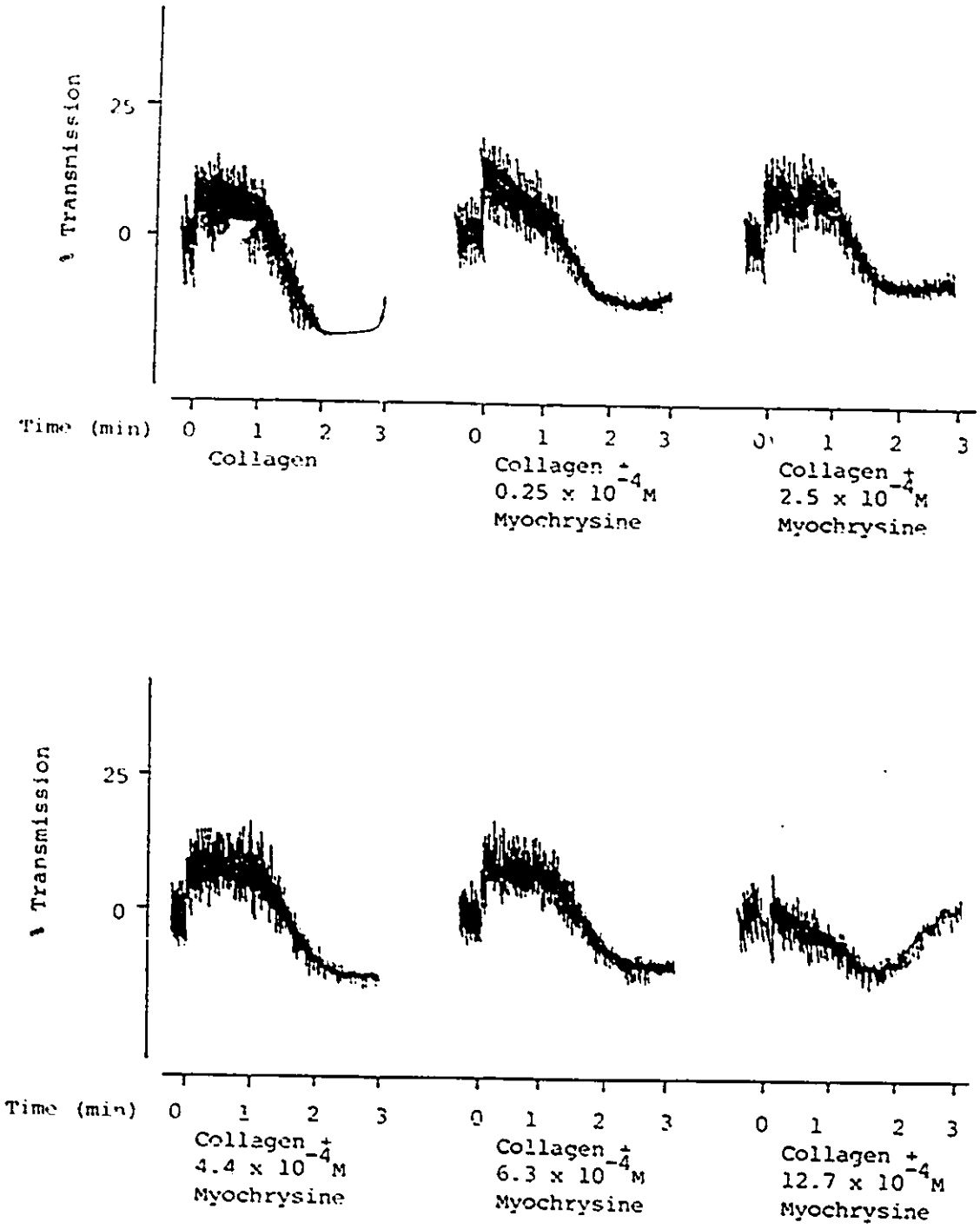
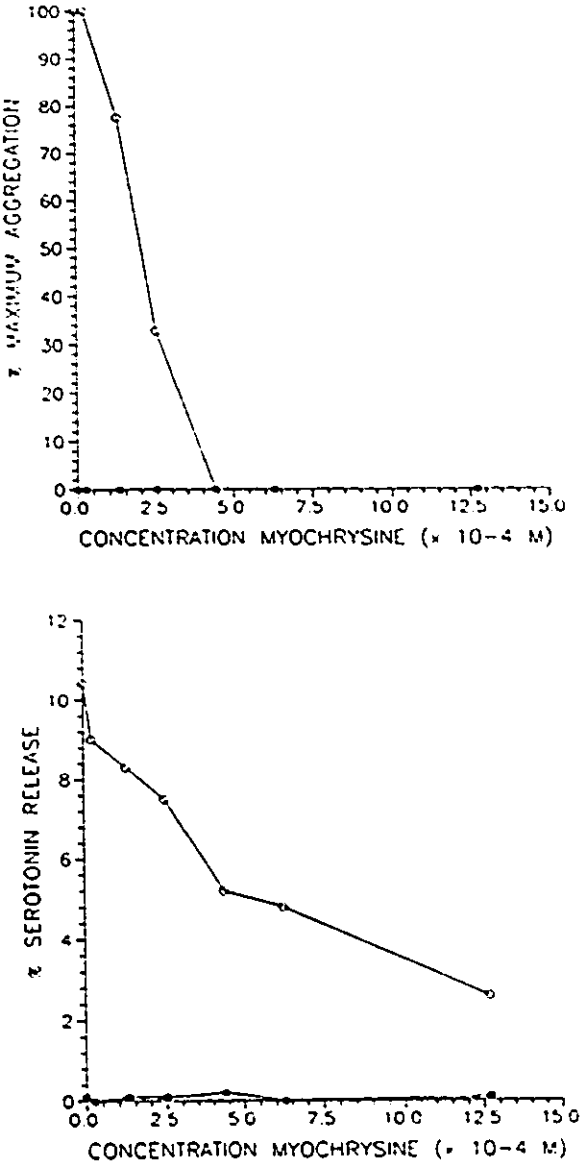
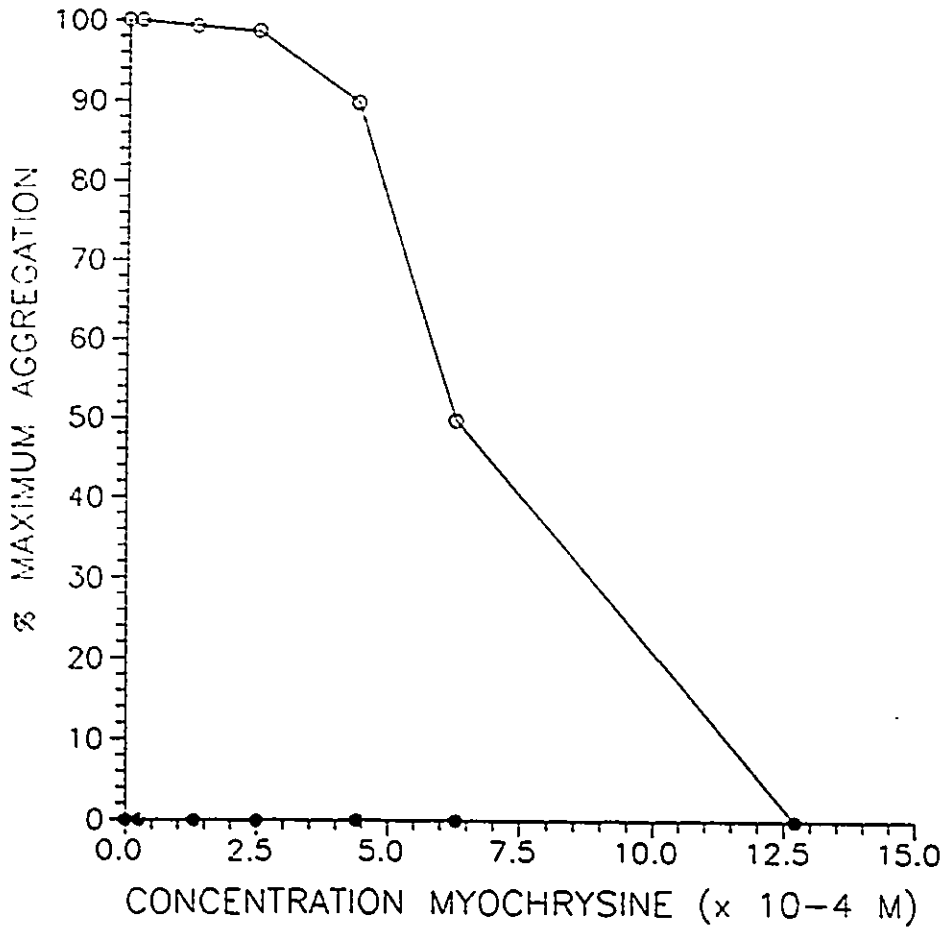


FIGURE 4.1.4 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED AGGREGATION AND SEROTONIN RELEASE OF RABBIT PLATELETS ISOLATED WITH TYRODES-GELATIN BUFFER



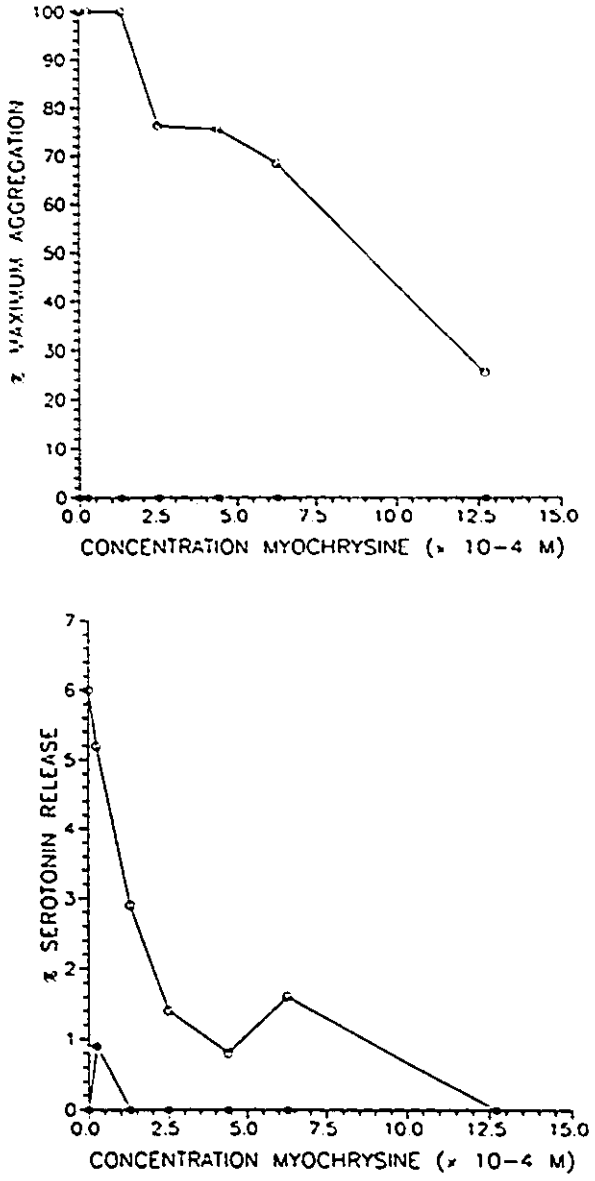
LEGEND: Closed circles represent Myochrysine alone. Open circles represent Myochrysine added together with collagen.

FIGURE 4.1.5 EFFECT OF MYOCHRYSINE ON ADP INDUCED RABBIT PLATELET AGGREGATION



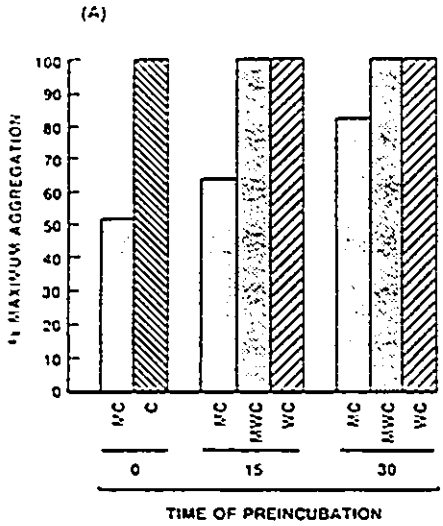
LEGEND: Closed circles represent Myochrysine alone. Open circles represent Myochrysine added together with collagen.

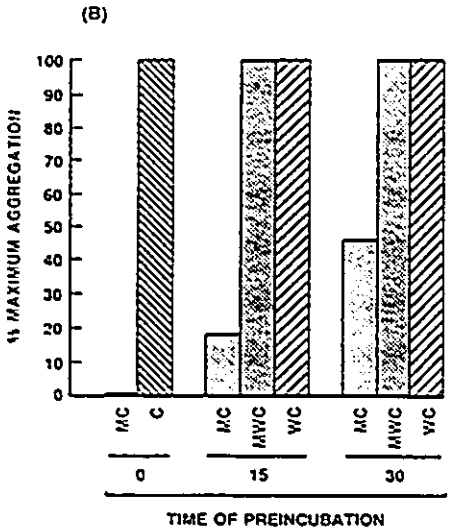
FIGURE 4.1.6 EFFECT OF MYOCHRYSINE ON U46619 INDUCED RABBIT PLATELET AGGREGATION AND SEROTONIN RELEASE



LEGEND: Closed circles represent Myochrysine alone. Open circles represent Myochrysine added together with collagen.

FIGURE 4.1.7 EFFECT OF PREINCUBATION OF RABBIT PLATELETS WITH MYOCHRYSINE ON COLLAGEN INDUCED AGGREGATION





LEGEND: (A) 4.4 x 10^{-4}M Myochrysine. (B) 6.3 x 10^{-4}M Myochrysine. M represents the addition of Myochrysine at time 0, W represents washing the platelets before stimulation with collagen and C represents stimulation with collagen.

4.2 DISCUSSION

Myochrysine inhibited collagen induced aggregation and serotonin release of rabbit platelets in a dose dependent manner. Collagen induced serotonin release also appeared to be slightly more sensitive to the effects of Myochrysine than did collagen induced aggregation. The ID_{50} for Myochrysine action on collagen induced serotonin release was significantly less than the ID_{50} for Myochrysine action on aggregation for $\alpha = 0.4$ (see Appendix 1). This was also seen in experiments in which human platelets were used. With human platelets, however, the variation between individuals was so great that it only appeared to be a trend and was not statistically significant. (Chapter 3)

Some variation in response was seen from day to day in the rabbit experiments but the variation was very small compared to that seen in the human experiments. Inhibition by Myochrysine of collagen induced activation occurred in all rabbits tested with the ID_{50} ranging from 1.2 - 12.5 x 10^{-4}M for aggregation and 2.2 - 7.3 x 10^{-4}M for release.

Some of the variation seen from day to day, may be a result of different amounts of collagen being used to activate platelets. In each experiment, the minimal amount of collagen required to induce full platelet aggregation within three minutes was determined by trial and error. In some cases, a low platelet yield resulted in this minimal concentration being an approximation. The effect of Myochrysine on different amounts of collagen is seen in Figure 4.1.1. When less collagen is used, smaller concentrations of Myochrysine were required to produce inhibitory effects.

Another difference between human and rabbit platelets with regard to their response to Myochrysine is that rabbit platelets appear to be slightly less responsive to the drug. The ID_{50} for the action of

Myochrysine on human platelet aggregation is significantly lower than the ID_{50} for the action of Myochrysine on rabbit platelet aggregation ($\alpha = 0.1$, see Appendix 1). Human platelets were also more responsive to the inhibitory action of Myochrysine on serotonin release than rabbit platelets, with a statistical difference seen for $\alpha = 0.2$ (see Appendix 1).

The fact that Myochrysine does not inhibit platelet shape change suggests that the drug is acting after collagen has bound to its receptor to initiate platelet activation. It is also consistent with the suggestion of Takahara *et al.* that platelet shape change operates through a biochemical mechanism which is different from aggregation and release.(163) If platelet shape change results from activation of the PK-C/Ca^{2+} pathway, then the results obtained can be explained by a time phenomenon. By the time Myochrysine interacts with platelets, shape change has already been initiated. If, however, platelet shape change is mediated by a different pathway, Myochrysine may not interact with this alternative pathway.

Myochrysine is known to bind albumin both *in vivo* and *in vitro*.(27,29,30) To determine if albumin in the buffer used to resuspend the platelets was affecting the action of Myochrysine, the effect of this drug on collagen induced activation was tested on platelets isolated with a gelatin based buffer. Gelatin has no reactive sulfhydryl groups.(196) It was found that Myochrysine inhibited collagen induced aggregation and release in a dose dependant manner in the gelatin system. The results from these experiments show that albumin may be interfering with the action of Myochrysine to a small extent. The ID_{50} for the action of Myochrysine on collagen induced aggregation in the gelatin system was significantly less than the ID_{50} for the albumin system ($\alpha = 0.1$, see Appendix 1). The same trend is seen for the action of Myochrysine on

serotonin release, but the number of experiments is too small to show a statistical difference. Shaw *et al.* have shown that albumin can bind Myochrysine through a sulfhydryl group on cysteine 34.(30) Binding of Myochrysine by albumin may be decreasing the effective concentration of the drug.

As was previously discussed in Chapter 3, the inhibitory action of Myochrysine on collagen induced aggregation and release gives evidence that Myochrysine inhibits the PK-C/Ca^{2+} pathway at some point. The action of Myochrysine on U46619 and ADP induced rabbit platelet activation was investigated to determine if the action of Myochrysine was specific to collagen induced activation. It was found that Myochrysine inhibited U46619 induced aggregation and release and ADP induced aggregation. The fact that Myochrysine inhibits U46619 and ADP induced activation gives further evidence that the site of Myochrysine action is some point within the PK-C/Ca^{2+} pathway. It also suggests that the action of the drug is at a point central in the activation of these agonists.

The ID_{50} for the action of Myochrysine on U46619 induced aggregation was found to be significantly higher than the ID_{50} for collagen induced aggregation ($\alpha = 0.3$, see Appendix 1). The difference in ID_{50}s suggests that perhaps Myochrysine is affecting U46619 in a different manner. It should be noted though, that the number of experiments which used U46619 as a platelet activator was very small.

Preincubation studies demonstrated that at intermediate concentrations, the longer the drug was incubated with the platelets, the less inhibitory it became. It is possible that over time the drug is being taken up in vacuoles by the platelets. Kassam *et al.* have previously shown that platelets are capable of phagocytosing Myochrysine and that both gold and sulphur are found within these vacuoles.(46) Since similar results were found in both the Tyrodes-albumin buffer system and

the Tyrodes-gelatin buffer system, the loss of drug activity could not be attributed to adsorption or binding to albumin.

In some cases, it was found that washing and centrifuging the platelets during the preincubation experiments resulted in the platelets being refractory to collagen activation. It has been shown that ADP is released from platelets during centrifugation.(197,198) This released ADP may partially stimulate the platelets during the washing procedure and leave them refractory to collagen activation.(183) The fact that Myochrysine protects against this is not surprising since it has been shown here that Myochrysine can inhibit ADP induced activation.

In summary, this work suggests that the site of action of Myochrysine is at some point within the PK-C/Ca^{2+} pathway. Myochrysine was able to inhibit rabbit platelet activation by collagen, U46619 and ADP, all of which exert their effects through receptor mediated PIP_2 hydrolysis, PK-C activation and Ca^{2+} mobilization. The fact that inhibition of Myochrysine can be removed by washing is evidence that the site of action of the drug is at the platelet membrane surface and the fact that Myochrysine does not inhibit collagen induced shape change indicates that inhibition occurs at some point after agonist-receptor binding.

CHAPTER 5

STUDIES TO DETERMINE THE SPECIFIC ACTION OF MYOCHRYSINE - GOLD OR LIGAND?

Myochrysine is a gold-based drug containing Au in the +1 oxidation state.(11) The chemistry of Au(I) predicts that it will react with a sulfhydryl group.(11) To test the possibility that interference of a sulfhydryl group could inhibit platelet activation, the actions of several sulfhydryl reacting compounds were tested on collagen induced aggregation and serotonin release in rabbit platelets.

Since thiomalic acid, the ligand of Myochrysine, can react with sulfhydryl groups, its action on collagen induced platelet activation was also tested and proven to be inhibitory. Evidence is presented, based on findings with "all d" Myochrysine and "all l" Myochrysine, which shows that the gold portion of Myochrysine also affects platelet function. Studies which used chloro(t-butylisocyanide)gold(I) and chloro[tris(2-pyridyl)phosphine]gold(I) support this finding.

5.1 RESULTS

5.1.1 THE EFFECT OF SULFHYDRYL REACTING AGENTS ON RABBIT PLATELET ACTIVATION

D-penicillamine, 0.67 - 13.4 x 10^{-4}M inhibited collagen induced aggregation and serotonin release in a dose-dependent manner, in rabbit platelets isolated with either Tyrodes-albumin buffer or Tyrodes-gelatin

buffer (see Figure 5.1.1). CPDS, 0.5 - 4 x 10^{-4}M, and ESSE, 0.1 -5 x 10^{-4}M, also inhibited collagen induced aggregation and serotonin release in a dose-dependent manner in rabbit platelets isolated with Tyrodes-gelatin buffer (see Figures 5.1.2 and 5.1.3).

5.1.2 THE EFFECT OF THIOMALIC ACID ON RABBIT PLATELET ACTIVATION

Thiomalic acid (TMA), 1.7 - 16.6 x 10^{-4}M, inhibited collagen induced aggregation and release in a dose-dependent manner in rabbit platelets isolated with either a Tyrodes-albumin buffer or Tyrodes-gelatin buffer (see Figure 5.1.4). The ID_{50} for the action of TMA on collagen induced aggregation was 3.13 ± 1.4 x 10^{-4}M, n = 4 in albumin ... 2.5 ± 0.62 x 10^{-4}M, n = 3 in gelatin. The ID_{50}s for the action of TMA on collagen induced release were 4.13 ± 0.87 x 10^{-4}M, n = 4 and 7.00 ± 2.83 x 10^{-4}M, n = 2 for albumin and gelatin, respectively.

When TMA was compared to Myochrysine under the same experimental conditions, it was found that the two compounds produced similar dose response curves (see Figure 5.1.5). When the ID_{50}s were compared, however, the ID_{50} for the action of Myochrysine on collagen induced aggregation was significantly greater, $\alpha = 0.1$, than the ID_{50} for the action of TMA on collagen induced aggregation (see Appendix 1). The ID_{50}s for the action of these two compounds on collagen induced release, however, were not significantly different.

Figure 5.1.6 shows a comparison of the action of the five different compounds tested on collagen induced rabbit platelet aggregation. Myochrysine, thiomalic acid, D-penicillamine, CPDS and ESSE all follow the same shape log dose - response curve, indicating a similar mode of action. The potencies of these compounds however, are different. From Figure 5.1.6, the descending order of potency is as follows: ESSE ≈

CPDS > TMA > Myochrysine > D-penicillamine.

5.1.3 THE EFFECT OF "all d" & "all l" MYOCHRYSINE AND d & l THIOMALIC ACID ON RABBIT PLATELET ACTIVATION

The action of "all d" and "all l" Myochrysine was tested in rabbit platelets as was d and l TMA. Results are shown in Figures 5.1.7, 5.1.8 and 5.1.9. Figure 5.1.7 shows that 1.7 - 16.6 x 10^{-4}M d or l TMA inhibited collagen induced aggregation and serotonin release in a dose dependent manner. It also shows that the action of these two compounds are essentially identical. The ID_{50} for the action of d TMA on collagen induced aggregation was 3.47 ± 1.10 x 10^{-4}M, n = 3, compared to an ID_{50} of 3.33 ± 1.06 x 10^{-4}M, n = 3 for the action of l TMA. These are not statistically different. The ID_{50}s for the action of TMA on collagen induced serotonin release were 4.27 ± 0.95 x 10^{-4}M, n=3 for d TMA and 4.20 ± 0.95 x 10^{-4}M, n = 3 for l TMA. Again, these are not statistically different. These ID_{50}s are also not statistically different from the ID_{50}s for the action of racemic dl TMA on collagen induced aggregation and serotonin release, 3.13 ± 1.4 x 10^{-4}M, n = 4 and 4.13 ± 0.87, n = 4, respectively.

The results obtained for the action of "all d" or "all l" Myochrysine are very different from those found with TMA. Figure 5.1.8 shows a comparison of the actions of commercial Myochrysine (Rhone-Poulenc Pharma) and three compounds, dl Myochrysine, "all d" Myochrysine and "all l" Myochrysine (made in the laboratories of Dr. C.J.L. Lock). Rhone Poulenc Myochrysine and dl Myochrysine behave in a similar manner with dl Myochrysine appearing to be slightly more potent. Neither of these compounds alone affect rabbit platelet function and they both inhibit collagen induced aggregation and serotonin release.

From Figure 5.1.8, it can be seen that "all d" and "all l" Myochrysine behave differently from each other and differently from dl Myochrysine. Both compounds cause platelet activation on their own, with "all d" Myochrysine being more potent than "all l" Myochrysine. The effects of these two compounds appear to be additive to the effects of collagen.

Figure 5.1.9 shows the actual aggregation tracings of the actions of these compounds. The tracings emphasize the findings in Figure 5.1.8 that Rhone Poulenc Myochrysine and dl Myochrysine behave similarly and that "all d" Myochrysine is different from "all l" Myochrysine, both of which are different from dl Myochrysine. Also shown in this figure is the action resulting from mixing "all d" Myochrysine with "all l" Myochrysine in a 1:1 ratio. It can be seen that this mixture is also clearly different from the other compounds and is not an averaging of the effects from "all d" Myochrysine with those of "all l" Myochrysine.

5.1.4 THE EFFECT OF NON-SULFUR CONTAINING GOLD COMPOUNDS ON RABBIT PLATELET ACTIVATION

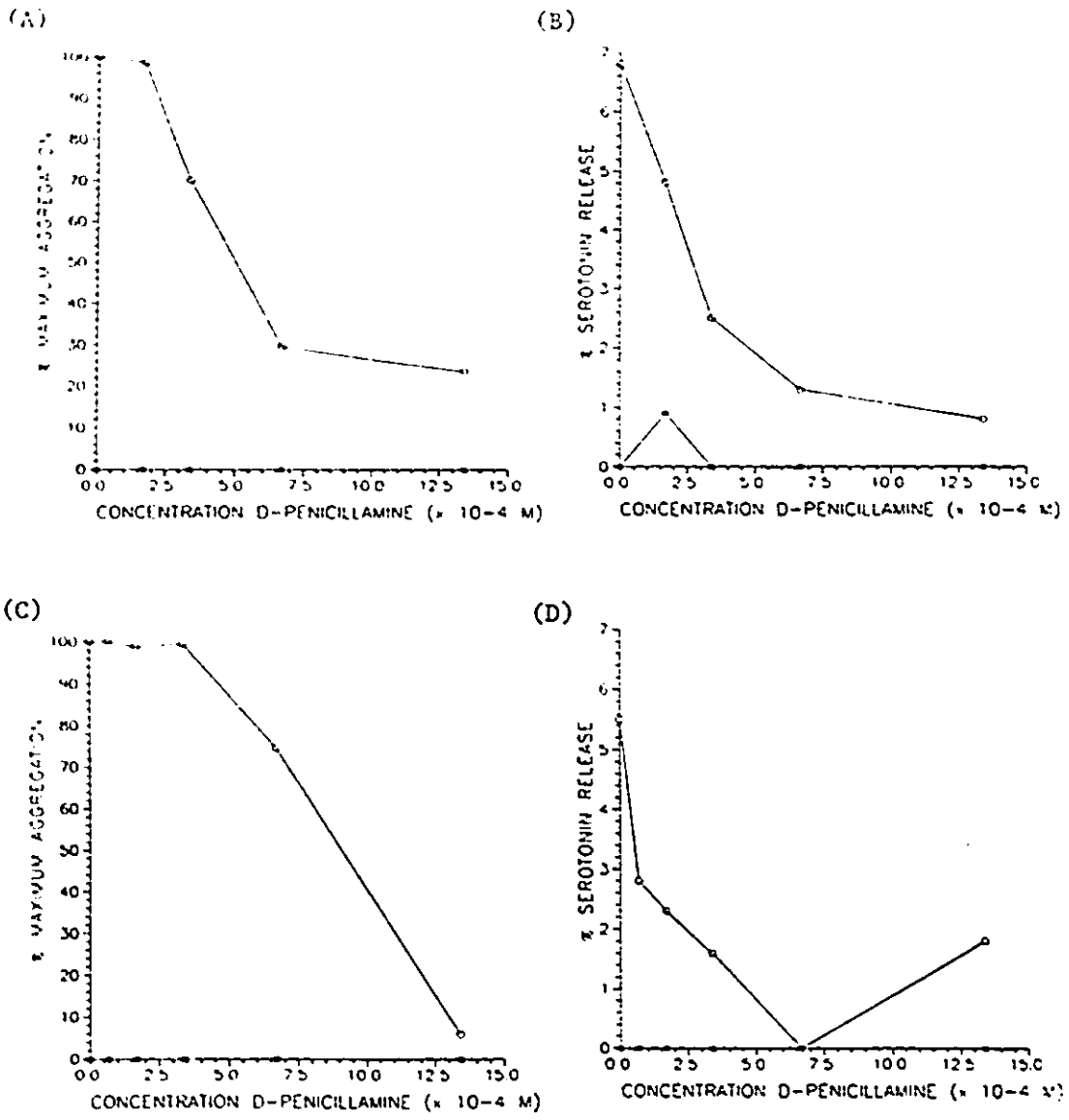
In order to confirm that the gold portion of Myochrysine can also be inhibitory to platelet function, two gold based compounds with non-sulphur containing ligands were tested. The two compounds, synthesized by Darren Leblanc and Katherine Warren were chloro(t-butylisocyanide)gold(I) which is soluble in acetone and chloro[tris(2-pyridyl)phosphine]gold(I) which is soluble in dimethylsulphoxide (DMSO). Figures 5.1.10 and 5.1.11 show that both chloro(t-butylisocyanide)gold(I) and chloro[tris(2-pyridyl)phosphine]gold(I) by themselves affect rabbit platelets. At low concentrations, 1.3 - 3.1 x 10^{-5}M, chloro(t-butylisocyanide)gold(I) appears to cause platelet shape change and a small degree of serotonin release.

At higher concentrations, 1.3-12.7 x 10^{-4}M, the amount of serotonin release is increased greatly. It is believed that this serotonin release is actually caused by lysis of the platelets. A similar finding is seen with chloro[tris(2-pyridyl)phosphine]gold(I). At 2.5 - 12.7 x 10^{-4}M, chloro[tris(2-pyridyl)phosphine]gold(I) was shown to cause platelet lysis as measured by ^{51}Cr release.

These two compounds, however, are capable of inhibiting collagen induced rabbit platelet aggregation and serotonin release. At low concentrations, 5.2 x 10^{-5}M chloro[tris(2-pyridyl)phosphine]gold(I) and 1.3 - 6.3 x 10^{-5}M chloro(t-butylisocyanide)gold(I) inhibit collagen induced activation without causing platelet lysis. In addition, both compounds inhibit collagen induced platelet shape change.

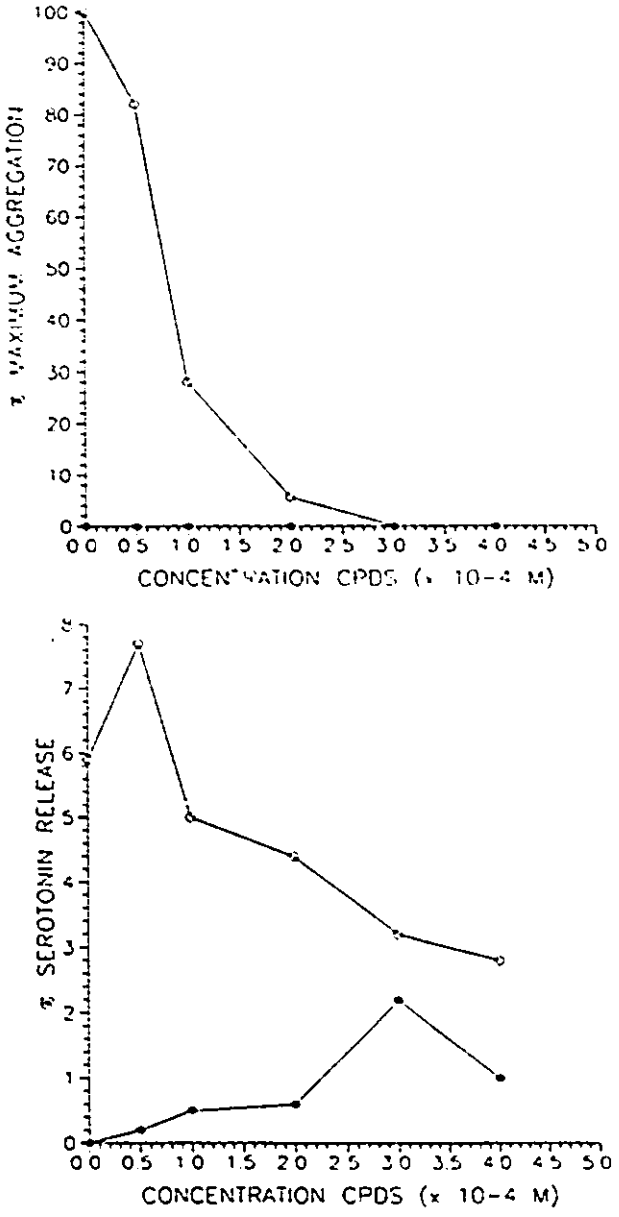
Inhibition of collagen induced platelet activation by these two compounds can be partly, but not completely, attributed to effects of the ligands, t-butylisocyanide and tris(2-pyridyl)phosphine. Figures 5.1.10 and 5.1.11 show that the ligands themselves cause some degree of inhibition of collagen induced aggregation and serotonin release. Neither ligand was able to inhibit collagen induced shape change and neither ligand by itself affected platelet function.

FIGURE 5.1.1 EFFECT OF D-PENICILLAMINE ON COLLAGEN INDUCED RABBIT PLATELET AGGREGATION AND SEROTONIN RELEASE



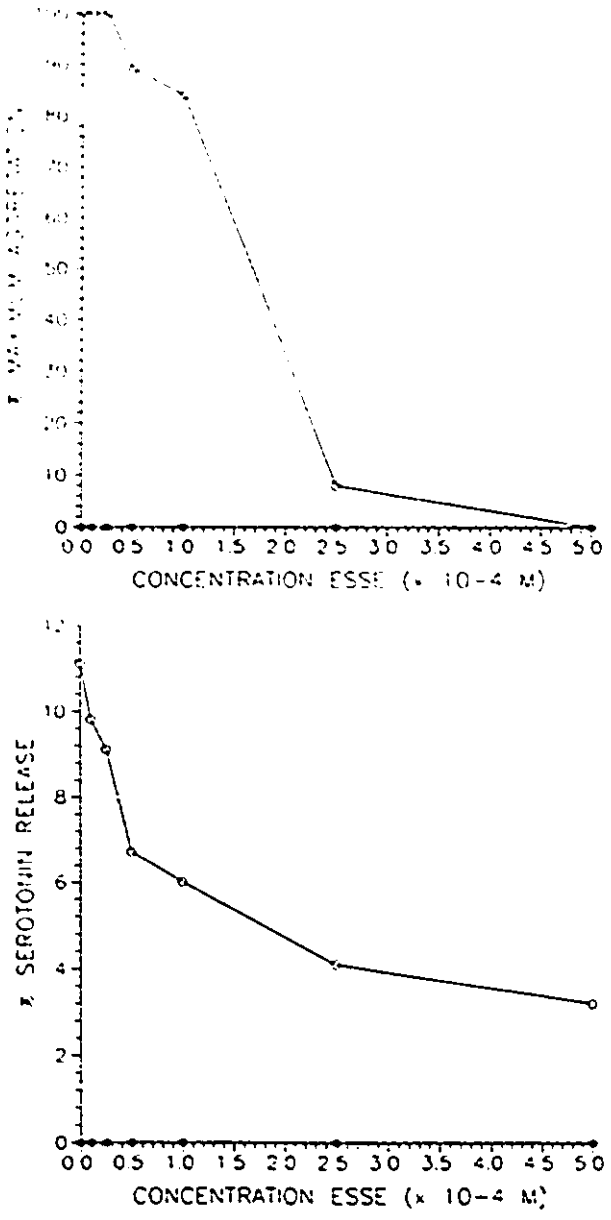
LEGEND: A and B represent experiments in which platelets were isolated with Tyrodes-albumin buffer. C and D represent experiments in which platelets were isolated with Tyrodes-gelatin buffer. Closed circles represent D-penicillamine alone. Open circles represent D-penicillamine added together with collagen.

FIGURE 5.1.2 EFFECT OF CPDS ON COLLAGEN INDUCED RABBIT PLATELET AGGREGATION AND SEROTONIN RELEASE



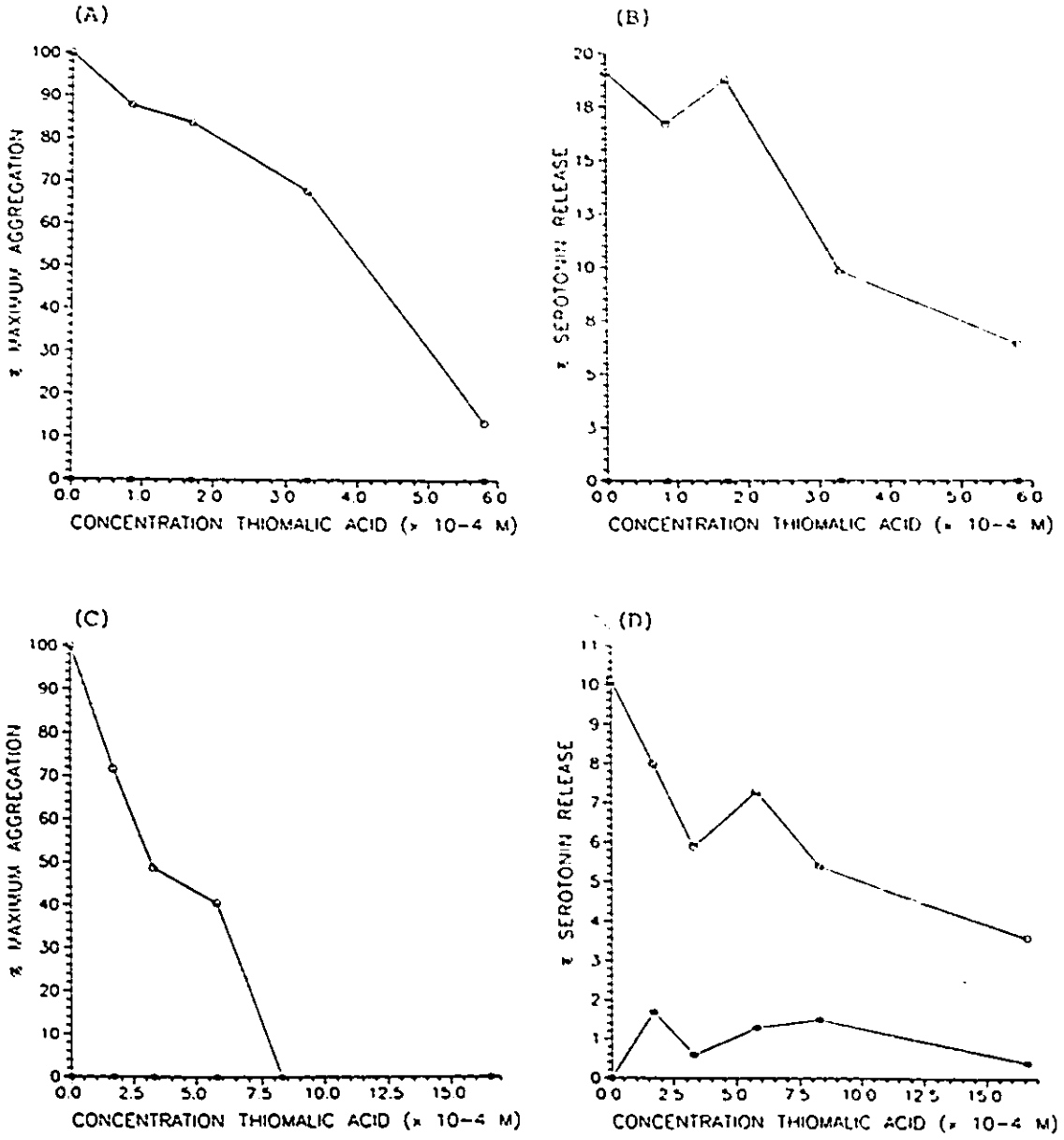
LEGEND: Open circles represent CPDS alone. Closed circles represent CPDS added together with collagen.

FIGURE 5.1.3 EFFECT OF ESSE ON COLLAGEN INDUCED RABBIT PLATELET AGGREGATION AND SEROTONIN RELEASE



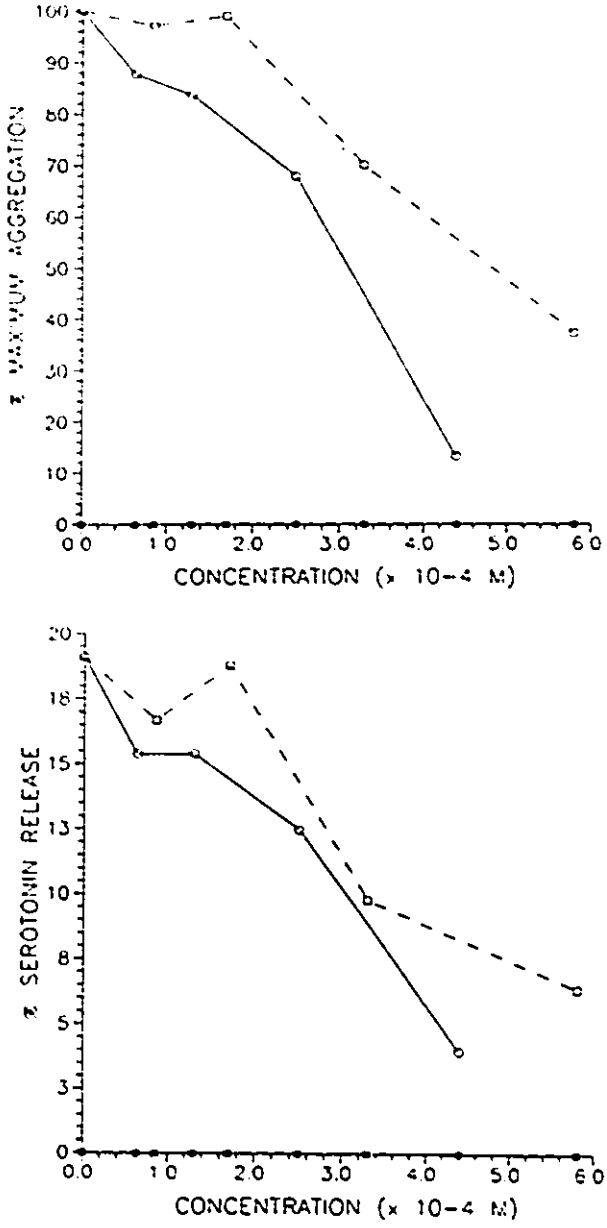
LEGEND: Open circles represent ESSE alone. Closed circles represent ESSE added together with collagen.

FIGURE 5.1.4 EFFECT OF TMA ON COLLAGEN INDUCE RABBIT PLATELET AGGREGATION AND SEROTONIN RELEASE



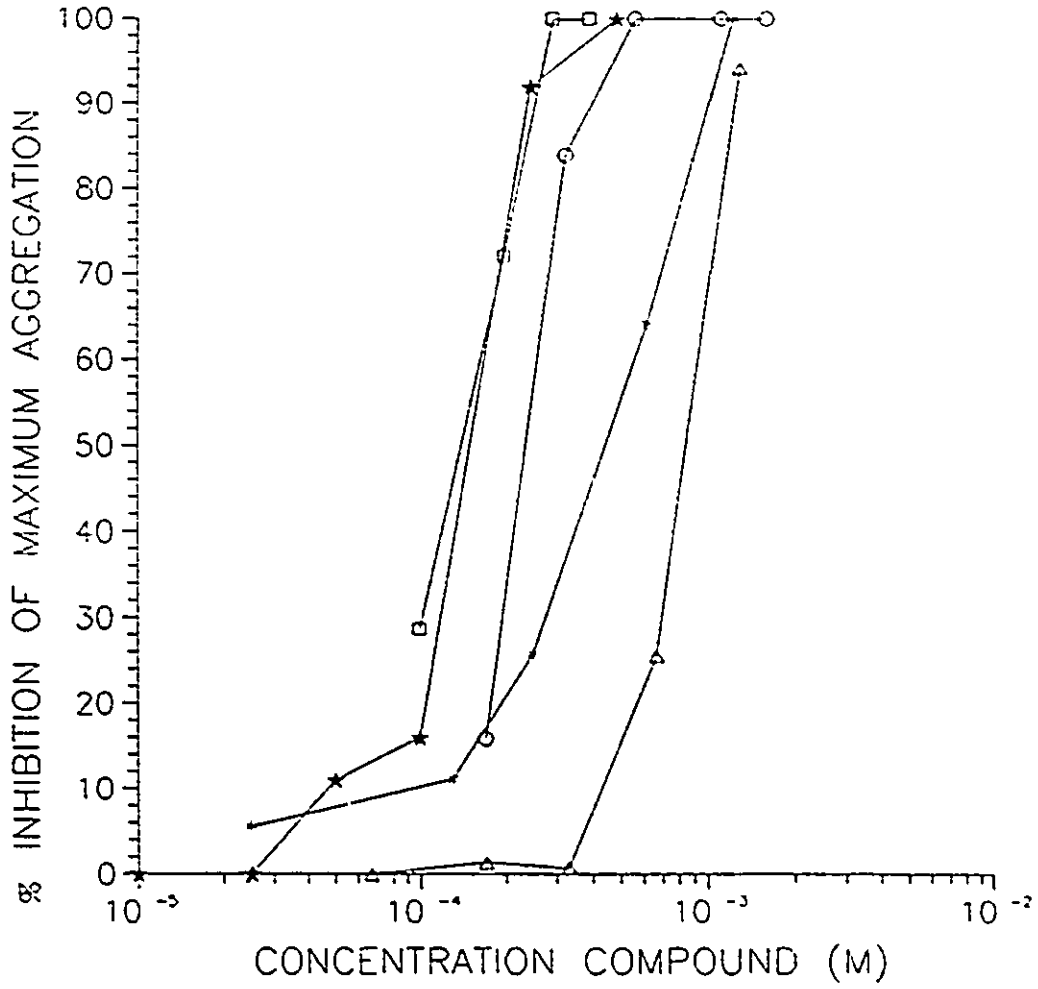
LEGEND: A and B represent experiments in which platelets were isolated with Tyrodes-albumin buffer. C and D represent experiments in which platelets were isolated with Tyrodes-gelatin buffer. Closed circles represent TMA alone. Open circles represent TMA added together with collagen.

FIGURE 5.1.5 COMPARISON OF THE EFFECT OF MYOCHRYSINE AND TMA ON COLLAGEN INDUCED RABBIT PLATELET AGGREGATION AND SEROTONIN RELEASE



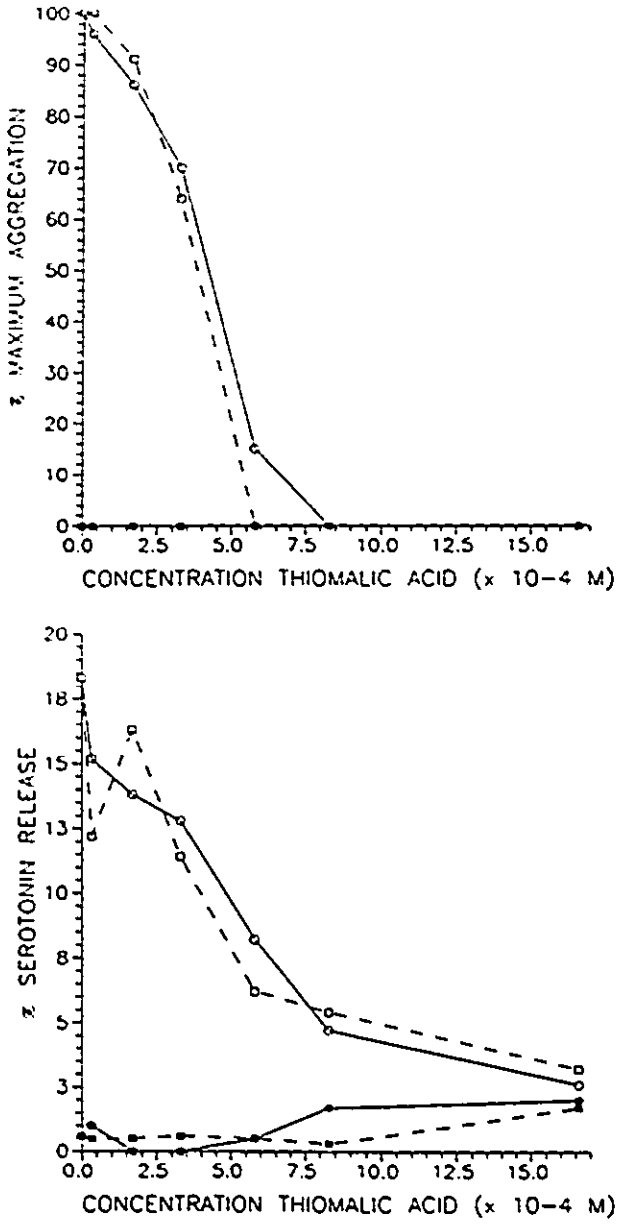
LEGEND: Closed circles represent Myochrysine alone. Closed Squares represent TMA alone. Open circles represent Myochrysine added together with collagen. Open squares represent TMA added together with collagen.

FIGURE 5.1.6 LOG DOSE-RESPONSE CURVES FOR THE ACTION OF SULFHYDRYL REACTING AGENTS ON COLLAGEN INDUCED RABBIT PLATELET AGGREGATION



LEGEND: All compounds were added together with collagen. * represents Myochrysine. O represents TMA. Δ represents D-penicillamine. □ represents CPDS. ★ represents ESSE.

FIGURE 5.1.7 EFFECT OF d & l TMA ON COLLAGEN INDUCED RABBIT PLATELET AGGREGATION AND SEROTONIN RELEASE



LEGEND: Closed circles and closed squares represent d TMA alone and l TMA alone, respectively. Open circles and open squares represent d TMA added together with collagen and l TMA added together with collagen, respectively.

FIGURE 5.1.8 EFFECT OF 4.4×10^{-4}M d,l MYOCHRYSINE ON COLLAGEN INDUCED RABBIT PLATELET AGGREGATION AND SEROTONIN RELEASE

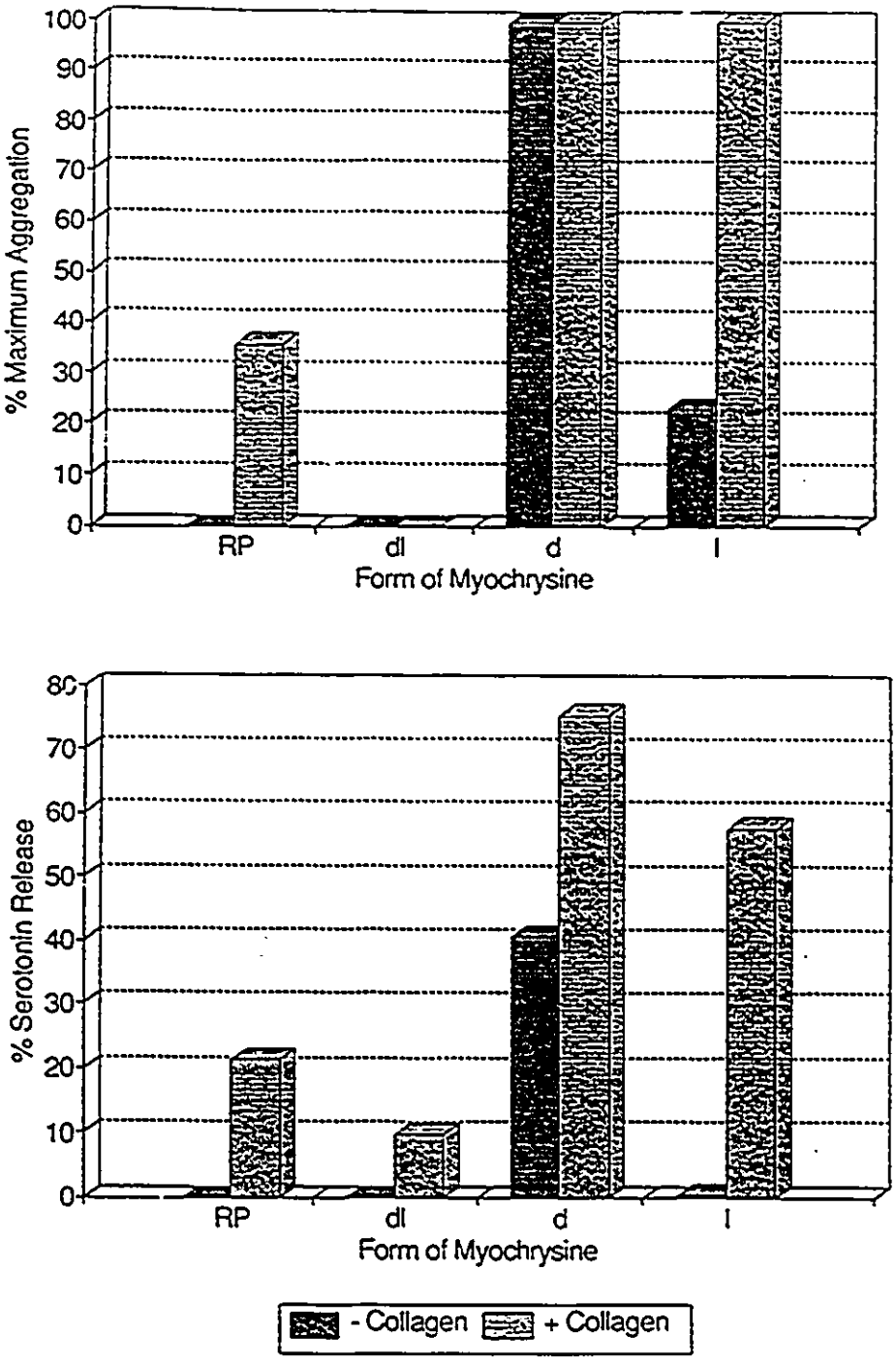
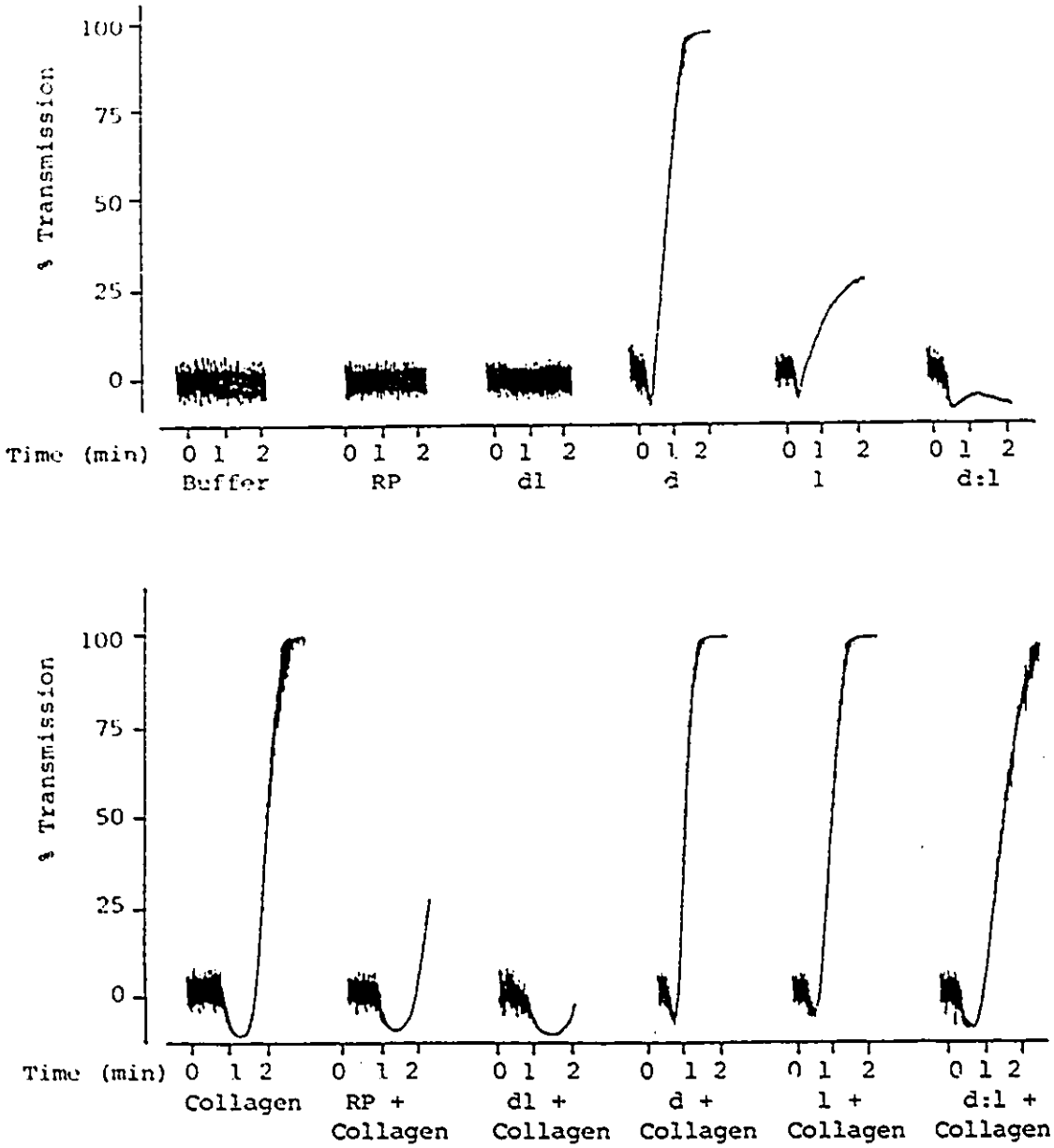
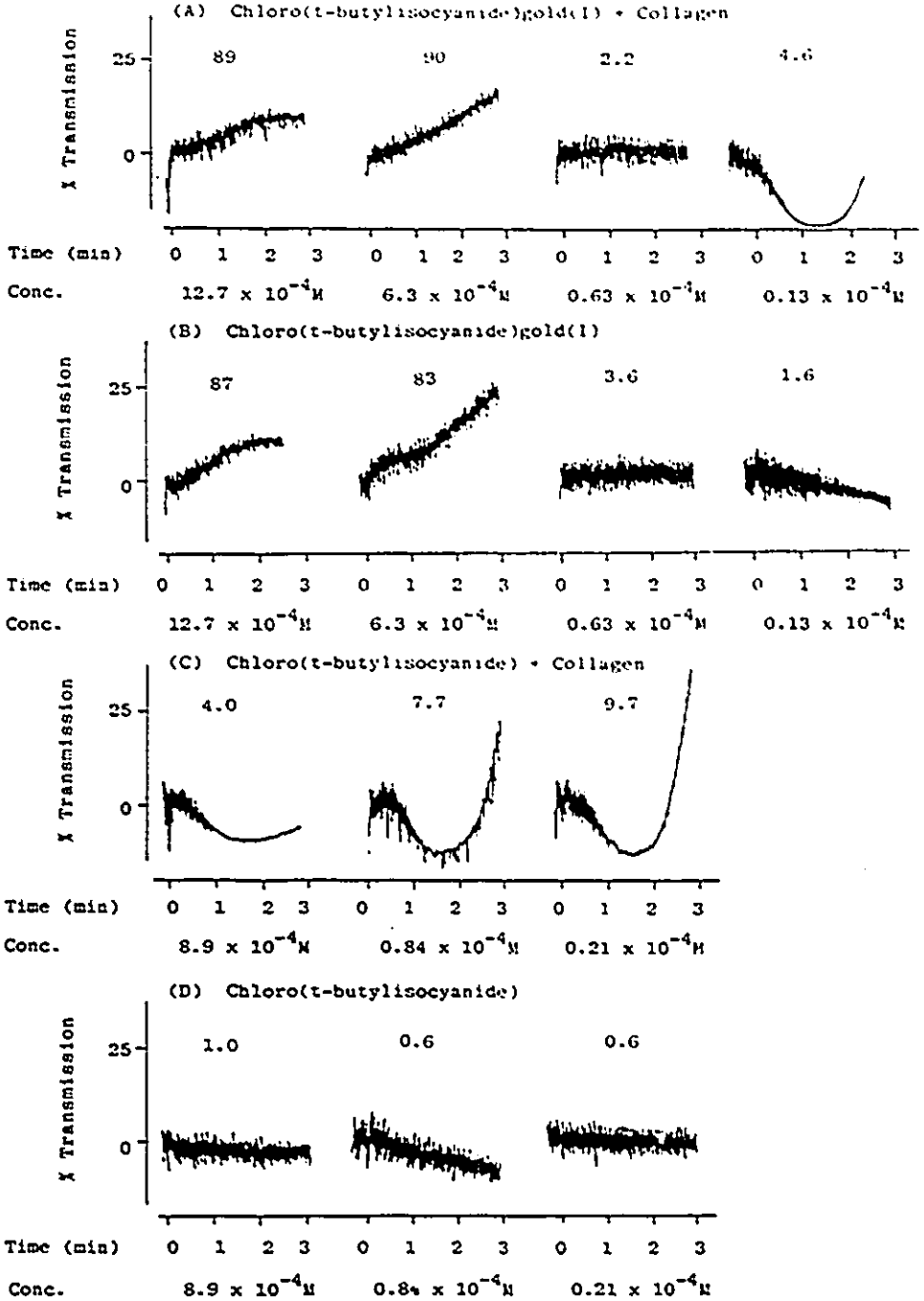


FIGURE 5.1.9 COMPARISON OF "all d" AND "all l" ENANTIOMERS OF MYOCHRYSINE



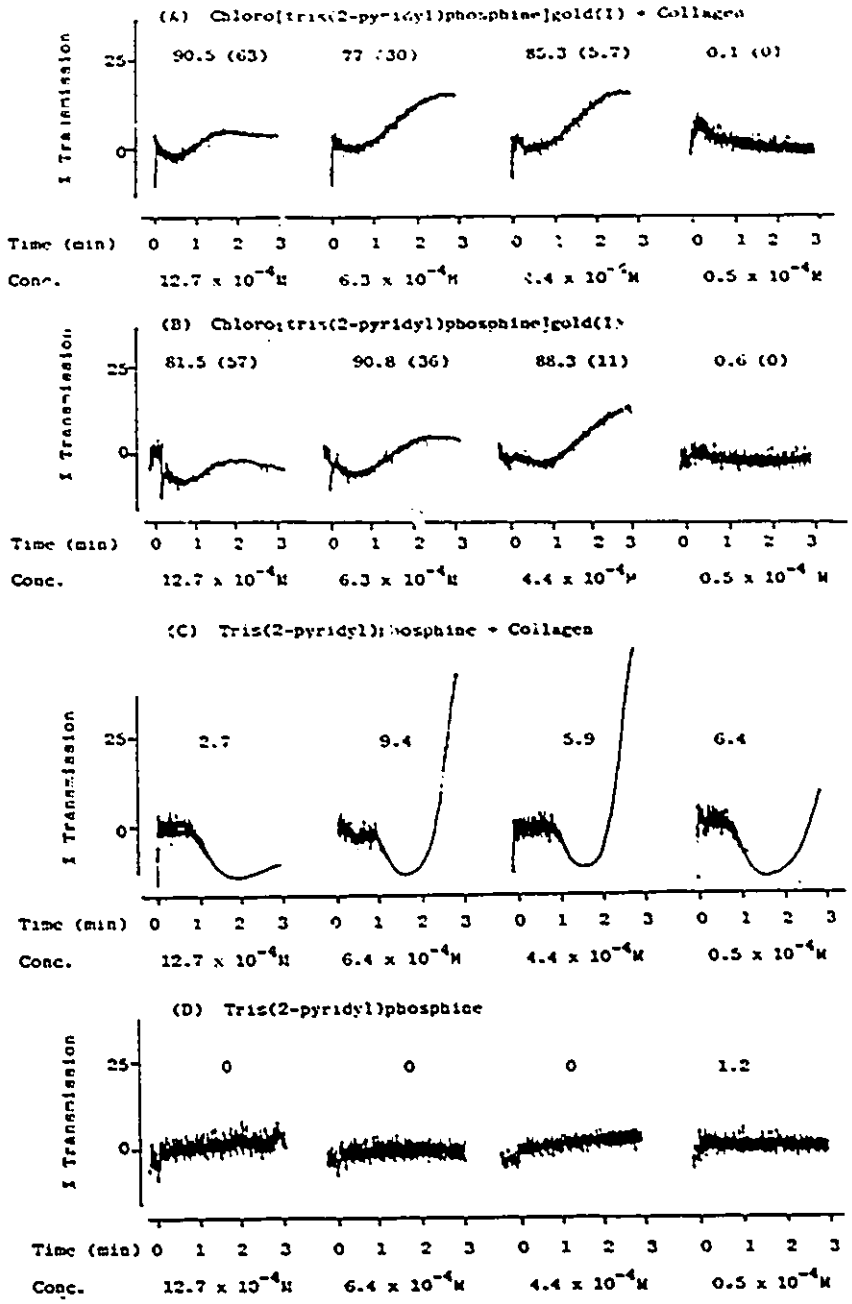
LEGEND: Figure shows aggregation tracings of various forms 4.4 x 10^{-4}M Myochrysine. RP = Rhone Poulenc Myochryisne. dl, d & l Myochrysine were made in the laboratories of Dr. C.J.L. Lock. d:l Myochrysine was mixed in the ratio 1:1 just before the experiment.

FIGURE 5.1.10 EFFECT OF CHLORO(t-BUTYLISOCYANIDE)GOLD(I) AND t-BUTYL ISOCYANIDE ON RABBIT PLATELET ACTIVATION



LEGEND: Figure shows aggregation tracings of various concentrations of chloro(t-butylisocyanide)gold(I) and t-butylisocyanide with and without the addition of collagen. Numbers above aggregation tracings represent % serotonin release.

FIGURE 5.1.11 EFFECT OF CHLORO[TRIS(2-PYRIDYL)PHOSPHINE]GOLD(I) AND TRIS(2-PYRIDYL)PHOSPHINE ON RABBIT PLATELET ACTIVATION



LEGEND: Figure shows aggregation tracings of various concentrations of chloro[tris(2-pyridyl)phosphine]gold(I) and tris(2-pyridyl) phosphine with and without the addition of collagen. Numbers above aggregation tracings represent % serotonin release and numbers in brackets represent % ^{51}Cr release.

5.2 DISCUSSION

Sulfhydryl reacting compounds have previously been shown to affect platelet function. N-ethylmaleimide (NEM) has been shown to block epinephrine mediated inhibition of PGE_1 induced adenylate cyclase activity, to inhibit platelet adhesion to connective tissue, to inhibit arachidonic acid and thrombin induced platelet secretion and to inhibit thrombin and collagen induced aggregation.(199-202) Diamide, para-bromophenacyl bromide, cysteine, 6-mercatopurine, and N-acetylcysteine, all of which react with sulfhydryl groups, have also been shown to inhibit platelet functions.(201,203-206) On the other hand, other sulfhydryl reacting agents such as dithiothreitol, β-mercaptoethanol and ESSE did not inhibit platelet function.(202,204)

The actions of three sulfhydryl reacting compounds, D-penicillamine, ESSE and CPDS on collagen induced rabbit platelet activation were tested here. The sulfhydryl group on D-penicillamine interacts with other sulfhydryl groups, the two sulfhydryl groups are oxidized to a disulfide. ESSE and CPDS both undergo exchange reactions to give mixed disulfides (see Figure 5.1.12). The difference between the two is that ESSE is cell impermeant while CPDS is cell permeant.(207,208) It was shown here that interference of a sulfhydryl group(s) by these compounds inhibited collagen induced rabbit platelet aggregation and serotonin release in a manner very similar to Myochrysine (see Figure 5.1.6). The activity of ESSE shows not only that interference with a sulfhydryl group can inhibit platelet activation but that interference must be with a surface sulfhydryl group. This is in agreement with the findings in Chapter 4 that suggest the action of Myochrysine is at the platelet membrane surface.

FIGURE 5.1.12: REACTIONS OF SULFHYDRYL SEEKING AGENTS

1. OXIDATION (e.g. D-penicillamine)

$$R\text{-}SH + HS\text{-}Protein + \tfrac{1}{2} O_2 \text{ -----> } R\text{-}S\text{-}S\text{-}Protein + H_2O$$

2. EXCHANGE (e.g. ESSE, CPDS)

$$R\text{-}S\text{-}S\text{-}R + HS\text{-}Protein \text{ ----> } R\text{-}S\text{-}S\text{-}Protein + R\text{-}SH$$

3. METAL BINDING

$$L_2Au + HS\text{-}Protein \text{ ----> } L\text{-}Au\text{-}S\text{-}Protein + LH$$

Thiomalic acid, the ligand of Myochrysine and a potential sulfhydryl reacting compound, was also shown to inhibit collagen induced rabbit platelet aggregation. Since Myochrysine dissociates almost completely after intramuscular injection into a human subject (24), it seemed possible that the thiomalate ligand may be pharmacologically active. Double label studies by Cottrill *et al.*, however, give evidence against this.(22) They found that very little of the labelled thiomalate in rat organs at 24-48 hours.(22) In addition, clinical studies in humans show that beneficial effects of Myochrysine treatment are not seen until 6-8 weeks.(11)

Results presented here suggest that both thiomalic acid and gold(I) are capable of inhibiting rabbit platelet function.

The fact that the dose response curves for the action of Myochrysine and thiomalic acid are almost completely identical suggest that thiomalic acid is the major contributor to inhibition of platelet function. On the other hand, the difference in ID_{50}s for the action of TMA and Myochrysine on collagen induced aggregation may suggest that the pharmacologically active component of Myochrysine and TMA are different. These results can be interpreted as a simple interaction with a protein site RSH, giving either RSSR' or RSAuSR', the nature of the interaction

being unimportant as long as a bond is formed, or alternatively, it may just mean that higher concentrations of Myochrysine are required to achieve high enough concentrations of dissociated TMA to identify an effect.

The experiments on d and l TMA and "all d" and "all l" Myochrysine give evidence that gold is also capable of affecting platelet function. This is explained in detail in a review paper by Kean *et al.*(41) Myochrysine is a polymer and because of the chiral nature of its ligand, TMA, is a mixture of a variety of different chiral combinations (see Figure 5.1.13).(41) If two chiral compounds are mixed to give a racemic mixture, the resulting biological property of the mixture is expected to be an average of the effects of the individual chiral compounds.(41) This is exactly what is seen with TMA. When d TMA is mixed with l TMA, the d:l mixture gives an average effect of the two individually (see Table 5.2.1).

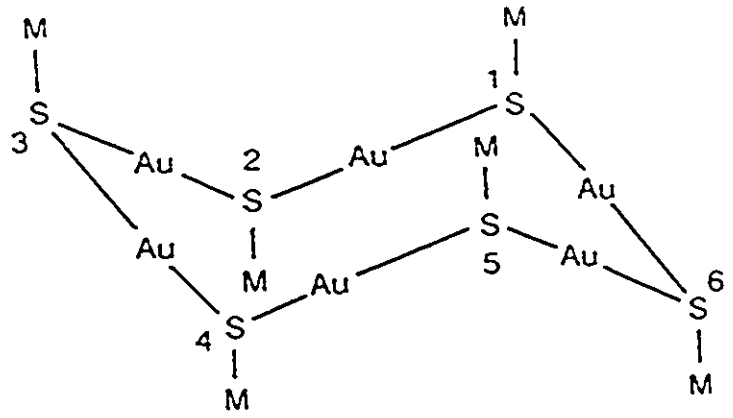
TABLE 5.2.1: EFFECT OF 5.8 X 10^{-4}M d & l TMA ON COLLAGEN INDUCED RABBIT PLATELET AGGREGATION AND RELEASE

COMPOUND	% Maximum Aggregation		% Serotonin Release	
	- Collagen	+ Collagen	- Collagen	+ Collagen
dl TMA	0	shape change only	1.1	2.8
d TMA	0	shape change only	1.0	3.1
l TMA	0	shape change only	0.4	2.6
d:l TMA	0	shape change only	0.5	3.8

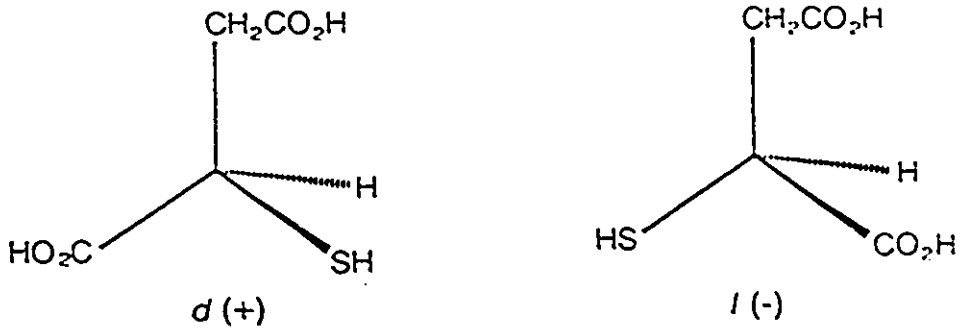
The reason for this, as explained in the paper by Kean *et al.*, is that when "all d" and "all l" Myochrysine are mixed, exchange occurs and a mixture as outlined in Figure 5.1.13, results.(41) The portion of "all d" and "all l" in this mixture is very small.(41) The difference in

FIGURE 5.1.13 THE POLYMERIC AND CHIRAL NATURE OF MYOCHRYSINE

(A)



(B)



(C)	MOLECULE	RATIO IN MIXTURE
	6d(+)	1
	5d(+),1l(-)	6
	4d(+),2l(-)	15
	3d(+),3l(-)	20
	2d(+),4l(-)	15
	1d(+),5l(-)	6
	6l(-)	1

Figure taken from W.F. Kean, C.J.L. Lock, H.E. Howard-Lock (1991) Lancet 338: 1565-1568.

(A) Numbers in hexamer denote sulphur groups about which the thiomalate ligand (M) is chiral.

(B) Shown are d(+) and l(-) configurations that may occur at these sulfur groups.

(C) Different chirality combinations and the ratios with which they occur.

activity between the expected averaging of activities and the actual activity obtained with d:l, suggests that TMA is not the only compound which is interacting with rabbit platelets.

The results obtained with the compounds chloro(t-butylisocyanide) gold(I) and chloro[tris(2-pyridyl)phosphine]gold(I) suggest that gold(I) is capable of interacting with rabbit platelets in an inhibitory fashion. The high level of toxicity seen with these compounds suggests that TMA is acting as a gold "buffer". In any mixture, an equilibrium exists (see Figure 5.1.14).

FIGURE 5.1.14: REACTIONS OF GOLD COMPOUNDS WITH PLATELET PROTEINS

(Ia) Protein-SH + chloro(t-butylisocyanide)gold(I)Cl ⇌ Protein-S-Au-Cl + t-butylisocyanide

(Ib) Protein-SH + chloro[tris(2-pyridyl)phosphine]gold(I) ⇌ Protein-S-Au-Cl + tris(2-pyridyl)phosphine

(II) Protein-SH + Au-TMA ⇌ Protein-Au + TMA

The gold atom must bind to another atom, but its nature is unknown. Nitrogen from histidine, other SH, disulfide or methionine groups are likely species. Since gold (I) preferentially reacts with sulphur, the reaction equilibrium for equation I will lie further to the right than for equation II. Thiomalic acid most probably prevents too high a concentration of gold from reacting with platelet protein sulfhydryls. This is consistent with findings by Turner, that gold compounds with non-sulfur containing ligands were highly toxic in Ames tests, while gold compounds with sulfur ligands were not.(211)

The fact that "all d" and "all l" Myochrysine cause rabbit platelet activation is interesting in that it may relate to the findings in human platelets. In the studies done with human platelets it was found

that in some individuals, Myochrysine caused platelet activation at high concentrations. It may be that these individuals are more sensitive to the action of "all d" and "all l" Myochrysine. At high concentrations, there may be enough "all d" and "all l" Myochrysine present to cause platelet activation in these individuals.

It is possible that this sensitivity to "all d" and "all l" Myochrysine relates to clinical findings that certain individuals are more sensitive to the toxic effects of Myochrysine. If certain adverse effects are produced by "all d" or "all l" or ratios of d and l Myochrysine, this creates the potential for the development of a specific, more efficacious compound with lower toxicity profile.

CHAPTER 6

THE EFFECT OF MYOCHRYSINE ON PROTEIN PHOSPHORYLATION AND CYCLIC NUCLEOTIDE LEVELS

The extent of phosphorylation of two platelet proteins, P20 or myosin light chain and P40 or pleckstrin, gives an indication of the extent of stimulation of the two arms of the PK-C/Ca^{2+} pathway (see Figure 1.4.1).(136) The degree of P20 phosphorylation gives an indirect determination of Ca^{2+} mobilization, while the degree of P40 phosphorylation gives an indirect measure of PK-C activation.(136) The effect of Myochrysine on collagen induced P20 and P40 phosphorylation was investigated in human and rabbit platelets.

TPA and A23187 are two chemical mediators which activate the separate arms of the PK-C/Ca^{2+} pathway, without activation of the initial receptor-mediated events.(151,150) TPA activates PK-C, which in turn results in P40 phosphorylation, while A23187, which is a Ca^{2+} ionophore, mimics Ca^{2+} mobilization and results in P20 phosphorylation.(151,153) The effect of Myochrysine on activation by TPA and A23187 was investigated.

Increases in the cyclic nucleotides, cAMP and cGMP, inhibit activation of the PK-C/Ca^{2+} pathway.(211,212) Levels of cAMP can be increased in the platelet by agents such as PGE_1 or adenosine through receptor mediated processes.(211,212) Levels of cGMP are increased through activation of the PK-C/Ca^{2+} pathway and this increase is believed to act as a negative feedback mechanism.(170,171) Levels of cGMP can also be increased in platelets by agents such as nitric oxide or sodium

nitroprusside (SNP), which are believed to act by stimulating guanylate cyclase.(212,213) It is possible that Myochrysine could be inhibiting platelet activation by a mechanism involving increases in cAMP and/or cGMP. The effect of Myochrysine on the levels of these two cyclic nucleotides was investigated in collagen induced rabbit platelets.

6.1 RESULTS

6.1.1 THE EFFECT OF MYOCHRYSINE ON HUMAN P20 AND P40 PHOSPHORYLATION

The effect of Myochrysine on collagen induced P20 and P40 phosphorylation was investigated in human platelets. The same 10 individuals who were studied in Chapter 3 were tested here. Results are shown in Figures 6.1.1, 6.1.2 and 6.1.3 and resemble those seen for the effect of Myochrysine on collagen induced human platelet aggregation and serotonin release (Chapter 3). Large inter- and intra- person variations were seen with regard to the effect of Myochrysine on P20 and P40 phosphorylation. Myochrysine, 0.25 - 38 x 10^{-4}M, inhibited collagen induced P20 and P40 phosphorylation in some individuals in a dose dependent manner, while in other individuals no inhibition of P20 and P40 phosphorylation was seen. In some individuals, Myochrysine induced P20 and P40 phosphorylation at higher concentrations. Figure 6.1.3 shows the intra-subject variation. Different results were found in experiments performed on different days.

6.1.2 THE EFFECT OF MYOCHRYSINE ON RABBIT P20 AND P40 PHOSPHORYLATION

The effect of Myochrysine on collagen induced P20 and P40 phosphorylation in rabbit platelets is shown in Figure 6.1.4.

Concentrations of 0.25 - 1.3 x 10^{-4}M Myochrysine appeared to enhance collagen induced P20 and P40 phosphorylation while concentrations of 2.5 - 12.7 x 10^{-4}M Myochrysine inhibited this response in a dose dependent manner. The ID_{50} for the action of Myochrysine on P20 and P40 phosphorylation was found to be greater than the ID_{50} for the action of the drug on collagen induced aggregation and serotonin release. The ID_{50} for the action of Myochrysine on collagen induced P20 phosphorylation was 10 ± 1.4 x 10^{-4}M, n=2. In 3 experiments in which inhibition was seen, 50% inhibition of P20 phosphorylation was not achieved by 12.7 x 10^{-4}M Myochrysine. The ID_{50} for the action of Myochrysine on collagen induced P40 phosphorylation was 6.4 ± 2.5 x 10^{-4}M, n=4. In 2 experiments in which inhibition was seen, 50% inhibition of P40 phosphorylation was not achieved by 12.7 x 10^{-4}M Myochrysine.

6.1.3 THE EFFECT OF MYOCHRYSINE ON TPA INDUCED RABBIT P40 PHOSPHORYLATION

The concentration of TPA which induced P40 phosphorylation to an extent similar to that induced by collagen was determined to be 1.6 x 10^{-9}M (see Figure 6.1.5). At this concentration very little P20 phosphorylation was induced and no platelet shape change, aggregation or serotonin release was seen. The effect of Myochrysine on TPA (1.6 x 10^{-9}M) induced P40 phosphorylation is shown in Figure 6.1.6. Concentrations 0.25 - 12.7 x 10^{-4}M Myochrysine did not inhibit TPA induced P40 phosphorylation in rabbit platelets. It appeared that at concentrations of 12.7 x 10^{-4}M Myochrysine enhanced TPA induced P40 phosphorylation.

6.1.4 THE EFFECT OF MYOCHRYSINE ON A23187 INDUCED RABBIT P20 PHOSPHORYLATION

Figure 6.1.7 shows that 0.5 x 10^{-6}M and 0.75 x 10^{-6}M A23187 induced P20 phosphorylation to the same extent as collagen. At these concentrations the extent of P40 phosphorylation was the same as the DMSO control, but there was platelet shape change at 0.5 x 10^{-6}M A23187 and a small degree of aggregation at 0.75 x 10^{-6}M A23187. A23187, 1.0 x 10^{-6}M caused an increase in the amount of P20 as well as P40 phosphorylation, serotonin release and caused 100% aggregation. The effect of Myochrysine on 0.5 – 1.0 x 10^{-6}M A23187 induced P20 phosphorylation in rabbit platelets was tested. In experiments in which the action of Myochrysine on 0.5 x 10^{-6}M and 0.75 x 10^{-6}M A23187 was tested, there was very little difference between DMSO and A23187 induced P20 phosphorylation (see Figure 6.1.8). In experiments testing the action of Myochrysine on 1.0 x 10^{-6}M A23187 induced P20 phosphorylation, aspirin was added to the platelet preparation to prevent formation of thromboxane A_2 and 100 µL apyrase was added to eliminate any released ADP. Figure 6.1.9 shows that under these conditions, Myochrysine did not inhibit A23187 induced P20 phosphorylation. On the contrary, Myochrysine enhanced A23187 induced activation. 1.0 x 10^{-6}M A23187 caused full aggregation. Addition of Myochrysine with A23187 decreased the time to reach full aggregation and enhanced A23187 induced P20 phosphorylation and serotonin release (see Figure 6.1.9 & 6.1.10). Myochrysine did not however, appear to affect P40 phosphorylation (see Figure 6.1.10).

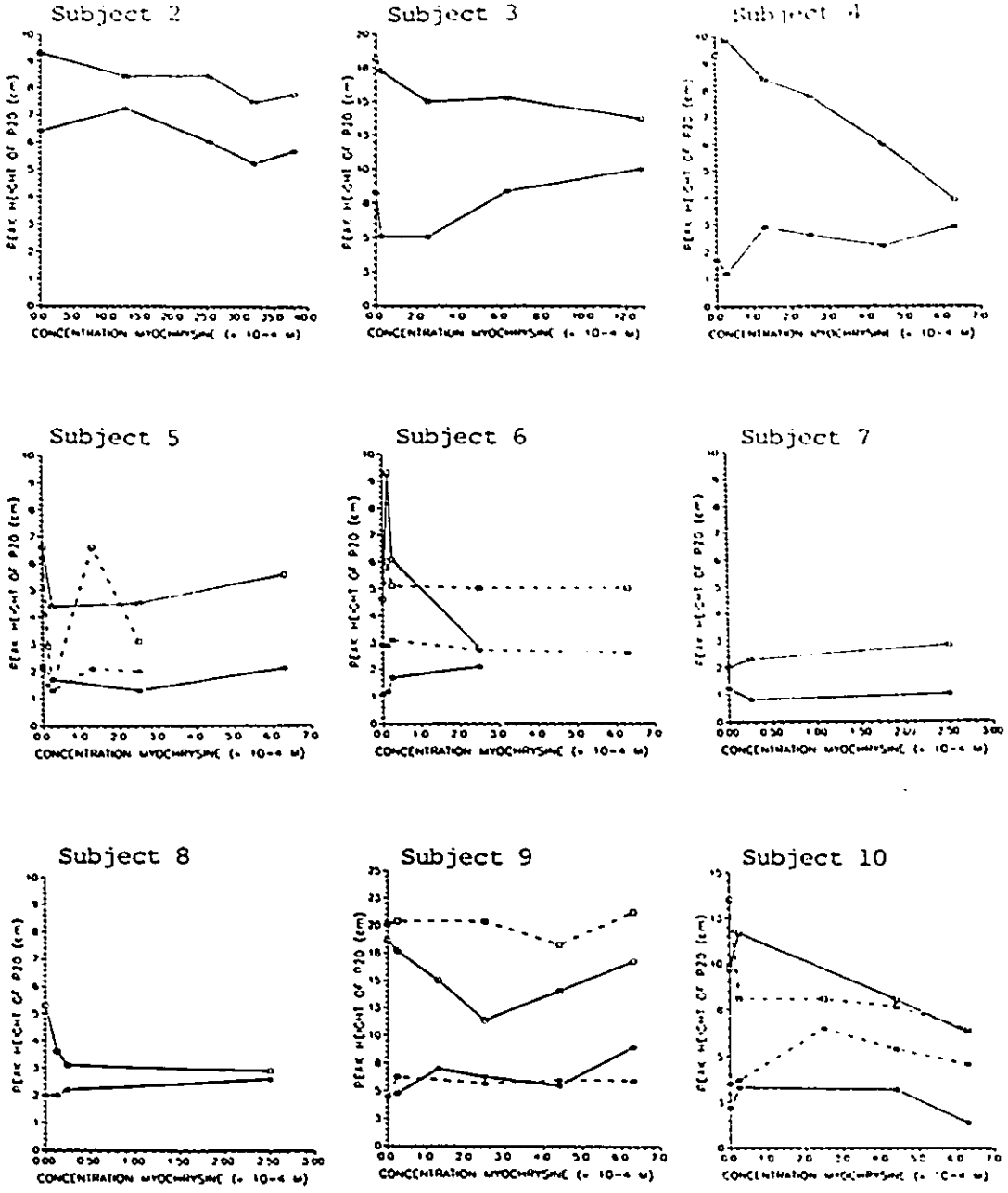
6.1.5 THE EFFECT OF MYOCHRYSINE ON cAMP and cGMP LEVELS

Figure 6.1.11 shows the effect of Myochrysine on the levels of

cAMP in rabbit platelets. From this figure it can be seen that there is very little difference in cAMP levels between resting platelets and collagen activated platelets. PGE_1 however, increased cAMP at concentrations of 10^{-7}M to 10^{-4}M. At these concentrations, PGE_1 inhibited collagen induced aggregation and serotonin release. It can also be seen from this figure that Myochrysine at concentrations of 0.25 - 12.7 x 10^{-4}M did not affect platelet cAMP levels when added to the platelets alone or when added to platelets with collagen.

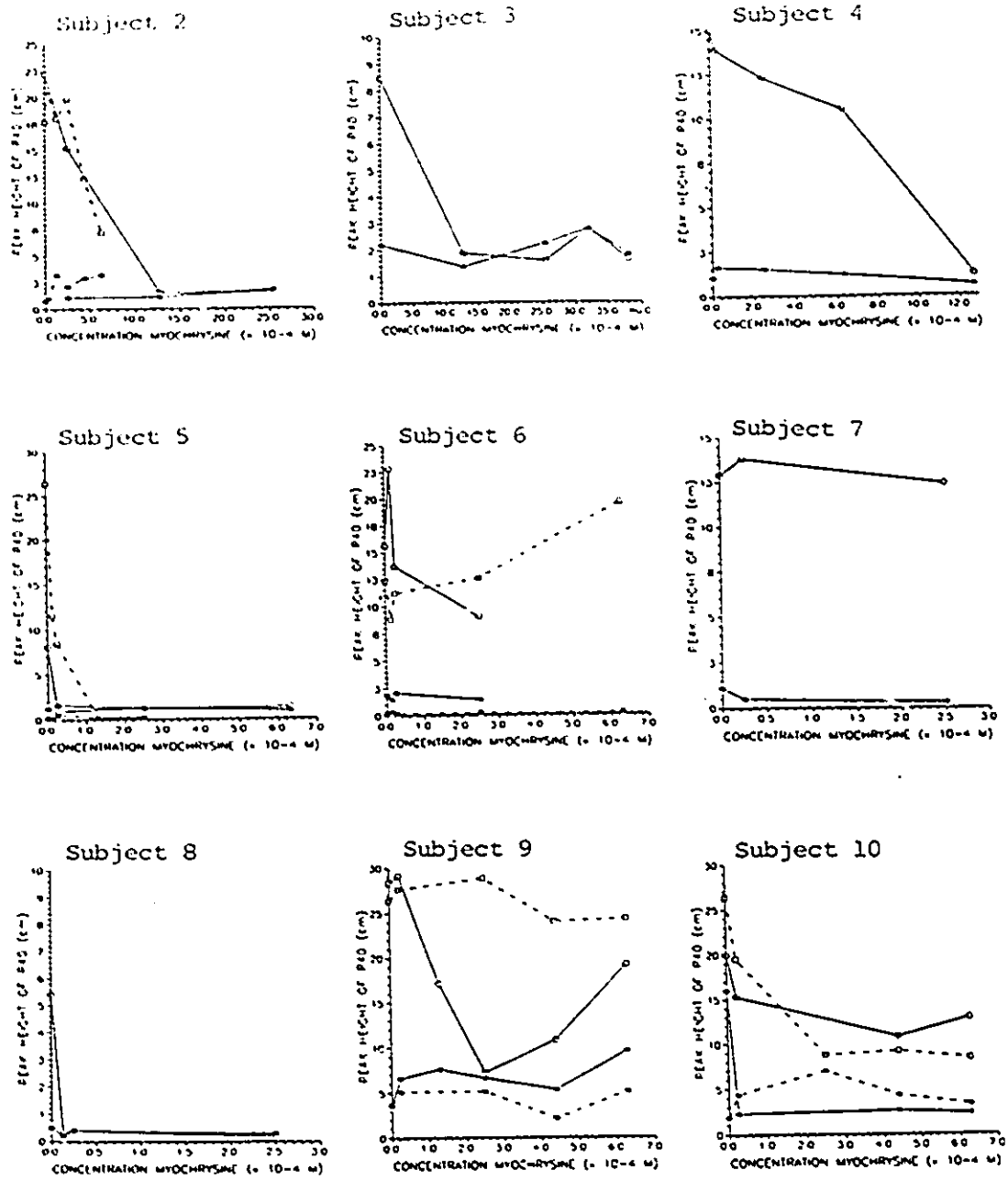
Figure 6.1.12 shows the effect of Myochrysine on the levels of cGMP in rabbit platelets. Collagen slightly increased the level of cGMP in rabbit platelets compared to the negative control. SNP at concentrations of 0.5 - 1.0 x 10^{-6}M caused increases in platelet cGMP. At these concentrations, SNP inhibited collagen induced rabbit platelet activation. In unstimulated rabbit platelets, Myochrysine, 0.25 - 12.7 x 10^{-4}M, appeared to have little effect on cGMP levels. However, in collagen stimulated rabbit platelets, Myochrysine, at these concentrations, appeared to decrease the levels of cGMP compared to the collagen control (see Figure 6.1.12).

FIGURE 6.1.1 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED MYOSIN LIGHT CHAIN (P20) PHOSPHORYLATION IN DIFFERENT INDIVIDUALS



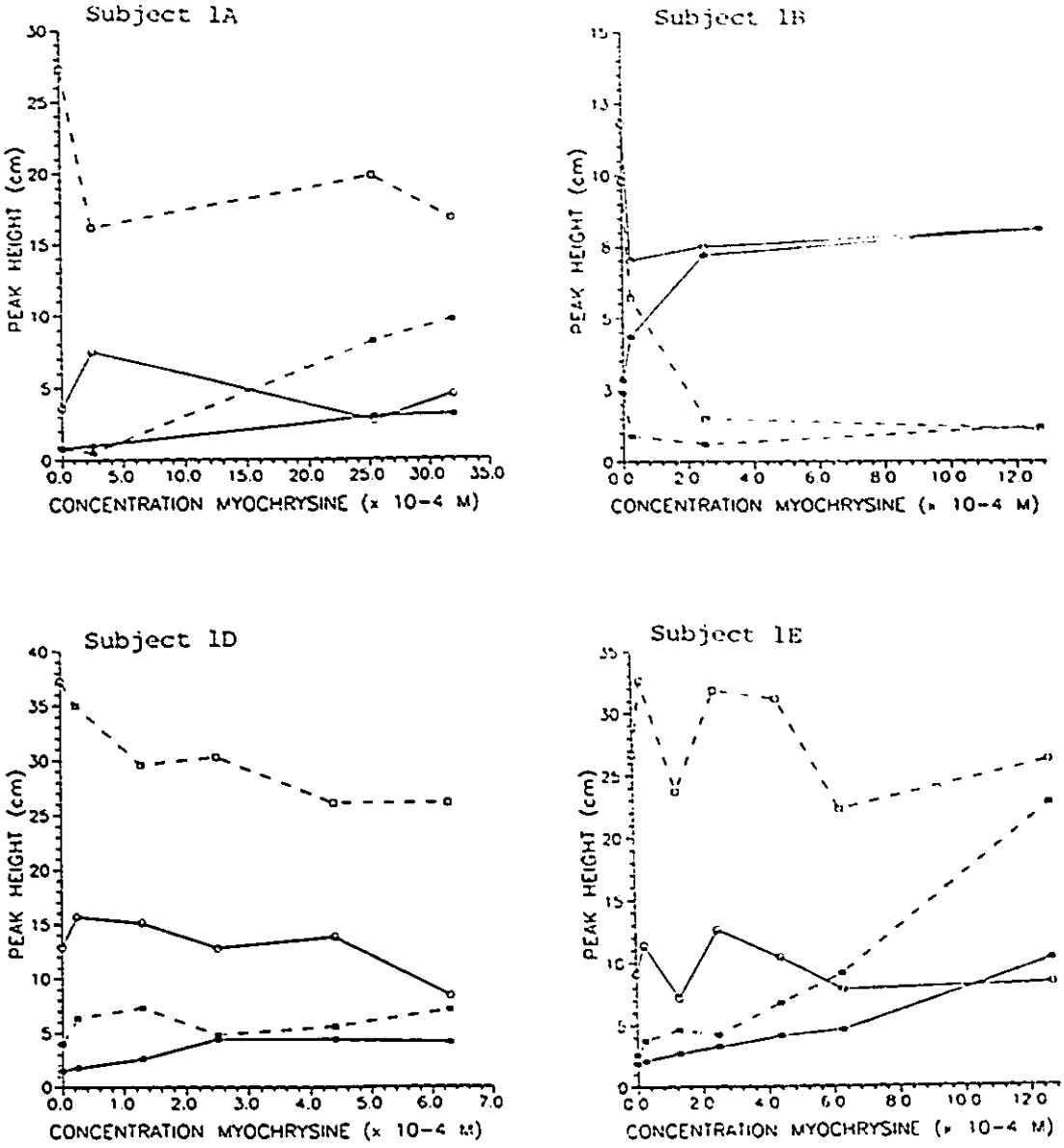
LEGEND: Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen. Different symbols represent different experiments.

FIGURE 6.1.2 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED PLECKSTRIN (P40) PHOSPHORYLATION IN DIFFERENT INDIVIDUALS



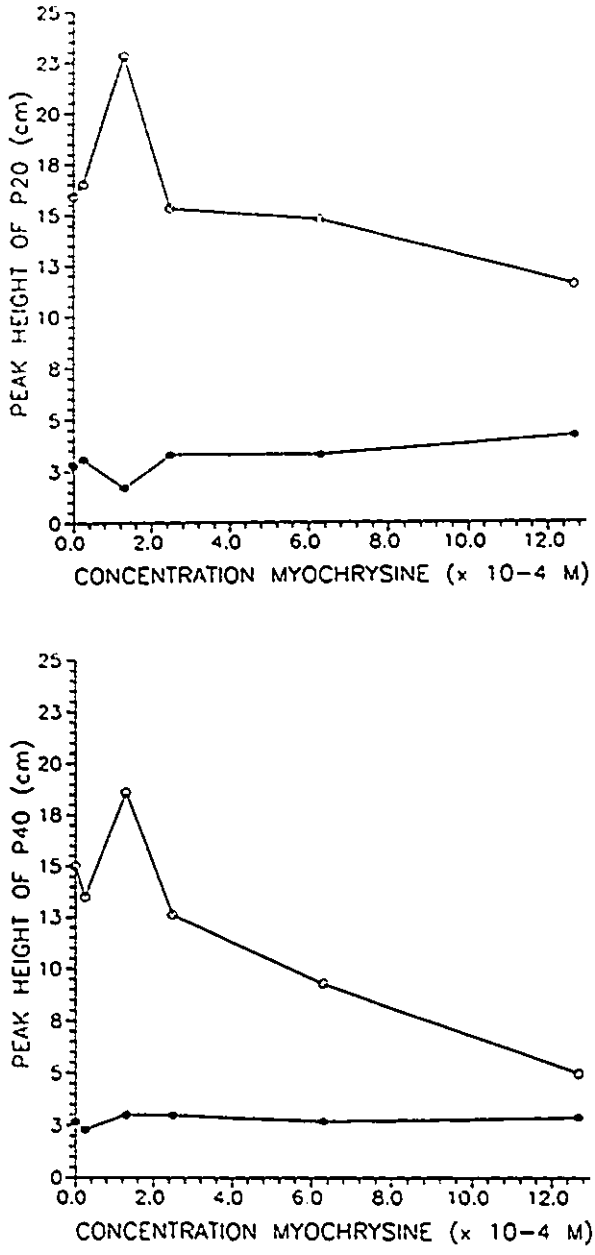
LEGEND: Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen. Different symbols represent different experiments.

FIGURE 6.1.3 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED MYOSIN LIGHT CHAIN (P20) AND PLECKSTRIN (P40) PHOSPHORYLATION WITHIN ONE INDIVIDUAL



LEGEND: Closed circles and closed squares represent P20 and P40 phosphorylation, respectively, by Myochrysine alone. Open circles and open square represent P20 and P40 phosphorylation, respectively, by Myochrysine added together with collagen.

FIGURE 6.1.4 EFFECT OF MYOCHRYSINE ON COLLAGEN INDUCED MYOSIN LIGHT CHAIN (P20) AND PLECKSTRIN (P40) PHOSPHORYLATION IN RABBIT PLATELETS



LEGEND: Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen.

FIGURE 6.1.5 TPA INDUCED MYOSIN LIGHT CHAIN (P20) AND PLECKSTRIN (P40) PHOSPHORYLATION IN RABBIT PLATELETS

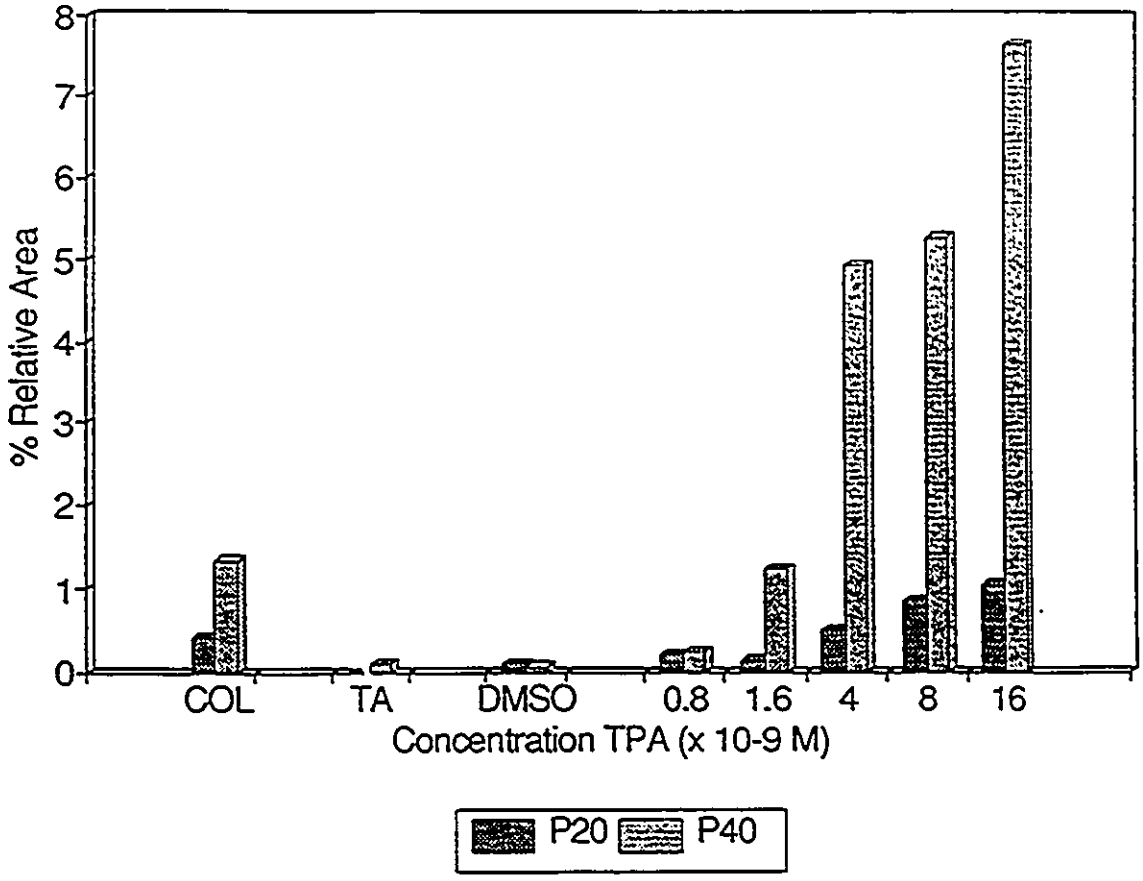
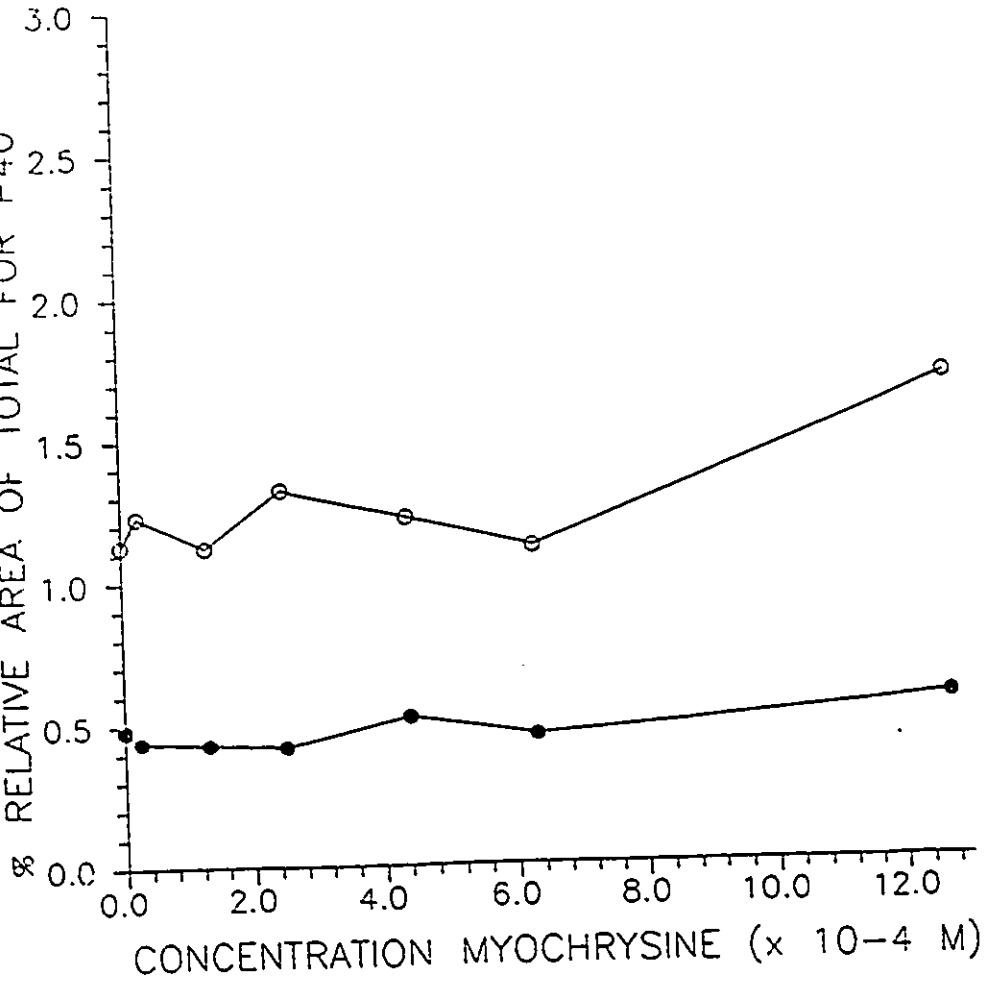


FIGURE 6.1.6 EFFECT OF MYOCHRYSINE ON TPA INDUCED PLECKSTRIN (P40) PHOSPHORYLATION IN RABBIT PLATELETS



LEGEND: Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen.

FIGURE 6.1.7 A23187 INDUCED MYOSIN LIGHT CHAIN (P20) AND PLECKSTRIN (P40) PHOSPHORYLATION IN RABBIT PLATELETS

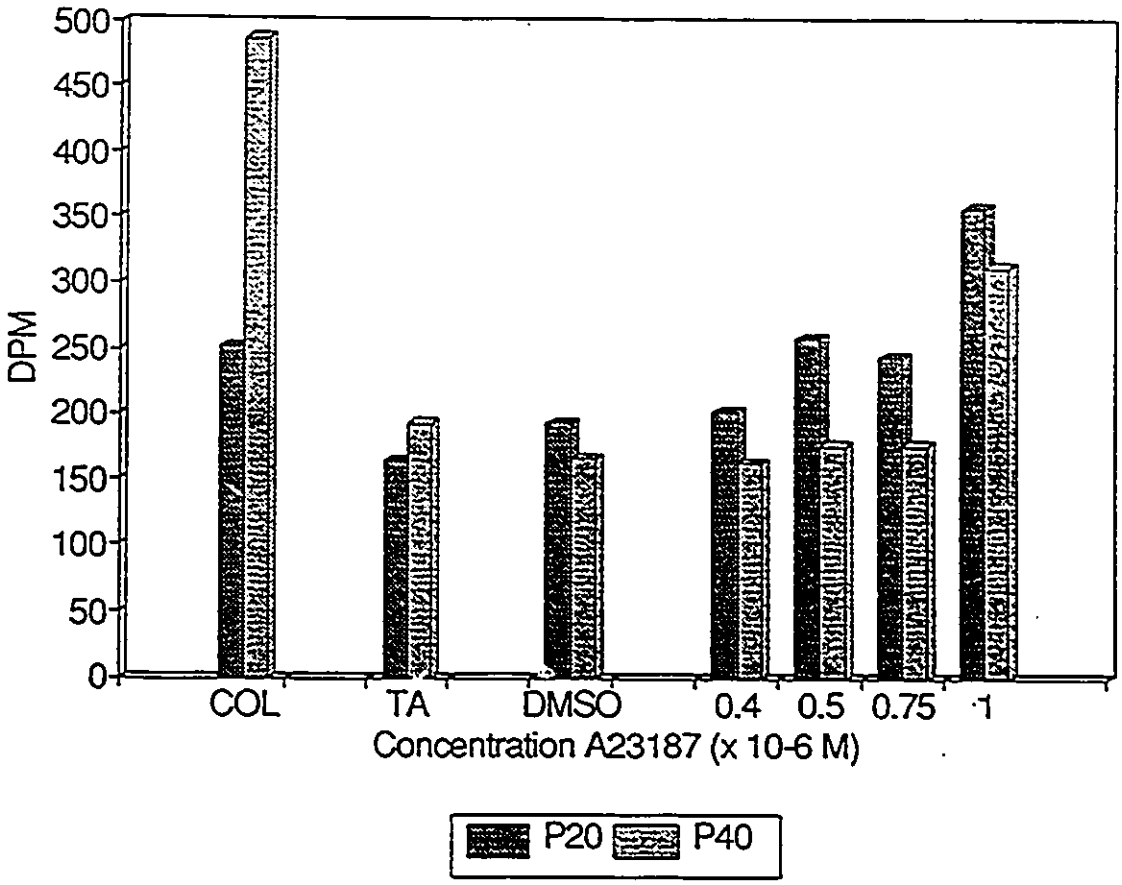
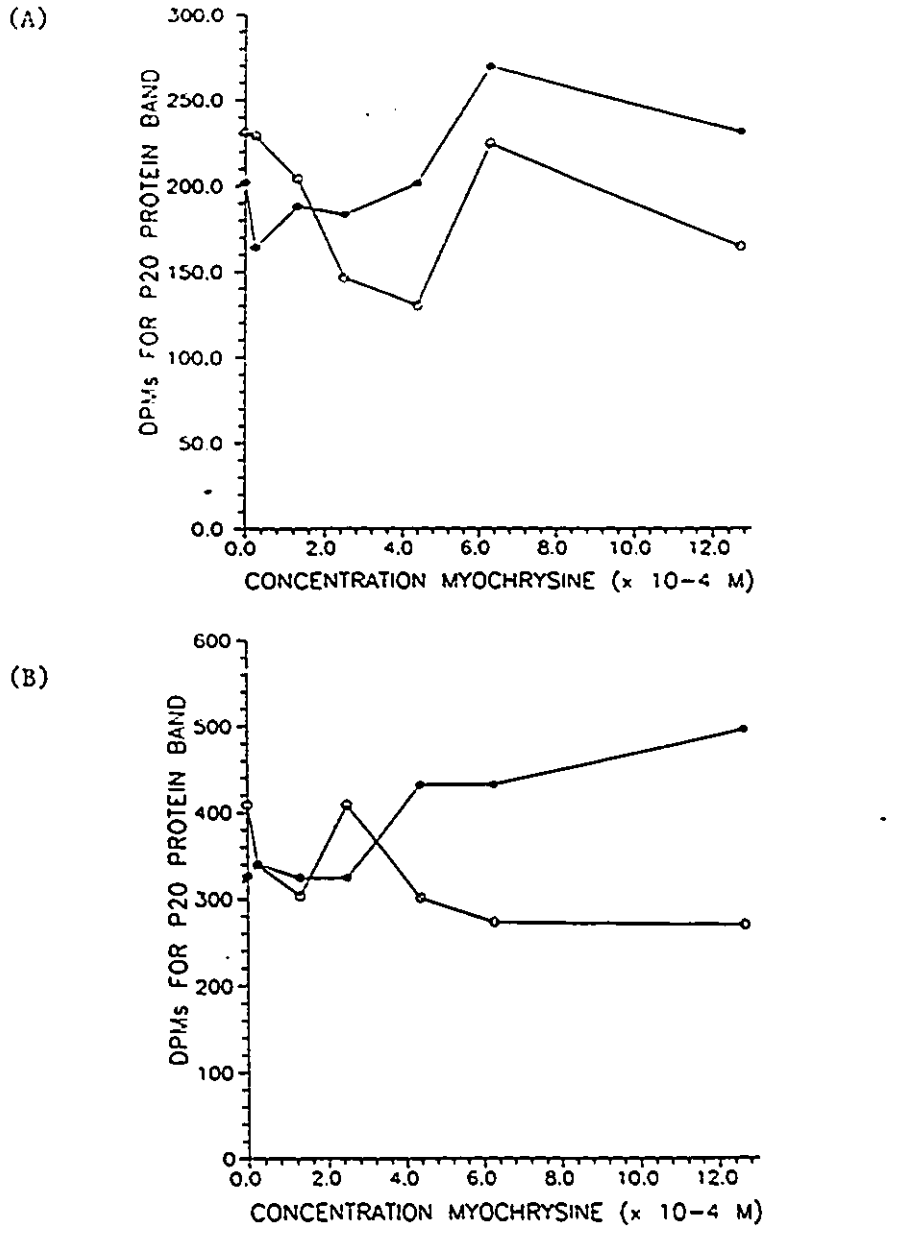
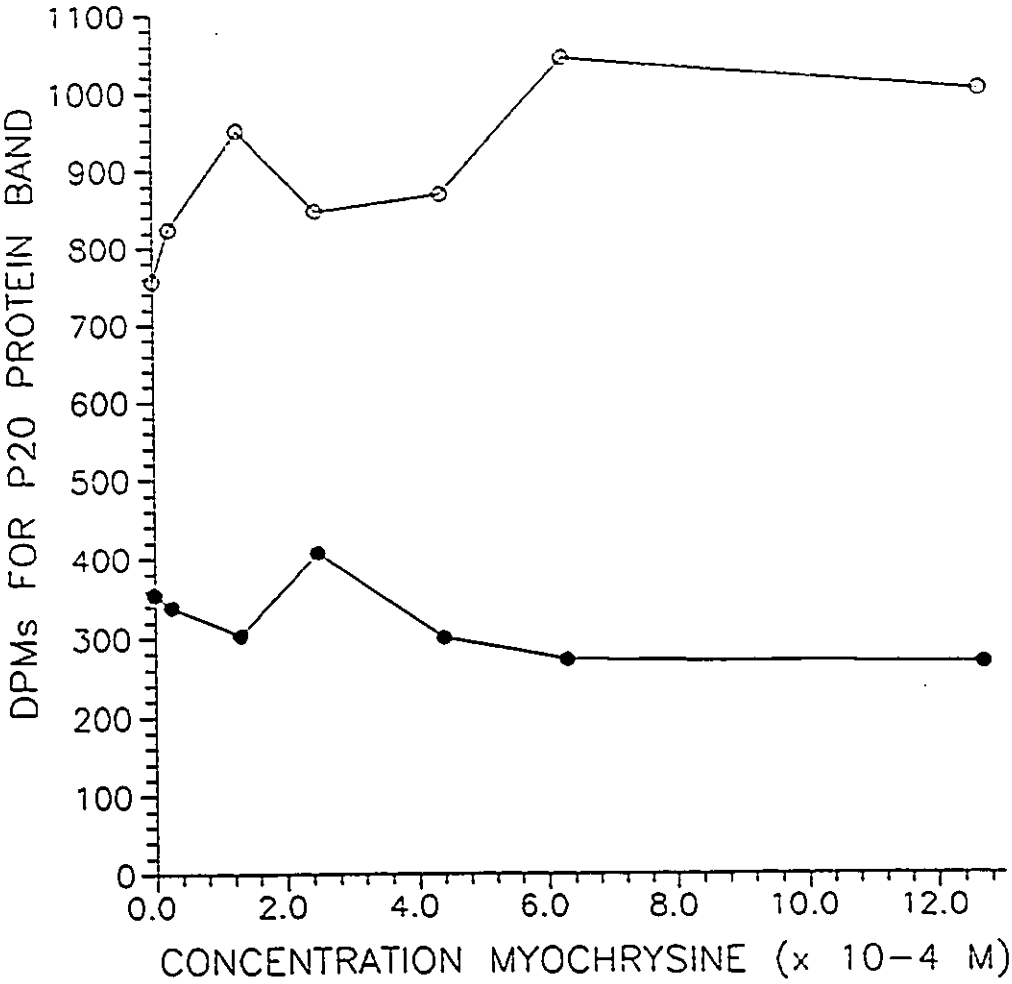


FIGURE 6.1.8 EFFECT OF MYOCHRYSINE ON 0.5 AND 0.75 µM A23187 INDUCED MYOSIN LIGHT CHAIN (P20) PHOSPHORYLATION IN RABBIT PLATELETS



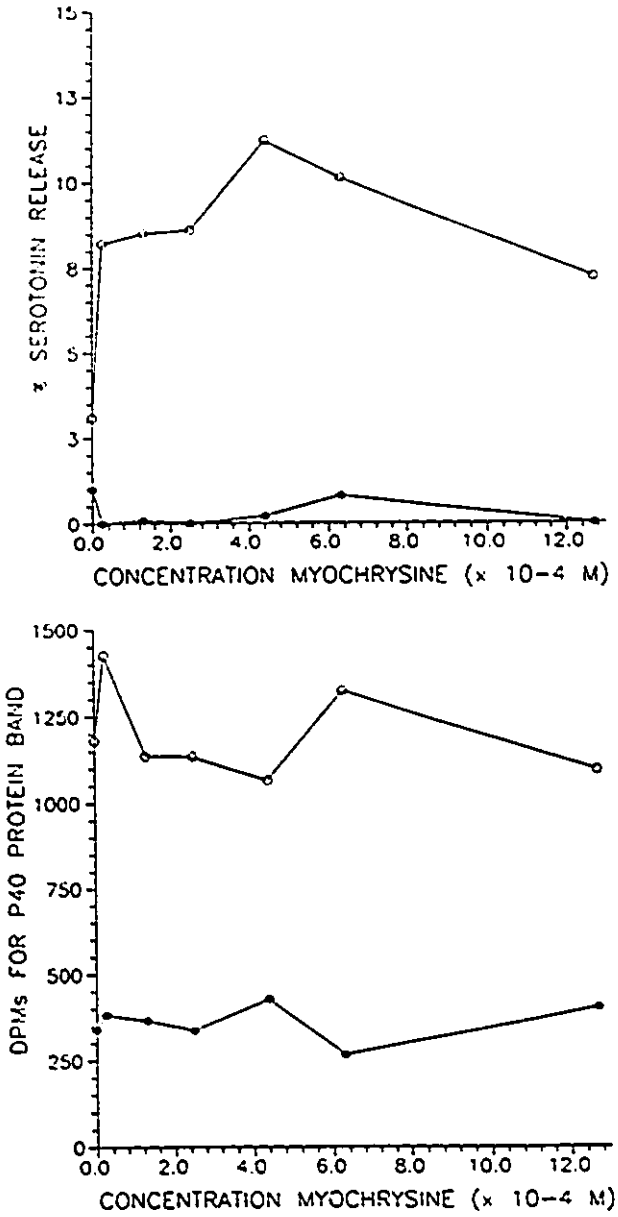
LEGEND: (A) represents 0.5 µM A23187 and (B) represents 0.75 µM A23187. Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen.

FIGURE 6.1.9 EFFECT OF MYOCHRYSINE ON 1.0 μM A23187 INDUCE MYOSIN LIGHT CHAIN (P20) PHOSPHORYLATION IN RABBIT PLATELETS



LEGEND: Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen.

FIGURE 6.1.10 EFFECT OF MYOCHRYSINE ON 1.0 μM A23187 INDUCED PLECKSTRIN (P40) PHOSPHORYLATION AND SEROTONIN RELEASE IN RABBIT PLATELETS



LEGEND: Closed symbols represent Myochrysine alone. Open symbols represent Myochrysine added together with collagen.

FIGURE 6.1.11 EFFECT OF MYOCHRYSINE ON cAMP LEVELS IN COLLAGEN ACTIVATED RABBIT PLATELETS

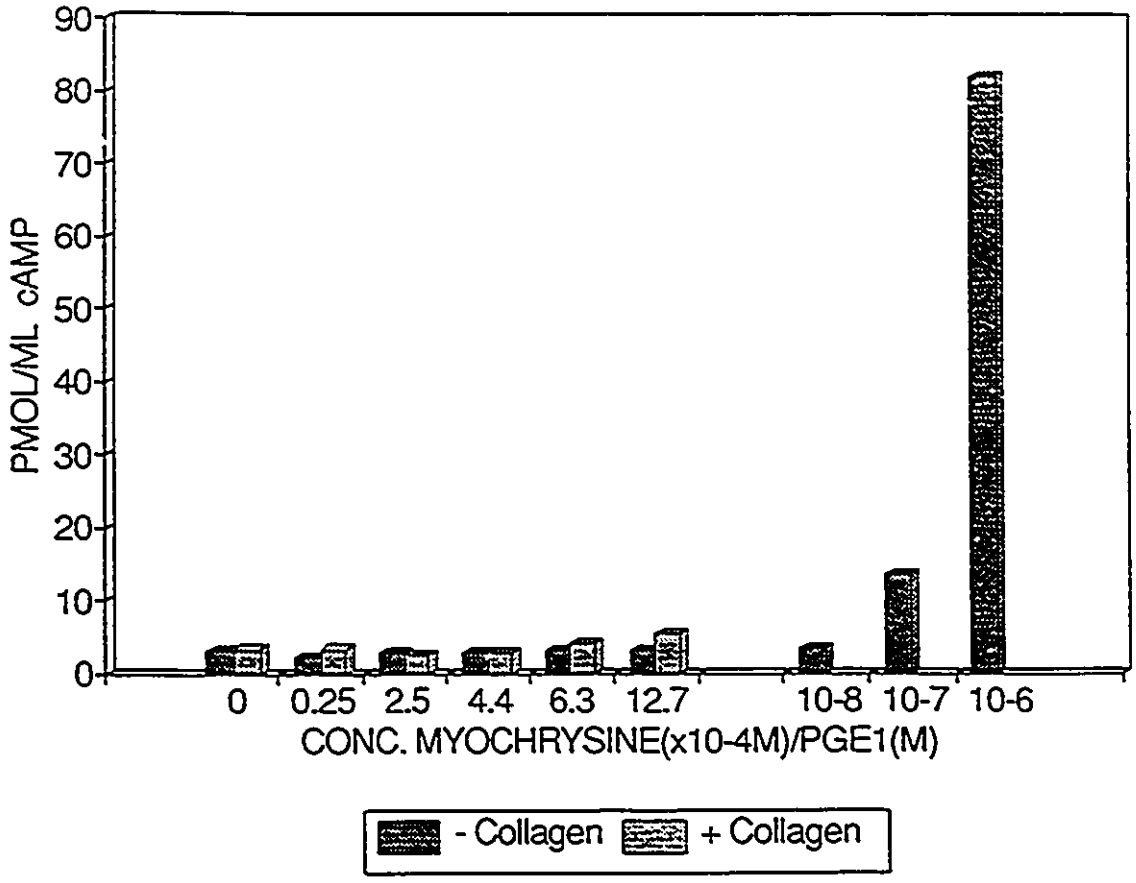
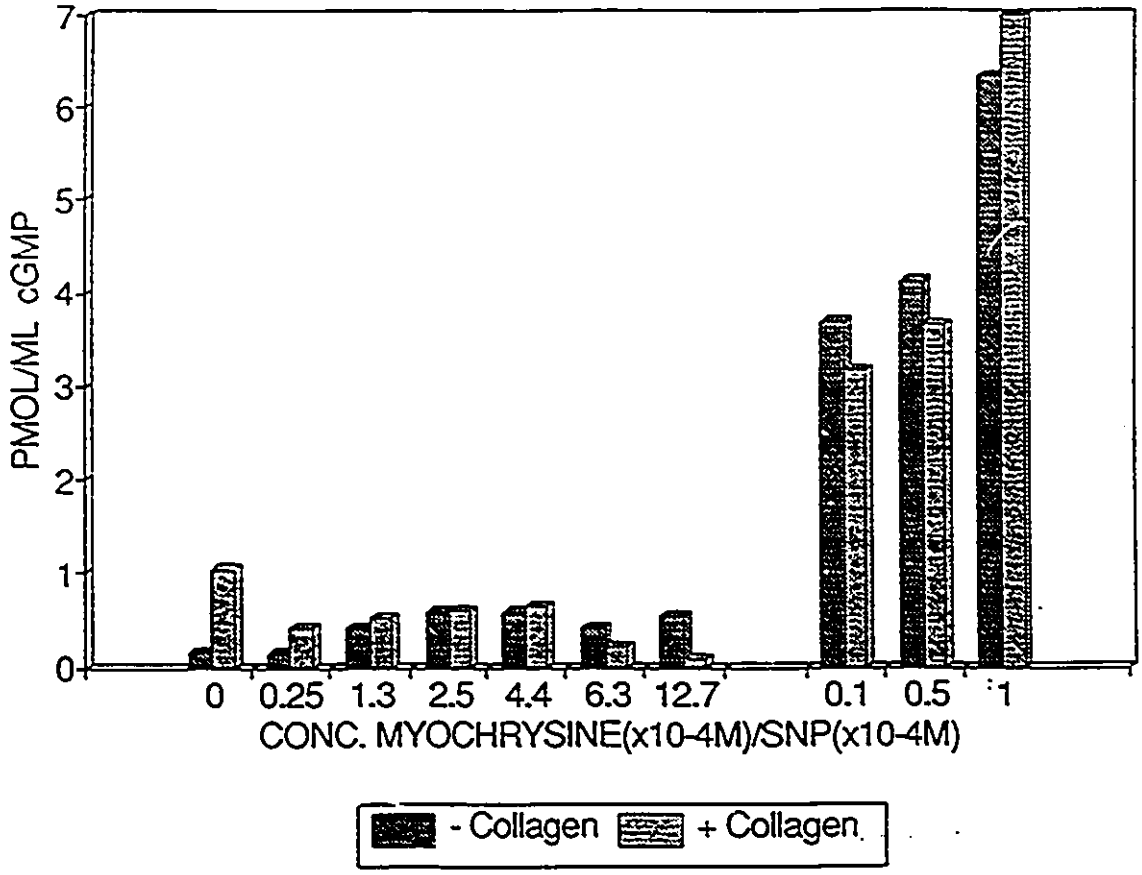


FIGURE 6.1.12 EFFECT OF MYOCHRYSINE ON cGMP LEVELS IN COLLAGEN ACTIVATED RABBIT PLATELETS



6.2 DISCUSSION

Activation of the PK-C/Ca^{2+} pathway in platelets by collagen results in phosphorylation of P20 or myosin light chain and of P40 or pleckstrin.(136) Myochrysine, 0.25 - 38 x 10^{-4}M, inhibited collagen induced P20 and P40 phosphorylation in human platelets and 2.5 -12.7 x 10^{-4}M Myochrysine inhibited collagen induced P20 and P40 phosphorylation in rabbit platelets. A large amount of inter- and intra- subject variation is seen in experiments examining the action of Myochrysine on human platelets. The results obtained here, however, mimic those seen in Chapter 3. That is, in those individuals in which Myochrysine inhibited collagen induced aggregation and serotonin release in Chapter 3, Myochrysine inhibited collagen induced P20 and P40 phosphorylation here. In those individuals in which Myochrysine did not inhibit collagen induced aggregation and serotonin release in Chapter 3, Myochrysine did not inhibit collagen induced P20 and P40 phosphorylation here.

Myochrysine, 0.25 - 1.3 x 10^{-4}M, caused slight stimulation of P20 and P40 phosphorylation in rabbit platelets. This is in contrast to results reported in Chapter 4 which showed that these concentrations of Myochrysine inhibited collagen induced aggregation and serotonin release. The reason for this stimulation is not known. That both phosphorylation of P20 and P40 were inhibited by Myochrysine is consistent with the action of this drug being at or before phospholipase C action (see Figure 1.1.2). Alternatively, Myochrysine may cause simultaneous inhibition of both sides of the pathway. To check this alternative, the action of Myochrysine on TPA and A23187 induced phosphorylation was examined. Results (Figures 6.1.6 and 6.1.9) show that Myochrysine did not inhibit either TPA induced P40 phosphorylation or A23187 induced P20 phosphorylation. This indicates that the action of Myochrysine is at or before bifurcation of the PK-C/Ca^{2+}

pathway.

Results from experiments examining the action of Myochrysine on A23187 induced activation showed that the drug enhanced platelet activation by this agonist. A23187 is the only agonist tested for which this was seen. Myochrysine inhibited the action of all the other platelet agonists tested.(Chapter 4) A23187 is different from the other agonists in that it does not act through receptor mediated events. Instead, the increase in intracellular calcium resulting from the ionophoric action of A23187, initiates platelet responses. It may be that as A23187 enters the platelet membrane, it disrupts the membrane in such a way as to unmask a sulfhydryl group involved in platelet activation. Myochrysine can then interact with this sulfhydryl group in a manner which results in enhanced platelet activation.

Figure 6.1.10 shows that Myochrysine did not appear to enhance P40 phosphorylation. The reason for this may be that P40 was fully phosphorylated by 1.0 μM A23187. Evidence which is consistent with this proposal is that Myochrysine, 6.3 - 12.7 x 10^{-4}M, enhanced 0.75 μM A23187 induced P40 phosphorylation.(data not shown)

One possible way in which Myochrysine could be inhibiting the PK-C/Ca^{2+} pathway, which is consistent with the findings presented here, is that Myochrysine may be causing increases in platelet cyclic nucleotides. Commercial RIAs were used to measure cAMP and cGMP levels in rabbit platelets exposed to Myochrysine with and without collagen stimulation. Cyclic nucleotide levels determined in controls from these experiments are in agreement with cyclic nucleotide levels reported in the literature (see Table 6.2.1).(212) Myochrysine, 0.25 - 12.7 x 10^{-4}M, did not increase either cAMP or cGMP levels in resting or in collagen activated platelets; thus Myochrysine does not inhibit platelet activation by increasing cyclic nucleotide levels.

TABLE 6.2.1: COMPARISON OF CYCLIC NUCLEOTIDE LEVELS DETERMINED WITH THOSE IN THE LITERATURE

Additions	Cyclic Nucleotide Levels (pmol 10^7 platelets^{-1})			
	Experimental cAMP	Literature cAMP[i]	Experimental cGMP	Literature cGMP[i]
Basal	13.39 ± 4.09 (3)[ii]	18	1.59 ± 1.07 (6)	0.9
Collagen	11.14 ± 2.62 (3)	18	2.70 ± 1.22 (6)	2.2
2 µM PGE_2	N.D.	159	N.D.	0.5
0.1 µM PGE_2	36.33 ± 6.45 (2)	34	N.D.	1.1
0.01 µM PGE_2	11.79 ± 2.31 (2)	N.D.	N.D.	N.D.
100 µM SNP	N.D.	37	15.73 ± 4.60 (2)	21.8
50 µM SNP	N.D.	N.D.	12.39 ± 3.19 (3)	N.D.
10 µM SNP	N.D.	N.D.	11.22 ± 0.17 (2)	N.D.
1 µM SNP	N.D.	33	N.D.	5.2

N.D. = Not Determined
i Taken from Haslam *et al.* (212)
ii Numbers in brackets represent the number of experiments performed.

Myochrysine, 0.25 - 12.7 x 10^{-4}M, inhibited slightly, cGMP levels in collagen activated rabbit platelets. This result is expected since collagen activation results in an increase in cGMP content within the platelet. The increase in cGMP is partly responsible for negative feedback inhibition.(170,171) Since Myochrysine inhibited collagen induced platelet activation, it is expected to inhibit collagen induced increases in cGMP levels.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE POSSIBILITIES

Myochrysine, a gold(I) based compound, has been shown to be an effective anti-rheumatic drug.(11) The precise mechanism of action of this drug is unknown. Evidence presented here provides new insight into the mechanism of action of Myochrysine.

Myochrysine inhibits collagen induced aggregation, serotonin release and phosphorylation of myosin light chain and pleckstrin in human and rabbit platelets in a dose-dependent mechanism. Since these cellular responses are mediated by the PK-C/Ca^{2+} pathway, it can be concluded that Myochrysine inhibits the PK-C/Ca^{2+} pathway at some point. The point of inhibition is a point which is common to several agonists of this pathway since Myochrysine inhibits ADP and U46619 induced activation as well as collagen induced activation. Kean *et al.* have shown that Myochrysine inhibits thrombin induced platelet activation.(88) Failure of Myochrysine to inhibit TPA induced pleckstrin phosphorylation and A23187 induced myosin light chain induced phosphorylation indicates that the action of Myochrysine is at or before phospholipase C action. Inhibition by Myochrysine occurs quickly, irreversibly and is similar to that caused by other sulfhydryl reacting compounds. This evidence suggests that the site of inhibition of Myochrysine is at a sulfhydryl group on the platelet membrane surface. Inhibition of collagen induced activation by the cell impermeant ESSE provides evidence that interaction of an extracellular sulfhydryl group can interfere with platelet activation. It has also been

shown that Myochrysine does not increase cAMP or cGMP levels in platelets. Thus, it is concluded that Myochrysine acts at the cell surface level to inhibit the PK-C/Ca^{2+} pathway by interfering with an essential sulfhydryl group in a manner which does not involve cyclic nucleotides.

Inhibition of phospholipase C or the putative G-protein which links phospholipase C to the membrane receptors by Myochrysine would be consistent with the phosphorylation data. Inhibition at either of these two sites would also be consistent with the finding that Myochrysine inhibits platelet activation by several platelet agonists.

G-proteins have been shown to be inhibited by sulfhydryl reacting compounds in other systems.(214,215) N-ethylmaleimide (NEM) has been shown to alkylate Gs in turkey erythrocyte membrane preparations.(215) Alkylation of Gs inhibits the ability of Gs to stimulate adenylate cyclase.(215) Inhibition of Gs by NEM was also found by Lipson *et al.* in rat liver plasma membrane.(214) The effect of Auranofin, an oral, gold-based, anti-rheumatic drug, and sulfhydryl reacting compounds were tested on purified PL-C activity and on PL-C activity in sonicated macrophages.(216) Auranofin was found to stimulate PL-C activity while neither NEM or glutathione had any effect on the enzymes activity.(216) The effect of this gold compound was therefore believed to be specific to Auranofin and not to be a thiol effect.(216)

It is highly unlikely that Myochrysine would have a direct effect on PL-C or the G-protein linking PL-C to membrane receptors in intact platelets since both these proteins are intracellular.(217) Although Korner and Lipson both reported reaction with a sulfhydryl group on Gs inhibited the function of Gs, these experiments used membrane preparations and not intact cells.(214,215) Since it has been shown that the action of Myochrysine is likely extracellular, inhibition of phospholipase C and/or G-proteins is not consistent with the data presented.

There is only one potential site at which Myochrysine may inhibit platelet activation which is consistent with the data presented. That site is platelet membrane receptors which are linked to the PK-C/Ca^{2+} pathway.

Several receptors which are linked to G-proteins in other systems have been isolated and cloned. All of these receptors have been found to share a common structure.(209) Common features of these receptors are seven transmembrane domains, an extracellular amino-terminal and an intracellular carboxy-tail.(209) In a review by Dohlman *et al.* it was reported that there are several cysteines in the extracellular regions linking transmembrane regions II and III and in the stretch linking transmembrane regions IV and V.(209) Recently, the thrombin receptor, which is linked to PL-C through a G-protein was also found to share the same structure as the other G-protein-linked receptors.(218) The thrombin receptor has an extracellular amino terminal, seven transmembrane regions, and an intracellular carboxy terminal.(218)

If all the platelet receptors linked to the PK-C/Ca^{2+} pathway share a similar structure and, more importantly, have a common important, extracellular, structural sulfhydryl group, it is possible that Myochrysine inhibits platelet activation through interaction with this sulfhydryl group. Interaction with the sulfhydryl could result in a transformational change of the receptor in such a way as to uncouple it from its G-protein and prevent activation of phospholipase C. This type of interaction is seen in turkey erythrocyte membrane preparations treated with NEM or ESSE.(214) Reaction of these compounds with the glucagon receptor at a sulfhydryl group uncouples the receptor from Gs and prevents activation of adenylate cyclase.(214) Interaction of Myochrysine with a common receptor sulfhydryl group resulting in inhibition of transmembrane signalling is consistent with all of the data presented.

The site of the reactive, extracellular sulfhydryl group is unknown but it is unlikely to be within the ligand binding site since Myochrysine does not inhibit platelet shape change. This indicates that inhibition is occurring after ligand binding and initiation of activation has occurred.

In addition to providing insight into the mechanism of action of Myochrysine, this report provides insight into the active component of the drug. Although results indicate that TMA is likely the portion of Myochrysine which is providing the majority of the inhibitory effect in terms of platelet aggregation and serotonin release, the gold(I) portion of the drug has also been shown to interact with the platelet membrane surface. Compounds such as chloro[tris(2-pyridyl)phosphine]gold(I) and chloro(t-butylisocyanide)gold(I) provide additional evidence that gold(I) is capable of interfering with platelet activation.

There are several possibilities for further research of the drug Myochrysine. Investigations of the action of Myochrysine on the PK-C/Ca^{2+} pathway with respect to the interaction of Myochrysine with platelet membrane receptors is warranted. Studies of the action of Myochrysine in other cell types involved in the mediation of rheumatoid arthritis are necessary.

Now that the thrombin receptor is isolated and cloned (218) it should be possible to investigate the interaction of Myochrysine with the purified thrombin receptor. Investigation of the interaction of Myochrysine with other platelet receptors may await identification, isolation and cloning of these receptors. Radioactive Myochrysine or radioactive sulfhydryl reacting compounds may be useful in identifying platelet receptors involved in PL-C activation. It may be possible to incubate platelets with radioactive sulfhydryl reacting compounds, expose the platelet proteins to SDS-PAGE, and through the use of autoradiography,

identify the proteins with which these compounds interact.

Another avenue of future research is to investigate the action of Myochrysine and/or components of Myochrysine in humans. Reaction to Myochrysine is variable between individuals with regard to platelet reactivity, clinical efficacy and toxicity. It would be of interest to investigate whether there is a correlation between Myochrysine induced platelet activation or Myochrysine inhibition of collagen induced platelet activation and clinical toxicity or efficacy to Myochrysine. If a correlation exists, a simple test for toxicity or efficacy would be generated.

In chapter 5, it was noted that dl Myochrysine (synthesized in the laboratories of Dr. C.J.L. Lock) was more potent than commercial Myochrysine. It would be of interest to determine whether the purer dl Myochrysine provides the same clinical benefit in patients at a lower dosage, or is less toxic than the currently used Myochrysine.

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APPENDIX 1

Calculations for the Determination of Statistical Significance

Calculations for the determination of statistical significance were made with the two-sample t test for indepedent samples with unequal variances as per pp. 247-248 of Fundamentals of Biostatistics by B. Rosner, Boston: Duxbury Press, 1982.

Hypothesis to be tested:

H_o: $x_1 = x_2$; H_1: $x_1 \neq x_2$, where x_1 and x_2 = mean

Test Statistic

$$\lambda = \frac{(x_1 - x_2)}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$, s = standard deviation

Approximate upper $\alpha/2$ percentile of $\lambda = y_{1-\alpha/2}$

$$y_{1-\alpha/2} = \frac{[(s_1^2/n_1)t_{n1-1,1-\alpha/2} + (s_2^2/n_2)t_{n2-1,1-\alpha/2}]}{(s_1^2/n_1) + (s_2^2/n_2)}$$

$t_{n-1,1-\alpha/2}$ are taken from Table III, pp 458 of Fundamentals of Biostatistics.

If $\lambda > y_{1-\alpha/2}$ or $\lambda < y_{1-\alpha/2}$, reject H_o.

If $-y_{1-\alpha/2} \leq \lambda \geq y_{1-\alpha/2}$, accept H_o.

1. ID_{50} for the action of Myochrysine on collagen induced rabbit platelet aggregation (x_1) versus ID_{50} for the action of Myochrysine on collagen induced rabbit platelet serotonin release (x_2).

$x_1 = 5.35 \times 10^{-4}M$ $x_2 = 4.33 \times 10^{-4}M$

$s_1 = 3.19 \times 10^{-4}M$ $s_2 = 2.28 \times 10^{-4}M$

$n_1 = 17$ $n_2 = 14$

$\alpha = 0.4$

$\lambda = 1.04 > y_{.80} = 0.87$, therefore reject $x_1 = x_2$.

2. ID_{50} for the action of Myochrysine on collagen induced human platelet aggregation (x_1) versus ID_{50} for the action of Myochrysine on collagen induced rabbit platelet aggregation (x_2).

$x_1 = 2.82 \times 10^{-4}M$ $x_2 = 5.35 \times 10^{-4}M$

$s_1 = 1.91 \times 10^{-4}M$ $s_2 = 3.19 \times 10^{-4}M$

$n_1 = 6$ $n_2 = 17$

$\alpha = 0.1$

$\lambda = 2.30 > y_{.90} = 1.88$, therefore reject $x_1 = x_2$.

3. ID_{50} for the action of Myochrysine on collagen induced human platelet serotonin release (x_1) versus ID_{50} for the action of Myochrysine on collagen induced rabbit platelet serotonin release (x_2).

$x_1 = 2.51 \times 10^{-4}M$ $x_2 = 4.33 \times 10^{-4}M$

$s_1 = 2.37 \times 10^{-4}M$ $s_2 = 2.28 \times 10^{-4}M$

$n_1 = 6$ $n_2 = 14$

$\alpha = 0.2$

$\lambda = 1.59 > y_{.90} = 1.44$, therefore reject $x_1 = x_2$.

4. ID_{50} for the action of Myochrysine on collagen induced rabbit platelet aggregation in an albumin buffer system (x_1) versus ID_{50} for the action of Myochrysine on collagen induced rabbit platelet aggregation in a gelatin buffer system (x_2).

$x_1 = 5.35 \times 10^{-4}M$ $\quad$ $x_2 = 1.57 \times 10^{-4}M$

$s_1 = 3.19 \times 10^{-4}M$ $\quad$ $s_2 = 1.12 \times 10^{-4}M$

$n_1 = 17$ $\quad$ $n_2 = 3$

$\alpha = 0.10$

$\lambda = 3.74 > y_{.95} = 2.23$, therefore reject $x_1 = x_2$.

5. ID_{50} for the action of Myochrysine on collagen induced rabbit platelet aggregation (x_1) versus ID_{50} for the action of Myochrysine on U46619 induced rabbit platelet aggregation (x_2).

$x_1 = 5.35 \times 10^{-4}M$ $\quad$ $x_2 = 9.70 \times 10^{-4}M$

$s_1 = 3.19 \times 10^{-4}M$ $\quad$ $s_2 = 2.47 \times 10^{-4}M$

$n_1 = 17$ $\quad$ $n_2 = 2$

$\alpha = 0.30$

$\lambda = 2.28 > y_{.90} = 1.82$, therefore reject $x_1 = x_2$.

6. ID_{50} for the action of Myochrysine on collagen induced rabbit platelet aggregation (x_1) versus ID_{50} for the action of TMA on collagen induced rabbit platelet aggregation (x_2).

$x_1 = 5.35$ $\quad$ $x_2 = 3.13$

$s_1 = 3.19$ $\quad$ $s_2 = 1.4$

$n_1 = 17$ $\quad$ $n_2 = 4$

$\alpha = 0.10$

$\lambda = 2.13 > y_{.95} = 2.02$, therefore reject $x_1 = x_2$.