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DEDICATION

To my family and friends for their support and encouragement

and

To the spirit, determination and perserverance of

Jerry Taichman.

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The University of Ottawa Department of Cardiothoracic Surgery is encouraging its residents to pursue careers which combine clinical and research responsibilities. An exposure to basic research methodology is essential to meet these goals. I would like to thank Dr. Wilbert Keon and the Department of Cardiovascular Surgery for providing me with the opportunity to spend a year in the Department of Physiology doing basic research.

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ABSTRACT

The complement system is made up of a series of self-assembling proteins which act together in the nonspecific arm of the immune defense system. Complement may be activated by artificial surfaces such as those found in the cardiopulmonary bypass machine (CPB) which may lead to post-CPB complications. Since it may function independently of antibody, complement activation may also be a factor in myocardial dysfunction seen after CPB.

To study this problem, human right atrial trabeculae contracting isometrically in vitro were used to assess myocardial function in four solutions: Tyrode's, autologous blood, plasma and denatured plasma. Muscle function was assessed by developed (DF) and resting force (RF) and mean rate of developed force (MRDF), all expressed as relative to control. Complement was activated with the addition of Zymosan in blood and plasma with Tyrode's solution and denatured plasma acting as controls. After initial control measurements were performed, the bath was emptied and replaced with the test solution. In the blood, plasma and denatured plasma groups, DF fell from 29.5 to 44.3% of control ($p < 0.001$). This was probably a result of lower ionized calcium concentrations within these solution compared to Tyrode's since the calcium concentration correlated with the percent drop in DF ($r = -0.94$).

The DF, RF and MRDF were similar between all groups at any given time by a 1-way ANOVA. The intercepts

and slopes of the decay in the preparation showed no statistically significant difference. Concentrations of C3 and C4 were not significantly different between most of the groups. However, CH50 fell during the blood and plasma experiments in control and Zymosan groups to the same extent ($p < 0.001$) indicating complement activation in both. Tyrode's solution and denatured plasma had no detectable complement activity.

Complement was activated by the experimental system as indicated by a fall in CH50 by 12% ($p < 0.01$) after 1 minute. In addition, dipyridamole is given to the patients prior to surgery which may have effects on complement. There was no statistically significant difference in degree of complement activation between patients who were receiving dipyridamole and those who were not.

It is concluded that complement activation does not directly affect myocardial performance. Dipyridamole does not effect complement activity. Foreign surfaces and oxygen bubbling activates complement.

INTRODUCTION

1.0 Preamble. Complement acts in the nonspecific arm of the immune defense system of the body. Clinical and laboratory studies have shown that activated complement may be responsible for some cardiopulmonary dysfunction following cardiopulmonary bypass (CPB) used for open heart surgery. There is a lack of information on how the myocardium is affected. The purpose of the experiments presented herein was to explore the direct action of complement on myocardium. This thesis will present an overview of the structure and function of the complement system, as well as the current state of knowledge of how the complement system may be implicated in causing myocardial dysfunction. The model used in the experiments will be assessed both in terms of reasons for use and muscle mechanical parameters measured. Finally, the experimental method and results will be presented and discussed.

1.1 The Complement System. In order to understand how the complement system may be implicated in myocardial dysfunction following CPB, it is necessary to discuss the normal physiology of the complement system. The following is a brief synthesis of various excellent reviews on the subject by Muller-Eberhard (1), Osler (2), Brown (3), Rother (4), Spitzer (5), and Roitt et al (6). Additional information not found above is referenced separately.

The complement system is made up of a series of self-assembling proteins which act as primary humoral

mediators of the immune response. Complement works via two separate pathways: classical and alternative.

The classical pathway (figure 1) is activated by IgG and IgM complexes (as well as nonimmunologic substances such as the acute phase reactants, urate crystals, heparin and protamine complexes and some bacterial glycolipids). It is divided into three functional units each consisting of various subunits: the recognition unit C1: C1q, C1r, C1s; the activation unit C2, C3, C4; and the membrane attack system C5, C6, C7, C8, C9. The cascade begins with the recognition of antibody by the C1q constituent of C1. It appears that C1q must interact with two or more antibodies for firm binding of C1. This enables it to undergo enzymatic activation with various structural changes occurring in C1r and C1s. The formed enzyme C1-esterase is capable of cleaving multiple C4 proteins thus leading to an amplification effect. The C4b fragment then binds C2 and in the presence of magnesium ion, is cleaved by C1-esterase. The C2a fragment and C4b then combine to form a new enzyme (C4b2a) which binds with and cleaves C3. C3b is formed and combines with C4b2a to form the C5 convertase. The steps that follow are common to both pathways and are discussed below.

The alternative (or properdin) pathway can be activated by IgA aggregates, polysaccharides (including zymosan and inulin) (7), lipopolysaccharides, endotoxin, bacterial and viral membranes, proteins such as

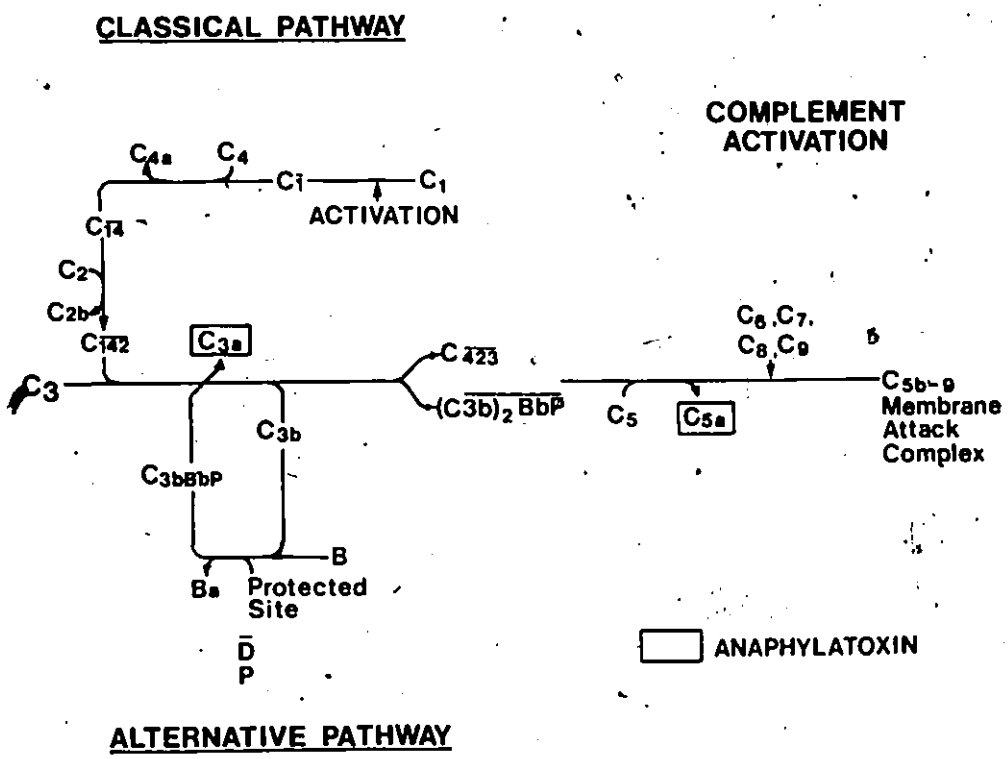

FIGURE 1: The Complement Cascade adapted from Brown et al (3). Shown are the pathways for complement activation: classical and alternative.

plasmin and thrombin, cobra venom factor and C3 nephritic factor (5), homogenized muscle (8), and heart subcellular membranes (9). The component with which the other factors interact is C3b. However, it is not well understood how the C3b is generated for this pathway. It may be the result of classical pathway activation or there may be another enzyme responsible for C3 cleavage. In the presence of magnesium ion, C3b binds to Factor B which exposes a site that acts as a substrate for the proteolytic enzyme, Factor D. The interaction results in a bimolecular complex, C3bBb which then acts as a C3 convertase. This causes further breakdown of C3 into its components. C5 convertase is a product of properdin (P), C3b and factor B: [P(C3b)*2Bb].

It is at this point that the two pathways converge. C5 is cleaved by the actions of C5 convertases produced by the classical and alternative pathways. In addition, trypsin, plasmin, and proteinases of polymorphonuclear leukocytes, macrophages, platelets and bateria may cleave C5, though the products do not have the same biological activity as those produced through pathway cleavage. C5b is liberated and binds C6 and C7. This complex allows for the absorptive binding of C8 which in turn binds three to six C9 components. It is the resultant C5-9 complex which is responsible for cytolysis.

1.2 Mechanisms of action. Once activated, complement may lead to dysfunction either locally at the cellular level or systemically to involve the entire organism. These actions

function for the defense of the organism against foreign substances (bacteria, viruses, toxins, etc.) however, the same mechanisms may be turned against the host under abnormal circumstances (discussed in Section 2).

1.2.1 Cytolysis. The cytolytic complement reaction is initiated with the attachment of antibody to a cell membrane (usually, an invading organism). As described above, the C1 recognition unit binds reversibly to the site. The C1s component activates C2 and C4 leading to the formation of C3 convertase at a topologically distinct site. C3 is cleaved and the C4b2a3b complex results in the cleavage of C5. At a different site, the C5-9 complex is formed which inserts itself either partly (by the C8 component) or completely into the cell membrane. The complex then allows exchange of intra and extracellular components (small molecules and ions, but not macromolecules) by means of an internal hydrophilic channel. The damaged membrane then allows increased water into the cell causing swelling and cytolysis. Conductivity changes have also been detected across the lipid bilayers attacked by C5b-9.

Although the cytolytic reaction usually occurs following C1 binding to antibody, C4 can bind to a cell in the absence of antibody, providing C1s is present in solution. There is then the potential for antibody-independent cytolysis.

1.2.2 Opsinization of foreign particles. Complement has

been suggested as having a role in phagocytosis by adhering to foreign agents such as bacteria to promote phagocyte recognition of the agent and their removal. The C3, C4 and C5 components are involved with C3b having been identified as having the major role (5).

1.2.3 Polymorphonuclear leukocyte activation. Activated complement has important actions on polymorphonuclear leukocytes (PMN's). Complement leads to sequestration and margination of PMN's as well as stimulating their release from the bone marrow. In particular, C5a is directly reactive with the PMN membrane to alter surface charge (10,11) and seems to be the most important component which results in greater adhesiveness. PMN's then aggregrate which leads to stasis and embolization in small vessels (12). C5a is also a potent chemoattractant for PMN's and stimulates the selective release of lysosomal constituents (4,13,14). These enzymes may lead to the production of toxic oxygen radicals which have damaging effects on endothelial cells (15,16,17) and the sarcolemma of myocardial cells (18,19).

1.2.4 Formation of anaphylatoxins. Whereas the above three modes of action work at the cellular level, anaphylatoxins may cause systemic reactions. Following enzymatic cleavage of C3, C3a is produced. Receptors for C3a have been identified on numerous cell types, the combination with which leads to various reactions. When active with mast cells and basophils, degranulation occurs

with release of histamine and other mediators of anaphylaxis. Smooth muscle contraction is also produced either by direct action of the anaphylatoxin on cellular receptors or secondary to histamine release. C3a suppresses T-cell mediated immunoglobulin secretion.

C5a is a component formed by C5 convertase. It is more potent in its anaphylactoid function than C3. In addition to the mast cell, basophil and smooth muscle effects, C5a leads to increased vascular permeability of endothelium which is histamine independent. Receptors for C5a are present on neutrophils and monocytes. When activated by C5a, directed locomotion or chemotaxis is induced. C5a also enhances immunoglobulin secretion.

Recently, C4a has been shown to have weak anaphylactoid characteristics.

1.2.5 Other roles of complement. The activation of complement has been associated with factors which lead to clotting, thrombolysis and release of kinins and prostaglandins (5,20). Hageman factor (factor XII) has been shown to be a common regulator in the these systems (20,21) and can be inhibited by C1 esterase inhibitor. They are all part of the nonspecific inflammatory response and it is logical to assume that if one component is activated, then all are affected. Release of kinins and prostaglandins have effects on the microvasculature of most organs including lungs (22).

1.3 Control of the Complement Cascade. The complement

cascade is controlled by two mechanisms. Firstly, the binding sites of C1 rapidly decay as do the enzymes containing C2 (ie. C4b2a and C4b2a3b). Secondly, there are many enzymes present in the serum which destroy activated complement. C1 esterase inhibitor and C3b/4b inactivator (Factor I) act on their respective components. Factor H accelerates the dissociation of Bb from C3b. Carboxypeptidase-N rapidly cleaves C3a and C5a anaphylatoxins. The complement reaction is therefore restricted in both the time and place it can occur. Heparin in high doses may also interfere with the alternative pathway by increasing the affinity of Factor H for C3b.

2.0 The roles of activated complement in disease.

Complement has a role in the nonspecific immune system and inflammatory response as discussed above. The immune system may turn against the host and cause multiple organ dysfunction including collagen vascular diseases (rheumatoid arthritis), immune complex disease (glomerulonephritis) and autoantibody diseases (Hashimoto's thyroiditis) (23). The use of artificial organs has provided another method for complement activation in which blood is exposed to man-made surfaces. This has led to the implication of complement in the pathogenesis of cardiopulmonary problems seen with the use of these devices. In addition, interest in various other diseases of the heart and lungs has resulted in experiments which implicate the complement system in the

pathophysiology of the following disorders.

2.1 Dialysis and Leukophoresis. In the early days of hemodialysis, cellophane was the material used for the dialyzing membrane. Mild to severe pulmonary dysfunction (including a drop in pO2 and decreased O2 diffusion) was noted in most patients during and shortly after dialysis. This was associated with a severe transient neutropenia of early onset. Experiments revealed that cellophane-incubated plasma transfused into animals produced neutropenia and pulmonary dysfunction associated with gross pulmonary vascular leukostasis. Zymosan (a complement activator) treatment of the plasma lead to the same results but heat treated plasma caused no neutropenia (11,24). It was concluded that cellophane activated complement which lead to the adverse reaction. Similar findings occurred in patients undergoing leukophoresis. C5a levels were found to be elevated in both patients undergoing nylon fibre ultrafiltration and in plasma incubated in nylon. Pulmonary dysfunction was felt to be caused by complement activation with the subsequent pulmonary sequestration of activated PMN's (25).

2.2 Cardiopulmonary Bypass. The cardiopulmonary bypass machine (CPB) is a device which artificially oxygenates blood and maintains nonpulsatile circulation, thus bypassing a patient's heart and lungs. The entire blood volume is exposed to many artificial surfaces including tubing and an oxygenation apparatus. Oxygen bubbles pass through the blood

which is circulated by a compression action of a roller pump. Kirklin and his group (26) feel that these factors lead to a whole-body inflammatory reaction which is variable in magnitude but whose effects are transient.

The bypass machine affects cellular components of blood. Red blood cells are lysed and platelet numbers are decreased and function altered (27). Plasma proteins are denatured probably at the gas/liquid interface which seems to affect globular proteins the most. The globular molecule unfolds thus creating changes in physical properties (28,29). Gamma immunoglobulins (IgG) have been noted to fall in concentration during CPB (30,31) and has been suggested as one of the reasons for impaired host defense after such surgery.

There has been a lot of interest in the effect of CPB on the complement system. Unfortunately, most of the studies are difficult to compare as they use different methods for measuring complement as well as complement related changes. However, most groups report a consumption of C3 and C4 during and after CPB (31,32,33,34,35,36,37,38) often with a recovery to normal levels within 24 hours after surgery (32,36,37). The CH50 assay reflects complement activity and this is reduced after CPB meaning that complement has been consumed (30,33,39). When specific components are assayed, C3a levels (21,40) and C3d levels (37) are elevated indicating activation. Complement activation may be either via the classical or alternate

pathways (37). Activators include denatured proteins (both complement and IgG) (29), the artificial surfaces of the CPB, and heparin/protamine complexes (41).

Although the phenomenon of complement activation probably occurs, the effects of this activation is less clear. Kirklin et al (26) showed that a high C3a level was a risk factor for postoperative cardiac and pulmonary dysfunction. PMN sequestration has been proposed as the mechanism for organ dysfunction particularly in the lungs. PMN's decrease in number while on bypass but this effect appears short-lived after CPB termination (16). The decrease in PMN's is caused by their sequestration mostly in the lungs and in clinical settings, this has not been found to change pulmonary function (42). Other groups which note complement activation are not able to correlate it with complications following CPB (33,38,40).

Steroids have been used to minimize complement activation. Cavarocchi et al (43) showed that methylprednisolone prior to CPB reduced both the amount of complement activated and the degree of pulmonary PMN sequestration. However, they were unable to show any benefit with respect to pulmonary function in the steroid treated group. Conversely, steroids were found to have no effect on complement activation by two groups (34,38).

Following CPB, toxic oxygen radicals may be produced by activated PMN's or ischemic cells. Myocardial dysfunction may be reduced by the addition of oxygen radical

scavengers (41,45,46).

2.3 Protamine/heparin reaction. In order to prevent activation of the clotting mechanism during CPB, heparin is given. Following surgery, protamine sulphate is used to complex and neutralize heparin. Unfortunately, severe reactions have been reported following protamine administration which include hypotension, anaphylactoid responses and pulmonary vasoconstriction (47).

Although heparin is known to inihibit the C3 convertase of the alternate pathway (3,48), the newly formed polyanion/cation complex of heparin/protamine causes complement activation (49) by the classical pathway (49,50,51). Complement is consumed (48) and there is an elevation of C3a and C4a (41,50). However, these last two reports do not identify any hemodynamic changes associated with complement activation.

2.4 The post-pump syndrome. The post-pump syndrome is characterized by coagulopathies, a systemic inflammation-like reaction and profound organ dysfunction (36,40). The syndrome of "Post-perfusion lung" is constituted by severe pulmonary dysfunction secondary to interstitial edema that leads to increased work of breathing and increased alveolar-arterial oxygen difference (21). These disorders are thought to be a result of complement activation by CPB.

2.5 The Postpericardiotomy and Post Myocardial Infarction Syndromes. Both the postpericardiotomy syndrome (PPS) and postmyocardial infarction syndrome (PMIS) are inflammatory

disorders. They are characterized by fever and pericarditis which occur from days to weeks after open heart surgery (52,53) or myocardial infarction (54,55). The presence of anti-heart antibodies has been found in patients with PPS (52,53,56,57,58). It has been suggested that the antibodies may be directly cytotoxic (57) or create an inflammatory response in the epicardium leading to pericarditis. In PMIS, complement activation has been noted by Earis et al (54) by the presence of an elevated C3d. They suggest that antiheart antibodies and cardiac antigens combine to form immune complexes which are deposited in various sites leading to complement mediated tissue damage.

2.6 Myocardial Infarction. With myocardial infarction (MI), complement is activated (59). Rossen et al (60) showed that C1q accumulated in areas of ischemic myocardium after 45 minutes with a concomitant rise in leukocyte concentration. This was thought to be due to a rise in C5a, though this was not actually measured.

Several groups have experimentally blocked C3 activation by cobra venom factor (CVF) and found that infarct size was reduced (61,62,63). Jacob (64) suggested that complement-induced leukoembolization may extend infarction. By inhibiting PMN aggregation with ibuprofen and methylprednisolone (65,66), they were able to show a decrease in infarct size. PMN damage is mediated by formation of toxic oxygen radicals which cause cell membrane breakdown with influx of sodium and calcium ions (19,67).

2.7 Adult respiratory distress syndrome. Following trauma, pancreatitis, CPB, surgery, and endotoxic shock, the adult respiratory distress syndrome (ARDS) may develop (68). ARDS is characterized by increased lung water with reduced compliance leading to arterial hypoxemia (69). All of the above conditions are known to activate complement with the production of C5a, a PMN chemoattractant. C5a activated PMN's in the lung release toxic oxygen species which leads to pulmonary endothelial damage, increased permeability and increased lung water (13,70,71). When rendered neutropenic, experimental animals are resistant to ARDS (68,72). Methylprednisolone reduces the pulmonary edema (73) by decreasing PMN fragmentation and enzyme release (69).

2.8 Other Tissue Lesions. When complement is activated experimentally and injected into the circulation of various organs, damage results. Seelig et al (74) showed that acinar necrosis occurred when the complement activators were injected into rat pancreas associated with a infiltration of PMN's. Similarly, hepatic cellular necrosis occurred when the activators were injected into the portal vein. They concluded that the complement system may exert a cytolytic effect on parenchymal cells which is antibody-independent, acting through the alternate pathway.

3.0 Experimental Model. Complement activation has been linked to the dysfunction of various organs as outlined above. This occurs nonspecifically without the need for

antibody recognition of foreign material. Complement appears to be able to localize in tissues and either directly or indirectly (via PMN's, etc.) cause damage. Since CPB leads to activation of the complement system, can myocardial dysfunction be explained by this phenomenon?

It is not possible to readily isolate the complement system and therefore it must be used within its blood or plasma medium. An isolated muscle preparation allows for the alteration of the bath solution in which the muscle is contracting. Blood or plasma can be used in the bath and complement can be activated in several ways to be discussed (see Methods section 2.0). The isolated muscle preparation has been generally accepted as a useful means to assess function.

To avoid possible species differences in immune systems and myocardial function, a human isolated myocardial muscle model was selected. Muscle has been taken from the atrium (trabeculae, atrial strips or whole atria) and ventricle (papillary muscles and muscle strips) taken from patients undergoing open heart surgery.

A brief review of basic myocardial structure and function is required to appreciate the isolated myocardial muscle model as well as to compare atrial and ventricular tissue.

3.1 Myocardial structure and function. The nature of the myocardium and its function have been well reviewed in works by Blinks and Koch-Weser (75), Sonnenblick (76), Braunwald

et al (77), Fabiato and Fabiato (78) and Alpert et al (79). The following is a brief synopsis of the main features.

The myocardium is mainly made up of interconnecting myocytes being somewhat smaller in size than those of skeletal muscle. They are organized in bundles called myofibrils which appear crossbanded or striated. The striations occur as a result of a repeating arrangement of contractile proteins which make up approximately 50% of the cell mass. Mitochondria, which are responsible for the oxidative phosphorylation processes within the cell, make up 25 to 30% of the myocyte mass. The remainder of the cell mass is made up of cytoplasm and other organelles. These include the sarcoplasmic reticulum which is important in calcium metabolism. The overall speed of contraction is related to the amount and structure of the sarcoplasmic reticulum within the cell. The myocyte is surrounded by the sarcolemma which consists of a plasma and basement membrane. The sarcolemma invaginates frequently, giving rise to a branching T-tubule network extending deep into the cell. Cells are interconnected via intercalated discs which are of three types. The fascia adherens serves to attach myofibrils to the cell membrane whereas the macula adherens fixes neighbouring cells together. Together, they allow myofibrils and myocytes to contract in series. The nexus is a gap junction which is a low resistance passage for electrical impulses between cells. The intercalated discs permit coordination of function resulting in a

functional syncitium of myocardial cells.

The sarcomere is the basic repeating segment along the myofibril bounded by 2 dark Z-lines every 1.6 to 2.2 um. The contractile proteins are arranged in 2 types of myofilaments. The thick filament is limited to the A-band within the sarcomere. It is composed of longitudinally stacked myosin chains made up of rodlike light meromyosin and the double globular headed heavy meromyosin. The thin filament consists of a double helix of actin and a double helix of tropomyosin adjacent to the actin. The regulatory protein troponin is associated with tropomyosin. The thin filaments are attached to the Z-line and interdigitate between the thick filaments.

The function of the myocyte is to contract and generate force. Calcium is essential for this to occur. Myocardium contains 2.5 mmole/l of calcium which is variably distributed between the sarcoplasmic reticulum, sarcolemma, mitochondria, myofibrils and nuclei. It is present in the resting state in the myoplasm at a concentration of 0.0001 mmole/l. Contraction may be elicited by electrical depolarization of the sarcolemma. This leads to an increase in intracellular calcium concentration by influx of calcium through sarcolemmal slow channels, the Na-Ca ATPase pump and release from sarcoplasmic reticulum stores.

Calcium then binds with great affinity to troponin. This results in a conformational change in tropomyosin which moves in relation to actin to reveal

binding sites that may interact with adjacent myosin strands. The heavy meromyosin chain has 2 globular heads which appear hinged on the light meromyosin chain. The protein of the head acts as an ATP-ase to cleave ATP and forms a cross-bridge with actin. Once the bond is formed, the myosin undergoes a conformational change which moves the the head in relation to the actin. The bond is then broken and ATP is regenerated to allow for further reactions as the myosin and actin slide upon each other, the so-called sliding filament mechanism. The process ceases once the membrane is repolarized, and calcium is either extruded from the cell or stored within the sarcoplasmic reticulum. The number of bonds formed and magnitude of force developed is therefore dependent on the influx of calcium in to the myoplasm.

3.2 Study of Myocardial Muscle Mechanics. A well-accepted method for studying myocardial muscle mechanics uses isolated myocardium contracting isometrically. This implies that the two ends of the muscle are fixed and contracting muscle exerts a force without changing length. Using this model, two properties of myocardium may be observed. Firstly, active developed force produced is a function of the length of the muscle prior to contraction, a statement of the Frank-Starling principle. The length at which developed force is greatest is the muscle's Lmax. Above or below Lmax, developed force will be less. Secondly, the property of contractility or inotropism is

unique to myocardium. It is characterized by a change in rate of force development at a given muscle length. An increase in velocity of contraction is usually accompanied by an increase in developed force. Increased intracellular calcium, sympathomimetic agents, cardiac glycosides and increasing frequency of contraction all result in increased velocity of contraction and developed forces. Increasing length will increase developed force but velocity of contraction will remain the same.

The solution in which the muscle is contracting is of great importance as it tries to simulate physiological conditions. A solution which produces vigorous contraction is not necessarily the most physiologic. Unfortunately, no artificial solutions will contain all substances carried in the extracellular fluid. Various solutions have been used with different electrolyte concentrations. Some have increased glucose uptake with the addition of insulin, while others have increased contraction by adding steroids. The temperature of the bath is important as the strength of contraction will increase with cooling over a considerable range. Since contraction relies on the availability of ATP, oxygen must be in good supply for oxidative phosphorylation. In crystalloid solutions, oxygen must be dissolved (0.02 ml O2/ml at 95% O2) by bubbling O2 gas through the solution. Hill (80) devised a formula for calculating the critical diameter for a muscle, beyond which the muscle core would be hypoxic. [redacted] calculation is very rough and a general rule

is to use muscles with the smallest possible diameters. The frequency of contraction is important as force will increase with frequency (ideal frequency for humans is 60 - 100 Hz) (81).

3.3 Atrial vs Ventricular muscle. The choice of source for myocardium is difficult. Normal human ventricular myocardium would be ideal however, it is not readily available. Autopsy material has been used but has questionable viability (82). There are inter-species differences in ventricular morphology and function, a list of which was prepared by Michael (83). Human right atrial appendages are available in good supply from centers doing open heart surgery. However, although this eliminates the inter-species comparison, atrial and ventricular muscle are different in several respects despite there common primordial origin.

Atrial and ventricular myocardium have been compared in different mammals including man. Atrial myocytes are generally smaller and have fewer T-tubules than ventricular myocytes (84,85,86). Atrial cells have unique granules which contain Atrial Natriuretic Factor and are important in fluid and electrolyte balance (87). Atrial layers are much thinner compared to ventricular with connective tissue forming a greater proportion of total wall thickness in the former (75). Atrial and ventricular muscle differ in myosin heavy chain constituents although the functional significance of this is not clear (88,89).

Atrial trabeculae are similar to ventricular papillary muscles in that fibers are oriented in a longitudinal axis (90,91) thus creating a more uniform contraction. The intercalated disc pattern in atria demonstrates a side-to-side connection in addition to the end-to-end arrangement seen between ventricular cells (86). Trabeculae are superior to muscle strips as they have a larger relative surface area permitting better oxygenation similar to papillary muscles (90).

Functionally, atrial muscles have a greater velocity of shortening compared to ventricular muscle probably due to a higher ATPase content in atrial muscle (89,92). Ventricular muscle creates a greater tension than atrial muscle (91,93,94). Ventricular muscle is also thought to be more stable and results more reproduceable than those with atrial muscle (95,96). Histamine has a similar effect on both ventricular and atrial muscle (96). Beta-blockade causes a greater developed force depression in atrial muscle compared to ventricular (97).

While accepting these differences but trying to avoid interspecies differences in both complement and myocardial function, a model using human right atrial trabeculae contracting isometrically <u>in vitro</u> was selected to demonstrate the effect of activated complement on myocardial performance.

STATEMENT OF PURPOSE

The complement system has been shown to be activated by the process of cardiopulmonary bypass. Serious disorders following CPB such as the post-pump syndrome and the adult respiratory distress syndrome have been linked to activated complement. In clincial practice, open-heart surgery is frequently followed by myocardial depression which is probably multi-factorial in origin. Activated complement can function nonspecifically which leads to the question: does activated complement affect myocardial function?

The known effects of activated complement are either direct or indirect. Complement may cause cytolysis acting by way of a membrane attack complex (C5b-9). Indirect effects include the chemotaxis and activation of polymorphonuclear leukocytes which may release toxic oxygen radicals that are damaging to most tissues. Histamine may be released from mast cells by anaphylatoxins (C3a and C5a) whereas kinins are released from indirect action on prekallikrien via Hageman factor. Any one of these actions could have detrimental effects on myocardial performance.

The purpose of this project was to assess the direct effects of activated complement on human right atrial trabecuale contracting isometrically <u>in vitro</u>. In order to identify a complement effect, several test solutions were used. Firstly, it was necessary to establish a control without complement in the system and to test the

complement activator (Zymosan) to assure the absence of its own negative effects. Human autologous blood was used to test the effects of complement on both cellular and humoral components of blood. In order to identify whether an effect was cell-mediated or not, autologous plasma was tested. Finally, to assess the effects of other plasma components other than complement as possible negative inotropes, denatured plasma was tested. Each solution was assessed using Zymosan suspended in saline as well as saline alone as a control.

Complement may be activated in many ways. It was therefore necessary to identify any complement activating factors in the equipment used for the experiment. Similarly, since human blood was taken from patients undergoing open heart surgery, the effects of drugs on complement activation had to be known. The only such drug with possible effects on complement is dipyridamole, a platelet inhibitor. Complement levels were compared between patients on the drug and control.

METHODS

1.0 Human right atrial trabeculae model. Human right atrial trabeculae have been used for the study of muscle mechanics (90,98,99), the effects of drugs on myocardium including morphine (100), histamine (101), beta-blockers (97,102), sympathomimetics (103) and steroids (104). The development of the human atrial trabeculae model used for the following experiments was described by Keon et al. (99). A major problem with other models using human atrial tissue is that the preparations lack uniformity in the variables which constitute the model. It was therefore the initial undertaking of Keon and his group to standardize the preparation in terms of stimulation rate, temperature and ionic composition of the bath solution so that maximum developed force would be produced consistently. The model was then adapted for use in the following experiments with changes being identified in the description of the individual experiments.

1.1 Adapted model. Prior to undergoing open heart surgery, patients signed a consent to enable the procurement of right atrial appendages at the time of cannulation for cardiopulmonary bypass (Ottawa Civic Hospital Research Ethics Committee, August 1984). Pediatric patients and those undergoing reoperation were excluded. Hemodynamic monitoring devices were placed at the time of surgery and patients were given a general anesthetic (a combination of high dose narcotic analgesic, muscle relaxant ±

inhalational anesthetic). CPB was established in the usual manner (105). The right atrial appendage was then clamped below the pursestring suture and then excised being careful to hold it by its edges. At the same time a syringe containing 10 cc of autologous arterial blood was prepared and the appendage was placed within it. The appendage was then taken to the laboratory as quickly as possible (10 mins).

In the laboratory, the appendage was placed on a piece of gauze and carefully inspected for suitable trabeculae. Once identified, the trabecula was removed and placed in a dish of Tyrode's solution. From past experience, trabeculae should be between 5 and 10 mm in length to be viable. Each appendage provides between 1 to 4 trabeculae. Patient data including identification numbers, age, sex, type of operation and surgeon were recorded.

The trabecula was then suspended between two stainless steel clips of the muscle stand (figure 2). The upper clip was attached to an isometric force transducer (Gould-Statham, model UC-2, Oxnard, California, USA) whose output was amplified and displayed on a direct writer-recorder (Grass driver amplifier model 7DA, low-level preamplifier model 7P1 and polygraph model 7, Quincy, Mass.,USA). The distance between the clips was adjusted to assure minimal tension of the muscle. The muscle was then submerged into a glass bath which was especially designed for this series of experiments. The average volume was 20

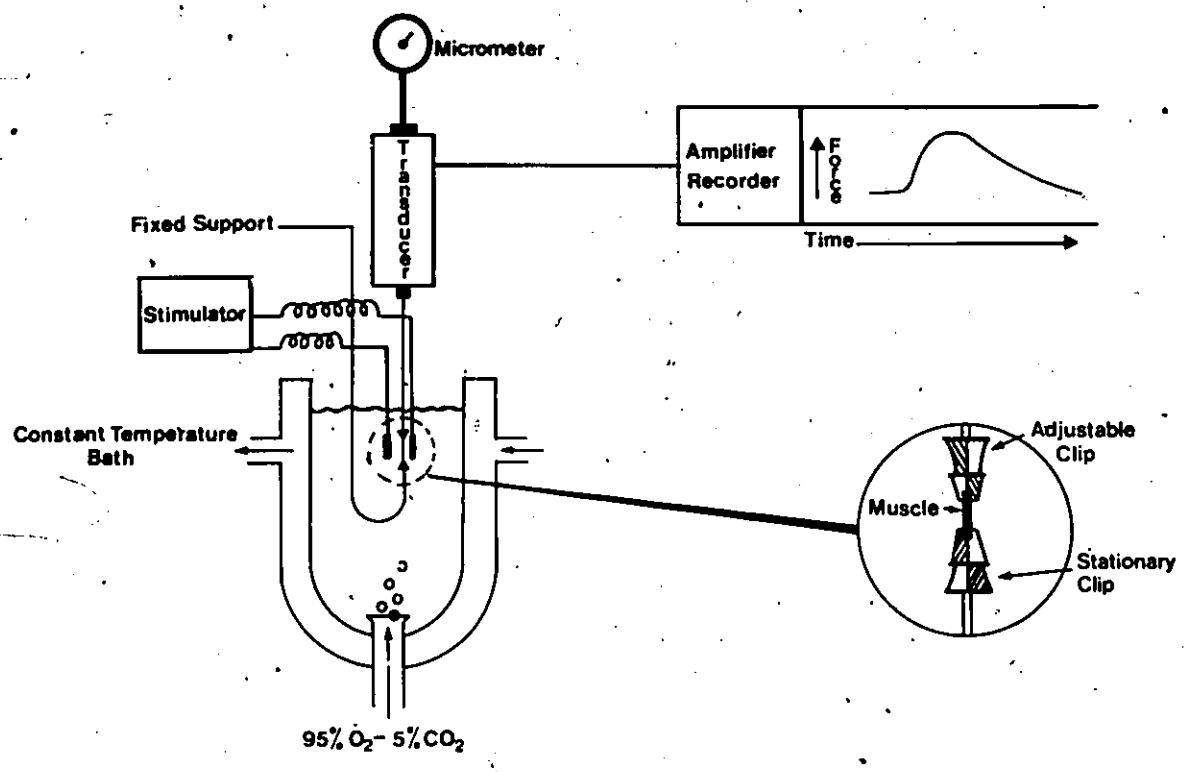

FIGURE 2: Human right atrial trabeculae contracting isometrically in vitro.

Schematic diagram of the muscle stand. The muscle is mounted between two clips (insert) and contraction is elicited by field stimulation at 1 Hz. The trabecula is placed in modified Tyrode's solution which is maintained at 34°C and aerated with a mixture of 95% O2/ 5% CO2.

mls (range = 18 - 20 mls) so as to minimize the amount of blood that had to be taken from individual patients. Contained in the bath was a modified Tyrode's solution with pH of 7.40 (NaCl 140, KCl 4.8, $MgSO_4$ 3.1, KH_2PO_4 1.2, $NaHCO_3$ 28, $CaCl_2$ 2.5, glucose 11 mmole/l) which was maintained at 34°C by a temperature regulated water jacket. The solution, was aerated with a 95% O_2/ 5% CO_2 mixture.

Field stimulation was then used to elicit contractions using platinum electrodes on either side of the trabecula. Stimulation pulses of 5 msec duration were produced by a Grass S88 stimulator (Grass Instrument Co., Quincy, Mass,USA). Both muscle length and stimulation voltage were increased alternatively (in increments of 0.03 mm and 2 volts, respectively) until muscle contraction began (Lo). Stimulation was then continued at 0.1 Hz at a voltage 20% above that required to elicit contraction for a period of 30 minutes. Then stimulation rate was increased to 1.0 Hz for the duration of the experiment. After 15 minutes allowed for equilibration, the muscle was then progressively stretched in 0.02 mm increments until Lmax was achieved (figure 3). Lmax was defined as the length at which maximum force was achieved thereby reaching the maximum of the Frank-Starling response curve. The muscle was then allowed to equilibrate for 10 minutes.

During the second equilibration period, muscle acceptability was evaluated. Muscles had to be contracting consistently without an irregular force/time contour with a

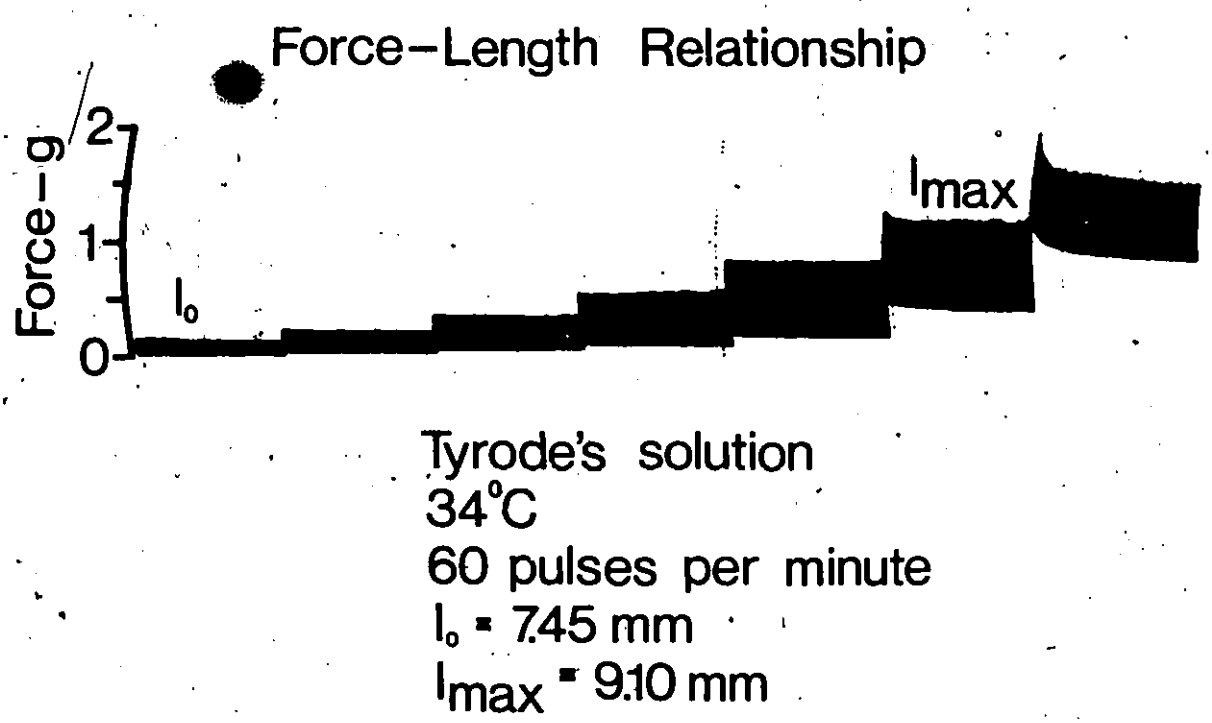

FIGURE 3: Force-Length Relationship.

Muscle length is increased until contractile force is maximized (Lmax), illustrating the Frank-Starling principle.

force greater than or equal to 0.8 gm. Muscles with contraction anomalies (multiple peak contractions, spontaneous electrical activity), low developed force or high resting force (greater than or equal to 1.0 g) were rejected. These restrictions were set arbitrarily based on previous experience.

The trabecula was then randomly assigned to one of eight groups outlined below in section 4.

2.0 Complement Activation. In vitro activation of the complement system in either blood or plasma has been commonly performed using one of two methods: Cobra Venom Factor or Zymosan. Cobra Venom Factor (CVF) is a 140,000 Dalton protein found in the venom of the Naja naja laoutha or Naja haje snakes (3). CVF is functionally analogous to C3b and on binding to Factor B, forms an alternate pathway C3 convertase. It cannot be inactivated and therefore the unregulated C3 convertase cleaves all C3 components until completely consumed. Some CVF's are able to act as C5 convertases so that all components are consumed. These factors have been used to deplete a given system of complement or to produce activated components (C3b) for in vitro or in vivo experiments (3,63,105,106).

Zymosan is a complex polysaccharide derived from yeast cell walls (107). When added to plasma, the complement system is activated via the alternate pathway leading to the formation of C3a and C5a anaphylatoxins and

other components. The Zymosan activated plasma (ZAP) may then be used to elicit various immune-related reactions.

Zymosan was chosen for the complement activator for its storability and ease of use.

2.1 Zymosan preparation. Zymosan A (from Saccharomyces cerevisiae, Sigma Chemical Co., St. Louis, Missouri, USA) was weighed and then added to 500 cc of distilled water. It was sterilized by boiling for 1 hour with constant stirring. The suspension was then placed into tubes for centrifugation (2000 rpm) for 10 minutes. The supernatant was decanted and discarded. The Zymosan pellet was then washed with saline and resuspended with vortex mixing. The washing and decanting was repeated five times. The final Zymosan pellet was resuspended in a known volume of saline to give a concentration of 20 mg/ml. This was kept refrigerated in a sterile container (7,11,15).

3.0 Complement Depletion. The removal of complement from blood or plasma may be done by one of two methods. As mentioned above, cobra venom factor depletes complement by acting as an unregulated convertase. Depending on the specific CVF, one factor or several could be selectively depleted. Activated subcomponents would be present for a short time. The plasma is usually kept for 3 days before use to assure the absence of these activated fragments. Since muscle and blood are brought together to the laboratory in this protocol, the muscle would not survive

the three day wait for complement depletion.

Complement components are proteins which are sensitive to heat. The common method for complement depletion is by heat inactivation at 56°C for 30 minutes (10). Although the proteins are still present (ie. they may still act as antigens for immune complexing), their biological function is destroyed.

4.0 Experimental Protocols. The experiments were divided into four groups differing only by their bath solution in which the muscles would be stimulated to contract: modified Tyrode's solution, autologous blood, autologous plasma and denatured autologous plasma. Within each group there was a Zymosan and control subgroup. In the Zymosan group, Zymosan (suspended in saline as discussed above) was added to activate complement whereas in the control group, saline alone was added to the bath. The general protocol followed for the groups is outlined in figure 4.

All groups shared common steps in the protocol. Once the trabecula was deemed acceptable, measurements of muscle mechanics (MMM) were then taken including developed force (DF), resting force (RF) and mean rate of developed force (MRDF) (see figure 5 for a description). The bath was then drained and refilled with the test solution. Following a ten minute equilibration period, MMM were taken again. Zymosan in saline (Zymosan group) or saline (Control group) was then injected into the bath. The amount of Zymosan

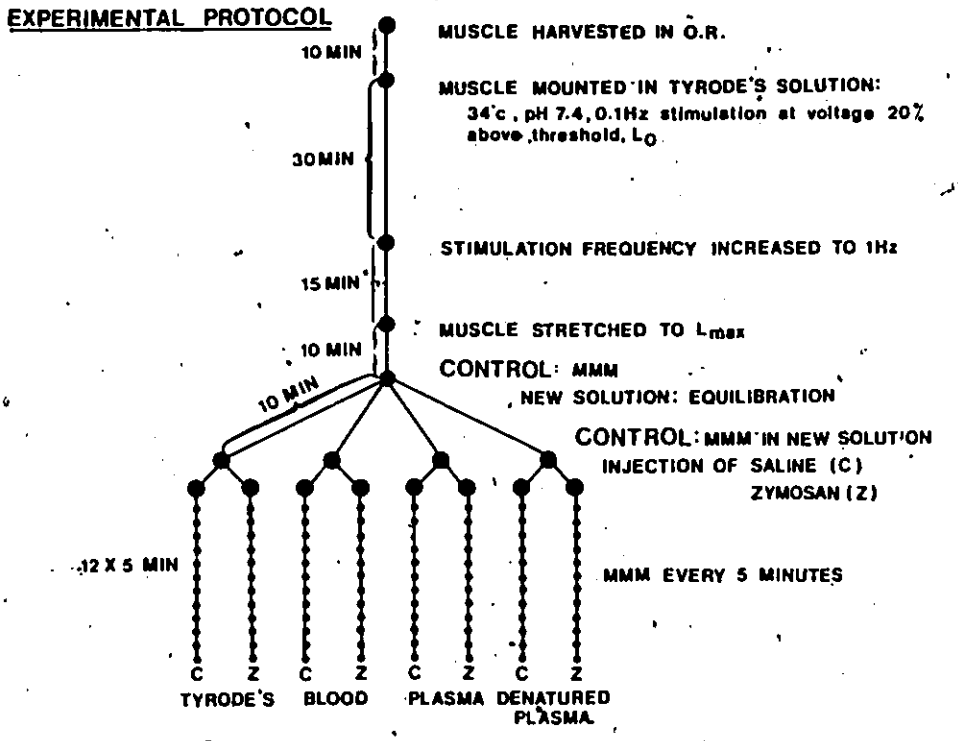

FIGURE 4: Diagramatic representation of the experimental protocol. (see text for details)

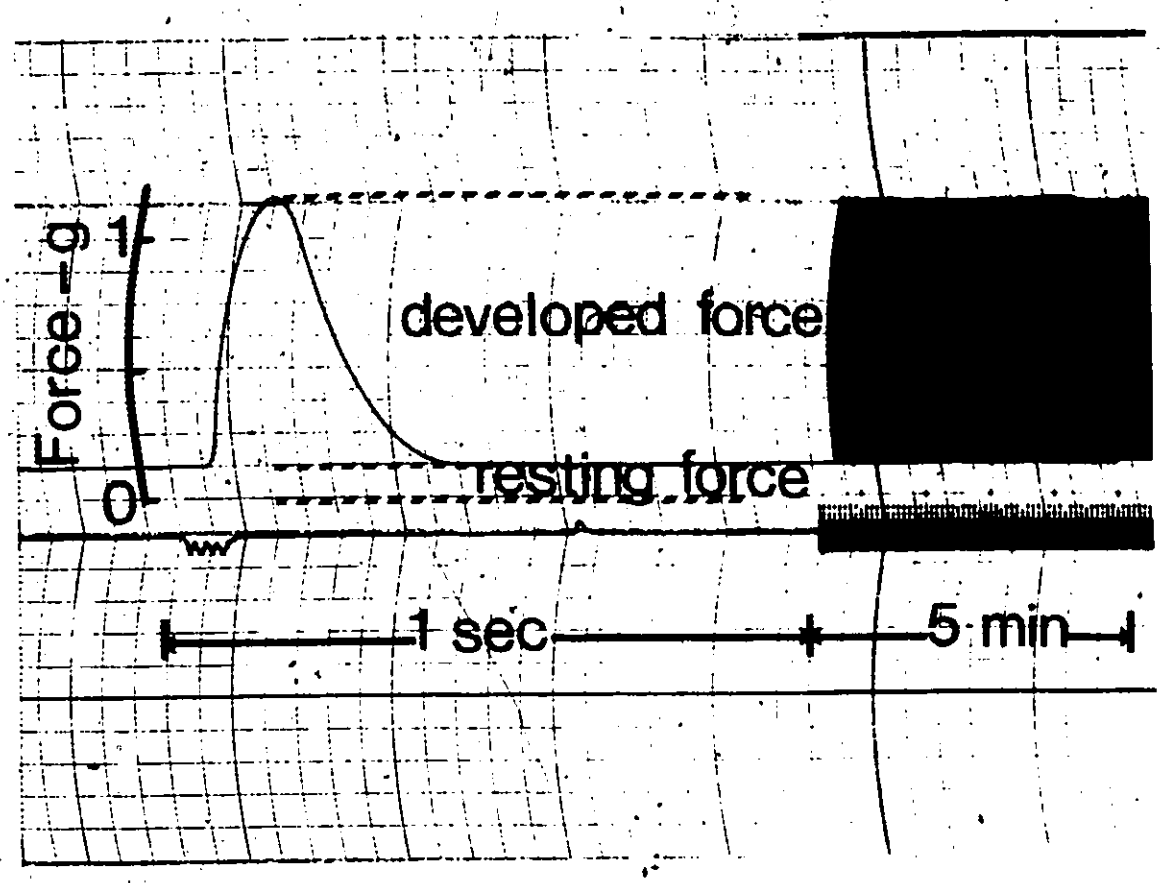

FIGURE 5: Myocardial Muscle Twitch: Measured Parameters

Developed force (DF) = (as shown above) 1 g

Resting force (RF) = (as shown above) 0.13 g

Time to maximum tension (TMT): time from onset of contraction to maximum DF = 0.11 sec

Mean rate of developed force (MRDF): DF/TMT = 9.09 g/sec

Other calculated parameters:

Cross-sectional area = Volume/length (mm2); where weight and length of the trabecula are measured, and volume is calculated using the equation: Weight/Density (where density is a constant = 1.056 g/mm2)

injected varied depending on the size of the bath (18 to 22 ml) so that a concentration of 2 mg/ml was achieved (ie. between 1.8 to 2.2 mls of .20 mg/ml stock solution was injected) (15). Similarly between 1.8 to 2.2 ml of saline was injected into the bath in the Control group. MMM were taken every five minutes for a one hour period. At the conclusion of the experiment, the muscle was weighed (wet weight) and frozen for drying later (dry weight) (see section 6.0). In the blood, plasma and denatured plasma groups, samples of the solution were taken before it was added to the bath (herein to be called the PRE sample). At the conclusion of the experiment, samples of solution were taken from the bath and sent for analysis (herein to be called the POST sample).

4.1 Tyrode's solution. Once the muscle bath was drained, it was refilled with Tyrode's solution. Zymosan in saline (Zymosan group) or saline (Control group) were added as above.

4.2 Autologous Blood. At the time of harvesting the atrial appendage, 60 ml of heparinized arterial blood (patients were given heparin 300 mg/kg body weight prior to CPB) was withdrawn from the same patient into a plastic syringe and transported with the appendage to the laboratory. It was kept at 37°C in a water bath until used in the experiment. PRE samples were taken for blood chemistry, hematology and immunology tests as described below (section 5.0).

The blood was used to refill the bath after

drainage. To prevent foaming due to bubbling of gas through the blood, 0.5 ml of a 1:30 Medical Antifoam AF Emulsion (Dow Corning Corporation, Medical Products, Midland, Mich, USA) was added to the bath. (NB: Antifoam has been tested in Tyrode's solution in unpublished experiments and has no direct effect on myocardial performance. In addition, muscles contracting in blood without Antifoam have similar mechanical performance compared to those with Antifoam. Antifoam was added to prevent loss of blood and plasma due to bubbling spillage.) Muscles were randomly divided into Zymosan and Control groups. At the end of the experiment, the blood was removed from the bath and POST samples were sent for biochemistry, hematology and immunology tests.

4.3 Autologous Plasma. Blood was taken from the patient following heparinization for cardiopulmonary bypass. It was taken to the laboratory where it was placed in 50 ml Falcon tubes and centrifuged at 2000 rpm for 15 minutes. The plasma was then placed in another tube and kept at 37 °C in a water bath. Plasma PRE and POST samples were sent for chemistry and immunology testing.

4.4 Autologous Denatured Plasma. Arterial blood was taken from the patient and sent with the appendage to the laboratory. The blood was centrifuged at 2000 rpm in Falcon tubes for 15 minutes and the plasma was separated into another tube. It was then placed in a water bath at 60 °C for 45 minutes. Following the denaturation, PRE samples

were sent for biochemistry and immunological testing. The plasma was cooled to 34°C prior to use in the bath. At the end of the experiment, the denatured plasma was recovered from the bath and POST samples were sent for biochemistry and immunological tests.

5.0 Blood analysis. Samples of blood, plasma and denatured plasma for biochemistry and hematology were cooled to 4°C and taken to the laboratories of the Department of Laboratory Medicine, Division of Biochemistry and Hematology, at the Ottawa Civic Hospital for analysis on the day of the experiment. Samples for immunology testing were taken to the Immunology Laboratory at the Ottawa Civic Hospital.

5.1 Biochemical tests. Heparinized plasma was placed in 10 ml Becton-Dickinson Vacutainer tubes (without additives). All blood samples were analyzed by the Technicon SMAC autoanalyzer (Tarrytown,New York,USA) on the day of the experiment. All tests had a coefficient of variance of 2 % or less.

5.1.1 Sodium. The method used by the autoanalyzer was a direct potentiometric measurement using a sodium selective glass electrode. The diluted serum from the sample riser was mixed with tris-tetramethyl ammonium choloride buffer and heated to 55°C before the sodium concentration was measured in the electrode flowcell. (normal (N) = 135 - 145 mmol/l)

5.1.2 Potassium. Analysis was performed by mixing diluted

serum with tris-potassium chloride buffer and measured in a flow cell fitted with a valinomycin membrane potassium electrode. (N = 3.6 - 4.8 mmol/l)

5.1.3 Chloride. The plasma was diluted and mixed with dilute nitric acid followed by dialysis. This was mixed with ferric nitrate and mercuric thiocyanate with the resulting red ferric thiocyanate measured by colorimetry at 480 nm. (N = 96 - 106 mmol/l)

5.1.4 Carbon Dioxide. The sample was diluted and mixed with sulphuric acid. The liberated carbon dioxide was dialyzed across a silicone rubber membrane into carbonate buffered phenolphthalein. A colorometric analysis was then performed at 550 nm. (N = 24 - 30 mmol/l)

5.1.5 Calcium. Dilute hydrochloric acid and 8-hydroxyquinoline were mixed with diluted plasma. The calcium was dialyzed into cresolphthaleine complexone reagent. Diethylamine base was added and the colored complex read at 570 nm. (N = 2.10 - 2.60 mmol/l)

5.2 Hematology analysis. Heparinized samples of whole blood were analyzed by the automated Coulter Counter Model S-Plus IV (Coulter Electronics Inc., Hialeah, Florida, USA). It counted and sized based on the detection and measurement of changes in electrical resistance produced by a particle which is suspended in a conductive liquid, traversing a small aperture. Red blood cells (RBC) and white blood cells (WBC) were counted independently, with the latter being counted following erythrocyte lysis.

5.2.1 Hematocrit. Cells that were greater than 36 µm3 in the RBC bath were classified as RBC (RBC count = n x 10E6 cells/mm3). Hematocrit was then calculated using the formula Hct (%) = RBC x MCV/10, where MCV was the mean corpuscular volume calculated based on the total number of counted cells and size of each cell.

5.2.2 White blood cell count. Cells greater than 45 µm3 following RBC lysis were classified as WBC (WBC count = n x 10E3 cells/mm3).

5.2.3 Hemoglobin. The reference and sample voltages generated by the photocurrent circuitry to measure transmittance (%T) were used in the following formula: Hemoglobin (Hgb) (gm/dl) = constant x log ref %T/ sample %T.

5.3 Complement Assays. Heparinized plasma (1 ml) was placed in three 5 ml Sarstedt plastic tubes and with another 2 ml into a 10 ml Becton-Dickinson vacutainer tube (without additives). Although heparin is a known inhibitor of complement activation, concentrations less than 6 mcg/ml have no effect (107). The coefficient of variance for these tests was less than 10%.

5.3.1 C3 and C4 determination. These tests assess absolute levels of specific complement proteins in a sample and do not determine biological activity. The 2 ml of plasma was cooled to 4 °C. A 1:36 dilution was made and anti-human C3 antibody (goat) (Beckman Co., Toronto, Ontario) was added. Antibody (Ab) and antigen (Ag) were allowed to react in solution with formed Ab/Ag aggregates causing light

scattering which was detected by the Beckman ICS Analyze II autoanalyzer (Beckman Instruments, Toronto, Ontario). (N = 0.8 - 1.8 gm/l).

C4 was determined in a similar fashion, except with the addition of anti-human C4 antibody (goat) (Beckman Co, Toronto, Ontario) to serum. (N = 0.15 - 0.45 gm/l)

5.3.2. CH50 assay. The assay tests the ability of the complement system to induce hemolysis of 50% of erythrocytes sensitized with anti-erythrocyte antibodies. It was a quantitative test for the biological activity of complement (108).

Sheep erythrocytes were sensitized with rabbit antibody (ShEA) and suspended in gelatin-veronal-buffered saline supplemented with magnesium (GVB+). The plasma was then diluted to 1:51 with GVB+. Five further dilutions were made and a known amount of ShEA was then added followed by incubation and centrifugation. The optical densities of the solutions were measured (Beckman Model 25 Spectrophotometer, Palo Alto, California, USA) and plotted for a sigmoidal hemolytic complement response curve. The CH50 was calculated based on the 50% lysis point. (N = 20 - 40 CH50 units/ml)

6.0 Wet and Dry Weights. Following the muscle experiment, the trabecula was removed from the muscle stand clips by sharp dissection and weighed (ie. using muscle between the clips which had been responsible for contractile force

generation) on a precision balance (Roller-Smith, Bethlehem, Pa, USA) resulting in a wet weight. The muscles were then frozen for drying at a later date.

All muscles were then dried at 60°C for 24 hrs and then weighed on a Mettler AE166 precision balance (Mettler Instruments, Griefensee, Switzerland). The dry/wet weight was then calculated as a percentage.

A control group was established for wet and dry weights. Eight trabeculae which were unsuitable for mounting were removed from appendages. They were weighed, dried and reweighed in a similar fashion to those above.

7.0 The effects of bubble oxygenation on complement. It is necessary to see how the muscle bath itself affects complement since it has been shown that artificial surfaces and gas bubbling cause activation of the complement system (40).

7.1 Bubble oxygenation (I). Heparinized arterial blood was taken from patients undergoing open heart surgery following anesthetic induction. The blood was sent to the muscle lab where samples were prepared for complement assay. Blood was then placed in the muscle bath (18 - 22 ml) and 0.5 ml of antifoam was added. The bath was then oxygenated by bubbling 95% O2/ 5% CO2 through the blood. Blood samples were taken at the end of 1.5 hours of bubbling.

7.2 Bubble oxygenation (II). Blood was obtained as above and placed in the muscle bath. Field stimulation

(4V) was maintained at 1 pulse/sec. 0.5 ml of antifoam was added. CH50 measurements were then taken at time 0, 1, 30 and 60 minutes to follow the change in biologic complement activity with time.

8.0 The effects of dipyridamole on complement. Dipyridamole (Persantine, Boehringer Ingelheim, Toronto, Ontario) is a platelet aggregation inhibiting agent which acts by reducing the phosphodiesterase breakdown of prostacyclin in platelets. It is routinely given to coronary artery bypass patients one day prior to surgery (109). There is some question that dipyridamole may affect the inflammatory response of which the complement system plays a part. As there is little in the literature to clarify the situation, the effects of dipyridamole on complement were tested.

Twenty patients who were scheduled for coronary bypass surgery (CABG) and ten patients who were to have non-CABG surgery were studied. Venous blood was taken preoperatively (herein known as the PREOP sample) for analysis including biochemistry, hematology and immunology. The CABG patients (Drug group) were then started on dipyridamole (50 mg three times daily) one day before surgery. On the day of operation, the patients were anesthetized and hemodynamic monitoring devices inserted. Arterial blood samples (herein known as the PERIOP sample) were then drawn prior to actual surgery and sent for testing. The non-CABG patients did not receive dipyrimadole

(Control group) but underwent the same blood testing and perioperative preparation for surgery as the CABG patients did.

At the time of anesthesia, patients are volume loaded which would reduce the hematocrit. Correction for this dilution was necessary based on relative changes of hematocrit. The complement values obtained from the second part of the experiment were multiplied by this correction factor. Complement levels between arterial and venous blood have been found to be the same (26,110).

9.0 Statistical Analysis. Muscle data including length (measured as the distance between the two stainless steel clips of the muscle stand, ie the working length of the muscle), weight (measured on the Roller-Smith precision balance) and cross-sectional area (calculated by converting the weight into volume using a density of 1.056, then dividing the volume of the muscle by the length) (figure 5) were compiled within each group. A 1-way analysis of variance (ANOVA) was performed to assess differences between groups. A p value of less than 0.05 was considered to be significant.

Muscle mechanical measurements included developed force, resting force and mean rate of developed force for each muscle. There were 14 separate measurements for each muscle. A 1-way ANOVA was performed on the means of the DF, RF and MRDF for all muscles in each group contracting in the

Tyrode's solution (CONTROL) to assess whether or not the muscles were similar at the beginning of the experiment. Following change of the muscle bath solution (TIME 0), absolute DF, RF and MRDF were again checked for any change by a 1-way ANOVA. Unpaired t-tests were then performed between Control and Zymosan groups for each solution.

The MMM for each muscle were then converted to relative forces as a percentage of force measured at TIME 0. A 1-way ANOVA was performed using all 8 groups at each time interval (TIME 0 to TIME 60) for relative DF, RF and MRDF (ie. a total of 39 ANOVA's were performed). To test the decay of the DF with time, a least squares regression analysis was performed for each muscle. The mean slopes and intercepts for each group were then compared with a 1-way ANOVA. Finally, percentage decay was calculated by expressing the final DF of each experiment in terms of the DF at time 0. A 1-way ANOVA was used to compare groups.

For the biochemical, hematological and immunological data, paired t-tests were used to compare the values before (PRE) and after (POST) each experiment. Unpaired t-tests were used to compare the means of POST samples between Control and Zymosan groups.

The results of the wet/dry weight measurements were expressed as % wet weight (ie. (dry weight/ wet weight) x 100). A 1-way ANOVA was used to test for differences between the groups.

For the oxygenation experiments, unpaired t-tests

were used to compare means of values at each time interval.

Results of the dipyridamole experiments were compared using a paired t-test between the same patient's PREOP and PERIOP results. An unpaired t-test was used to compare the PERIOP values between patients in the Control and Drug groups.

RESULTS

1.0 Trabeculae results. There was a total of 128 trabeculae that were successfully mounted, although numerous appendages were rejected prior to trabeculae dissection. Of these, only 65 trabeculae were accepted based on the criteria described in the Methods section. The mean patient age was 55.5 with a range of 33 to 71 years. Males represented 76.5% of the total number of muscle donors. Some appendages provided multiple trabeculae accounting for the discrepancy between appendage and trabeculae number (51 versus 65, respectively) (table 1). Coronary artery bypass grafting was the most common procedure performed (92.2%), with mitral valve replacements (2.0%), atrial septal defect repairs (2.0%) and open mitral commisurotomies (3.8%) being performed less often. Previous statistical analysis (unpublished data) suggest that age and type of operation are not predictors of acceptibility or performance of muscles.

The mean trabeculae length, weight and cross-sectional areas (mean ± SEM) for all muscles were 5.79 ± 0.24 mm, 5.88 ± 0.28 mg and 0.95 ± 0.03 mm2 respectively. Group means are shown in table 1. A 1-way ANOVA revealed that there was no statistically significant difference between the groups.

The first control muscle mechanical meaurement (MMM) was made in Tyrode's solution. The average developed force (DF), resting force (RF) and mean rate of

TABLE 1

PATIENT DATA (n = 51)
(mean ± SEM)

sex: 39 males
12 females
age = 55.5 ± 1.4 years
age range = 33 - 71 years

operations: 47 coronary artery bypass procedures
1 mitral valve replacement
1 atrial septal defect repair
2 open mitral commissurotomies

TRABECULAE DATA (n = 65)
(mean ± SEM)

wet weight = 5.9 ± 0.3 mg
length = 5.8 ± 0.2 mm
cross-sectional area = 0.95 ± 0.03 mm2

developed force = 1.06 ± 0.02 g
resting force = 0.29 ± 0.03 g
mean rate of developed force = 11.31 ± 0.30 g/sec

EXPERIMENT (mean ± SEM)		n	WEIGHT mg	LENGTH mm	X-SECTIONAL AREA mm2
Tyrode's	control	8	6.1 ± 0.9	6.3 ± 0.6	0.91 ± 0.07
	Zymosan	8	6.4 ± 1.1	6.1 ± 0.7	0.98 ± 0.09
Blood	control	9	5.1 ± 0.6	5.0 ± 0.6	0.98 ± 0.07
	Zymosan	8	5.6 ± 1.0	6.0 ± 0.6	0.87 ± 0.08
Plasma	control	8	5.3 ± 0.7	5.9 ± 0.7	0.89 ± 0.11
	Zymosan	8	5.6 ± 0.6	5.2 ± 0.4	1.00 ± 0.05
D-Plasma	control	8	7.0 ± 0.9	6.6 ± 0.9	1.00 ± 0.03
	Zymosan	8	6.1 ± 0.8	6.0 ± 0.6	0.97 ± 0.09

developed force (MRDF) for all muscles together are shown in Table 1. Tables 2, 3 and 4 give the mean absolute value for DF, RF and MRDF for each group. A 1-way ANOVA showed that there was no statistically significant difference between the groups.

Following the change in bath solution, a second MMM control was performed. When replaced by Tyrode's solution, there was a minimal change in DF (1.5% drop in the Control group and 3.6% drop in the Zymosan group). However, in the autologous blood, plasma and denatured plasma, there were decreases in DF of 29 to 44% (table 5, figure 6). Although the 1-way ANOVA showed an overall difference in percentage decrease in DF, individual unpaired t-tests failed to show any differences between Control and Zymosan groups among the different solutions. A 1-way ANOVA between the six blood/plasma groups showed no statistically significant difference in fall of DF. In an attempt to determine the reason for the drop, the total calcium concentrations in Tyrode's solution, blood, plasma and denatured plasma were compared to the percentage decrease in DF following solution change. There was a high degree of correlation between calcium and fall in DF ($r = -0.94$, $p < 0.001$).

The next analysis used relative DF, RF and MRDF as a percentage of the value at Time 0. A 1-way ANOVA between groups at each different time from 5 mins to 60 mins (at 5 minute intervals) was performed for relative DF, RF

TABLE 2

CONTROL: TYRODE'S SOLUTION
DEVELOPED FORCE (absolute values)

	TYRODE'S CONTROL (n = 8)	TYRODE'S ZYMOSAN (n = 8)	BLOOD CONTROL (n = 9)	BLOOD ZYMOSAN (n = 8)
mean (g)	1.04	1.04	1.18	0.95
Var	0.03	0.01	0.05	0.01
SD	0.16	0.12	0.22	0.09
SE	0.06	0.04	0.07	0.03

	PLASMA CONTROL (n = 8)	PLASMA ZYMOSAN (n = 8)	DENATURED PLASMA CONTROL (n = 8)	DENATURED PLASMA ZYMOSAN (n = 8)
mean (g)	1.10	1.01	1.08	1.08
Var	0.06	0.02	0.08	0.03
SD	0.24	0.15	0.28	0.18
SE	0.08	0.05	0.10	0.06

ANOVA TABLE

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
1.05	7	57	2.31	0.26	2.04

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
3.56 E-02	4.46 E-03	0.41

TABLE 3

CONTROL: TYRODE'S SOLUTION
RESTING FORCE (absolute values)

	TYRODE'S		BLOOD	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 9)	ZYMOSAN (n = 8)
mean (g)	0.47	0.21	0.29	0.27
Var	0.06	0.02	0.03	0.03
SD	0.25	0.16	0.18	0.18
SE	0.09	0.06	0.06	0.06

	PLASMA		DENATURED PLASMA	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (g)	0.24	0.35	0.28	0.26
Var	0.02	0.08	0.06	0.04
SD	0.15	0.28	0.24	0.20
SE	0.05	0.10	0.09	0.07

ANOVA TABLE

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
1.19	7	57	2.86	0.36	2.49

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
4.39 E-02	6.51 E-03	0.32

TABLE 4

CONTROL: TYRODE'S SOLUTION
MEAN RATE OF DEVELOPED FORCE (absolute values)

	TYRODE'S		BLOOD	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 9)	ZYMOSAN (n = 8)
mean (g/sec)	10.77	10.69	13.31	9.86
Var	2.34	1.03	10.51	2.08
SD	1.53	1.02	3.24	1.44
SE	0.54	0.36	1.15	0.51

	PLASMA		DENATURED PLASMA	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (g/sec)	11.94	10.12	11.83	11.48
Var	4.69	2.27	13.00	6.43
SD	2.17	1.49	3.61	2.54
SE	0.77	0.53	1.27	0.90

ANOVA TABLE

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
1.99	7	57	381.64	74.92	306.72

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
5.29	1.26	0.07

TABLE 5

CONTROL #2: TEST SOLUTION
% DROP IN DEVELOPED FORCE FROM CONTROL #1

	TYRODE'S		BLOOD	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 9)	ZYMOSAN (n = 8)
mean (%)	1.51	3.63	38.39	29.49
Var	29.13	70.93	265.20	990.16
SD	5.40	8.42	16.28	31.47
SE	1.91	2.98	5.43	11.13

	PLASMA		DENATURED PLASMA	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (%)	36.78	30.33	44.25	43.17
Var	300.96	92.44	790.89	187.27
SD	17.35	9.61	28.12	13.68
SE	6.13	3.40	9.94	4.84

ANOVA TABLE #1: TYRODE'S, BLOOD, PLASMA, DENATURED PLASMA

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
6.71	7	57	35297.87	15943.86	19354.01

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
340.87	282.97	8.23 E-06

ANOVA TABLE #2: BLOOD, PLASMA, DENATURED PLASMA

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
0.71	5	43	20203.94	1550.34	18653.59

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
437.82	38.72	0.62

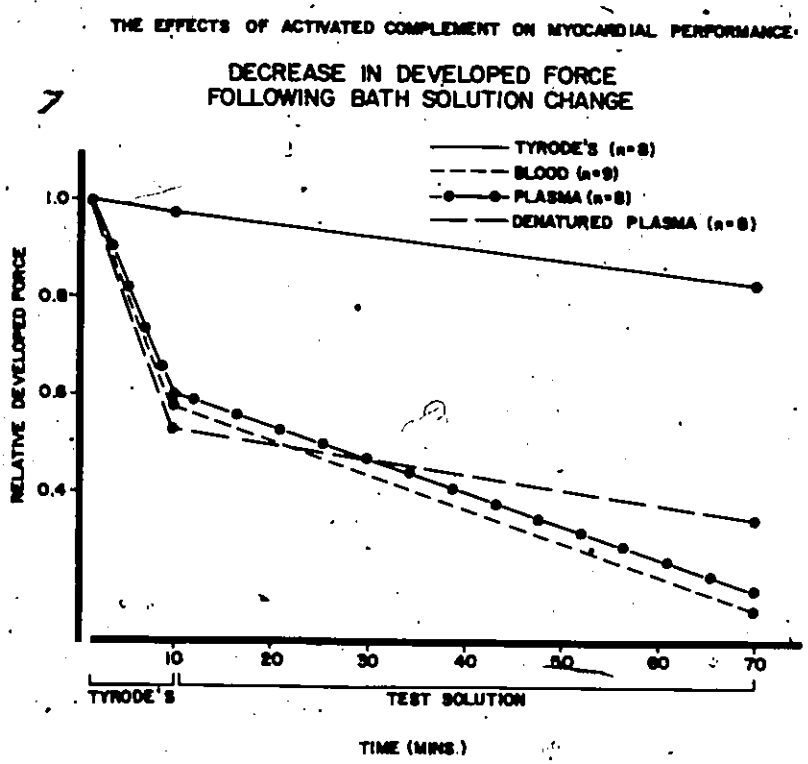

FIGURE 6: Decrease in relative developed force following solution change. Control measurements are taken at time Ø in Tyrode's solution. Following drainage at 1Ø minutes, either Tyrode's, blood, plasma or denatured plasma is placed into the bath. This diagram shows developed force before and after solution change for the control (saline injected bath) muscles.

and MRDF. There were no statistially significant differences between any of the groups. A total of 39 ANOVA analyses were performed, however for the sake of brevity, the results for the analysis at 60 minutes is shown for relative DF (table 6), RF (table 7) and MRDF (table 8) including the ANOVA tables as a representative of the total body of the results.

To calculate the rate of decay of the preparation in each solution, a least squares regression analysis was performed on each set of data produced by individual trabeculae. The slopes and y-intercepts were then averaged for each group and are presented in tables 9 and 10 respectively. The mean slopes and y-intercepts are shown in tables 9 and 10. The mean and range of correlation coefficients are presented as well. A 1-way ANOVA between the slopes showed no statistically significant difference. The lines produced are shown in figures 7 to 10. The decay was also expressed as a percentage using the final value for DF relative to initial DF (table 11). By a 1-way ANOVA, there were no statistically significant differences between the 8 groups.

2.0 Biochemistry, Hematology and Immunology results.

2.1 Blood Control and Zymosan. In both groups, there was no significant difference in hemoglobin concentration, so correction for dilution was not applied. Using a paired t-test between PRE and POST values (as described in the

TABLE 6

RELATIVE DEVELOPED FORCE: 60 MINS

	TYRODE'S		BLOOD	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 9)	ZYMOSAN (n = 8)
mean (g)	0.74	0.74	0.63	0.66
Var	0.06	0.01	0.05	0.08
SD	0.25	0.10	0.21	0.28
SE	0.09	0.03	0.07	0.10

	PLASMA		DENATURED PLASMA	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (g)	0.71	0.67	0.84	0.84
Var	0.02	0.06	0.09	0.16
SD	0.16	0.25	0.30	0.40
SE	0.06	0.09	0.11	0.14

ANOVA TABLE

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
0.75	7	57	4.15	0.35	3.79

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
0.07	6.11 E-03	0.63

TABLE 7

RELATIVE RESTING FORCE: 60 MIN

	TYRODE'S		BLOOD	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (g)	0.84	1.31	0.87	0.76
Var	0.01	2.28	0.05	0.04
SD	0.11	1.51	0.23	0.20
SE	0.04	0.53	0.08	0.07

	PLASMA		DENATURED PLASMA	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (g)	0.96	0.92	4.49	1.64
Var	0.12	0.01	92.89	1.70
SD	0.34	0.10	9.63	1.31
SE	0.12	0.03	3.41	0.46

ANOVA TABLE

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
1.06	7	57	768.26	88.46	679.81

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
12.14	1.57	0.40

TABLE 8

RELATIVE MEAN RATE OF DEVELOPED FORCE: 60 MIN

	TYRODE'S		BLOOD	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 9)	ZYMOSAN (n = 8)
mean (g/sec)	0.76	0.73	0.69	0.74
Var	0.06	0.01	0.06	0.09
SD	0.25	0.11	0.24	0.29
SE	0.09	0.04	0.08	0.10

	PLASMA		DENATURED PLASMA	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (g/sec)	0.71	0.69	0.89	0.87
Var	0.02	0.06	0.10	0.17
SD	0.16	0.24	0.32	0.42
SE	0.06	0.09	0.11	0.15

ANOVA TABLE

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
0.69	7	57	4.44	0.34	4.10

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
0.07	6.07 E-03	0.68

TABLE 9

DEVELOPED FORCE: SLOPES

	TYRODE'S		BLOOD	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 9)	ZYMOSAN (n = 8)
mean (%/min)	- 0.003	- 0.004	- 0.005	- 0.006
Var	0.00004	0.000002	0.00001	0.00002
SD	0.006	0.002	0.003	0.004
SE	2.09 E-03	5.34 E-04	1.07 E-03	1.47 E-03
mean r	- 0.66	- 0.89	- 0.64	- 0.79

	PLASMA		DENATURED PLASMA	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (%/min)	- 0.005	- 0.005	- 0.002	- 0.003
Var	0.00002	0.00002	0.00002	0.00004
SD	0.004	0.004	0.005	0.006
SE	1.44 E-03	1.48 E-03	1.74 E-03	2.12 E-03
mean r	- 0.75	- 0.70	- 0.28	- 0.08

ANOVA TABLE

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
0.71	7	57	1.22 E-03	9.78 E-05	1.12 E-03

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
1.99 E-05	1.72 E-06	0.67

TABLE 10

DEVELOPED FORCE: INTERCEPTS

	TYRODE'S		BLOOD	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 9)	ZYMOSAN (n = 8)
mean (%)	1.00	0.98	1.00	1.02
Var	0.01	0.002	0.02	0.003
SD	0.07	0.05	0.15	0.06
SE	0.03	0.02	0.05	0.02

	PLASMA		DENATURED PLASMA	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (%)	1.02	0.94	0.96	1.01
Var	0.01	0.004	0.02	0.01
SD	0.14	0.07	0.13	0.10
SE	0.05	0.02	0.05	0.04

ANOVA TABLE

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
0.69	7	57	0.66	0.05	0.61

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
0.010	9.25 E-04	0.66

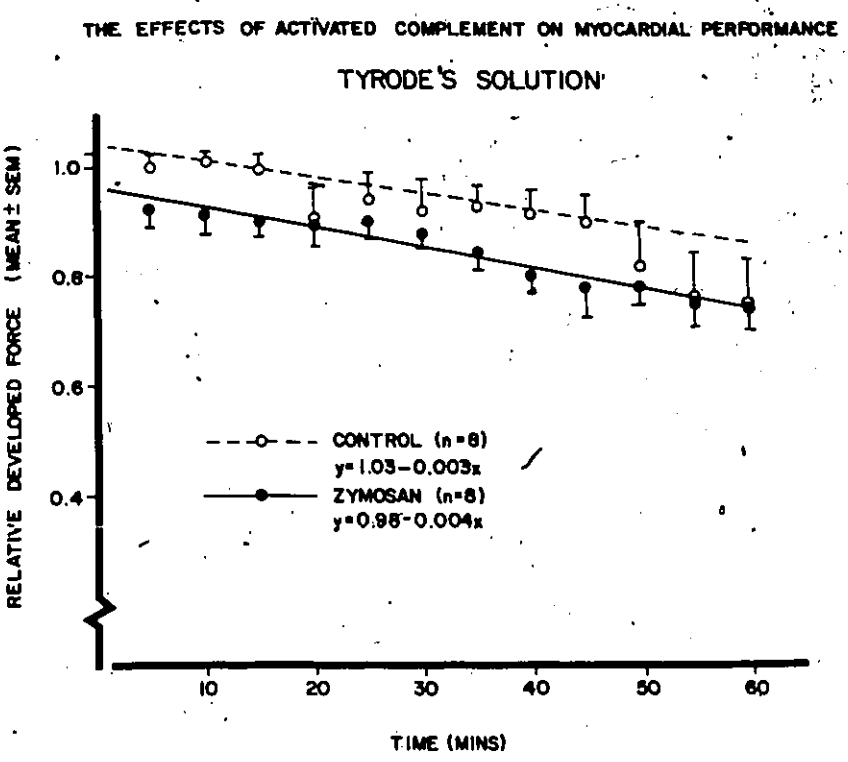

FIGURE 7: Shown are the resultant lines from regression analysis of the change in relative developed force with time of muscles contracting in Tyrode's. Open and closed symbols represent mean ± SEM at those given times.

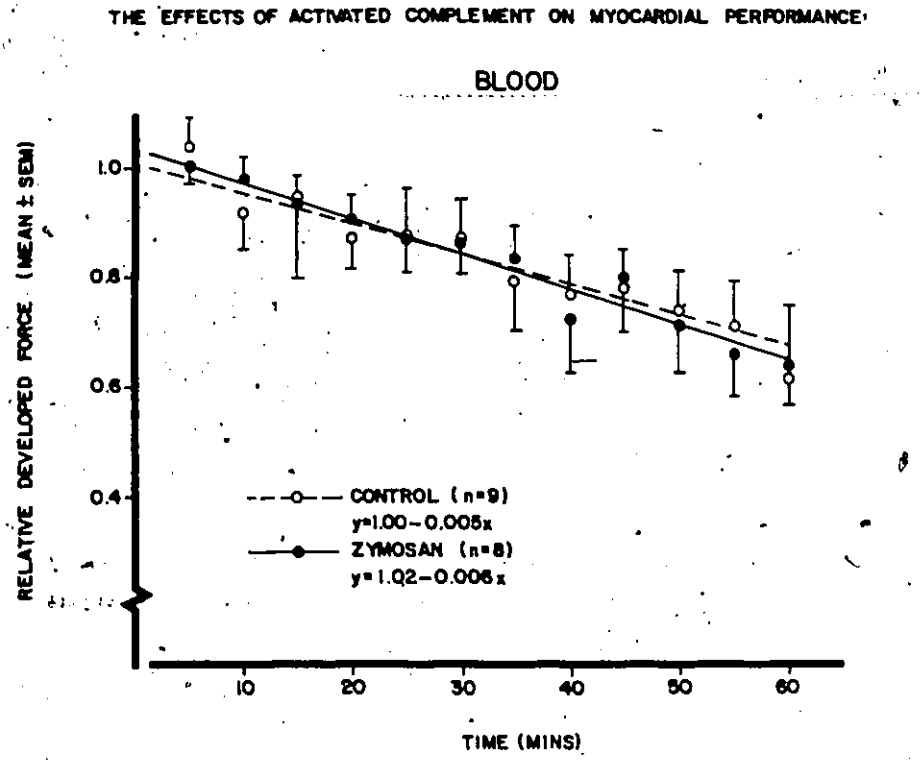

FIGURE 8: Shown are the resultant lines from regression analysis of the change in relative developed force with time of muscles contracting in blood. Open and closed symbols represent mean ± SEM at those given times.

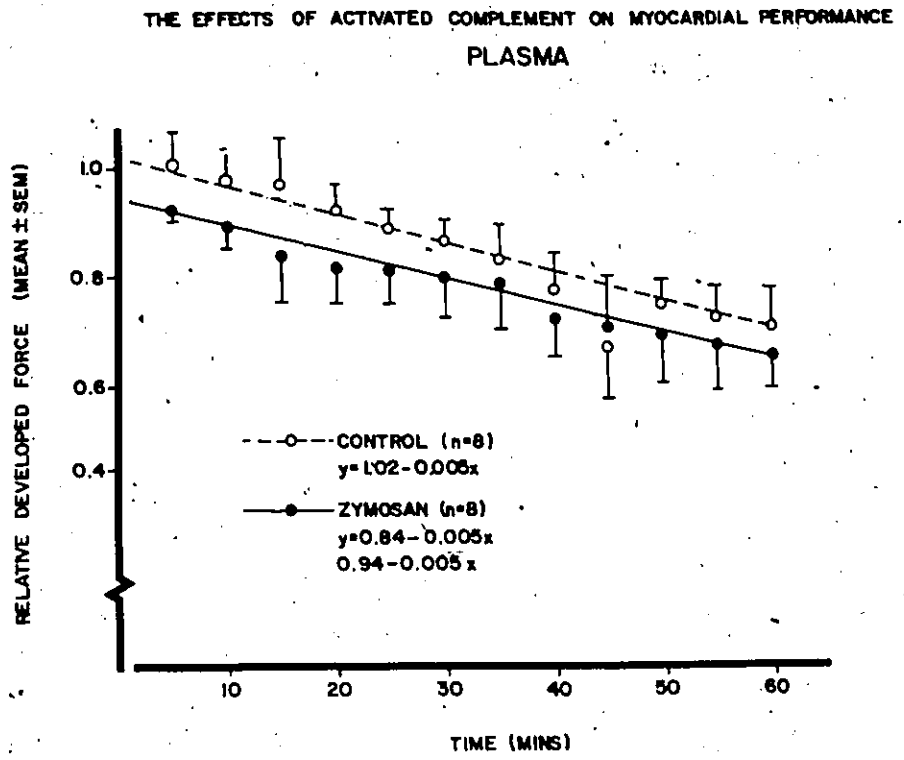

FIGURE 9: Shown are the resultant lines from regression analysis of the change in relative developed force with time of muscles contracting in plasma. Open and closed symbols represent mean ± SEM at those given times.

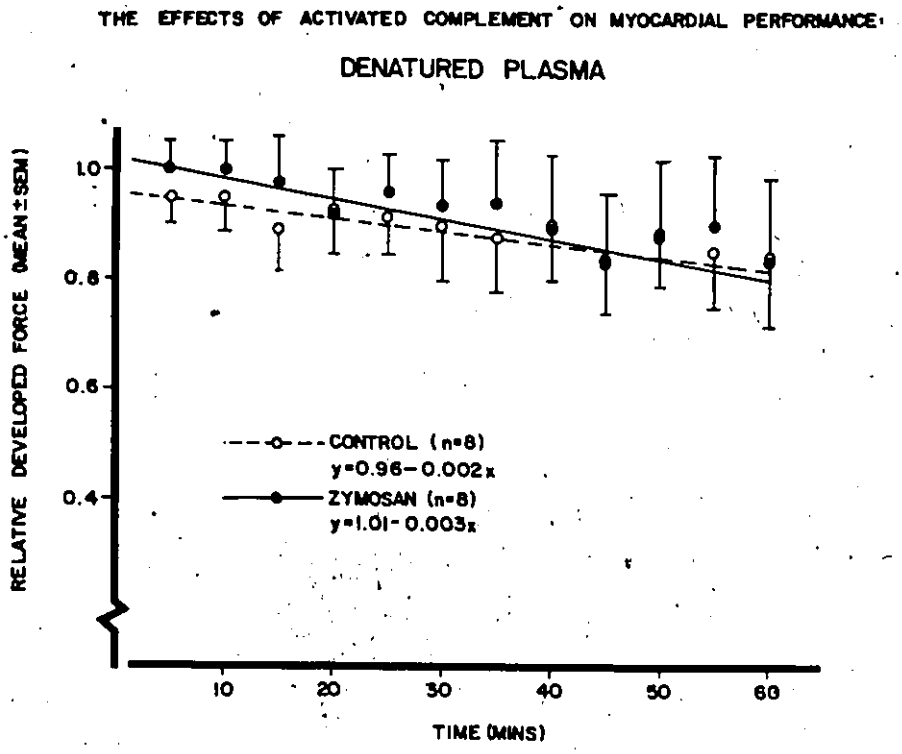

FIGURE 10: Shown are the resultant lines from regression analysis of the change in relative developed force with time of muscles contracting in denatured plasma. Open and closed symbols represent mean ± SEM at those given times.

TABLE 11

DEVELOPED FORCE: % DECAY IN 1 HOUR

	TYRODE'S		BLOOD	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 9)	ZYMOSAN (n = 8)
mean (%)	25.88	26.25	37.44	34.25
Var	615.55	90.50	454.53	783.93
SD	24.81	9.51	21.32	28.00
SE	8.77	3.36	7.11	9.90

	PLASMA		DENATURED PLASMA	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (%)	29.25	32.63	16.50	16.13
Var	244.79	602.27	925.71	615.55
SD	15.65	25.54	30.43	40.47
SE	5.53	8.68	10.76	14.31

ANOVA TABLE

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
0.76	7	57	41468.06	3525.72	37942.35

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
669.43	61.15	0.63

Methods section), there were significant differences in total CO2 (23.5 PRE and 21.5 POST, $p < 0.05$) and in CH50 (16.3 PRE and 7.9 POST, $p < 0.001$) for the Control (saline) group. In the Zymosan group, there were significant differences in sodium (137.3 PRE and 144.5 POST, $p < 0.001$), potassium (4.7 PRE and 5.1 POST, $p < 0.05$), chloride (111.6 PRE and 120.3 POST, $p < 0.001$), total CO2 (23.1 PRE and 20.1 POST, $p < 0.01$) and CH50 (14.6 PRE and 3.5 POST, $p < 0.001$).

Comparing the Control and Zymosan groups POST values by an unpaired t-test, there were no statistically significant differences in any value except for white blood count which was significantly higher in the Zymosan group ($p < 0.05$) (tables 12 and 13).

2.2 Plasma Control and Zymosan. PRE and POST values were again compared in both Control and Zymosan groups. In the control group, there were significant differences in mean sodiums (140.0 PRE and 145.4 POST, $p < 0.05$), chlorides (112.0 PRE and 117.1 POST, $p < 0.05$), total CO2's (23.6 PRE and 25.0 POST, $p < 0.001$), C3 levels (0.5 PRE and 0.6 POST, $p < 0.5$) and CH50's (16.8 PRE and 7.8 POST, $p < 0.01$). The Zymosan group had significant differences in mean sodium (138.9 PRE and 147.3 POST, $p < 0.001$), chloride (110.4 PRE and 120.6 POST, $p < 0.001$), and CH50 (15.9 PRE and 0.0 POST, $p < 0.001$).

The POST values for both Control and Zymosan were compared and no statistically significant differences were

TABLE 12

BIOCHEMISTRY, IMMUNOLOGY AND HEMATOLOGY RESULTS: BLOOD

GROUP: CONTROL PRE (n = 9) TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50	Hb	Hct	WBC
mean	137	4.5	109	23.5	1.81	0.67	0.12	16.3	90.0	26.2	4.1
SD	4.0	0.5	2.2	2.6	0.06	0.14	0.04	3.0	10.8	3.4	1.3
SE	1.7	0.2	0.9	1.1	0.02	0.05	0.01	1.0	4.4	1.4	0.5

GROUP: CONTROL POST (n = 9) TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50	Hb	Hct	WBC
mean	141	4.9	115	21.5	1.96	0.68	0.12	7.9	88.7	25.9	3.7
SD	8.6	0.7	7.7	1.6	0.20	0.15	0.05	5.0	14.9	4.5	1.3
SE	3.5	0.3	3.2	0.7	0.08	0.05	0.02	1.7	6.1	1.9	0.5

GROUP: ZYMOSAN PRE (n = 8) TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50	Hb	Hct	WBC
mean	137	4.7	111	23.1	1.77	0.56	0.11	14.6	75.3	22.2	3.8
SD	5.8	0.4	2.1	2.1	0.08	0.11	0.04	3.5	13.1	3.9	1.6
SE	1.8	0.1	0.8	0.7	0.03	0.04	0.01	1.2	6.5	1.9	0.7

GROUP: ZYMOSAN POST (n = 8) TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50	Hb	Hct	WBC
mean	145	5.1	120	20.1	1.85	0.58	0.12	3.5	78.3	22.8	8.4
SD	7.9	0.4	3.3	1.3	0.19	0.13	0.03	4.1	4.9	1.5	4.1
SE	2.8	0.1	1.2	0.4	0.07	0.05	0.01	1.4	2.4	0.8	2.1

UNITS

Na, K, Cl, CO2, Ca = mmole/I
C3, C4 = g/l
CH50 = CH50 units/l
Hb = g/l
Hct = %
WBC = 10E3 cells/mm3

TABLE 13

t-TESTS: BIOCHEMISTRY, IMMUNOLOGY AND HEMATOLOGY RESULTS BLOOD

PAIRED t-TEST: (CONTROL: PRE AND POST)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50	Hb	Hct	WBC
t	-1.7	-1.9	-2.1	3.2	-2.0	-0.6	-0.01	4.83	0.6	0.4	0.9
df	5	5	5	5	5	8	8	8	5	5	5
p	0.2	0.1	0.1	0.03	0.1	0.6	0.6	0.001	0.6	0.7	0.4

PAIRED t-TEST: (ZYMOSAN: PRE AND POST)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50	Hb	Hct	WBC
t	-5.6	-2.7	-5.9	5.0	-1.3	-0.4	-1.2	7.3	-0.5	-0.3	-2.5
df	7	6	7	7	7	7	6	6	3	3	3
p	0.001	0.04	0.001	0.002	0.2	0.7	0.3	0.001	0.4	0.8	0.1

UNPAIRED t-TEST: (CONTROL VS ZYMOSAN POST)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50	Hb	Hct	WBC
t	-0.3	-0.3	-0.8	0.7	0.4	0.5	0.0	0.7	0.5	0.5	-3.1
df	12	11	12	12	12	15	15	15	10	10	7
p	0.8	0.8	0.4	0.5	0.7	0.6	1.0	0.5	0.6	0.6	0.01

found by unpaired t-tests (tables 14 and 15).

2.3 Denatured plasma Control and Zymosan. There were significant differences between most PRE and POST values in the Control group. These included sodium (141.4 PRE and 149.6 POST, $p < 0.001$), potassium (4.6 PRE and 4.8 POST, $p < 0.01$), chloride (113.7 PRE and 119.6 POST, $p < 0.01$), total CO2 (23.1 PRE and 24.1 POST, $p < 0.05$), and calcium (1.8 PRE and 2.0 POST, $p < 0.05$). In the Zymosan group significant differences occurred between sodium (141.9 PRE and 152.3 POST, $p < 0.001$), potassium (4.0 PRE and 3.9 POST, $p < 0.05$), and chloride (111.8 PRE and 122.5 POST, $p < 0.001$).

A comparison by unpaired t-tests between the two group's POST values showed no statistically significant differences (table 16 and 17).

3.0 Wet and Dry weights. Dry weight is expressed as a percentage of wet weight. A 1-way ANOVA between all eight groups and control showed no statistically significant difference (table 18).

4.0 Effects of oxygen bubbling on complement. In the first series of experiments, bubbling through blood took place over 1.5 hours. C3 and C4 levels were not significantly changed but CH50 decreased significantly from 15.1 to 10.4 ($p < 0.05$) (table 19).

In the second series of experiments, CH50's were measured over a 1 hour period. They decreased significantly

TABLE 14

BIOCHEMISTRY AND IMMUNOLOGY RESULTS: PLASMA

GROUP: CONTROL PRE (n = 8).

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
mean	140	4.5	112	23.6	1.82	0.51	0.10	16.8
SD	5.0	0.6	3.6	1.1	0.12	0.10	0.02	2.8
SE	1.8	0.2	1.3	0.4	0.04	0.04	0.01	1.1

GROUP: CONTROL POST (n = 8)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
mean	145	4.7	117	25.0	1.93	0.61	0.10	7.8
SD	2.8	0.5	2.9	1.1	0.22	0.11	0.03	4.8
SE	1.0	0.2	1.0	0.4	0.08	0.04	0.01	1.8

GROUP: ZYMOSAN PRE (n = 8)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
mean	138	4.3	110	22.9	1.78	0.55	0.11	15.9
SD	4.7	0.7	4.1	1.3	0.07	0.14	0.04	4.1
SE	1.8	0.3	1.6	0.5	0.03	0.05	0.02	1.5

GROUP: ZYMOSAN POST (n = 8)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
mean	147	4.1	120	22.3	1.90	0.58	0.14	0
SD	4.9	0.7	4.3	2.0	0.21	0.15	0.08	0
SE	1.8	0.3	1.6	0.8	0.08	0.05	0.03	0

UNITS

Na, K, Cl, CO2, Ca = mmole/l
C3, C4 = g/l
CH50 = CH50 units/l

TABLE 15

t-TESTS: BIOCHEMISTRY AND IMMUNOLOGY RESULTS PLASMA

PAIRED t-TESTS: (CONTROL PRE AND POST)

TEST:	Na	K	CL	CO2	Ca	C3	C4	CH50
t	-2.6	-1.7	-2.9	-5.2	-1.5	-3.1	-0.3	4.6
df	7	7	7	7	7	7	7	7
p	0.03	0.1	0.02	0.001	0.2	0.02	0.8	0.003

PAIRED t-TESTS: (ZYMOSAN PRE AND POST)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
t	-14.82	1.6	-6.8	0.8	-2.2	0.5	-0.7	-7.8
df	7	7	7	7	7	7	7	7
p	0.001	0.2	0.001	0.5	0.06	0.6	0.5	0.001

UNPAIRED t-TESTS: (CONTROL VS ZYMOSAN POST)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
t	-0.3	0.6	-0.7	1.2	0.1	0.6	-0.4	1.6
df	14	14	14	14	14	14	14	14
p	0.7	0.6	0.5	0.3	0.9	0.6	0.7	0.1

TABLE 16

BIOCHEMISTRY AND IMMUNOLOGY RESULTS: DENATURED PLASMA

GROUP: CONTROL PRE (n = 8)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
mean	141	4.6	113	23.1	1.78	0.30	0.10	0
SD	3.4	0.5	1.8	0.9	0.08	0.16	0.04	0
SE	1.3	0.2	0.7	0.3	0.03	0.06	0.01	0

GROUP: CONTROL POST (n = 8)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
mean	150	4.8	120	24.1	2.04	0.30	0.11	0
SD	5.2	0.5	3.6	0.7	0.19	0.16	0.03	0
SE	2.0	0.2	1.3	0.3	0.07	0.06	0.01	0

GROUP: ZYMOSAN PRE (n = 8)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
mean	142	4.0	112	23.9	1.81	0.37	0.10	0
SD	4.2	0.4	3.2	1.3	0.09	0.24	0.03	0
SE	1.5	0.1	1.2	0.4	0.03	0.09	0.01	0

GROUP: ZYMOSAN POST (n = 8)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
mean	152	3.9	123	23.5	1.92	0.39	0.10	0
SD	4.8	0.3	3.3	1.2	0.16	0.27	0.03	0
SE	1.5	0.1	1.2	0.4	0.06	0.10	0.01	0

UNITS

Na, K, Cl, CO2, Ca = mmole/l
C3, C4 = g/l
CH50 = CH50 units/l

TABLE 17

t-TESTS: BIOCHEMISTRY AND IMMUNOLOGY RESULTS DENATURED PLASMA

PAIRED t-TESTS: (CONTROL PRE AND POST)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
t	-5.6	-2.9	-4.2	-3.2	-3.5	0.0	-0.23	0.0
df	6	6	6	6	6	6	5	7
p	0.001	0.003	0.006	0.02	0.01	1.0	0.8	1.0

PAIRED t-TESTS: (ZYMOSAN PRE AND POST)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
t	-20.8	3.1	-21.9	1.4	-2.0	-0.6	0.0	0.0
df	7	7	7	7	7	6	6	7
p	0.001	0.02	0.001	0.2	0.1	0.6	1.0	1.0

UNPAIRED t-TESTS: (CONTROL VS ZYMOSAN POST)

TEST:	Na	K	Cl	CO2	Ca	C3	C4	CH50
t	-0.4	1.5	-0.6	0.4	0.5	-0.3	0.2	0.0
df	13	13	13	13	13	12	11	14
p	0.7	0.2	0.6	0.7	0.6	0.8	0.8	1.0

TABLE 18

WET AND DRY WEIGHT: DRY/WET %

	TYRODE'S		BLOOD	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 9)	ZYMOSAN (n = 8)
mean (%)	28.30	20.96	25.73	32.95
Var	246.83	79.34	26.21	499.04
SD	15.71	8.91	5.12	22.34
SE	5.55	3.37	1.71	7.90

	PLASMA		DENATURED PLASMA	
	CONTROL (n = 8)	ZYMOSAN (n = 8)	CONTROL (n = 8)	ZYMOSAN (n = 8)
mean (%)	24.79	23.76	25.10	20.83
Var	87.52	50.36	78.97	4.59
SD	9.36	7.10	8.89	2.14
SE	3.54	2.68	3.14	0.76

	UNMOUNTED CONTROLS (n = 8)
mean (%)	22.38
Var	5.50
SD	2.34
SE	0.83

ANOVA TABLE

F	df N	df D	TOTAL SS	TRTMT SS	ERROR SS
0.95	8	61	8275.06	917.48	7357.58

MEAN VARIANCE	VARIANCE OF MEANS	p VALUE
119.82	14.61	0.48

TABLE 19

EFFECT OF OXYGEN BUBBLING ON COMPLEMENT

	C3		C4	
	PRE Ø2 (n = 8)	POST Ø2	PRE Ø2 (n = 8)	POST O2
mean (g/l)	Ø.06	Ø.06	Ø.11	Ø.11
SD	Ø.14	Ø.13	Ø.03	Ø.03
SE	Ø.05	Ø.05	Ø.01	Ø.01
t	Ø.Ø		Ø.Ø	
df	7		7	
p	1		1	

	CH5Ø	
	PRE O2 (n = 8)	POST O2
mean (CH5Øu/l)	15.14	1Ø.39
SD	4.26	4.42
SE	1.5Ø	1.56
t	2.65	
df	7	
p	Ø.Ø2	

from a mean of 14.3 at time 0 to 12.0 at 1 min ($p < 0.01$), 10.1 at 30 mins ($p < 0.001$) and 9.5 at 60 mins ($p < 0.001$). The decrease in CH50 with time is shown in figure 11.

5.0 Clinical Dipyridamole Study. There were 7 females and 3 males in the control group compared to 5 females and 15 males in the experimental group. The average age of control patients was 46.8 whereas patients in the drug group had a mean age of 57.1 ($p < 0.05$).

At the time of surgery, the patients are hemodiluted prior to CPB. The PERIOP samples were taken at this time and therefore there is a significant dilution compared to pre-operative values. All PERIOP complement values were corrected appropriately for dilution based on individual change in hemoglobin between PREOP and PERIOP measurements.

Using an unpaired t-test, complement levels were compared between the Drug group (those who received dipyridamole) and the Control group (those who did not receive the drug). There were no significant differences in CH50, C3 and C4 levels between the two groups.

However, paired t-tests within the Drug or Control groups showed a significant difference between PREOP and PERIOP complement levels. In the Drug group, C3 differed (1.4 PREOP and 1.3 PERIOP, $p < 0.001$), as did C4 (0.29 PREOP and 0.26 PERIOP, $p < 0.001$) and CH50 (27.1 PREOP and 24.8 PERIOP, $p < 0.01$). The corrected C3 (1.3 PREOP and 1.1

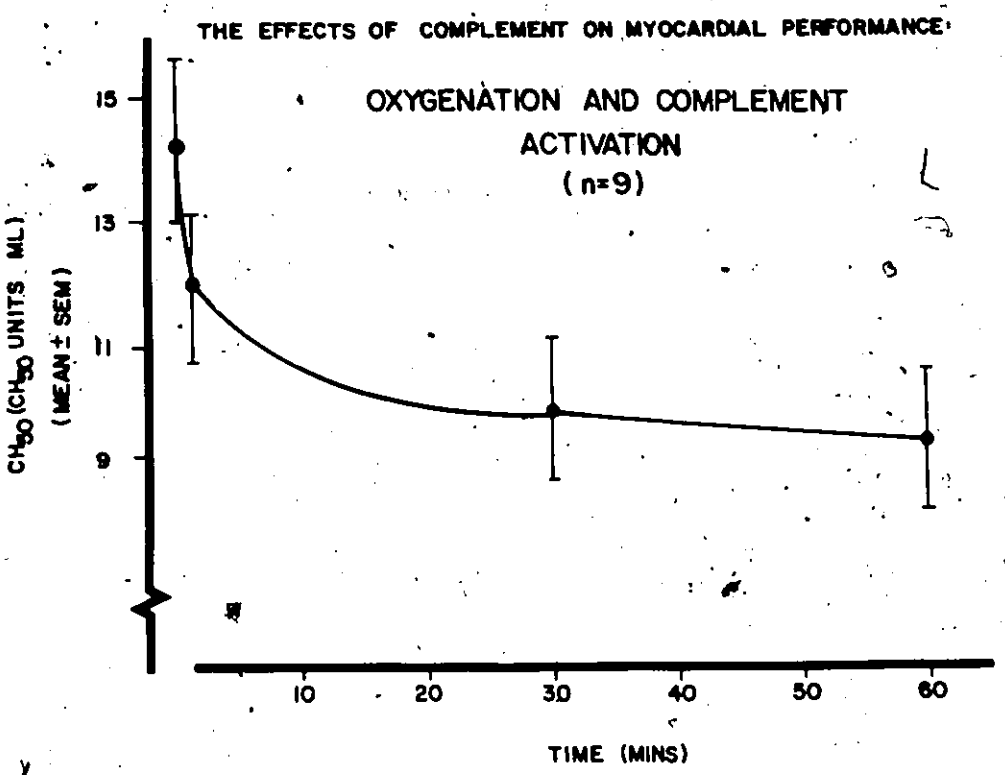

FIGURE 11: Oxygenation and complement activation. The change in CH50 (indicative of the biological activity of complement) with time is shown. CH50 decreases following exposure to the muscle bath system (glass and metal surfaces and oxygen bubbles). (see text for discussion)

PERIOP, $p < 0.05$) and CH50 (29.0 PREOP and 24.7 PERIOP, $p < 0.05$) differed significantly (table 20).

6.0 Summary. The following is a brief review of the major findings of the study:

1. On comparing the initial physical characteristics of weight, length and cross-sectional area of the muscles, there were no statistically significant differences between any group.

2. Assessing the initial control values for DF, RF and MRDF, there were no statistically significant differences between the muscles in any of the eight groups.

3. Following the addition of the test solution, there was a 29.5 to 44.3% drop in DF for muscles contracting in blood, plasma and denatured plasma compared to those in Tyrode's solution. This drop was inversely related to the calcium concentration in the bath ($r = 0.94$).

4. The muscles were allowed to contract in the test solution and the muscle parameters were followed every 5 minutes for 1 hour. A 1-way ANOVA was used to compare the data at each time interval between groups and showed no statistically significant difference.

5. The muscles have a natural decay within the solution as seen in the Tyrode's control group. The data for each muscle was transformed into a line by linear regression analysis. The slopes and intercept were compared by a 1-way ANOVA which showed no statistically significant

TABLE 20

DIPYRIDAMOLE AND COMPLEMENT

GROUP: CONTROL (n = 10)

TEST:	PREOP				PERIOP (corrected)			
	Hb	C3	C4	CH50	Hb	C3	C4	CH50
mean	137	1.3	0.25	29.0	118	1.1	0.24	24.7
SD	10.6	0.3	0.08	5.0	7.9	0.3	0.05	3.9
SE	3.4	0.1	0.02	1.7	2.4	0.1	0.02	1.3

PAIRED t-TEST:

	Hb	C3	C4	CH50
t	7.2	2.8	0.55	2.4
df	9	9	9	8
p	5.2E-5	0.02	0.6	0.05

GROUP: DRUG (n = 20)

TEST:	PREOP				PERIOP (corrected)			
	Hb	C3	C4	CH50	Hb	C3	C4	CH50
mean	139	1.4	0.29	27.1	126	1.2	0.24	22.7
SD	15.2	0.3	0.07	5.8	20.4	0.2	0.07	5.8
SE	3.4	0.1	0.02	1.3	4.6	0.1	0.01	1.3

PAIRED t-TEST:

	Hb	C3	C4	CH50
t	3.7	4.1	5.06	3.3
df	19	19	19	19
p	0.002	0.001	0.001	0.004

UNPAIRED t-TEST: (CONTROL VS DRUG)

	PREOP			PERIOP		
	C3	C4	CH50	C3	C4	CH50
t	0.4	0.4	0.20	0.1	0.0	0.22
df	28	28	27	28	28	27
p	0.7	0.7	0.84	0.9	1.0	0.83

UNITS

Hb, C3, C4 = g/l
CH50 = CH50 units/l

difference between the groups.

6. Immunological tests on samples of blood, plasma and denatured plasma before the solution was placed in the bath (the PRE sample) and at the conclusion of the experiment (the POST sample) showed that the levels of C3 and C4 remained fairly constant between PRE and POST samples. However, CH50 decreased in blood and plasma for both Control and Zymosan groups indicating that complement had been activated to a similar extent in both preparations. There was no complement activity in either PRE or POST sample of the denatured plasma.

7. The wet to dry weight ratio was determined for all muscles and compared to a control group. There was no statistically significant weight gain in any of the groups.

8. The muscle bath system's ability to activate complement was assessed. CH50 levels were followed over a 1 hour period which showed a significant drop in complement activity from control ($p < 0.001$) suggesting that complement had been activated by the system itself.

9. The muscles and blood were taken from patients undergoing cardiac surgery. Dipyridamole is an anti-platelet agent which is given to some cardiac patients preoperatively. It may interfere with complement activity so complement levels (C3, C4 and CH50) were compared between cardiac patients who were receiving the drug and those who were not. There were no statistically significant differences between the two groups.

DISCUSSION

1.0. Complement in perspective. The complement system is a part of the non-specific immune system which is important in inflammatory and infectious processes. It is linked with other systems including the thrombotic, fibrinolytic and kallikrein cascades (3,6) which together serve as protective mechanisms against either physical, chemical or infective injury. It is not unexpected that the complement system may be activated by many mechanisms as outlined previously (see Introduction section 1.1). There has been considerable interest in complement activation and its effects on body function. The cardiopulmonary bypass machine has been one device that has been shown to activate complement (26,32,33,37,38,39,43). This activation has been implicated in the mediation of various post-pump syndromes which have a propensity for affecting the lung. Myocardial dysfunction is also frequently seen postoperatively which relates to multiple factors. Since complement activity is non-specific, it is reasonable to pose the question whether some of the myocardial problems can be related to complement induced reactions, either by direct or indirect means. It was the goal of this project to assess the effects of complement activation on myocardial mechanical performance.

2.0 Methodology.

2.1. The Model. As previously outlined in the Introduction

(sections 3.0 and 3.3), an isolated human right atrial trabeculae model has numerous advantages. In general, an isolated preparation allows manipulations of conditions not possible in other models. In particular, the complement system is difficult to isolate and is probably only biologically active in blood or plasma. The experimental system described previously permits the activation of complement and subsequent observation of its effects. The trabecula itself provides a simple, reliable tool for the assessment of myocardial function with ease of preparation and availability (90,99).

The use of a human preparation is attractive in that it avoids the problems of interspecies differences in both complement and myocardial structure and function. The source of most human myocardial tissue must be patients who undergo open heart surgery. The majority of these patients require surgery for ischemic heart disease. Despite intrinsic disease, the right atrial muscle is likely the least affected compared to left and right ventricular muscle since its oxygen supply is relatively less compromised. Previous experiments (unpublished data) have shown that initial developed force does not vary greatly with age or type of disease. In addition, the criteria for acceptance of trabeculae outlined previously are more stringent than for others using either animal or human preparations. This may reduce the likelihood of using diseased tissue. Accepting this and the differences between atrial and

ventricular muscle discussed previously (Introduction section 3.4), the human right atrial trabeculae model is a reasonable approximation of human ventricle for the purposes of this study.

2.2 Muscle Oxygenation. The trabeculae which were accepted into the protocol were similar in weight, length and cross-sectional area. The cross-sectional area is of extreme importance since the muscle is depending on diffusion for its oxygen supply. Controversy surrounds the question of what is the critical diameter for the adequate oxygenation of isolated muscles (75,111). The oxygen consumption of the muscle depends on its thickness and frequency of contraction. Blinks and Koch-Weser (75) stress that the muscle be as thin as possible to allow for adequate oxygen supply. They suggest that unless the muscles are less than the critical diameter for diffusion, small variations in pO2 at the surface of the muscle will be reflected in changes in the mechanical performance. Unfortunately, critical diameter is difficult to calculate and subject to many assumptions. Approximations are available in the literature which vary from 0.64 to 1.1 mm (75,111).

The muscles used in the experiments for this thesis had a mean cross-sectional area of 0.95 mm2. This is less than 1 mm2 which is generally considered to be the critical cross-sectional area for adequate muscle oxygenation.

2.3 Model Controls.

2.3.1 Oxygenation and Foreign surfaces. These studies were designed to assess complement activation by the experimental equipment itself. Clearly, there was complement activation which appeared to be greatest within the first minute of placing the blood in the muscle bath (figure 11). The factors possibly responsible for this include the foreign surface of the bath; bubble oxygenation and the antifoam solution. This accounts for the observation that complement was activated to almost the same extent in both Control and Zymosan groups. However, the two other solutions, Tyrode's and denatured plasma, acted as controls for blood and plasma. Complement is not present in Tyrode's solution and is present but not functional in denatured plasma. It does not seem possible to isolate complement without activating it. Therefore with the use of these additional groups both with and without the addition of Zymosan, adequate controls were assured.

2.3.2 Dipyridamole experiments. Dipyridamole is the only medication that preoperative cardiac patients take which has anti-inflammatory properties that could interfere with complement activation (see Methods section 8.0). Since the blood of these patients was used in these experiments, it was important to assess any effect that dipyridamole might have had on complement activation.

The patient age and sex distribution were different which reflects the type of patients having CABG

and non-CABG cardiac surgery. Although the patients in the Control and Drug groups are not well matched for sex and age, there is no evidence to suggest that the differences should interfere with interpretation of complement results.

On comparing complement values between preoperative and perioperative samples, both groups showed a decrease in C3 levels and CH50 (after correction for dilution). There was a small but statistically significant decrease in C4 for the Drug group which was not evident for the Controls. There were no differences between perioperative C3, C4 and CH50 levels between the two groups. These data suggest that there was consumption of complement as a result of perioperative manipulations (including insertion of hemodynamic monitoring devices via venipuncture and anesthetic induction) which occured before the actual surgery. Complement consumption (shown by a fall in CH50) was accompanied by a drop in C3 and/or C4 which indicated that complement protein fragments probably disappeared from the circulation. A fall in C3 and C4 was not seen in *in vitro* experiments because there was no scavenger system for the removal of protein fragments. There was no apparent effect exerted by the drug on complement activity and therefore blood taken from patients taking this drug may be used for these experiments.

2.4 Choice of Solutions. To assess the effects of complement on myocardial performance, four different solutions were used. This particular trabeculae model was

developed using Tyrode's solution. Therefore, muscles contracting in the latter were used as the standard against which others could be compared. It was necessary to test the effects of Zymosan alone on muscle performance in the absence of complement. A Control group (using saline) was established for comparison to the Zymosan group in Tyrode's solution (see Methods section 4).

Complement is present in the plasma phase of whole blood. Whole blood was used to determine whether or not the cellular components of blood were necessary to cause any change in muscle performance. Plasma, the acellular fraction of whole blood, was used to compare with whole blood so as to clarify the role of the individual components in causing any complement mediated effect. As described in the Results (tables 12-15), both the blood and plasma Control and Zymosan groups showed similar activation of complement. This demonstrates that complement activation is sensitive and would probably occur in any <u>in vitro</u> system. It is unlikely that functional complement could be isolated without causing its activation.

Denatured plasma however, contains the complement proteins (as indicated by the C3 and C4 levels in table 16) but the complement activity is lost when the plasma is heated. Therefore, along with the Tyrode's solution groups, the denatured plasma Control and Zymosan groups act as the true controls for these experiments.

3.0 Results. The possible mechanisms of action of complement on myocardial tissue are discussed in the Introduction (section 1.2). It was postulated for this study that complement would either have an early or late effect. Complement could affect the muscle immediately after it was placed in the blood or plasma solutions. This was expected to manifest itself as either a fall in DF or MRDF or a rise in RF. Alternatively, the effect may have taken time to develop and so the muscle would be allowed to contract in the solution with mechanical parameters measured every 5 minutes for 1 hour.

3.1 Early Muscle results. Following acceptance of muscles into the protocol, there were no differences between groups in control DF, RF, or MRDF. When the bath solutions were changed, there was a minimal drop in DF when Tyrode's solution was added to the bath. However, there was a dramatic drop in DF of most muscles when blood, plasma and denatured plasma were added. The response was immediate and did not affect the decay of the muscle performance later in the experiment. The total calcium levels in these solutions (1.77 - 1.82 mmole/l) differed from Tyrode's solution (2.5 mmole/l). There was a high correlation between total calcium concentration and the percentage drop in DF ($r = -0.94$, $p < 0.001$). Although this is the most likely reason for the fall in DF, other factors may be involved.

It is unlikely that complement had any role to play in the acute fall in DF. Denatured plasma showed the

largest fall in DF however, it had no complement activity. All patients were anesthetized (essentially with narcotics alone) at the time of muscle harvesting. However, these agents at clinical levels have been shown not to interfere with muscle performance (unpublished data for morphine and fentanyl) using this model. Blood, plasma and denatured plasma were anticoagulated with heparin however, clinical doses of the drug have been shown not to alter myocardial contractility (unpublished results). The pH of solutions varied somewhat from 7.4 in Tyrode's solution to as high as 7.8 in plasma. The higher pH in blood and plasma is the result of loss of CO2 caused by bubbling the solutions with 95% O2/ 5% CO2. This simulates a respiratory alkalosis and leads to a relative increase in bicarbonate concentration. A rise in pH up to 7.9 does not severely affect muscle performance (99). Thus the main reason for the fall in DF is probably secondary to a change in calcium concentration.

3.2 Late muscle results.

3.2.1 DF Decay. The data collected over the course of the one hour observation period was analyzed in several ways to assess differences between muscles contracting in the four solutions. Firstly, the values of MMM observed at each 5 minute interval were compared using a 1-way ANOVA. Secondly, the natural decay of the preparation was assessed in each group. As demonstrated by the muscles contracting in Tyrode's solution, DF slowly falls off with time. It does this with an apparently uniform linear pattern. A

linear regression analysis was then used on the data for each muscle. The slopes and intercepts were then averaged within each group. They were then compared between groups by a 1-way ANOVA to establish significant differences. A longterm effect on DF could then be assessed by differences in slope values. Finally, the maximum drop in DF was calculated using the values observed at 60 minutes relative to the initial control values. Again differences were assessed by a 1-way ANOVA.

There were no significant differences between the groups in MMM taken at any 5 minute interval. Similarly, the slopes of the lines indicating DF decay were all similar. The drop in DF after 60 minutes in the solutions showed no statistically significant difference. Therefore, activated complement does not seem to affect muscle function. In addition, there were no differences in muscle function between blood and plasma groups. Since whole blood contains a cellular fraction, it may be concluded that these cells (PMN's in particular) (see Introduction section 1.2.3) do not give rise to an indirect effect on myocardial performance resulting from complement activation.

3.2.2 Complement Activation. In order to make the above conclusion, it must be determined whether or not complement was actually activated. Tyrode's solution does not contain any complement and was used as the control. Zymosan was added to Tyrode's to assess its effects on the trabeculae.

There were no changes in MMM which suggests that Zymosan has no direct effect itself on myocardial contraction.

Both the CH50 of blood Control (16.3 u/ml) and Zymosan groups (14.6 u/ml) were below normal (N = 20 - 40 CH50 u/ml) because of perioperative dilution. A similar dilution was seen in plasma (Control = 16.8 CH50 u/ml, Zymosan = 15.9 CH50 u/ml). In both Control groups, CH50 decreased significantly during the experiment. This is probably a result of activation due to contact with foreign surfaces (glass, metal electrodes) and gas bubbles passed through the solutions. In the experimental groups in which Zymosan had been added to the bath, complement was consumed to a greater extent (completely in the plasma experiments). Due to the moderate variability, there were no statistically significant differences between Zymosan and Control CH50 levels at the end of the experiments.

Complement byproducts such as C3a, C3d, C5a can be determined using radioimmunoassay. However, since other groups have shown conclusively that the cascade is activated by the methods outlined above, it is assumed that they follow the same pattern in these experiments. C3 and C4 levels remained unchanged before and after the experiments suggesting that the protein fragments are still present and able to react with the antibody reagents for the nephelometry tests. Although complement was activated to a similar extent in both Control and Zymosan groups of blood and plasma, there were no differences in muscle performance

compared to Tyrode's solution.

Denatured plasma served as another control whereby complement was inactivated by heat but the proteins and other constituents of plasma were present. Again, muscle performance in this solution was not different from that in the other three solutions. Complement was completely deactivated as shown by absence of hemolytic activity.

The fall in CH50 levels seen in the blood and plasma experiments following the use of recognized complement activators are evidence for the presence of activated complement fragments. Despite this, no effect on muscle performance could be demonstrated using human right atrial trabeculae contracting isometrically *in vitro*.

3.3 Biochemistry and Hematology results. Although there were some statistically significant differences in some of the electrolyte concentrations between PRE and POST samples, the differences were not large when absolute values were assessed. Changes in concentration of sodium, potassium and bicarbonate are known to influence myocardial contractions (75) which may explain some of the variability in mechanical parameters between muscles in each group. Aside from the dramatic drop in DF with the initial change of bath solution which correlated with calcium concentration, there were no other major changes that could be attributed to changes in ionic composition of the bath solution.

Hematology results for the blood experiments

revealed similar hemoglobin concentration and hematocrit between Control and Zymosan groups. However, the WBC for the POST sample in the blood Zymosan group was significantly higher than the PRE sample. Due to incomplete data (some WBC results were not performed in some experiments), the significance of these results cannot be properly assessed.

3.4 Weight results. The purpose of this determination was to assess whether there was any appreciable weight gain by the muscles following exposure to activated complement. If muscle function was adversely affected, edema due to increase membrane permeability might serve as an explanation for the deterioration. However, there were no differences in dry to wet weight ratios between any of the groups including a control group. This supports the conclusion that activated complement does not affect myocardial performance directly.

4.0 A Critical Appraisal of the Model: Why is there no effect? Upon accepting the above conclusion, why were there no changes in myocardial contractile performance? It does not seem likely that myocardium should be immune from the effects of activated complement as have been shown in the lung and other organs. Indeed, complement seems to be important in myocardial infarction, the size of which can be reduced by complement depletion (61,62,63).

4.1 Time for the Complement Reaction. A consideration is that the damage created by complement in these experiments

may take longer than 1 hour to occur. This is unlikely since other groups have shown that the mechanisms described previously take minutes to occur. Hammerschmidt et al showed that PMN's are activated and aggregate immediately (10,112). Similarly, cellular necrosis takes only 15 minutes to occur when complement is locally injected (74). With such a rapid onset of action, dysfunction should have been demonstrated within an hour period.

4.2. Complement Survival in vitro. Activated complement fragments have a very short lifespan *in vivo* as do by-products of complement activity such as PMN-produced toxic oxygen radicals. These may have been depleted quickly in this protocol by enzymes and scavengers in the blood and plasma and therefore did not have time to accumulate in the myocardium. The *in vitro* model is limited in that there is only a finite amount of complement in the 20 ml of blood used for the experiment. The muscle occupied a relatively small volume in a large pool of solution so that the complement fragments may not have been physically close enough to the muscle to affect it before their decay. With a constant and more localized blood flow present *in vivo*, a continuous supply of complement may be provided to enhance the inflammatory reaction.

With this in mind, two additional models may be suggested. Firstly, following control measurements in Tyrode's solution, oxygenation could be withheld for a period of time to render the muscle hypoxic. The solutions

could then be tested in a controlled hypoxic model which would simulate the post-perfusion heart or ischemic zone about a myocardial infarction. Secondly, an in vivo model could be developed whereby a coronary artery is isolated and perfused with activated blood or plasma. A larger volume of activated complement would be available to perfuse the myocardium. Myocardial performance could be assessed by wall motion and regional flow studies.

4.3 Vascular Role. This in vitro model did not assess the possible role the vasculature might play. Leukosequestration and embolization (12) may cause reduced blood flow and tissue ischemia resulting in deleterious effects on myocardial activity. Similarly, complement activation leads to kinin and histamine release from mast cells, basophils, etc. which would culminate in changes in vascular tone and permeability both locally and systemically which might alter myocardial function. These indirect effects of complement activation could only be studied using an intact circulatory system which was not possible in our model.

4.4 Electrical Stimulation. Muscle contraction was elicited by field stimulation. The two platinum electrodes were in close proximity to the muscle. In the blood experiments, a small buildup of blood cells and protein was seen on one electrode in each experiment. It may be that as a protein, complement factors could be diverted away from the muscle and attracted to either plate. However, this was not

observed in the other solutions.

4.5 Effects of Heparin. The thrombotic mechanism was blocked by the addition of heparin prior to procurement of the blood. Heparin acts to potentiate antithrombin III, which prevents the action of thrombin on fibrinogen. Although heparin is known to activate complement, this occurs at plasma concentrations in excess of 10 u/ml. The normal plasma level of heparin in patients undergoing cardiopulmonary bypass (from whom we took the blood for these experiments) is 6 u/ml (107). The pathways for clotting, fibrinolysis, kinin production and complement activation are linked via Hageman factor and plasmin (6). They all share common means of activation and may each act to amplify one anothers reactions. The blockage of thrombotic arm may reduce the magnitude of the complement reaction. Fibrin, kinins and other by-products may have additive effects though this is not well understood. Complement seems to have been activated adequately in this system as indicated by the significant drop in CH50.

4.6 Defects in Complement Regulation. Although most patients undergoing cardiopulmonary bypass show some complement activation, it is difficult to predict who will develop the severe complications described previously. Assuming that complement activation is responsible for these problems, then there must be a reason for the variability among patients. Some may have defective inflammatory control mechanisms such as C1 esterase inhibitor deficiency

or have circulating anti-tissue antibodies which could help mediate damage (ie anti-heart antibodies seen in PMIS or PPS). If these abnormalities are necessary for an adverse complement-mediated response, then the patients used in these experiments may not have been affected. Therefore reactions would not be seen in vitro or in vivo.

4.7 Statistical Variability in Results. The last area of concern is the variability in the results. Some of the results showed a high degree of scatter, especially in the series utilizing denatured plasma in which correlation was poor ($r < 0.3$) and standard errors high. This may be due to factors which include hormone levels, medications, etc. Although uniformity of sampling was attempted, each clinical situation would have been unique. For instance, at the time of sampling the patient may have just had a dose of narcotic or other drug thus creating a blood level that would be different from other patients being studied. As a response to the trauma of surgery, levels of adrenaline, glucagon and cortisol would be increased to varying extents in different patients (113). These differences in patients would be difficult to control. Concerning the denatured plasma in particular, it would have contained proteins with conformational changes which may have lead to the release of hormones and other bound substances that would affect the results. Although human blood is a complex suspension of a myriad of components, a more constant solution may have decreased variability. Blood taken from only one donor

would not be practical as there might be immune reactions between blood and tissue of different subjects. Also, the problems of maintaining a supply of the same blood would be numerous. There are also intrinsic differences between individual muscles from the same or different individuals. The complement system is a difficult one with which to work because it cannot be isolated by itself and it is activated so easily.

5.0 Conclusions and Relevance. Activated complement does not appear to have any direct or indirect effect on myocardial performance *in vitro*. If these effects are not important, then the post-CPB effects seen clinically may be due to complement mediated reactions which were not tested by this model. These include the role that the coronary vasculature *in vivo* might play in myocardial dysfunction associated with conditions which may activate complement. Complement could mediate the release of vasoactive substances which might act locally to alter blood flow to the heart directly, causing a change in contractility or systemically by changing peripheral resistance. Both of these effects could lead to an alteration in cardiac output. PMN sequestration and embolization is affected by activated complement and may have a role in myocardial dysfunction in terms of creating a physical obstruction to blood flow and/or a release of destructive enzymes and toxic oxygen radicals.

Bubble oxygenation and foreign surfaces activate complement and emphasize the difficulty of isolating and using complement *in vitro*. Dipyridamole does not affect complement activation *in vivo*.

Complement functions as part of the body's defense system. This may be activated in numerous ways including cardiopulmonary bypass which may lead to organ dysfunction. This study has shown that the myocardium is not directly affected by activated complement. Other mechanisms for alterations in heart function may be the focus for future research.

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