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Effect of Acid Rain and Humic Substances on Aluminum Toxicity (And); A Comparative Study of Chemical Composition of Old and Newly Deposited Plaque From Heart Patients.

Jane Igoki Murungi
Louisiana State University and Agricultural & Mechanical College

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Effect of acid rain and humic substances on aluminum toxicity [and] A comparative study of chemical composition of old and newly deposited plaque from heart patients

Murungi, Jane Igoki, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1990

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EFFECT OF ACID RAIN AND HUMIC SUBSTANCES ON ALUMINUM TOXICITY

A COMPARATIVE STUDY OF CHEMICAL COMPOSITION OF OLD AND NEWLY DEPOSITED PLAQUE FROM HEART PATIENTS

A Dissertation

Submitted to the graduate faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirement for the degree of Doctor of Philosophy in The Department of Chemistry

by

Jane Igokí Murungi
M.S. Louisiana State University, 1981 August 1990
DEDICATION

To the almighty God who makes
all things possible

and

to my husband and children

who had to bear the pain of long separation.
ACKNOWLEDGEMENTS

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A preliminary study was initiated to compare the chemical composition of the newly deposited atherosclerotic plaque after a bypass operation and rigorous treatment, and the material that had been depositing since childhood of heart patients. The lipid composition was determined using HPLC, TLC and MS, after extraction from the plaque using 2:1 Chloroform:methanol. The metal composition was determined using ICP, after acid digestion.

The results indicated a significant difference between old and new deposits in both metal concentration and the lipid composition. The number and type of lipids in the old and the new deposits from the same individual were different. Metal ratios and concentration, especially calcium, sodium, and phosphorus, were also different.

The results indicated that a more extensive and controlled study would be useful in understanding the etiology of heart diseases.
A study was carried out to find if leaf extracts could be used to counteract the effect of acid rain and aluminum in soils and lakes.

Tilapia, and common shiners fish, and alligator grass plant were used to study the effects of pH and aluminum. The effect of dried leaves on the pH and the buffering capacity of deionized water and the effect of the presence of cations such as iron, calcium and magnesium on aluminum toxicity were investigated.

The results of our investigation indicated a synergistic toxic effect of aluminum and acidity, with the gills' endothelia and the roots of alligator grass being the most affected by aluminum exposure at pH below 5, while the presence of leaves increased the pH of water, decreasing the toxic effect.

Study revealed that the leaves that decomposed easily to form humic and fulvic acids were most effective in reducing the aluminum toxicity. Pine leaves did not mitigate the toxic effect. The presence of iron and/or phosphorus reduced the toxic effect of aluminum to fish while magnesium and/or iron reduced the toxicity of aluminum to alligator plant.
PART 1

A COMPARATIVE STUDY OF CHEMICAL COMPOSITION OF OLD AND NEWLY DEPOSITED PLAQUES FROM HEART PATIENTS FROM NEW DEPOSITS (BYPASS ARTERIES) AND OLD DEPOSITS (NON-BYPASSED ARTERIES) OF HEART PATIENTS
CHAPTER 1

GENERAL INTRODUCTION

The proper functioning of the cardiovascular system is essential for the survival of all animals including man, since all other organs depend on the heart and vascular system to circulate blood and enable them to function properly.

Diseases of the cardiovascular system have become major causes of death in the industrialized as well as the developing countries. Where death does not occur the individual may be crippled for a long time or for life. It is believed that about 25% of the American population has some form of cardiovascular disease (1-6).

A lot of time and resources have been spent in research to reveal the causes of heart diseases and to find ways to eradicate them or reduce their effect. Much information has been obtained about cardiovascular diseases and the increased knowledge has led to some reduction in death rate. For example, in 1963 coronary heart disease alone accounted for 30% of all deaths in the USA whereas in 1985 the same disease accounted for 26% of all deaths in the USA (3). The decline in death rate may be due to a better understanding of risk factors in cardiovascular diseases, leading to better diet, as well as better treatment. Improvement in patient care and reduction in associated risk factors may also have contributed to the decline in death rate(4-7).

Despite the reduction in death rate, cardiovascular diseases are still among the leading causes of death in the industrialized nations and therefore continued research is needed to understand the causes and to find better ways of treating heart patients so that the disease can be eradicated or minimized.

The diseases of the cardiovascular system are many and the causes may be many, but they all affect the performance of the heart. The malfunctioning of the heart and
vascular system is indicated by such conditions as hypertension, coronary heart disease, cardiomyopathy, atherosclerosis, myocardial infarction, angina pectoris, heart arrhythmias and ischaemic heart disease.

The symptoms of heart attack include severe chest pain, accompanied by sweating that is not reduced by resting. The sensation of pain may spread to the left arm and neck (8, 91).

A FORMS OF CARDIOVASCULAR DISEASES

1 Hypertension

Hypertension or high blood pressure is one of the principal risk factors associated with cardiovascular diseases (10-17). It is called a silent killer because it does not produce tangible symptoms that alert the patient or distinguish it from other illness. Symptoms which may indicate the possibility of hypertension include emotional and physical fatigue, with occasional headaches, dizziness and lightheadedness(8).

There are two phases of hypertension: the essential hypertension, which seems to have no known causes, and the severe hypertension, which causes severe damage to blood vessels, and may produce poor vision, with blood spots (9,18-21). If high blood pressure is left undetected or is left untreated, it can lead to sudden death due to heart failure.

a Effect of High Blood Pressure on the Cardiovascular System

The actual effect of hypertension on the cardiovascular system depends on the health of the heart, the frequency and the severity of the high blood pressure, and the ability of the blood vessels to withstand high pressures. Recent studies with experimental animals, have shown that hypertension increases the thickening of arterial wall, by increasing the rate of proliferation of endothelial cells [20, 22]. Changes in size and shape
of the cells were also observed, with increased pressure (23). There were also changes in permeability of the cells (24). Chronic hypertension in rats was shown to change DNA, and this change was not reversed by reduction in the blood pressure (25). Studies with monkeys, showed that hypertension, which is not accompanied by hyperlipemia, does not induce atherosclerosis (18, 26, 27,).

With age, and continued high blood pressure, the arteries may harden, scar and sometimes break off causing the blood to leak and form a clot (10, 21, 28). If the formed clot lodges itself in an important artery of the heart or the brain, death of the heart muscle or the affected part of the brain may result due to blockage of blood flow. Sometimes the clot may partially block the artery servicing the heart. The heart muscle may enlarge in an effort to cope with increased demand to pump harder through the choked artery. This may finally lead to heart failure due to increased demand and decreased nutrients (2, 10, 11).

High blood pressure can cause closure or rupture of a small or large artery. This may cause severe temporary or permanent injury to the heart depending on the amount of heart muscle that is cut off from its supply of nutrient (8).

Hypertension can cause rupture or blockage of a renal artery leading to kidney failure. Renal failure can rapidly lead to electrolyte imbalance resulting in edema and heart failure (8)

b Causes of Hypertension

The causes of hypertension are not well understood. It is believed that some forms of hypertension, especially essential hypertension, may have genetic origin (8, 10, 12, ). Hypertension may be a result of metabolic disorder or it may be due to the lack of some essential nutrients.
c The Role of Metals in Hypertension

Studies with humans and animals have shown that low intake of calcium and magnesium can cause hypertension (10, 13, 29, 42, 43 - 48). Humans with hypertension have been shown to have increased excretion of calcium in the urine (9, 14, 29). Many experimental and epidemiological studies have shown an inverse relationship between water hardness and development of hypertension (50, 51). People living in soft water areas have been shown to have higher blood pressure than those living in hard water areas (50, 52 - 59). Water hardness is determined by the concentrations of both calcium and magnesium.

The Maasai, a tribe in Kenya, whose diet is mostly meat and whole milk (28, 60), do not have hypertension, even though this kind of diet is believed to contribute to development of coronary heart diseases including hypertension (61). It is possible that calcium and magnesium in milk protect the Maasais from heart disease.

In animal experimental and human hypertension, disordered calcium metabolism was observed in different organs (14, 62, 49, 63, 46). In experimental animals, vascular tissues showed increased membrane permeability to calcium, altered kinetic binding in cell membrane and accumulation of calcium in the intercellular fractions including mitochondria (29).

Epidemiological and experimental studies indicated cadmium in the development of hypertension (44, 46, 52-55, 63 - 69, 70-72), Cadmium has been shown to interfere with absorption of calcium in the intestinal mucosa and to replace calcium in the mitochondria (62). It is possible that cadmium induces hypertension by its effect on calcium metabolism.
d  The Role of Neurotransmitters in Hypertension

It is believed that the catecholamines may play a role in hypertension (73, 74). These catecholamines affect the blood pressure by their neurotransmitter function in the central nervous system. The most active catecholamines are histamine, norepinephrine, and epinephrine. They are released at sympathetic nerve endings or from adrenal medulla, and they have a direct effect on the kidney, where they influence blood flow and renin production (73, 75). Studies with experimental animals, especially rats, showed that blood pressure correlated with catecholamines release (74). Hypertensive rats were shown to have a higher rate of release of dopamine than non-mortensive rats. Adrenaline and noradrenaline levels were reduced in hypertensive rats (74).

Studies have also shown that emotions, especially those that produce anger, depression and hostility, increase blood pressure and increase death due to cardiovascular disease (76, 77, 78). Emotions are controlled by a sympathetic system that controls the release of neurotransmitters. These neurotransmitters, or catecholamines, control the blood flow by acting as either vasoconstrictors such as serotonin, or vasodilators such as histamine. The catecholamines play a major role in hypertension. Drugs that are useful in treatment of high blood pressure, influence deposition, metabolism, or actions of neurotransmitters.

2  Arteriosclerosis

Arteriosclerosis is a chronic disease that results from abnormal thickening and hardening of arterial wall resulting in loss of elasticity (8, 79, 80). The thickening increases resistance to blood flow, making the heart work harder, to pump the blood through the narrowed arteries. Continued high blood pressure may play a role in the etiology of
arteriosclerosis by causing injury to the artery, either by hardening it or causing it to tear. The most common site of arteriosclerosis is the aorta.

The causes of arteriosclerosis are not well understood, but is believed that it is a normal process of aging.

3 Atherosclerosis

Atherosclerosis is a condition in which the arteries are abnormally thickened, and become partially or wholly clogged with atheromas, or plaques, that are composed mainly of lipid. The major components of these plaques are believed to be cholesterol and cholesterol esters (61,81-84).

It is believed that deposition of lipids may start as a result of injury in the interior wall of the artery. This injury may result in production of thromboxane which causes platelets to aggregate and trap lipid components (85-88). Some people believe that the lipids start a chain of events that increases smooth muscle cells, and the movements of these cells from arterial media to the intima (see figure 1)(89). Others believe that thickening of the arteries (arteriosclerosis) results in accumulation of lipids (84, 87, 90-92).

Some also believe that the increased lipids in the blood, especially low density lipoprotein cholesterol, causes damage to the cell wall (78, 79, 90-98) Proteins referred to as lipoprotein carry fatty acids, cholesterol, and triglycerides to and from the liver. These lipoproteins are classified according to their densities in water. The very low density lipoproteins have a density of 0.97g/ml, while low density lipoproteins (LDL) have a density of 1.03g/ml. High density lipoproteins (HDL) have a density of 1.121g/ml (97). The LDL carries cholesterol from the liver to other parts of the body, through the blood, while the HDL removes excess cholesterol from the blood to the liver, where it is converted
to excretable substances such as bile acids. It is believed that high ratio of LDL/HDL, above 1, results in accumulation of cholesterol in the blood resulting in injury of the blood vessels (96 - 99). This injury then changes membrane permeability resulting in accumulation of the lipids in the intima of the artery (80 - 84). There are some who also speculate that arteriosclerosis is an expression of a deficiency state in the specialized endothelial cells (102). This could be a deficiency of essential fatty acids such as linoleic acid, and arachidonic acid or minerals such as potassium, magnesium, calcium zinc, etc. (100-106).

A Schematic Diagram of Formation of the atheroma is shown in fig. (1).

A person with atherosclerosis may live a normal life as long as there is no impairment of blood flow to vital organs. In most cases, the hardening of the arteries with partial occlusion results in impedance of blood flow. This forces the heart to pump harder and faster, which may lead to weakening of heart muscles and may result in heart failure.

Atherosclerosis may cause ulceration and damage of the lining of the artery. This may lead to the formation of a clot or thrombus (2,8,95,). The formation of a clot may cause the arteries to contract, leading to severe chest pain or it may totally block a major artery of the heart, leading to a heart attack (92,95).

4 Coronary Heart Diseases

Coronary heart diseases occur as a result of malfunctioning of the arteries that provide the necessary nutrients to the heart muscle. The coronary artery may be partially or wholly blocked by a clot or as a result of atheroma which accumulate and choke off the artery (8). The muscle of the heart served by the blocked artery may die due to lack of nutrient. This results in a condition known as myocardial infarction (4,8). If an extensive area of heart muscle is affected, it may lead to death due to heart failure (11,16,24, ).

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FIG. 1 DEVELOPMENTAL STAGES OF ATHEROMATOUS PLAQUE
Sometimes, the coronary artery may be only partially blocked. If sufficient blood moves through the occluded artery to reach the heart muscle, then the patient may not have any symptoms and may live a relatively normal life.

When obstruction of the blood supply reaches about 70%, supply of the blood to the heart muscle is inadequate, the patient begins to feel chest pains with exercise. This is called angina pectoris. This pain may extend all over the chest and may move to the neck and the arms. The pain may be associated with the feelings of pressure, heaviness, lightness or obstruction of the chest. The pain may be sometimes brought about by conditions such as anger or anxiety or after the consumption of a heavy meal. (8, 107)

5 Irregularities in Heart Rhythms

The insufficient supply of nutrients to the heart muscle, may weaken the heart, resulting in an irregular heart beat (108,109). The heart may either slow down or accelerate. In either case, the heart fails to pump sufficient blood to the brain and this may result in dizziness or unconsciousness.

A healthy heart beats 60-90 times per minute at rest but it may reach 150 beats per minute during exercise or excitement (110-112). The electrical impulses of the heart are produced in a collection of cells called sino-arterial nodes. These cells are controlled by the autonomic nervous system (110-112). In coronary arterial disease, the conduction of electrical impulses may be affected. This may in turn lead to a disturbance in pulse rate or uncoordinated contraction of the heart (110-112)

Partial failure in sino-arterial nodes, may result in changes in the electrical generation which may produce irregular heart rhythms. Complete failure of sino-arterial nodes forces the atrium of the heart to take over conduction of the electrical impulse. This conduction results in the stimulated heart rate which may reach as much as 350 beats per minute (110-
As the rate increases, contraction of the heart muscle becomes chaotic, resulting in atrial fibrillation (110-112) (quick erratic contractions which do not produce enough force to push the blood through the atrial chamber). In coronary arterial diseases, the conduction system may be injured, preventing the electrical current from reaching all parts of the heart. This may occur after injury and swelling of the heart muscle. Partial or complete blockage may occur. When all connections are blocked it may cause the heart to stop beating for a few seconds or the heart rate may go as low as 35-45 beats per minute (110-112).

Sometimes the irregularity of the heart may be caused by muscles of the ventricular wall of the heart. This may be as a result of damage to ventricular muscles. The ventricular chamber may enlarge to compensate for the weaker muscles. The conduction of the impulse in the chamber may become irregular due to enlarged muscles (11). This results in ventricular fibrillation where the heart beats very rapidly in an uncoordinated way. It is fatal if not treated immediately.

Potassium controls the movements of ions and it also controls membrane potential of heart muscles. This potential is necessary for impulse conduction which causes the heart to contract. If the concentration of potassium is low, especially intercellular potassium, movement of ions may be erratic, resulting in uncoordinated contraction of ventricular muscles. Low potassium concentration in serum is believed to play a role in ventricular fibrillation (110-113).

6 Cardiomyopathy

This is a structural and functional weakening of the heart muscle, especially the ventricular muscles. This abnormality includes damage to the ventricular cells, resulting in enlargement of the ventricular chamber. The injection force of the blood is reduced due to weaker contraction of the affected muscles. This may result in blood accumulation in the ventricular chamber, leading to congestive heart failure. The other form of cardiomyopathy
is thickening or excessive growth of heart muscles. This affects the relaxation and filling of the ventricular chamber (11).

Causes of cardiomyopathy are not well understood. However, it is believed that viruses, bacteria, spirochetes, fungal or protozoa infection may destroy heart muscle, resulting in cardiomyopathy (11). Extensive muscle weakening can lead to death unless a heart transplant is performed (2, 11).

B THE ROLE OF METALS IN CARDIOVASCULAR SYSTEM

There are many forms of cardiovascular diseases and therefore, the causes may be multifactorial. Genetic, as well as metabolic disorders, may be responsible for some or all of cardiovascular diseases. It is believed that interference with cell metabolism of essential elements that play a key role in maintaining proper functioning of the cardiovascular system may be one of the causes of heart diseases (31-49, 62, 101, 110-113, 115, 116). Therefore, it is important to understand the roles played by metal ions and lipids in order to understand their role in the etiology of cardiovascular diseases.

1 Role of Calcium and Magnesium

Calcium and magnesium are elements of relatively high concentration and of major importance in the body. They each play a vital role in the cardiovascular system (27-47, 56, 114-120). Most body cells including heart muscle cells, maintain high concentrations of magnesium and potassium and low concentrations of calcium and sodium inside the cell. The reverse is true in the extracellular space. Active transport as well as diffusion of the metal ions through the cell membrane plays a key role in controlling the contraction or
relaxation of the heart muscles (29, 30 - 34, 48, 71, 113-119, 121, 123). These movements result from changes at the membrane. These changes may be due to alteration of binding sites at the membrane or changes in the electrical potential that selectively allows only certain ions to move across the cell membrane (46, 121, 124). It is believed that calcium and magnesium control the permeability of the cardiovascular membrane system (31, 47,51, 114-118). Interactions of calcium and magnesium with the receptor sites at the membrane are believed to control the movement of sodium and potassium ions (48,109,116,).

Studies with animals showed that a progressive increase of extracellular magnesium concentration above physiological level, (0.7-1mM/l) (125), increased inhibition of most contractile substances such as acetylcholine and angiotensin, which results in relaxation of heart muscle. The relaxation of the muscle allows the blood to flow especially if the muscle spasms were responsible for the blockage of blood. It is believed that magnesium stabilizes the membrane and blocks calcium entry (48).

Increased intercellular Ca$^{2+}$ causes the membrane to be more permeable to K$^+$ (48, 126 - 128). The transfer of electrolytes through the membrane is controlled by external and internal concentration of magnesium. Magnesium regulates sodium and potassium channels, which control the electrical conduction of the heart (48).

It is believed that Ca$^{2+}$ ions regulate the contraction of heart muscles. The regulation mechanism is believed to be located on the thin filament proteins of myocardial muscle (48). Modification of the proteins is believed to be affected by phosphorylation and dephosphorylation of myosin (128 - 132).

The basic requirement for the regulation mechanism in smooth muscle is that the Mg$^{2+}$ ATPase activity of actomyosin is activated in the presence of Ca$^{2+}$ at concentrations necessary to start contraction. No contraction is observed in the absence of Ca$^{2+}$, in
Experiments involving heart muscle (114, 121, 109). When the muscle cells are stimulated
to contract, calcium is set free and is taken up by endoplastic reticulum, which binds
calcium strongly in the presence of ATP (106). The uptake of Ca\textsuperscript{2+} by reticulum may be a
physiological function or releasing factor (133).

Research showed that an increase in extracellular Mg\textsuperscript{2+} as little as 2.4 mM inhibited
the spontaneous mechanical activity of the heart (48). The data obtained showed that
extracellular magnesium played an important role in the regulating membrane permeability
to Ca\textsuperscript{2+} (48). It is believed that magnesium occupies sites in the membrane of arterial and
venous smooth muscles which are exchangeable with membrane bound Ca\textsuperscript{2+} in arterial
and venous smooth muscles (48, 121, 134, 135).

In vitro studies with heart muscles of animals showed that withdrawal of Ca\textsuperscript{2+}
impairs excitation-contraction coupling of heart muscles and reduces contractile force (117,
134). The study showed that complete withdrawal of Ca\textsuperscript{2+} slowed the heart rate and
finally led to cardiac arrest (1, 121).

Deficiency in magnesium results in edema of the vascular endothelium, calcification
and cardiac necrosis (33, 48, 105, 117, 119, 137) Magnesium is required for oxidative
phosphorylation which provides energy for the maintenance of strength of cardiac muscle
(48, 117). Contraction and relaxation of myofibrilles depend on magnesium ions and ATP.
This may in turn be due to the former's control of release of calcium ions from sarcotubules
(117, 133). Magnesium is believed to prevent heart damage by inhibiting calcium
stimulated contractions of the injured myocardial muscles (33, 47, 48).

Creatine phosphokinase plays an important role in the transfer of intercellular
energy from ATP of the mitochondria to the cytoplasmic creatine (47). Magnesium ions
regulate the action of this enzyme and therefore play an important role in providing energy
to the cardiovascular system (115, 117).
Studies with rat hepatic cells showed that magnesium deficiency resulted in cells losing intercellular K\(^+\) and Mg\(^{2+}\) and increased intracellular Na\(^+\) and Ca\(^{2+}\), resulting in a decoupling of energy process mediated by ATP (48,56,57, 121).

Magnesium is also believed to play a role in blood pressure. Acute hypomagnesium (chronic low serum magnesium) is associated with elevation in blood pressure and increased resistance to blood flow through the blood vessels (87). Studies with animals showed that magnesium prolonged intraventricular conduction of the heart impulses (48, 117). Clinically, magnesium shows vasodilation effects and increases cardiac output (48). Studies show dependence of myofibrillar contraction and relaxation on magnesium ATP which in turn controls the uptake and release of calcium (121, 133). Studies have also shown that supplementation of diet with magnesium salts lowers total serum lipids and atherosclerosis induced by diet (48, 119,121).

2 Role of Sodium and Potassium

Propagation of impulses, which cause the heart muscle to contract and relax, is controlled by movement of ions to and from the cell. This movement maintains the membrane potential. Potassium and sodium ions are primarily responsible for maintenance of cell membrane potential of the heart. The movement of the ions is controlled by both the concentration gradient and active transport (120,121, 126).

Serum concentrations of potassium are normally between 3.8-4.0 meq/l and intracellular levels are about 110.0 meq/l (132). This gradient is maintained by Na\(^+\) which pumps K\(^+\) into the cell in exchange for Na\(^+\). Resting heart muscles are more permeable to K\(^+\) than to other ions (120). Entry of cations into the cell appears to be in stages which in turn control the potential, rhythmicity and length of contraction of the heart. Na\(^+\) enters cells during rapid current increase and fast impulse conduction in the heart muscle, while Ca\(^{2+}\) enters during slow impulse that maintains steady current. K\(^+\) leaves the cell during
repolarization (43, 120). The K+ effuse through potassium selective channels serving to polarize the membrane. If extracellular K+ goes below 3 mM, the permeability of K+ is decreased.

Low intake of potassium results in heart arrythmias, due to the impairments of the mechanical functions of the heart muscle (132, 137). Studies with experimental animals showed that depletion of potassium in the heart resulted in an increase in binding of calcium to mitochondria and a decrease in uptake of calcium by sarcoplasmic reticulum (121, 132). It is believed that a decrease in contractile forces of the heart muscle caused by potassium depletion in myocardopathy may be due to shifting of intracellular Ca\textsuperscript{2+} from sarcoplasmic reticulum to mitochondria (48, 110, 133 - 139). Increased binding of calcium to mitochondria may be as a result of change in phospholipid composition of mitochondrial membranes (138). Patients with hypokalemic cardiomyopathy were shown to have decreased cardiac performance.

In vitro studies with amphibian hearts, where the heart was perfused in a controlled medium, showed that Na\textsuperscript{+} / Ca\textsuperscript{2+} exchange removes Ca\textsuperscript{2+} after contraction, and a Na\textsuperscript{+} free environment results in a large Ca\textsuperscript{2+} loading of heart fiber and contraction in many fiber types (43, 68).

Na\textsuperscript{+} is the major determinant of volume in the extracellular space. Exchange that results in leakage of Na\textsuperscript{+} followed by Cl\textsuperscript{-} and H\textsubscript{2}O into intracellular space, leads to intracellular edema if the extra ions and H\textsubscript{2}O are not pumped out by active transport. This happens when the ATPase dependent electrochemical gradient is disturbed (106, 132). Calcium moves continuously into the cells down its concentration gradient. To prevent intracellular overload, it must be continuously pumped out of the cells (106). There are two pumping systems for Ca\textsuperscript{2+}, a specific ATPase and Na\textsuperscript{+} / Ca\textsuperscript{2+} pump, whose maximum pumping velocity is 30 times slower (132, 138). This is useful when massive
Ca\textsuperscript{2+} must be transported rapidly through the membrane. It is believed that even Na\textsuperscript{+} / Ca\textsuperscript{2+} exchange is dependent on ATPase (110, 121).

Studies have shown that the Na\textsuperscript{+} / Ca\textsuperscript{2+} exchange operates electronically with a possible stoichiometry of 3 Na\textsuperscript{+} for 1 Ca\textsuperscript{2+}. Charge compensation is probably carried out by movement of Cl\textsuperscript{-} and Na\textsuperscript{+} together, and movement of K\textsuperscript{+} and Ca\textsuperscript{2+} in the opposite direction (118, 132, 140).

It is believed that the ATP dependent Ca\textsuperscript{2+} pumping system transports Ca\textsuperscript{2+} into a department from which it can be removed by Na\textsuperscript{+} / Ca\textsuperscript{2+} exchange which is present in the sarcolemmal fraction of the heart (106, 121, 140).

3 Role of Copper and Zinc

The actual role of zinc and copper in the cardiovascular disease is not very clear. They seem to have opposite effects. The zinc concentration in the ventricular muscle is approximately 141 ppm and does not change with age. There is evidence that copper plays a vital role in maintenance of a healthy heart muscle (141, 142, 143). Serum zinc levels are decreased in peripheral atherosclerotic diseases, but in myocardial ischaemia serum zinc is elevated (104). In myocardial infarction there is a tendency for low zinc.

It is believed that increased concentrations of zinc serum in some heart diseases may be due to breakdown of zinc enzymes in the muscle (141). This may result from the death of heart muscle. In cases where there has been heart injury, serum zinc may be transferred to the injured area, resulting in a decreased level in the serum (142).

Copper plays a role in the cardiovascular system. Feeding chicks with low copper diet resulted in rupture of the arteries because of lack of collagen cross-linking (112). Defects in arterial walls were also observed in experimental animals fed on low copper diets (101). It is believed that the ratio of zinc/copper does play a role in atherosclerosis. The
role is controversial (103, 104, 142-147). Klevay (144) believes that high zinc/copper ratio results in atherosclerosis. This was as a result of observation that high zinc/copper ratios increased cholesterol blood levels. However, other studies showed that the HDL cholesterol is increased more than LDL cholesterol in high zinc/copper ratios (101).

Studies showed that substances that decreased cholesterol increase copper absorption from the intestine whereas those which increase copper intake decreases cholesterol in the plasma. It also is believed that calcium lowers cholesterol by decreasing zinc/copper ratio in the liver (101). The role of zinc/copper ratio in pathogenic of atherosclerosis is still controversial since only total cholesterol was studied.

Zinc plays a role in stabilization of membrane. It is believed that combination of zinc with phospholipids in the cell monolayer prevents temperature induced phase transition of the monolayer (140, 142, 149).

Polyunsaturated fatty acids are important in cell membrane. Zinc is involved in desaturation of fatty acid and incorporation of fatty acid to other lipids (87,140,150). Zinc is involved in synthesis of prostaglandins (127) which are powerful cell regulators.

Zinc is also shown to inhibit some enzymes such as phospholipases, and thromboxane synthentase and therefore may play a role in platelet aggregation (140, 151, 152). Zinc is present in relatively high concentration in the liver microsomes which are responsible for desaturation of fatty acids (104, 152). It is believed that the essential fatty acids increase absorption of zinc in the gut.

4 Role of Manganese

Manganese is required for lipid and glucose metabolism and in oxidative phosphorylation. Manganese activates enzymes that use vitamin C, Choline and B vitamins. Inability to use choline properly results in low production of acetylcholine which
Fig. 2 Structures of Common lipids.
is a neurotransmitter in the brain (101). Deficiency in acetylcholine results in loss of muscle strength. Low manganese may affect heart muscle by reducing the amount of acetylcholine produced. It may also play a role in atherogenesis by its effect on lipid metabolism.

C The Role of the Lipids in the Cardiovascular System

Plasma membrane cells as well as other cells, including heart muscles cells contain about 35% lipids, 15% of which are neutral lipids and 20% phospholipids (12). Of the lipids portion 15-30% is believed to be cholesterol and cholesterol esters and the rest are phospholipids (12, 124, 138). Storage lipids contain more saturated and monosaturated fatty acid whereas structural lipids generally have a higher level of polyunsaturated fatty acids (207).

Lipids control cell function because they can be broken down and synthesized from small molecules (12, 98).

Lipids control membrane permeability to ions (105,140). Electrical impulses in the nerve and muscles are produced by changes in the relative permeabilities of surface membrane to some ions (132,183). It is believed that the lipid component of the membranes allows for conformation changes that lets ions move in and out of the cells (124).

1 The Importance of Cholesterol

Cholesterol is a sterol found in man and animals. It is a solid alcohol of high molecular weight, and contains a tetracyclic perhydropentanophenanthrene skeleton. The structure of cholesterol is shown in fig (2). It has 27 carbon atoms. Cholesterol is a
Fig. 3 Conversion Of Cholesterol to Cholesterol Esters and Bile Acids in the Liver.

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component of all body cells, but more is found in brain, adrenal gland, ovary, testes, placenta, and liver tissue (24, 61, 83). In the liver cholesterol is used to manufacture transverse sterol hormones of the adrenal cortex, bile acid, vitamin D, and water balancing steroids (83). The equation that shows conversion of cholesterol to bile acids in the liver, is given in fig. (3).

NMR and electron microscopy studies have shown that cholesterol has a special ability to cause conformational changes in the membrane (124). It is believed that the presence of cholesterol in the membrane increases thickness of membrane bilayers especially those consisting of phospholipids with hydrocarbon chains up to sixteen carbons in length. With phospholipids whose carbon chain length is higher than 18 carbons, the cholesterol decreased the width of the bilayer (124). Cholesterol also causes conformational changes in the membrane proteins. Cholesterol increases aggregation of membrane protein and this interaction may play a role in membrane permeability (124).

It is believed that cholesterol controls fluidity of the phospholipids moiety (124). Cholesterol stimulates ATP-ADP exchange and may play a role in energy processes of the cardiovascular system (124).

Although cholesterol is vital to the maintenance of cell integrity, it has been implicated in the etiology of cardiovascular diseases (20,24,90 - 95, 99,184,185). This results from the fact that most people dying of cardiovascular diseases or having high risk of getting some form of heart diseases are found to have elevated cholesterol in their blood (186-190).

2 Role of Cholesterol in Cardiovascular Diseases

Cholesterol is maintained at an acceptable level through a feedback mechanism. Most body cells are able to synthesize their own cholesterol. If too much cholesterol is
absorbed from food, then the body stops manufacturing cholesterol and increase its breakdown until a balance is reached (98). Failure of the control mechanism or faulty breakdown may result in excessive cholesterol in the blood. Excessive cholesterol is believed to cause or contribute to the genesis of atherosclerosis. This hypothesis is supported by finding of high levels of cholesterol in patients with cardiovascular diseases (12,90, 93, 95,148, 187).

The cholesterol hypothesis is further supported by research that showed that feeding excess cholesterol to animals resulted in atherosclerosis (12, 148,184, 190 -195). Studies also revealed that there are many people with very high cholesterol in their blood who do not develop cardiovascular disease (60,171, 179). This led to the hypothesis that the total cholesterol may not be a good indicator of cardiovascular disease. The ratio of HDL (high density lipoprotein) to LDL cholesterol is important in determining the potential for developing atherosclerosis (12,93,94,97, 99, , 181, 182, 195, ), as described below.

3 Role of Lipoproteins in Cardiovascular System

The low density lipoproteins carry cholesterol through the blood to all parts of the body where it is needed for cell formation or for production of steroids. High density lipoproteins, on the other hand, are believed to remove excess cholesterol from the blood and take it to the liver where it is broken down into bile acids for excretion or for solubilizing fats in the intestines. Recent studies have shown that LDL cholesterol increases platelet aggregation and prevents membrane permeability, whereas HDL cholesterol increases membrane fluidity and permeability (196). Experimental as well as epidemiological studies showed that high levels of HDL in the plasma protect against cardiovascular diseases whereas high levels of LDL cholesterol increase the likelihood of cardiovascular diseases (187,191,194, 197, 198, )
The role of HDL cholesterol in protecting against coronary heart diseases has become controversial. Cross cultural studies failed to produce statistically significant protection by HDL against cardiovascular disease (184,201).

Many older people were found to have lower plasma HDL cholesterol than people in their 40s (202). Cross culture studies revealed a group of people with low HDL cholesterol who did not have cardiovascular diseases and some with high HDL cholesterol who had some form of cardiovascular diseases (12, 177, 184). This led to the idea that diet and genetic factors may play a major role in cardiovascular diseases. (12, 200).

4 The Role of Fatty Acids in Cardiovascular System

Fatty acids especially polyunsaturated fatty acids play a role in membrane permeability (203-208). The fatty acid in conjunction with phospholipids, cholesterol and proteins, control permeability of cells to selected ions (121,124,149,204). Biological membranes involved in signal transmission are believed to have a high requirement of polyunsaturated fatty acids (201,206,209,210,). For example, the synaptic junctions have high concentrations of polyunsaturated fatty acid (204, 208).

Powerful cell regulators such as prostaglandins and leukotrienes are made from arachidonic acid (20:4 group) (203). Thromboxane which is also produced from long chain polyunsaturated fatty acid is important in platelet formation and in blood clotting (75). Prostacyclin produced from polyunsaturated acids is believed to suppress platelet aggregation (78,204,).

Studies with both animals and humans have shown that diets high in saturated fats produce atherosclerosis especially where HDL cholesterol is much lower than LDL (12,193, 207,). Studies have shown that increasing polyunsaturated fats in the diet lowers cholesterol in the serum (193,195,206, 207, ). This is believed to be due to increased
excretion of steroids including cholesterol. Omega-3 fatty acids from fish reduce plasma lipids and reduce cardiovascular diseases by reducing platelet aggregation (206).

The omega-3 fatty acids are believed to prevent formation of thromboxane A₂ which causes platelet coagulation (75, 185, 205). Arachidonic acid, which is a precursor for thromboxane A₂, is decreased while eicosapentenoic acid is increased in fish diets.

Although polyunsaturated fatty acids play a role in reducing cholesterol levels, excess eicosapentenoic acid and linoleate reduce phospholipid arachidonate levels. This decreases endothelial synthesis of prostacyclin (208). Studies have also shown that polyunsaturated fatty acid can be oxidized to peroxides or hydroperoxides and these metabolites of polyunsaturated fats could damage the endothelial layer of the plasma membrane, and this damage is believed to be the initiator of atherogenesis (211). Animal and plant fats usually contain linoleic acid and arachidonic acids. Too much linoleic acids (18.2) inhibits production of prostacylin (PG). This results in elongation of arachidonic acid to 22:5 and to formation of hydroxyperoxide by cyclooxygenase in blood vessel (211).

5 Role of Phospholipids

Phospholipids are compounds of lipids, that are derivatives of glycerol phosphates. They are large molecules containing both a polar and a non polar part. They are found in all living organisms, with high concentrations in the liver, brain, and spinal tissues. They are found in outer membranes of most cells (212).

Phospholipids play a major role in regulation of cell function. They play a role in the structural and functional formation of the membranes (212, 213). They form the hydrophobic backbone of the cell membrane and they actively participate in signal transfer (210). Metabolically active substances are mostly associated with phospholipid aggregates.
For example hydrophobic chemicals such as enzymes, hormones, and neurotransmitters, are found inside the cell as part of phospholipids moiety (211). It is believed that phospholipids control the fluidity and permeability of cell membranes including those of heart muscles (132,214). Interaction between phospholipids, fatty acids and cholesterol results in the formation of micelles that prevent precipitation of cholesterol in aqueous media (62). It is believed that phospholipids either alone or in conjunction with protein, act as a binding sites for ions in the membranes, and this binding is the first step in the movement of ions across the membrane. (124).

Studies with phospholipids, especially soy lecithins, show that plasma cholesterol is lowered by feeding soy lecithins and this decreased cholesterol level does not affect the concentration of high density lipoprotein (210).

D RISK FACTORS IN CARDIOVASCULAR DISEASES

Although the causes of cardiovascular diseases are not known, there are risk factors that predispose one to contract heart diseases. These factors include genetic, diet, obesity, cigarette smoking, age and environmental factors.

1. Genetic Factors

Most cardiovascular diseases run in families, and people with relatives who have had some form of heart disease have a higher chance of getting cardiovascular disease than the general population. Conditions such as hyperlipemia, hypertension etc. seem to have familial predisposition. (12, 184,215).
Studies with twins raised in different environmental conditions showed that some form of cardiovascular diseases have genetic predisposition, since twins raised in different environment developed the same type of heart disease. (17).

2. Environmental Factors

Some people, even in cases where family history indicates a strong possibility of having some form of cardiovascular diseases, never show any symptoms of the disease, whereas people with no history of heart diseases may develop some form of cardiovascular disease by moving to an area with a high prevalence of heart diseases. Therefore, environmental factors may play a role in heart diseases (192).

Epidemiological studies showed that people living in areas with soft water have a higher incidence of heart disease than those living in areas with hard water (55-58). Studies also showed that when people moved from a low incidence area to areas with higher prevalence of cardiovascular diseases, the risk of getting heart diseases also increased. The classic example is that of Japanese who migrate to the USA. Japanese generally have low incidence of heart disease, but when they migrate to USA, their risk of contracting cardiovascular diseases increases (12).

3. The Role of Diet

People who migrate to higher risk areas usually increase their risk when they adapt to the diet of the people in high risk areas. Diet plays a great role in cardiovascular diseases. Epidemiological and experimental research using animal and humans from different environmental backgrounds have shown that diets high in saturated fats, simple carbohydrate and high cholesterol increase the risk of cardiovascular diseases (12,84,192,201, 216,247).
The effect of diet is more pronounced in obese people, especially those who are diabetic (10,12). Studies with both humans and animals have shown that obese people have a higher risk of dying of cardiovascular disease than those who are not fat, all other factors being equal. The study also showed that obese people had higher levels of plasma insulin, aldosterone and norepinephrine than normal people [246].

There is evidence that diets high in polyunsaturated fat and low in saturated fats reduce the risk of heart diseases (60,73,124,172,179, 180,191-193, 200, 201, 217). However, recent studies have shown that the benefits of polyunsaturated may be offset by their tendency to be oxidized to hydroxyperoxides which may be more harmful than saturated fats, since hydroxyperoxides are believed to be atherogenic and may increase the risk of cancer (124).

Eskimos of Alaska who eat diets high in cholesterol do not develop cardiovascular diseases. There is evidence that the fish oils which are polyunsaturated protect the Eskimos. Polyunsaturated fats are also believed to increase the HDL cholesterol which reduces the risk of cardiovascular diseases (201).

4. The Role of Smoking

Smoking seems to increase the risk of cardiovascular diseases. Studies have shown that smokers have a higher incidence of hypertension than non-smokers. Studies have also shown that cigarette smoking affects hormone metabolism, and lowers HDL cholesterol (170). Cigarette smoking increases platelet aggregation, leading to atherosclerosis(213).
5. The Role of Age

The deleterious effect of cardiovascular diseases are not manifested until after the age of forty, except in rare cases. Hardening of the arteries increases with age. Age weakens the body's defence mechanism and therefore may play a role in the etiology of heart disease, or may accelerate the injurious effect of the diseases especially to the susceptible people. Animal studies in Japan showed that development of atherosclerosis plaques in rats fed cholesterol diet was dependent on the age of the animal. Older rat were more affected. (243)

6. The Role of Physical Activity

Many studies with both animals and humans have shown that physically active people have a lower risk of dying from cardiovascular diseases than sedentary people (114, 204, 206, 227). The actual role of exercise in prevention or reduction of mortality from cardiovascular diseases is not well understood, although, studies with runners and joggers have shown that they have higher HDL than people who do not exercise (107, 200, 220). Regular exercises over extended period of time was shown to lower cholesterol, reducing the risk of cardiovascular diseases. (200).

Although exercise increases HDL and reduces cholesterol and LDC cholesterol, some believe that the actual benefits of exercises are as a result of weight loss. Exercise increases production of epinephrine and norepinephrine, which directly activate hormone sensitive triglycerides lipases (200). The lipases break down fat (43). Some believe that exercise helps the body to build natural bypasses so that the blood is able to reach vital areas of the heart even when the coronary arteries are blocked (200). It is therefore possible that natural bypasses help physically active people with blocked coronary arteries live a relatively normal life.
7. The Role of Emotions

Studies have shown that people with certain personality traits are more prone to cardiovascular diseases than others. The impatien, anxious, angry depressed personality is more prone to coronary heart disease than the relaxed type personality (75, 79, 107, 200, 220).

The effect of emotion on the cardiovascular system may be due to the fact that emotions are controlled by a sympathetic system, which control adrenergic hormones. These hormones influence the heart mechanisms (75, 138).

In cases where patients have some form of cardiovascular diseases, physiological factors can determine how well and fast the patient recovers. Anxiety, anger, depression or any extreme emotions worsen the conditions.

F TREATMENT OF CARDIOVASCULAR DISEASE

There are many forms of heart diseases and therefore treatments vary. The extent of disease also determines the mode of treatment. For essential hypertension, reduction in salt with mild diuretics has been used. In severe hypertension, accompanied by atherosclerosis, other methods are used. These methods include use of B-blockers, calcium blockers and cholesterol lowering drugs such as cholestyramine, and clofibrate (187-190,221-223). An example of B-blocker used is propranolol. These B-blockers were shown to increase PGI formation (prostacyclin), which prevents platelet aggregation (224).

Calcium blockers include verapamil, nefidine etc. Most of these drugs work as vasodilators(138). They also relax the heart muscles (66,132). These drugs are believed to work by blocking slow calcium entry into the cell through calcium channels (66). Some
calcium blockers also work by interacting with calcium dependent regulator proteins which play multiple calcium dependent roles (66). Calcium blockers bind with high specificity to calcium channels. The binding, blocks calcium currents, resulting in relaxation of tensions (138).

In conditions where hearts are severely damaged, heart transplants become the only way to save the patients.

B-blockers and calcium blockers are used to treat heart arrhythmias, and other forms of cardiovascular diseases. In irregular heart beat, if the beats are not regulated with drugs, a heart pace maker is implanted in the patients to restore regular heart rhythm (14). Aspirin has also been used to treat heart patient. It is believed that aspirin prevents production of thromboxine A2, which is a coagulating factor (245).

In some patients, the coronary arteries that feed the heart muscles may be severely blocked. Treatment with drugs alone is not sufficient to prevent damage and suffering. These patients undergo a bypass operation where veins from legs or stomach are grafted in the arteries to bypass the blocked area and provide nutrients to the deprived area of the heart (225, 254-256).

Sometimes the bypasses become clogged even after application of rigorous treatment with calcium blockers, low cholesterol diets and exercises.

Histological studies of bypasses and the arteries show that the material in the arteries and bypasses are similar. The methods that have been used to analyze cholesterol are subject to interferences from other factors. Not much work has done compare the chemical compositions of the plaques in the bypasses, and arteries whose deposits started since childhood. This is the subject of this study.
E PURPOSE OF STUDY

1 Analytical Procedure

There were two primary goals to be achieved in this study. When this project started, the normal analytical techniques utilized colorimetric or enzymatic methods for lipid characterization and either colorimetric or atomic absorption spectroscopy for trace metal determination. Each of these techniques was analytically inadequate. The lipid determination was subject to interference from closely related compounds which may be present in the sample. The metal analysis determined only one metal at a time per sample, and multi-element analysis on the small sample was impossible.

Both methods were very time consuming and required a high degree of skill on the part of the analyst to obtain reliable data.

A new analytical approach for each determination was essential. The first objective was therefore to develop analytical methods capable of qualitatively and quantitatively determining the lipids present in the plaque. Also it was highly desirable to perform simultaneous multi-element determination of the pertinent trace metals in the plaque.

The work was done in collaboration with a surgeon with an unusual approach to heart surgery. His patients were people who had had bypass surgery in the past, but the bypasses had experienced accelerated plaque deposits and had refilled over a period of a few years. In each case, further bypass surgery was not possible. As a result, the heart arteries were reopened by surgically drilling out some plaque. In the process, both bypasses and non-bypassed arteries were drilled out as necessary.

The plaque in the non-bypassed arteries presumably had been forming over the lifetime of the patient, but the plaque in the bypasses had deposited since the original bypass operation. These two types of plaque were named 'old' and 'new' material respectively.
Palmatate: $X = \text{CH}_3(\text{CH}_2)_{14}$
Olate: $X = \text{CH}_3(\text{CH}_2)_7\text{CH}=\text{CH}(\text{CH}_2)_7$
Stearate: $X = \text{CH}_3(\text{CH}_2)_{16}$
Linoleate: $X = \text{CH}_3(\text{CH}_2)_4\text{CH}=\text{CHCH}_2\text{CH}=\text{CH}(\text{CH}_2)_7$

Fig. 4 Cholesterol Esters Commonly Found in Atherosclerotic Plaque of Heart Patients.
Characterizations Of the Composition of the Old and New Lipids in the Plaque

The second objective was to characterize the old and new plaque for major differences and to ascertain if further systematic and extensive studies in collaboration with the surgeon were justified.

a Lipid Characterization

The lipids constitute a major part of the plaque. These lipids are thought to be made up of primarily cholesterol and four cholesterol esters. The esters are cholesterol palmitate, cholesterol stearate, cholesterol oleate, and cholesterol linoleate. Their structures are shown in fig 2.

These lipids are very similar in structure which presents a difficult analytical problem. It was decided that the most likely method for separating them was HPLC. However, separation would hinge on developing a mobile and stationary phase capable of separating these large and very similar compounds. Initial Studies were therefore undertaken to ascertain the best stationary phase and mobile phase for the separation.

b Use Of Thin Layer Chromatography

A problem with carrying out such studies directly using HPLC is that it is not possible to determine if the components progress down the column unless they have a reasonably short retention time. This problem was overcome using thin layer chromatography. Numerous stationary and mobile phases could be quickly tested for cholesterol and cholesterol esters separation. If the components were not mobile, it was immediately obvious on developing the thin layer plate, since they remained at the origin. This would not have been clear using HPLC directly, but it is clear with the thin layer chromatography. Using this technique, numerous combinations of stationary and mobile phases were tried.
After selecting the best mobile and the best stationary phases, HPLC columns were made up using the best stationary phase. The mobile phase was used as the solvent and the combination used for lipid characterization of the plaque.

3 Component Detection Using HPLC

Two methods of detection are commonly used in HPLC. These are based on (1) UV absorption and (2) changes in refractive index. With the refractive index equipment available, the sensitivity was very poor. The studies were therefore carried out based on the use of a UV absorption detector.

It can be seen from the structure (fig.-) that these compounds are primarily paraffinic with only small amount of unsaturation. Paraffinic compounds show little UV absorption even at the lowest wavelength (200nm).

Attempts were made to increase the UV absorption coefficient by treating the sample with silver which adds to double bonds. Briefly, the approach was to add silver nitrate to the mobile phase containing water and methanol anticipating an addition of silver at the double bond. This would result in a large increase in UV absorption coefficient and the compounds would be detected. Reverse phase columns were used in this case. Unfortunately, the results were unsatisfactory. The reaction with silver was seldom complete. Analysis of the products lead to more confusion than enlightenment.

The problem was overcome by using a mobile phase that absorbed in the UV (not too strongly) and gave a baseline at 47-80% absorption. When a non absorbing component reached the detector, there was a decrease in absorption and a reverse peak recorded. The components were located thereby and an HPLC trace was obtained. Location of the esters was performed by injecting standard pure esters into the column and determining their retention time. Presumably the retention of the esters in the sample were the same as the injected standards. Identification was therefore made based on retention time. This is notoriously unreliable for positive identification, but can be used to identify peaks with a different retention time from the standard and which are therefore not the standard.
The identification of peaks with the same retention time as the standard should be confirmed by some other techniques (HPLC-MS) or by using a different mobile phase and stationary phase for separation. It would be unusual for the same compound to have the same retention as the standard using two column systems.

4 Use of ICP for metal analysis

The samples were small in size and it was essential to use a method providing simultaneous multi-element analysis. The analytical techniques capable of this analysis include x-ray fluorescence, emission spectroscopy, and ICP. X-ray fluorescence is not sensitive for the determination of trace quantities of elements below calcium in the periodic chart. Emission spectroscopy is suitable for solid samples, but we were contemplating using liquid samples (aqueous solutions). Fortunately ICP was quite satisfactory for this purpose. It is capable of simultaneous multi-element analysis of aqueous samples. Importantly, it was available on campus operating on a routine mode. The elements in which we were interested were calcium, manganese, magnesium, sodium, potassium, phosphorus, iron, zinc, and copper because of their biological significance. They were discussed above.

Similarly, ICP data showed that the old and the new material were different from each other, particularly the calcium and magnesium contents. The precision of the analytical procedure was satisfactory (5% relative standard deviation). However, the analysis of the samples showed considerable variation from one patient to another. With such a small sample, it was not possible to draw statistical conclusions from these results. However, it was clear that the old and the new plaque were different from each other in composition. This suggests a change in the deposition process in the old and the new deposits and possibly a change in the metabolism of the patient in the later life.
EXPERIMENTAL

1 Instrumentation

Atomic absorption; Perkins Elmer atomic absorption spectrophotometer model 403 equipped with a flame atomizer was used.

ICP a quantometer model no. 34-100, made by Applied Research Laboratories, was used.

b Reagents; Analytical grade nitric acid from Baker was used.

2 EXPERIMENTAL PROCEDURE

a Acid Digestion of the Sample

The samples were collected and stored as described in chapter two. A weighed portion of the clogged artery was placed in acid cleaned glass beaker. A known volume of 70% analytical grade nitric acid was added to the sample. All samples were in triplicates. The samples were covered with acid cleaned watch glasses and were heated on a hot plate until all the material dissolved. Heating was continued until the volume was reduced to about 3 mls.

The contents of the beaker were transferred into a 10mls volumetric flask. The beaker and the watch glass were rinsed several times with very small volumes of deionized water. All the washings were transferred to the volumetric flask. The sample volume was made up to the mark with deionized water. All the washings were transferred to the volumetric flask. The sample volume was made up to the mark with deionized water. The volumetric flask was shaken for a few minutes to allow proper mixing before the contents were transferred into acid cleaned polyethylene bottles for storage. When possible, the samples were analyzed immediately. When not possible, the digested samples were stored in the refrigerator until analyzed.
The blanks were prepared by putting the same amount of acid in the beakers as was added to the samples. The beakers containing only acid, were heated under the same conditions as the samples. The same procedure used for the samples was followed.

3 Analysis of the Sample Using AA

Standard samples for calcium and magnesium were prepared. These were used to construct a calibration curve. The standards in the concentration ranges of 1 ppm to 10 ppm were initially prepared. The standards were aspirated into the flame and the absorption readings were taken.

The samples were run under the same conditions as the standards. The concentrations were determined from the calibration curve. The concentration of the metals in the blanks were determined. The blank readings were subtracted from the sample readings to obtain accurate results.

The flame analysis was slow because the metals could only be analyzed sequentially and by changing the lamp each time a new element was to be analyzed. The samples were also very small and only a few metals could be analyzed.

After analyzing a few samples, it was realized that the deposits from bypasses contained much higher concentrations of calcium than the plaques from non-bypassed arteries (24.42 mg/g and 1.48 mg/g respectively). It was then decided that ICP would be used for the samples so that all the metals present in the plaques could be determined simultaneously.
4 Determination of Various Metal Concentrations in the Plaque Using ICP

a. Procedure

Samples were prepared as described for atomic absorption analysis. Before each sample was run in the ICP, the standards containing all the metals of interest at various concentrations, were analyzed. The concentrations of metals in each sample solution were determined by the computerized instrument. The results obtained were used to calculate the concentration of the metals in the original samples.

The results obtained are shown in table 1 and 2

5 Analysis of Metals in the Residue After Lipid Extraction

A known weight of the plaque was treated with 2:1 chloroform: methanol mixture to remove all the lipids. The residue was dried to remove any traces of the solvents. The residue was then acid digested and the concentration of the metals determined as before using ICP. The results obtained are presented in table 3.

6 Determination of Metals in the Lipid Portion

To determine the concentration of metals in the lipid portion, duplicates of each sample were taken. One sample portion was analyzed for metals after acid digestion. The other portion had the lipids extracted before the residue was acid digested. The difference in metal concentration between the two sample was taken as the concentration of the metals in the lipid portion. The results are shown in table 2 and 3
Table 1

AVERAGE CONCENTRATION OF ALL METALS FOUND IN THE PLAQUE (mg/g)

<table>
<thead>
<tr>
<th>METAL</th>
<th>NEW MATERIAL</th>
<th>OLD MATERIAL</th>
<th>NEW/OLD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>0.010 ± 0.001</td>
<td>0.008 ± 0.001</td>
<td>1.3</td>
</tr>
<tr>
<td>Zn</td>
<td>0.045 ± 0.040</td>
<td>0.018 ± 0.012</td>
<td>2.5</td>
</tr>
<tr>
<td>Cd</td>
<td>0.003 ± 0.006</td>
<td>0.001 ± 0.001</td>
<td>3</td>
</tr>
<tr>
<td>Pd</td>
<td>0.170 ± 0.010</td>
<td>0.008 ± 0.006</td>
<td>21.3</td>
</tr>
<tr>
<td>Cr</td>
<td>0.009 ± 0.012</td>
<td>0.027 ± 0.029</td>
<td>0.3</td>
</tr>
<tr>
<td>Ni</td>
<td>0.012 ± 0.017</td>
<td>0.017 ± 0.020</td>
<td>0.7</td>
</tr>
<tr>
<td>As</td>
<td>0.002 ± 0.001</td>
<td>0.002 ± 0.001</td>
<td>1</td>
</tr>
<tr>
<td>Fe</td>
<td>0.190 ± 0.230</td>
<td>0.220 ± 0.212</td>
<td>0.9</td>
</tr>
<tr>
<td>Mn</td>
<td>0.001 ± 0.001</td>
<td>0.002 ± 0.004</td>
<td>0.5</td>
</tr>
<tr>
<td>Mo</td>
<td>0.001 ± 0.000</td>
<td>0.001 ± 0.000</td>
<td>1</td>
</tr>
<tr>
<td>Al</td>
<td>0.001 ± 0.003</td>
<td>0.014 ± 0.013</td>
<td>2.8</td>
</tr>
<tr>
<td>Ca</td>
<td>21.090 ± 12.20</td>
<td>2.44 ± 3.52</td>
<td>8.65</td>
</tr>
<tr>
<td>Mg</td>
<td>0.47 ± 0.33</td>
<td>0.076 ± 0.100</td>
<td>6.12</td>
</tr>
<tr>
<td>Na</td>
<td>4.85 ± 1.33</td>
<td>3.66 ± 1.56</td>
<td>1.33</td>
</tr>
<tr>
<td>K</td>
<td>0.41 ± 0.17</td>
<td>0.011 ± 0.085</td>
<td>0.89</td>
</tr>
<tr>
<td>P</td>
<td>4.14 ± 0.19</td>
<td>3.3 ± 1.89</td>
<td>4.98</td>
</tr>
</tbody>
</table>

Table 2

CONCENTRATION OF METALS IN THE LIPID PORTION OF THE PLAQUE

<table>
<thead>
<tr>
<th>METAL</th>
<th>NEW %</th>
<th>OLD %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>0</td>
<td>0.007</td>
</tr>
<tr>
<td>Zn</td>
<td>0.001</td>
<td>0.000</td>
</tr>
<tr>
<td>Cd</td>
<td>0</td>
<td>0.003</td>
</tr>
<tr>
<td>Pd</td>
<td>0.017</td>
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</tr>
<tr>
<td>Cr</td>
<td>0.005</td>
<td>0.022</td>
</tr>
<tr>
<td>Ni</td>
<td>0.001</td>
<td>0.000</td>
</tr>
<tr>
<td>As</td>
<td>0.001</td>
<td>0.049</td>
</tr>
<tr>
<td>Fe</td>
<td>0.044</td>
<td>0.001</td>
</tr>
<tr>
<td>Mn</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>Ca</td>
<td>0.43</td>
<td>0.001</td>
</tr>
<tr>
<td>Mg</td>
<td>0.018</td>
<td>0.005</td>
</tr>
<tr>
<td>Mo</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>Si</td>
<td>0.023</td>
<td>0.0036</td>
</tr>
<tr>
<td>P</td>
<td>0.46</td>
<td>0.75</td>
</tr>
<tr>
<td>Al</td>
<td>0.074</td>
<td>6.1</td>
</tr>
<tr>
<td>K</td>
<td>0.484</td>
<td>0.13</td>
</tr>
<tr>
<td>Na</td>
<td>1.649</td>
<td>0.906</td>
</tr>
</tbody>
</table>

Note: The % concentration of the total metal concentration in the lipid compared to total metal concentration in the whole plaque, is shown in table 2.
### Table 3

**CONCENTRATION OF METALS IN MATCHED AND UNMATCHED SAMPLES**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Matched</th>
<th></th>
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<th></th>
<th></th>
<th>Unmatched</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>New/Old</td>
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<td>New/Old</td>
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<td>New/Old</td>
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<tr>
<td>Calcium</td>
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<tr>
<td></td>
<td>New</td>
<td>Old</td>
<td>New/Old</td>
<td>New</td>
<td>Old</td>
<td>New/Old</td>
<td>New</td>
<td>Old</td>
<td>New/Old</td>
<td>New/Old</td>
</tr>
<tr>
<td>Matched</td>
<td>21.09 ± 12.20</td>
<td>24.4 ± 3.52</td>
<td>4.6 ± 0.35</td>
<td>8.65 ± 1.25</td>
<td>26.42 ± 0.54</td>
<td>0.076 ± 0.10</td>
<td>1.13 ± 0.53</td>
<td>4.4 ± 0.24</td>
<td>0.24 ± 0.15</td>
<td>0.15 ± 0.13</td>
</tr>
<tr>
<td>Unmatched</td>
<td>22.17 ± 10.88</td>
<td>5.5 ± 1.54</td>
<td>3.31 ± 0.09</td>
<td>0.096 ± 0.07</td>
<td>0.083 ± 0.09</td>
<td>0.11 ± 0.08</td>
<td>0.97 ± 0.07</td>
<td>3.63 ± 0.27</td>
<td>1.92 ± 0.06</td>
<td>0.15 ± 0.08</td>
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<tr>
<td>Total</td>
<td>18.5 ± 9.01</td>
<td>3.5 ± 1.89</td>
<td>1.28 ± 0.04</td>
<td>0.094 ± 0.07</td>
<td>0.86 ± 0.03</td>
<td>0.03 ± 0.07</td>
<td>2.42 ± 0.14</td>
<td>4.4 ± 0.21</td>
<td>1.52 ± 0.15</td>
<td>0.51 ± 0.07</td>
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<tr>
<td>Magnesium</td>
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</tbody>
</table>

The concentration is in Mg/g wet weight. The standard deviation was based on too few samples to be reliable statistical data. Also it is probably that these new data represent different set or data such as different age, medical treatment etc. However, invariably the new material was different from the old material.
Table 4

AVERAGE CONCENTRATION RATIOS OF METALS IN THE PLAQUE OF ALL SAMPLES

<table>
<thead>
<tr>
<th>Ratio</th>
<th>New</th>
<th>Old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu/Zn</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Na/K</td>
<td>50</td>
<td>35</td>
</tr>
<tr>
<td>Ca/Mg</td>
<td>45</td>
<td>32</td>
</tr>
<tr>
<td>Ca/P</td>
<td>1.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Mg/P</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Mg/K</td>
<td>5</td>
<td>0.7</td>
</tr>
<tr>
<td>Ca/Na</td>
<td>4.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

TABLE 4a

AVERAGE CONCENTRATION RATIOS OF MATCHED AND UNMATCHED SAMPLES

<table>
<thead>
<tr>
<th>Matched Unmatched</th>
<th>Matched</th>
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<th>Matched</th>
<th>Unmatched</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>New</td>
<td>Old</td>
<td>New</td>
<td>Old</td>
</tr>
<tr>
<td>Cu/Zn</td>
<td>0.36</td>
<td>0.44</td>
<td>0.17</td>
<td>0.43</td>
</tr>
<tr>
<td>Na/K</td>
<td>62.26</td>
<td>25</td>
<td>50.52</td>
<td>40.12</td>
</tr>
<tr>
<td>Ca/Mg</td>
<td>77.25</td>
<td>28.07</td>
<td>41.06</td>
<td>11.51</td>
</tr>
<tr>
<td>Ca/P</td>
<td>2.66</td>
<td>0.23</td>
<td>1.21</td>
<td>0.15</td>
</tr>
<tr>
<td>Mg/P</td>
<td>0.034</td>
<td>0.041</td>
<td>0.029</td>
<td>0.013</td>
</tr>
<tr>
<td>Mg/K</td>
<td>3.87</td>
<td>1</td>
<td>5.63</td>
<td>0.86</td>
</tr>
<tr>
<td>Ca/Na</td>
<td>4.8</td>
<td>1.12</td>
<td>4.57</td>
<td>0.002</td>
</tr>
<tr>
<td>TABLE 5</td>
<td>CONCENTRATION OF METALS IN THE PLAQUE OF UNMATCHED SAMPLES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Copper</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New</td>
<td>0.005</td>
<td>0.02</td>
<td>0.015</td>
<td>0.017</td>
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<tr>
<td>Old</td>
<td>0.004</td>
<td>0.002</td>
<td>0.002</td>
<td>0.001</td>
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<tr>
<td><strong>Zinc</strong></td>
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<tr>
<td>New</td>
<td>0.076</td>
<td>0.106</td>
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<tr>
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<td>0.084</td>
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<td>0.064</td>
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<td>0.015</td>
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<tr>
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<td>0.001</td>
<td>0.005</td>
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<td></td>
<td></td>
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<tr>
<td>New</td>
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<td>0.0003</td>
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<tr>
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<td>0.004</td>
<td>0.005</td>
<td>0.005</td>
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<td></td>
<td></td>
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<tr>
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<td>0.128</td>
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<td>0.086</td>
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<td>ND</td>
<td>0.004</td>
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<td>0.008</td>
<td>0.001</td>
<td>0.001</td>
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<td>Old</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td><strong>Aluminium</strong></td>
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<tr>
<td>New</td>
<td>0.009</td>
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<td>0.007</td>
<td>0.001</td>
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<td>0.002</td>
<td>0.023</td>
<td>0.003</td>
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<tr>
<td><strong>Calcium</strong></td>
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<td></td>
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<tr>
<td><strong>Magnesium</strong></td>
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<tr>
<td>New</td>
<td>0.587</td>
<td>0.268</td>
<td>0.208</td>
<td>0.222</td>
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<tr>
<td>Old</td>
<td>1.384</td>
<td>0.504</td>
<td>0.432</td>
<td>0.26</td>
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<tr>
<td><strong>Phosphorus</strong></td>
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<td></td>
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<td></td>
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<tr>
<td><strong>Phosphorus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Old</td>
<td>5.335</td>
<td>4.93</td>
<td>4.285</td>
<td>2.589</td>
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</table>

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Table 6

CONCENTRATION OF METALS IN MATCHED SAMPLES (mg/g wet sample)

<table>
<thead>
<tr>
<th>Metal</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>New</td>
<td>Old</td>
<td>New</td>
<td>Old</td>
<td>New</td>
<td>Old</td>
</tr>
<tr>
<td>Cu</td>
<td>0.001</td>
<td>0.001</td>
<td>0.003</td>
<td>0.005</td>
<td>0.02</td>
<td>0.005</td>
</tr>
<tr>
<td>Zn</td>
<td>0.011</td>
<td>0.001</td>
<td>0.007</td>
<td>0.007</td>
<td>0.003</td>
<td>0.023</td>
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<tr>
<td>Cr</td>
<td>0.007</td>
<td>0.003</td>
<td>0.001</td>
<td>0.002</td>
<td>0.012</td>
<td>0.003</td>
</tr>
<tr>
<td>Ni</td>
<td>0.003</td>
<td>0.003</td>
<td>0.001</td>
<td>0.005</td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>Fe</td>
<td>0.141</td>
<td>0.081</td>
<td>0.114</td>
<td>0.254</td>
<td>0.027</td>
<td>0.024</td>
</tr>
<tr>
<td>Mn</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ca</td>
<td>9.546</td>
<td>7.143</td>
<td>0.173</td>
<td>0.41</td>
<td>18.68</td>
<td>14.112</td>
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<tr>
<td>Mg</td>
<td>0.276</td>
<td>0.219</td>
<td>0.013</td>
<td>0.706</td>
<td>0.476</td>
<td>0.424</td>
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<tr>
<td>P</td>
<td>5.889</td>
<td>4.922</td>
<td>1.762</td>
<td>0.612</td>
<td>11.165</td>
<td>8.081</td>
</tr>
<tr>
<td>K</td>
<td>0.098</td>
<td>ND</td>
<td>0.156</td>
<td>ND</td>
<td>0.039</td>
<td>0.297</td>
</tr>
</tbody>
</table>

The old and old material were from the same patient.

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Results and Discussion of the Metal Analysis

The results of metal analyses are shown in tables 1-4. The principal metals found in both old and new plaque were calcium, phosphorus, sodium and magnesium. Most metals were found in trace amounts. There were a number of samples in which the levels of copper, chromium, manganese, molybdenum and potassium were undetectable. Metals present in old plaque, were also found in the new plaque. There were increased concentrations of metals in the new plaque compared to the old plaque with the exception of chromium, potassium, iron and nickel which had decreased concentration in most of samples in the new plaque. Toxic metals such as arsenic, lead, calcium and aluminum were found in some samples. Aluminum and silicon were found in most of the samples analyzed.

The concentrations of the metals found in the plaques were significantly different from what would be expected in normal blood. Assuming that the metals deposited in the plaque were from the blood, then the results indicate that the metal composition of the blood of heart patients may be significantly different from the blood of normal people.

The metal ratios were different from what would be expected in the normal blood. The copper/zinc ratio was less than one in all the samples analyzed, including old and new. The average ratio of Na/K was found to be 40/1 in non-bypassed plaque (old), while it was 51/1 in the new plaque. The ratio of Ca/Mg was more than 20/1 in most of the samples. The ratio of Ca/P was close to 2 in both the new and the old plaque.

A most significant observation was that the concentrations of calcium and phosphorus were found to be 16 times higher in the new material than the old material. The increased deposition of calcium and phosphorus after treatment indicate that the current method of treatment may be treating symptoms rather than the cause of the problem. It also
shows that the current hypothesis about cholesterol and fats as being the cause of atherosclerosis and other coronary heart diseases may not be correct.

Cholesterol and calcium seem to play complimentary roles in their biological functions, and changes in concentration of one, seem to affect the other. Factors that control the levels of calcium also seem to regulate cholesterol levels (170).

It may be that the increased deposition of calcium may be due to changes in regulation mechanisms. Calcium plays very vital role in the regulation of cell processes, especially the cells of the heart muscle. (113-119) Changes in membrane can result in changes in calcium ions flow. Cholesterol and other lipids play a vital role in membrane fluidity (132). Oxidation of lipids may alter membrane fluidity, resulting in changes in calcium ions flow. If calcium slow channel is blocked, decreased calcium entry into the cell may result in increased mobilization of calcium from the storage areas especially inside the cell or the from sarcol plasmic reticulum (171). Calcium may also be mobilized from the bones. This may lead to excess calcium in the blood leading to deposition inside the arterial lumen.

The ratio of calcium/magnesium in the blood is usually (3:1). (97) Magnesium is believed to be natures' calcium blocker (48,135). It is possible that in heart patients there is a deficiency in magnesium, or there is a change in metabolism that alters magnesium function so that it is not able to block the influx of calcium into the cell. It has been shown that increased calcium in the intracellular space interferes with the contraction-relaxation mechanisms of the heart and can lead to heart attack. (121,122)

There has been a suggestion that aluminum may play a role in heart diseases (172). Studies of the dialysis patients who developed dementia showed that their risk of dying from heart disease was 14 times more than those who did not develop demential (172). Aluminum is believed to play a major role in dialysis demential (173). Aluminum is known
to bind strongly to the phosphate group of ATP (173). It could therefore interfere with the energy processes of the heart. Aluminum is not known to have any useful role in the body however, there were significant amounts of aluminum found in almost all the plaques analyzed. Aluminum is insoluble and is therefore not easily absorbed through the intestinal mucosa. However it has been shown that chelating agent increase the absorption of aluminum (175). Citric acid was shown to increase the absorption of aluminum in experimental animals (175). Tea contains about 30 mg of aluminum /g dry tea. Iced tea with lemon (acidic) can increase the amount of aluminum absorbed to dangerous levels if taken for a long time. A comparative analysis of aluminum in the blood of healthy and heart patients may reveal the role of this metal in heart diseases.

8 Problems Encountered

The major problem was that the sample size was relatively small. The number of samples where the new and old plaques were from the same individual was only five because the number of patients who undergo a second or third bypass operation is small. There were only a total of seven samples from non-bypassed arteries. There were more samples from the bypasses (new material) than from the old plaque. This is again due to delicate operation performed to obtain the old plaque. The surgeon has to send an instrument through the vessel to the blocked area to scrape off the plaque without injuring the blood vessel. As a result, the statistical analysis could not be used to show significant differences. There were a lot of individual variation in the metal concentrations although the general pattern was the same for all the sample analyzed. This resulted in the standard deviation being higher than the mean in some metals. The deposition of the plaque is not uniform. This was shown by the fact the two segments obtained from the same individual had different concentration although the general pattern was similar. The same sample was measured at different times to check the instrument precision. The results were
consistent indicating that the variation was not due to the instrument. The instrument precision was 5% relative standard deviation of the method.

9 Conclusion

The results show that there is a difference in the concentrations and ratios of metals found in the old and the newly deposited material. Increased deposition of metals in the newly deposited material may indicate possible changes in the metabolism of these metals either as a result of the disease, or the changes may be the cause of the disease. Further research is necessary to find the role metals play in the heart disease. Study of metal ratios, especially zinc/copper, calcium/magnesium, and calcium/phosphorus in heart patients and healthy individuals may give some clue. Also, there is a need to look at the role of aluminum.
CHAPTER 2

ANALYSIS OF THE LIPIDS IN THE ATHEROSCLEROTIC PLAQUE

A. INTRODUCTION

Lipids, especially cholesterol, play a vital role in cell membranes and in the cell processes. They are major structural components of cell membrane. Despite their vital roles in the body, lipids have been implicated in the etiology of cardiovascular diseases, especially atherosclerosis (8,12,97). Great emphasis has been laid on reduction of plasma cholesterol and saturated fats by cutting down the intake of foods high in these lipids. (176 - 181) It is believed that by cutting down cholesterol intake, the heart diseases can be reduced, or eliminated.

These drastic measures were recommended because most people suffering from cardiovascular diseases, have elevated cholesterol in their blood, and also because the atherosclerosis plaques, contain high proportions of cholesterol and cholesterol esters. Fig: () show the representative structures of lipids found in the atherosclerotic plaques.

The lipids are vital in the maintenance and survival of cells. Without them, the cells cannot function properly. In fact, most cells are able to manufacture their own lipids from simple molecules (12).

Although cholesterol is believed to play a role in heart diseases, its actual role in the etiology of the atherosclerosis has become controversial. Some studies showed formation of atherosclerosis in experimental animals fed diets high in cholesterol (181, 182). Other studies did not produce atherosclerosis by feeding animals cholesterol rich diet (60, 70).

This study was initiated to find out if there is significant difference in lipid composition between atherosclerosis plaques deposited since childhood, and the material
deposited after the first bypass operation, and rigorous treatment with cholesterol lowering diets.

B ANALYSIS OF LIPIDS USING CHROMATOGRAPHIC METHODS

1 Introduction

Chromatographic methods are useful for separation and identification of complex mixtures with minimal sample preparation. They are non-destructive, simple to use, relatively inexpensive and they facilitate sample separation into components for further analysis using other methods.

The detectors used in chromatography are very sensitive and only a small sample is required. This is extremely useful in analysis of biological and environmental materials where the concentration of the element of interest may be in parts per million and/or the sample size may be in microgram levels.

Chromatographic methods can handle different types of samples, i.e. large molecules, small molecules, liquid samples solids, gases. The only requirement is that the solid samples be either easily volatilized or be soluble in a suitable solvent.

Chromatographic methods were chosen for this analysis because of the above reasons, and also because they have been used extensively in the analysis of biological samples including lipids. Theory and basic principles of chromatographic methods can be found in the following references: (130, 165, 229, 230-237)
C  ANALYSIS OF LIPIDS USING THIN LAYER CHROMATOGRAPHY

1  Introduction

Thin layer chromatography has been extensively used for the analysis of biological compounds. This method has been used for separation and identification of complex and simple lipids (1). We used this method to enable a rapid selection of stationary and mobile phase to be used for separation. It was presumed that separation performed on thin layer chromatography, would also be possible in HPLC. It further enabled improved separation of the compounds by using mobile phases that could not be used with UV detectors.

a  Experimental Parameters

i  Reagents

Hexane, heptane, chloroform dichloromethane,
acetone, acetonitrile, cyclopentane, pentane,
ethylacetate, ethanol, isopropyl alcohol.

i  Equipment

TLC, Reverse phase plates, Whatman
KC-18 without fluorescent, Whatman Silica
plates with K5 gel.

Formulation: LK5D Cat. NO. 4855-821 (20 x 20 cm)
Developing Chambers: Class chambers 17 cm x 8 cm x 17 cm, (iii) spotting capillaries.
Developing reagents: (1) Ferric chloride (50 mg) in 5% H₂SO₄
b Experimental Procedure

The lipids were extracted as described in HPLC section. Hexane crude extracts were used for this study.

The samples were applied to the plate using Drummond micro cap spotting capillaries. Samples and standards were applied on the same plates and the spots were air dried before the plates were placed in the developing chamber that contained 100 mls of developing solvents. The samples were eluted until the mobile phase had covered 80% of the plate.

The plates were removed from the developing chamber, dried, and then sprayed with identification reagents. The plates were then heated in the oven at 200°C until the spots appeared, which was about 5 to 10 minutes. The Rf (which is the ratio of distance traveled by sample over distance travelled by mobile phase) of sample spots were compared with those of the standards. Copies of developing thin layer plates with sample spots are shown on fig. 7 and 8.
2. **TLC Results and Discussion**

The results are shown on tables 8-9 and fig5-6. The results obtained using 66:1 heptane: ethyl acetate as the mobile phase are shown on table 7. The first two pairs of samples from two patients have compounds that have Rf which is different from those of cholesterol and cholesterol standards used. The new material from the two individuals seem to have same number of compounds indicated by the proximity of the compounds Rf. The old material on the other hand seem to be different from each other and also different from the new material from the same individual. For example the first patient has the old material containing all compounds found in the new material and two extra compounds. Only two compounds seem to have Rf close enough to those of the standard cholesterol and cholesterol esters in the first patient.

There were more compounds in the new material of the second patient than in the old material. Two of the compounds in the new material have Rfs that are close to that of the standards, while only one compound in the new material is close.

6:4 cyclohexane: chloroform was used as the mobile for next 5 pairs of samples. The samples from patient (5) show that the old material has more compounds. The new material seem to have cholesterol stearate (0.800) at Rf 0.83 and cholesterol (0.051) at Rf 0.051. The compound with Rf 0.319 was not identified. The old material on the other hand seems to have cholesterol stearate at Rf 0.814 and three unidentified compounds.

Patient no. 6 seem to have cholesterol and cholesterol stearate in the old material and only cholesterol stearate in the new material. The number of spots were identical but Rf were different.

Patient no. 7 seem to have material that have Rf that is slightly different from that of cholesterol which indicate that the compounds may have structure that is close to that of
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<tr>
<th></th>
<th>Mobile Phases Tried for TLC</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>chloroform : cyclohexane</td>
</tr>
<tr>
<td>2</td>
<td>cyclohexane : acetonitrile : ethanol</td>
</tr>
<tr>
<td>3</td>
<td>heptane : ethyl acetate</td>
</tr>
<tr>
<td>4</td>
<td>chloroform : methanol : water</td>
</tr>
<tr>
<td>5</td>
<td>heptane : isopropyl ether</td>
</tr>
<tr>
<td>6</td>
<td>heptane : isopropyl alcohol</td>
</tr>
<tr>
<td>7</td>
<td>chloroform : acetonitrile : isopropyl</td>
</tr>
<tr>
<td>8</td>
<td>ethanol : acetonitrile : chloroform</td>
</tr>
<tr>
<td>9</td>
<td>hexane : dichloromethane : ethanol</td>
</tr>
<tr>
<td>10</td>
<td>chloroform : ethanol : water</td>
</tr>
<tr>
<td>11</td>
<td>heptane : ethyl acetate : acetic acid</td>
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</tbody>
</table>
FIG. 5 TLC CHROMATOGRAMS OF EXTRACTS IN HEXANE
PLATE: SILICA GEL
MOBILE PHASE: 70:20:10 HEXANE: CHLOROFORM: ETHANOL
DEVELOPING REAGENT: 50:50 water : sulphuric acid
N = NEW MATERIAL
O = OLD MATERIAL
C = CHOLESTEROL
CE = CHOLESTEROL AND ITS ESTERS
FIG. 6 TLC CHROMATOGRAMS OF LIPID EXTRACTS IN HEXANE
66:1 HEPTANE:ETHYL ACETATE AS MOBILE PHASE
SILICA PLATES. 50:50 SULFURIC ACID:WATER WAS
THE DEVELOPING REAGENT.
<table>
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<th>Patient 1</th>
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<th>Standards</th>
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<td>New</td>
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<td>New</td>
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<tr>
<td>0</td>
<td>0</td>
<td>0.012 0.19</td>
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<td>0.176 0.28</td>
<td>0.271 0.141</td>
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<td>0.447 0.294</td>
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<td>0</td>
<td>0</td>
<td>0.018 0.015</td>
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<td>0.036 0.036</td>
<td>0.427 0.087</td>
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<tr>
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<td>0.468 0.606</td>
<td>0.525 0.642</td>
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<td>New</td>
</tr>
<tr>
<td>0.051 0.021</td>
<td>0.319 0.257</td>
<td>0.833 0.364</td>
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<th>Standards</th>
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<td>New</td>
<td>Old</td>
<td>New</td>
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<td>0.073 0.094</td>
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<td>0.942 0.913</td>
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</thead>
<tbody>
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<td>0</td>
<td>0</td>
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<tr>
<td>0.29 0.216</td>
<td>0.577 0.797</td>
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### Table 8a

**TLC RF OF UNMATCHED SAMPLES**

<table>
<thead>
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<th>Sample 1 (old)</th>
<th>Sample 2 (old)</th>
<th>Sample 3 (new)</th>
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<tr>
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<td>0</td>
</tr>
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<td>0.277</td>
<td>0.196</td>
<td>0.19</td>
</tr>
<tr>
<td>0.864</td>
<td>0.544</td>
<td>0.519</td>
</tr>
<tr>
<td>0.97</td>
<td>0.937</td>
<td>1</td>
</tr>
<tr>
<td>0.985</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Standard**

- Cholesterol: 0.046
- Stearate: 0.855
- Linoleate: 0.865
- Palmitate: 0.899
- Arachidonate: 0.947
- Olate: 0.947

### Table 8b

**TLC RF RESULTS OBTAINED USING CYCLOHEXANE: CHLOROFORM (4:6)**

<table>
<thead>
<tr>
<th>Old</th>
<th>New</th>
<th>New</th>
<th>New</th>
<th>New</th>
<th>New</th>
<th>New</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.024</td>
<td>0.022</td>
<td>0.021</td>
<td>0.021</td>
<td>0.021</td>
<td>0.027</td>
<td></td>
</tr>
<tr>
<td>0.427</td>
<td>0.315</td>
<td>0.329</td>
<td>0.314</td>
<td>0.301</td>
<td>0.466</td>
<td></td>
</tr>
<tr>
<td>0.516</td>
<td>0.359</td>
<td>0.414</td>
<td>0.289</td>
<td>0.343</td>
<td>0.527</td>
<td></td>
</tr>
<tr>
<td>0.565</td>
<td>0.444</td>
<td>0.464</td>
<td>0.324</td>
<td>0.42</td>
<td>0.571</td>
<td></td>
</tr>
<tr>
<td>0.605</td>
<td>0.489</td>
<td>0.504</td>
<td>0.408</td>
<td>0.462</td>
<td>0.607</td>
<td></td>
</tr>
</tbody>
</table>

**Standard**

- Cholesterol: 0.024
- Stearate & Palmitate: 0.473
- Linoleate & Olate: 0.532
- Arachidonate: 0.622

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cholesterol. The new material seem to have cholesterol oleate while the old material seem to have both oleate and palmitate. The old material has more compounds than the new material. Patient No. 8 probably had Cholesterol, stearate, and linoleate in new material, while old material has cholesterol Other material were present in both material and stearate.

The patient 9 has the same number of compounds in the old and the new but the Rfs are different except for one compounds that seem to be close. The new material seem to have arachidonate, and probably cholesterol while the old material has probably arachidonate and stearate.

The unpaired samples also show similar differences. There were differences in the old material from different individuals, and also new material from different individuals.

3 Separation Of Phospholipids From Other Lipids Using Two Consecutive Solvents

a Introduction

Our chromatographic study of the lipid composition of the plaque from the heart patients showed that cholesterol and cholesterol esters were present in the plaque. There were other materials present in these plaques that were not identified. Apart form cholesterol and cholesterol esters, triglycerides and phospholipids are believed to be also present. Our attempt to separate triglycerides from cholesterol esters was futile. The triglycerides eluted together with the cholesterol and also had the same Rf in TLC.

It has been shown that phospholipids can be separated from other lipids by use of two consecutive mobile phases TLC (234). The purpose of this study is to use this method to find out if phospholipids are present in the plaque.
b Experimental

i Equipment;  TLC plate (silica gel), Developing chamber spotting capillary tubes.

ii Reagent;  Hexane, chloroform, diethyl ether, methanol. Deionized water, cholesterol and cholesterol esters triglyceride mixture (palmitoyl, palmitic acid cholesterol, tripalmitate) developing reagent;  50:50 sulphuric acid : water

c. Experimental Procedure

Two developing chambers were set up. The first chamber had chloroform: methanol:water at the ratio of 65: 25:4 respectively. The second chamber had hexane: diethyl ether 4:1 respectively. 100 mls were added to each chamber and the lids were put in place. The chamber were allowed to saturate with the vapour for 30 minutes before the plates with the sample sports were put into the first chamber. The samples were placed into the plate as described earlier. The solvent front was allowed to move for about 5 cms and the plate was removed. The plate was allowed to dry before it was placed in the second chamber with the hexane:ether mixture. The second solvent was allowed to move 3/4 of the plate i.e about 10 cms. the plate was removed from the chamber dried and then sprayed with the developing solution. The plate was heated at 120°C for ten minutes. The Rf of the sports were calculated and compared with the Rf of standards. The Rf of phospholipids were compared to those given in the literature (234).

d Results

The results are shown on table 9 Rf of the new and old material from different individual

Results are shown in table 9
Table 9

TLC RESULTS OBTAINED BY USING TWO CONSECUTIVE SOLVENTS

<table>
<thead>
<tr>
<th>New Triglycerides</th>
<th>Cholesterol</th>
<th>Linoleate</th>
</tr>
</thead>
<tbody>
<tr>
<td>New 0.14 0.13 0.61</td>
<td>Old 0.12 0.11 0.62</td>
<td>New 0.85 0.91</td>
</tr>
<tr>
<td>0.21 0.4 0.85</td>
<td>0.22 0.24 0.43</td>
<td></td>
</tr>
<tr>
<td>0.65 0.62 0.36</td>
<td>0.9 0.64 0.81</td>
<td></td>
</tr>
<tr>
<td>0.88 - 0.81</td>
<td>- 0.91</td>
<td></td>
</tr>
</tbody>
</table>

Table 9a

TLC Rf VALUES OF STANDARD PHOSPHOLIPID OBTAINED USING TWO CONSECUTIVE SOLVENTS

<table>
<thead>
<tr>
<th>PHOSPHOLIPID Rf</th>
<th>Lysophosphatidyl choline 0.06</th>
<th>Sphingomyline 0.12</th>
<th>Phosphatidyl serine 0.13</th>
<th>Phosphatidyl inositol 0.13</th>
<th>Phosphatidyl choline 0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHOSPHOLIPID Rf</td>
<td>Phosphatidyl ethanolamine 0.4</td>
<td>Cerebroside 1 0.48</td>
<td>Cerebroside 2 0.51</td>
<td>Cerebroside 3 0.58</td>
<td>Cholesterol 0.79</td>
</tr>
</tbody>
</table>
D ANALYSIS OF LIPIDS USING HPLC

The need to analyze non-volatile biological compounds, combined with increased performance of high pressure liquid chromatography, made the technique very popular, and it has become one of the most powerful separation tools in chemistry and biochemistry. Interfacing HPLC with mass spectrometry, has increased its power for separation and structural identification. The name was also changed from high pressure liquid chromatography to high performance liquid chromatography (HPLC). HPLC can be operated in either reverse or normal phase.

1 Components of HPLC

HPLC consists of a solvent reservoir, a high pressure solvent delivery pump, pulse dampener, and injection valve for sample introduction, the separation column, the detector, and the data output system. Schematic diagram of HPLC instrument is shown in Fig 9.

a The Pump

There are two basic types of pumps in use in HPLC, constant pressure and constant volume pumps. These pumps can reach a maximum pressure of up to 6000 psi, and can increase solvent flow rate up to 10mls per minute, with an accuracy of better than 0.5% (230). Sometimes a pulse dampener comes with the pump, to maintain smooth delivery. Some pumps also have dual delivery system which enable them to carry out continuous solvent composition change.

The constant volume pump is made in such a way that whenever there is a fluctuation in the flow rate, due to viscosity changes of the mobile phase, swelling of the
packing material, etc, the pump automatically changes its pressure, to maintain constant flow rate. It is very useful in gradient elution.

The constant pressure pump has some form of pneumatic device for direct pressurization of the mobile phase with an inert gas. It gives pulse free flow, is of low cost, and is easy to use, but can not compensate for any changes in the column that change the flow rate. It is not very useful when reproducibility and accuracy are important.

b Sample Introduction System

It is important to introduce the sample onto the column as a very small band, to minimize peak broadening. This is made possible by use of septum injector of small internal volume that allows sample to be placed at the column where it can be completely swept by the mobile phases. Special syringes that can withstand high pressures are used.

Sometimes a valve-loop injector that can withstand high pressure is used, eliminating the use of septum. When the valve-loop is used, the solvent flow is bypassed into the column and an external or internal loop of volume ranges of 10 to 500 microliter is filled with the sample. The sample is introduced into the column by switching a valve. Loop-valve injection has the advantage of being reproducible, and having the capability of using varying volumes of sample. It also reduces plugging problems (230).

c Column

HPLC uses columns made of stainless steel tubing. The column packing material used in HPLC may have particle sizes ranging from 5 microns to 30 microns. The optimum diameter of the column depends on sample volume and column and column packing. Commonly, columns have internal diameters of about 5mm and 25 cm length.
Microbore columns of 1.0 mm internal diameter and 100 cm length have also been introduced. They are packed with 5 to 10 mm particle sized with theoretical plate counts of up to 25,000 per meter. They use low flow rate of mobile phase, which reduces dilution increasing sensitivity and can be directly interfaced with mass spectrometer (242).

**d Detectors In Liquid Chromatography (HPLC)**

There are many detectors in use in liquid chromatography but the most common are the photometric and the refractive index detectors. Detectors that are capable of differential measurement are refractometers, conductivity and dielectric constant detectors. Photometric, polarographic and radioactivity detectors measure sample properties that are different from that of the mobile phase. Flame ionization and electron capture detectors can be used in HPLC but mobile phase must be removed first. Since these detectors cannot distinguish solute and solvent properties.

**i Photometric Detectors**

These detectors are based on absorbance of light either in the UV or the visible region of the spectrum. They measure the changes in absorption of light as a sample component passes through the flow cell of the detector. A schematic diagram of a photometric detector is shown on fig; 10.

There are two types of photometric detectors, the fixed wavelength photometer and the variable wavelength photometer. The fixed wavelength detector uses low pressure mercury vapor lamp with a predominant wavelength at 254 nanometers. Mercury pressure can be changed to enable other lines either in the UV or visible regions to be observed.

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Fig: 7 Block Diagram of HPLC System
Variable wavelength photometric detector use deuterium lamp in the UV range of 190-400 nanometers and Quartz-Iodine or tungsten-halogen ranges of 350-700 nanometers. (238)

The use of the diode array in photometric detectors has increased the speed of HPLC analysis since components of the sample can be analyzed at different wavelengths simultaneously. The sensitivity of photometric detectors is about \(1 \mu l/\text{ml}\) of effluent (230). Most organic compounds do not absorb in these regions and have to be derivatized with a chromophore, which absorbs in the UV or visible in order to be detected photometrically. Mobile phases used should not absorb the wavelengths of the sample. A schematic diagram of UV detector is shown on fig.

2 Experimental Parameters

a Equipment

(a) Solvent delivery system: Perkin Elmer HPLC pump model series 2 equipped with two sinusoidal dual piston pump.

(b) Columns: Normal phase Altech silica columns 5\(\mu\), 25 cms Bondapak ODS 3.9 mm \(\times\) 15 cm, Lichrosorb C2, C8, C18, diameter 3.9 mm, length 25 cm

(c) Sample Injection: Rheodyne 7125 injector with 100 \(\mu l\) sample loop. A 100\(\mu l\) Hamilton Syringe was used for sample injection.

(d) Detector: UV/VIS Perkin Elmer variable wavelength (190 - 800 nm) detector model LC75.

(e) Reagents: HPLC Grade chloroform, methanol hexane, acetonitrile, isopropyl alcohol, ethanol, heptane, cyclopentane, 1-pentanol, isooctane. All from Mallinckrodt.
Fig. 8 Schematic Diagram Of a Dual Beam UV Detector.
Reagents: cholesterol, cholesterol linoleate, cholesterol arachindonate, cholesterol Palmitate, cholesterol stearate, cholesterol oleate from Aldrich.

3 Experimental Procedure

a Sample Collection

The arteries were obtained from a surgeon. They were removed from the patient during bypass operation. Two samples were obtained from each patient. One sample from the bypass and the other from an old, clogged artery. Each sample was placed in a plastic vial containing 10% formalin solution. The samples were stored at -20°C until analyzed.

b Extraction of Lipids from the Sample

A weighed portion of each sample was ground using a hand held grinder. The lipids were extracted at 40°C under nitrogen, for about three hours using 2:1 chloroform: methanol. The extracted lipids were dried under nitrogen and then redissolved in hexane. The hexane extract was either analyzed immediately, or was stored at -20°C in glass vials until analyzed.

c Analysis of Lipids Using Normal Phase

Before the samples were analyzed, the conditions necessary to obtain separation had to be determined. This was done by using standards containing cholesterol and cholesterol esters. Mixtures containing different esters of known concentration were prepared. A mixture of isooctane and ethanol was chosen as the beginning mobile phase for silica columns. Initial mixture of 80% isooctane and 20% ethanol was tried. The
mobile phase was degassed by passing helium through for about 20 minutes. The column was conditioned by running the mobile phase through the column, for about thirty minutes. Before the sample was injected into the column, it was filtered using 0.45 micrometer nylon filters from Waters Corporation. Between 10 and 50 microliter samples were injected each time. The lipids were also hydrolyzed using potassium hydroxide in ethanol. The resulting lipids were extracted into hexcane and were run through the HPLC column.

d Determination of Optimum Flow Rate

To determine the best flow rate, the mobile phase was initially set at 0.7 mls/min. A standard mixture was injected and components allowed to elute. Increments of 0.2 mls/min were made before each injection until the flow rate was 1.5 mls/minutes. A Van Deemter plot of plate height vs. flow rate was used to determine the best flow rate. A flow rate of 1 ml/minutes was determined to be the best flow rate, and this flow rate was kept constant while different mobile phase combination were tried to obtain best separation. None of the ethanol isooctane mixture tried gave complete separation of all the esters. Other solvents and solvent combinations were tried but none gave complete separations of all the cholesterol esters tried. The list of solvents tried is given in Table 1.

Mixtures of hexane and ethanol in the ratio of 9:1, and cyclohexane and ethanol in the ratio of 280:20 and separation and were chosen for the sample analysis. The results obtained with the standards and samples are shown in figures and Table 10.

e Analysis of Lipids Using Reverse Phase Column.

Procedure

Reverse phase columns were studied to find out if they would provide an improvement in sample separation. Acetonitrile /water in different proportions were tried.
This mobile phase gave very long retention times and the separation was not very good. Silver nitrate was added to the mobile phase to find out if the resolution would improve, since Ag$^+$ ion combines with olefins to give silver compounds, which would have different retention times from the paraffinic and can be located and detected. Silver ion does not react with paraffinic compounds. Linoleate, oleate, and arachindonate eluted at the same time. The retention time for palmitate was very close to that of stearate. It was also found that individual esters gave slightly different retention times when they were injected as a mixture and when injected into the column individually.

Silica columns were used for most of the analysis because mobile phases that dissolved sample readily, could be used. The list of mobile phases tried is given in Table 7.
The results of standards run individually under the same conditions. Stearate 3.85, palmitate 3.85, linoleate 4.0, arachidonate 4.0 oleate 3.9 and cholesterol 4.7 conditions. Column silica (econosil) mobile phase. Heptane: isopropyl 9:1, flow rate 0.9 mls/minute chart speed 1cm/minute.

3; HPLC results obtained by using 9:1 isoctane: isopropyl alcohol as mobile phase. Flow rate 0.5 mls/minute.
Results of HPLC Analysis

Tables 10-13 and Fig 9-15 show the HPLC results obtained using a silica column. The number of compounds in the new and old sample from the first patient are the same but the retention time was different indicating that the compounds may be different. This patient seems to have all the standard cholesterol and cholesterol esters in the new material (4 compounds), while in the old material, only two compounds resemble that of the standards. The retention times of stearate and palmitate were the same, while that of the oleate and arachidonate were also very close, i.e., 3.85 minutes to 3.90 minutes. Linoleate and arachidonate were also inseparable, at a retention time of 4.0 minutes. The standard gives slightly different retention times when injected as a mixture and when injected alone. For example, in using heptane:ethanol as mobile phase, stearate had retention time of 2.9, while palmitate had retention time of 3.0. When the two were injected as a mixture, they eluted together at 3.1 minutes. This phenomena may be due to the fact that these compounds are very similar in structure so that their interaction with the mobile phase and stationary may be the same and a result the come out together.

Patient 2 also shows different compounds in the new material from those found in the old material. The new material had more compounds than the old material. Only one compound in the new material seemed to be present in the old material. This patient seems to have material that is different from the standard.

Patient no 3 had more compounds in the old material than in the new material. Only one compound seemed similar in both materials.

The HPLC results of the hydrolyzed material show significant differences. The results obtained using isooctane:isopropyl alcohol also show that there is a difference in
FIG. 9  HPLC TRACES OF PLAQUE EXTRACT IN HEXANE.
CONDITIONS:
COLUMN: 5U ECONOSIL SILICA
MOBILE PHASE: 9:1 HEXANE:ETHANOL
FLOW RATE  1ml/minute
WAVELENGTH 200 nanometers

Old Material

New Material

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FIG. 10  HPLC TRACES OF PLAQUE EXTRACTS IN HEXANE
COLUMN: 5u ECONOSIL SILICA 25 cms
MOBILE PHASE: HEXANE : ETHANOL 28: 2
FLOW RATE: 1ml/minute
CHART SPEED: 1cm/minute
WAVELENGTH: 200 nanometers.

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FIG. 11 HPLC CHROMATOGRAMS OF NEW MATERIAL

CONDITIONS: COLUMN ECONOSIL SILICA
MOBILE PHASE ISOOCTANE: ETHANOL 9:1
FLOW RATE 1ml/minute
WAVELENGTH 200nm

Retention Time in Minutes

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Fig. 12 HPLC CHROMATOGRAM OF OLD MATERIAL.

Fig. 13: HPLC CHROMATOGRAMS OF STANDARD CHOLESTEROL, CHOLESTEROL ESTERS, AND TRIGLYCERIDES.
Fig. 12 HPLC CHROMATOGRAM OF OLD MATERIAL.

Fig. 13: HPLC CHROMATOGRAMS OF STANDARD CHOLESTEROL, CHOLESTEROL ESTERS, AND TRIGLYCERIDES.
Fig. 14 HPLC CHROMATOGRAMS OF STANDARD CHOLESTEROL ESTERS. COLUMN: SILICA 5μ
MOBILE PHASE: HEXANE: ETHANOL 28:2
FLOW RATE: 1ml/minute
FIG. 15 HPLC CHROMATOGRAMS OF LIPID EXTRACTS
MOBILE PHASE: HEXANE:ETHANOL 28:2 SILICA
COLUMN 5μ, FLOW RATE: 1ml/minute.
### TABLE 10

**MOBILE PHASES TRIED USING SILICA COLUMNS**

<table>
<thead>
<tr>
<th>Mobile Phase</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane: ethanol</td>
<td>5.0:50, 70:30, 99:1</td>
</tr>
<tr>
<td>Hexane: acetonitrile</td>
<td>99:1, 99:05, 90:10</td>
</tr>
<tr>
<td>Isooctane: ethanol</td>
<td>70:30, 80:20</td>
</tr>
<tr>
<td>Hexane: methylene chloride: acetonitrile</td>
<td>10:3:3</td>
</tr>
<tr>
<td>Hexane: isopropyl alcohol</td>
<td>3:2</td>
</tr>
<tr>
<td>Pentanol:acetonitrile:isoctane:water</td>
<td>17.5:6:62:1</td>
</tr>
<tr>
<td>Ethyl acetate: hexane</td>
<td>1:0</td>
</tr>
<tr>
<td>Hexane: Isopropanol</td>
<td>9:0</td>
</tr>
<tr>
<td>Heptane:ethanol</td>
<td>9:0</td>
</tr>
<tr>
<td>Methanol: methylene chloride</td>
<td>2:23</td>
</tr>
</tbody>
</table>

### TABLE 11

**MOBILE PHASES TRIED USING REVERSE PHASE COLUMNS**

<table>
<thead>
<tr>
<th>Mobile Phase</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>50:50, 20:80-1:99</td>
</tr>
<tr>
<td>Water: methanol</td>
<td>10:1-1:10</td>
</tr>
<tr>
<td>Acetonitrile: water</td>
<td>94:3:3</td>
</tr>
<tr>
<td>Acetonitrile: methanol:water +5 mM silver nitrate</td>
<td>65:35</td>
</tr>
</tbody>
</table>
the number and type of compounds eluting from the old material and the new material. Table 12 show the results.

g. Conclusion

The HPLC results show significant difference in the number and type of compounds found in the new and old material. From the same individual.

E SUMMARY AND DISCUSSION OF THE CHROMATOGRAPHIC ANALYSIS OF THE PLAQUE FROM HEART PATIENTS

The results of TLC and HPLC analyses of the plaque are shown in fig. 5-6 and 9-15.

The results show a lot of individual variation in terms of the number and composition of lipids while others show presence of cholesterol, cholesterol esters as well as phospholipids and possibly triglycerides. The triglycerides were very difficult to separate from the cholesterol esters with our system. There were significant differences in the composition of the lipids in the new and the old deposits from the same individual.

The ratios of the different lipids vary from one individual to the next but despite these variations, the results show clear differences between the old and the new material in terms of lipid composition. Cholesterol or cholesterol type material appear to be present in most samples both old and the new. There were a number of samples that did not seem to have detectable amount of cholesterol. The saturated fatty acid esters of cholesterol were observed more frequently than unsaturated fatty acid esters. Triglycerides were very difficult to separate from the cholesterol esters with our system. There were significant difference in the composition of the lipids in the new and the old deposits from the same individual.
TABLE 12

HPLC RETENTION TIMES OF LIPIDS EXTRACT AND STANDARD CHOLESTEROL ESTERS 1

<table>
<thead>
<tr>
<th>Cyclohexane : ethanol (2:2)</th>
<th>Heptane : isopropyl alcohol (9:1)</th>
<th>Isooctane : isopropyl alcohol (9:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>Patient 2</td>
<td>Patient 1</td>
</tr>
<tr>
<td>New Old</td>
<td>New Old</td>
<td>New Old</td>
</tr>
<tr>
<td>2.5 2.4</td>
<td>3.9 4</td>
<td>2.9 3.2</td>
</tr>
<tr>
<td>3 2.9</td>
<td>4.3 4.5</td>
<td>3.2 3.4</td>
</tr>
<tr>
<td>3.2 3.1</td>
<td>4.7 4.7</td>
<td>3.4 4.3</td>
</tr>
<tr>
<td>3.5 3.5</td>
<td>4.8 4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>4 3.8</td>
<td>6.3 4.7</td>
<td>4.7</td>
</tr>
<tr>
<td>4.1 4.1</td>
<td>8.1</td>
<td>8.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Standard mixture</th>
<th>Stearate &amp; Palmitate 3.0</th>
<th>Stearate &amp; Palmitate 3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>oleate</td>
<td>oleate 3.5</td>
<td>oleate 3.5</td>
</tr>
<tr>
<td>linoleate &amp; arachidonate 4.0</td>
<td>linoleate &amp; arachidonate 4.0</td>
<td>linoleate &amp; arachidonate 4.0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.1</td>
<td>Cholesterol 4.5</td>
</tr>
</tbody>
</table>

TABLE 13

HPLC RESULTS OF HYDROLYZED MATERIAL

<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Standard mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>New</td>
<td>Old</td>
</tr>
<tr>
<td>1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>1.6</td>
<td>2.3</td>
</tr>
<tr>
<td>3.6</td>
<td>4.6</td>
</tr>
</tbody>
</table>

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The material from the bypasses was deposited after rigorous treatment with cholesterol lowering diet and or calcium blockers. Assuming that these patients followed the diet rigorously, it can be said from the results that treatment changes the composition but does not prevent plaque formation. The results indicate the current methods of treatment may be inadequate.

The increased cholesterol found in heart patient may be a symptom of a problem rather than the cause of the problem. The results show individual variations in terms of the material deposited as the plaque. This indicates that treatment should be individualized.

Emphasis on the dietary cholesterol as the main cause of the disease may be obscuring other factors that may be involved in the heart disease. The result show that treatment does not prevent clogging of the artery. The increased cholesterol in heart patient could be as a result of deficiency of important hormones and or enzymes that regulate synthesis and breakdown of cholesterol. There is need for further research to find why some people are not affected by dietary cholesterol while others continue to have elevated cholesterol despite low cholesterol diet.

Our research was basically designed to find out if there is a difference between the composition of the material that deposits since childhood and that deposits at a later date. Our result show that there is a significant difference in number and type of compounds deposited in the old and the new material.

HPLC and TLC results show significant differences in both ratios and types of lipids deposited in the new and old material. The individual variations observed may be due to the diet and drugs that the patient were given. This shows the need for
individualized screening and treatment. It also indicates that generalized implications of cholesterol as a major cause of heart diseases may not be accurate.

Research should be directed towards finding the cause of increased levels of lipids in heart patients.

1 Problems Encountered In The Separation Process

One of our major problems was that we were limited to using UV/VIS detector because the refractometer was insensitive to the low concentration of the organics in these samples. We could not use most of the solvents recommended in the literature for separation of lipids because they absorbed strongly in the wavelength of interest.

Cholesterol and cholesterol esters do not absorb strongly in the UV. The samples had to be analyzed at wavelength below 200nm in order to observe the signal from these lipids. This again limited the number of mobile phases that could be used. Many substances absorb in this region and therefore any impurities in the sample can absorb very strongly giving false readings.

2 Conclusion

The TLC and HPLC indicate that there is a difference in number and proportions of lipids found in the newly deposited plaque and the plaque that had been depositing since childhood.
F. ANALYSIS OF LIPIDS EXTRACT USING MS

1. Introduction

Mass spectrometry has become a very powerful tool for structural identification of organic molecules. Interfacing liquid chromatography with mass spectrometry has enabled separation and identification of large, biologically important molecules that are nonvolatile and are thermally labile.

2. Principles of MS

Mass spectrometry is an analytical technique that can be used for qualitative and quantitative identification of different elements and molecules. It is a very sensitive technique, which can give structural information that can be used either alone or in conjunction with other information to obtain complete identification of unknown pure compound.

In mass spectrometry, the sample to be analyzed is introduced into the inlet chamber where it is vaporized. The produced gas is directed to the ionization chamber, where the sample is ionized and fragmented, depending on the mode of ionization.

The ionized molecules are accelerated towards the detector by a magnetic field and applied voltage. The velocity of the ionized molecules depend on their mass, the magnetic field and voltage. Controlling the voltage and magnetic field determines the masses reaching the detector(165). Theory and principals of mass spectrometer can be found in the following references(165,258,259).
3 Components Of A Mass Spectrometer

Mass spectrometer consists of inlet system, ionization chamber, accelerating chamber, the drift chamber, the detector and the read out system, as shown in figure.

a Inlet System

The inlet chamber or sample introduction system is made to accommodate different types of sample. There are different type of sample introduction devices.

i Bach type

In this type of device, the sample is volatilized externally and is then allowed to leak into the ionization chamber. The chamber is kept at a temperature of up to 500°C, to volatilize the sample.

ii Direct probe

This is a type of sample introduction device which is used for non-volatile or solid samples. The sample is placed in a removable small cup or glass capillary tube and the probe is placed a few millimeters from the ionization source. The probe has a mechanism for rapid cooling and heating, that enables the sample to be vaporized directly into the ionization chamber.

4 Ionization Chamber

The ionization chamber consists of an anode and cathode. A potential difference is applied that causes the electrons to be accelerated toward the anode. As the sample molecules enter this chamber they collide with electrons and they become ionized.
\[ M + e^- = M^+ + 2e^- \]

If the speed of an electron is increased by increasing the voltage, the sample molecules are shattered into reproducible fragments that are also ionized. The chamber is maintained at low pressure to prevent recombination of fragments.

The extent of fragmentation can be controlled by controlling the method of ionizing the sample. If information on the parent mass is important, soft-ionization modes can be used to produce mostly the parent ion without further fragmentation. This can be done by photoionization using tunable lasers whose energy can be controlled.

a. Electron Impact

The common method of producing ions for mass spectrometry, involves bombardment of the gaseous sample with a beam of energetic electrons. The electrons are produced at heated tungsten or rhenium wires. The electrons are produced at an energy of about 70 eV by an applied potential across the filament and an anode. The electrons pass through a stream of the high energy electrons with the sample molecules results in ionization and shuttering of the molecules into different ionized fragments. Each molecule fragments in a definite pattern. The complex mass patterns that results, can help with the identification of the sample molecules. [165]

5. Accelerating Chamber

As the ionized molecules enter the accelerating chamber, their speed is increased by an applied electric field at a particular voltage. The speed of the molecules depend on their masses and therefore reach drift chamber at different times depending on the masses.
FIG. 16 SCHEMATIC DIAGRAM OF A MASS ANALYZER SHOWING THE FOUR ELECTRICALLY CONNECTED RODS.
6 Drift Chamber

After the particles are accelerated, they enter a magnetic field that causes the molecules to move in circles around it against the centrifugal force. When the magnetic force and the centrifugal forces are equal, the particles travel uniformly.

The radius of the circular path of the particle depends on m/e, the magnetic field and the accelerating voltage. When accelerating voltage and the magnetic field are kept constant, the masses of particles can be calculated using equation E, if charge is unity and radius is known.

Voltage and magnetic field are used to separate particles of different masses before they enter the detector.

7 Quadrupole Accelerators

The quadruple has four short parallel metal rods instead of an accelerating chamber. The poles are symmetrical with opposing rods electrically connected, as shown in fig 21a.

One pair of rods is attached to the negative side of a variable DC source and the other to the positive terminal. In addition, variable radiofrequency AC potentials which are 180 degrees out of phase, are applied to each pair of rods. The sample molecules oscillate back and forth along the rods, as a result of the influence of the combined fields (165). Only those ions whose frequency is in resonance with the Rf frequency get to the mass analyzer. Mass scanning is done by varying the frequency of AC supply while keeping potential constant, or by varying the potential of the two sources, while keeping their ratio and frequency constant (165).
8 Detector

a Electron Multiplier

The detector used in the MS analysis was electron multiplier. It consists of an electron emissive dynode. The collision of the charged masses with the dynode releases electrons, which are accelerated to another surface. This surface also releases electrons after the impact after multiple stages, a cascade of electrons is produced by a single collision. These electrons are collected and counted by electron collector.

9 Read Out System

Modern mass spectrometers have several types of read out systems such as analog or digital recorders, oscilloscopes, photographic recording galvanometers and computerized printers, which produce bars (165).

G EXPERIMENTAL

Instruments - Mass spectrometer; Finnigan model TSQ-70 and Waters HPLC system were used.

1 Experimental Procedure

Although the MS instrument available in the department had the capability of interfacing with HPLC, the interfacing device was not operating at the time of these analyses. HPLC was run under conditions described previously.

Each peak eluting from the HPLC column was collected individually. Each sample was injected into the column repeatedly until enough material for each eluting peak was collected. The solvent was dried under nitrogen and the residues were analyzed by mass
spectrometer using direct probe for sample introduction into the ionization chamber. Electron impact mode of ionization was used in the MS analysis.

The mass spectra of the peaks from the bypasses and the non bypassed arteries were compared.

2 Results and Discussion

Representative mass spectras are shown on figures 17-20. The eluting peaks may not have been pure since separation of closely related lipids was difficult and complete identification of separated lipid was not possible. The samples seemed to be contaminated with plasticizers such as phthalates. The source of these plasticizers was not found, but was probably in the mass spectrometer used. Attempts to remove these contaminants proved futile. The samples were shipped to us in plastic containers. We soaked these containers in hexane and analyzed the spectra of the resulting solution. The spectra of this blank was very different from the spectra obtained with samples, and was not a source of contamination. However, despite these problems, the cracking pattern resembled that of cholesterol and cholesterol ester standards.

The mass spectra of the separated lipids does not resemble any one of the cholesterol esters or cholesterol, although the general pattern is similar for some compounds. For example, the fig. 19 which is the mass spectra of the first compound eluting from HPLC of new material, has the abundant peak at 368. And has a small peak at 386. The mass pattern resembles that of cholesterol esters. On the other hand, fig. 18 which is the mass spectra of the first compound of the old material does not have the abundant peak at 368, but instead, it has abundant peaks at 279 and 149. It also has a peak 386.3 and at 391. This indicates that the first eluting compounds from the old material is different from that eluting from the new material. The retention times were also different indicating that the compounds were different.
FIG. 17 MASS SPECTRA OF SECOND SOLUTE (NEW MATERIAL)
FIG. 18 MASS SPECTRA OF FIRST SOLUTE
(old material)
FIG. 19  MASS SPECTRA OF FIRST SOLUTE (new material)
FIG. 20 MASS SPECTRA OF THIRD SOLUTE
(old material)
Separation of closely related cholesterol esters was not possible with our system and therefore the purity of the separated compounds was not known. Several compounds may have eluted together making it impossible to identify the compounds. Also, collection of samples by following UV traces, may have introduced error, since the emergence of the peak may not be an accurate measure of time the sample reached the collecting tubes.

Despite these problems, the results show that the old material is significantly different from the new material from the same individual.

3. Conclusion

The mass spectra data show significant difference between newly deposited plaque and old plaque.

H. ANALYSIS OF LIPIDS USING NMR

1 Introduction

Nuclear magnetic resonance has become a very powerful tool for structural identification of pure organic compounds. Recent improvements in NMR techniques have made it possible to identify components of a mixture without separation, especially when two dimension NMR is used. Development of solid state NMR has made it possible to determine structures of substances that are not soluble in common solvents used in NMR. Use of other nuclei apart from proton has made it possible to analyze large biological
molecules. For example protein structure can elucidated by looking at the carbon NMR rather than proton

Nuclear magnetic resonance involves interaction of a spinning nucleus with radiofrequency radiation. In order for the nucleus to interact with the radio waves, it must have an odd number of protons or a odd number of neutrons or both.

The unpaired proton or neutron act as a magnet as it spins. It also has momentum which is determined by spin number.

When a nucleus is placed in a strong magnetic field it orients itself in certain directions relative to the direction of the magnetic field. These orientations are determined by the spin number I. The nucleus can absorb the radio waves and go to a different orientation or to an excited state. The energy of this transition is quantized. It depends on the strength of the magnetic field. Principals of NMR can be found in most analytical books. (260)

2 Chemical Shift

Structure determination of NMR is based on the fact that the effective magnetic field depends on the other groups surrounding the nucleus. Some groups shield the nucleus from the magnetic field and a higher magnetic field is required to obtain resonance frequency.

If the magnetic field is kept constant the nuclei will absorb at different frequencies depending on their environment. The difference between the applied and the effective magnetic field is called the chemical shift.

\[
\text{Chemical shift} = \frac{B_s - B_r}{B_r}
\]
Bs = Magnetic field at which samples absorbs

Br = Magnetic field at which the reference (i.e. TMS absorbs)

3 Experimental

All NMR spectra were obtained on chemistry departments Brunker WP-200 FT-NMR spectrometer.

Chemicals: Deuterated chloroform

Methanol Chloroform from Aldrich

4 Experimental Procedure

The lipids were extracted with chloroform methanol as described previously. The liquid was evaporated under nitrogen, and the residue was redissolved in deuterated chloroform. The resulting solution was placed in NMR tubes and proton NMR was run using the WP 200 FT-NMR spectrometer. The crude mixture was run first to find the major functional groups. The HPLC separated compounds were also run, but the concentration was too low for any significant detection. Even after preconcentration of the separated peaks the concentration was too low, and no peaks were observed.

5 Results and Discussions

The NMR spectra of the crude lipids show major peaks in the aliphatic region but the general pattern closely resembles the spectra of steroids (261). This is especially shown by sharp narrow peaks at region between 0.5-1.5 ppm and the broad peaks at region between 3-4 ppm. Also the narrow peaks at 0.86, 1.21 and 0.69 are indicative of steroid structure (261). The peak at 0.69 is probably due to C-18, while the peak at 0.83 may probably be due to C-19 of the steroid skeleton. The actual identification of the
compounds by NMR was not possible because the samples were very small and attempts to separate the individual components for analysis failed because of the low concentration. The NMR spectra of the new material resembled that of cholesterol and cholesterol like substances, such as the hydroxycholesterol. The spectra of the old material is not very different from the that of the new material. The main difference was the peak heights and areas and the splittings. This indicates possibility of differences in types of compounds present and or the relative amounts of the different compounds. The HPLC results showed that there was proportional differences between the two sets of samples. It is also possible that the peak height differences may be due to proportional differences. The NMR spectra of lipids extract is shown in figures 21-24.

6 Conclusion

The NMR results show that the compounds present in the lipid extracts are very similar in structure but the proportion of the different components may be different. The NMR spectra resemble that of steroids.
FIG. 21 PROTON NMR OF CRUDE LIPID EXTRACT (old material)
FIG. 22 NMR SPECTRA OF CRUDE LIPID EXTRACT (new material)
FIG 23 NMR OF CRUDE LIPID EXTRACT
(OLD MATERIAL PATIENT 2)
FIG. 24 NMR SPECTRA OF CRUDE LIPID EXTRACT (new material patient 2)
I. SUMMARY OF RESULTS OF THE ANALYSIS OF THE OF THE PLAQUE FROM HEART PATIENTS.

1. Cholesterol and cholesterol esters were found in both old and newly deposited plaque but not all samples contained cholesterol.

2. Phospholipids and triglycerides were also found in the plaque but the type of phospholipids and triglycerides varied from one patient to the next.

3. The new and old deposits from the same individual were significantly different both in the type of lipids present and the proportions of the lipids. TLC and HPLC results showed that the number of lipids also differed significantly.

4. Although most samples contained saturated fatty acid cholesterol esters, there were some samples that contained cholesterol oleate, linoleate and arachidonate.

5. The results of metal analyses also indicated differences in terms of concentration of the metals and the metal ratios. There was increased deposition of metals in the newly deposited plaque compared to the old plaque, with the exception of nickel, chromium, iron and potassium which showed decreases in concentration in the new material.

6. Calcium and phosphorus were almost sixteen times higher in the new plaque compared to the old deposits.

7. Ca/Mg ratio was much higher in the new material than the old plaque and the ratio in both the old and the new was much higher than would be in the normal blood. For example the average Ca/Mg ratio in the new plaque was found to be about 77,
while in the old plaque it was found to be 28 in matched samples. The normal ratio in the blood plasma is around 3. The Ca/Na ratios were also much higher in the new material than the old material or in blood plasma of normal people.

8. The copper/zinc ratios were less than one in all the samples analyzed including the old and the new, and the ratio in the new material was lower than in the old.

**Conclusion**

The result of lipid analysis showed that the material that deposited in the new plaque was different from that which had been depositing since childhood. This indicates that there may be more to heart disease than lipid levels. Further research is needed to find what causes the difference in the chemical composition of the deposits. Further research in the regulation mechanisms of cholesterol and changes in the regulation that result in cholesterol accumulation needs to be investigated. Also there is need for further research to find the actual role of free radicals in the etiology of atherosclerosis. It may be that decreased levels of antioxidants in susceptible individuals may be playing a major role in the etiology of heart diseases.

**GENERAL DISCUSSION OF THE RESULTS OF THE LIPID ANALYSIS**

The NMR and the mass spectra data show that the general pattern of the lipids deposited in both new and the old deposits are similar with minor differences in peak heights or the peak pattern. The differences may be due to the composition of the lipids. TLC and HPLC results indicated that the differences were not only due to the ratios of the different lipids but the number of lipids were also different. In some cases more lipids were found in new material than in the older material. There were a lot of individual variation in terms of the proportions of the different lipids, but cholesterol and or cholesterol esters were present in most samples. HPLC and TLC results also showed
presence of phospholipids and triglycerides but not all samples contained phospholipids and triglycerides. Their were a lot of individual variations. Separation of the individual cholesterol esters was very difficult since a number of them eluted at the same retention time. Our chromatographic system was not able to separate closely related cholesterol esters. Despite these difficulties, it was clearly shown that the lipid composition in the newly deposited plaque was different from the old material (i.e. material that has been coating since childhood) from the same individuals.

The fact that the arteries became clogged even after rigorous treatment with cholesterol lowering diets or drugs, indicated that the dietary cholesterol may not be the major culprit. There may be other metabolic changes that results in excessive accumulation of the lipids.

This was a preliminary research whose purpose was to find out if there were significant differences. Further research needs to be done to find out why current methods are not effective in treating heart diseases. Emphasis on dietary cholesterol may prevent change in direction of research that may bring some enlightenment on the actual causes of heart diseases

Our study and other studies have shown that other factors may be involved (263-266). It has been shown that hyperlipemic people have a genetic defect that reduces their livers' ability to remove LDL cholesterol from the blood (225). Recent studies with healthy human volunteers have shown that feeding a high cholesterol diet does not change the blood cholesterol levels and there was no significant difference between those fed high cholesterol diet and the control fed low cholesterol high complex carbohydrate diet, high in fiber (178, 180, 201).
Study in Europe showed that when blood levels of cholesterol were decreased in heart patients death due to coronary arteries disease, decreased, but there was no difference in overall mortality rate when compared to the group that was not treated (180, 187).

Recent studies have shown that exercise reduces the death due to heart disease by increasing the levels of HDL cholesterol and that of prostacyclin, which inhibits platelet aggregation (200, 224). It has been shown that factors that increase platelet aggregation also increase the risk of heart diseases. Recent studies have shown that platelet count can be an independent risk factor (267). Exercise is also believed to increase the lipase activity which increases the break down of fat and carbohydrate (200). It is also believed that the exercise relaxes an individual, which in turn reduces the levels of neurotransmitters (200). Exercise was shown to increase synthesis of cholesterol in the liver without affecting the intestinal cholesterol (251). In fact, it has been postulated that cholesterol may play a protective role against cancer, and that the increased levels of cholesterol in heart patients may due to lack of receptors of LDL cholesterol at the membrane (180).

It may be that modern conveniences, that have reduced the levels of physical activity, coupled with diets high in fats and refined sugar may be playing a major role in etiology of heart diseases by increasing the levels of lipids in the body without increasing the factors that prevent the accumulation of these lipids. Inactivity has been shown to weaken heart muscles (241). It is possible that decreased levels of physical activity at early age could lead to changes in the body that may predispose some individuals to heart diseases. Inactivity may also increase production of free radicals that may result in injury to the heart muscle (252, 253).

Negative emotions and pressures of modern living are believed to play a role in heart diseases by altering the release of neurotransmitters (220). Neurotransmitters control
nerve impulse and therefore control heart rhythms (220). All these factors could be contributing to the heart diseases.

a Conclusion

Both metal and lipid results indicated that the material that deposited after rigorous treatment with cholesterol lowering diets or drugs was different from the material that deposited since childhood. The results indicated that cholesterol alone may not be the cause of the problem. Other factors may be involved.
K  GENERAL DISCUSSION OF THE CHEMICAL
COMPOSITION OF THE PLAQUE

The results of the metal and the lipid analysis of the plaque showed that there was a significant difference in the metal and lipid composition of the new deposits compared to that of the material that had been deposited since childhood. Although there was individual variations in terms of the metal concentrations and lipid composition, there was increased metal deposition in all new materials that were analyzed. The average concentration of potassium, chromium and nickel decreased in the new deposits. Chromium and potassium play a very important role in the cardiovascular system. It may be that their decrease in the new deposits indicate changes in the levels of these metals as the disease progresses. Also, the increased deposition of the other metals in the new material compared to the old deposits indicates possibility of metabolic changes either as a result of heart diseases, or as a results of increased concentration of the metals. The ratios of the metals in the old and the new deposits were relatively similar but very different from what would be expected in blood of normal people. Assuming that the ratio of these metals reflected the ratios in the blood of these patients, this may indicate that the changes in the metal ratios may play a role in the etiology of heart diseases or that the heart diseases lead to changes in metal composition.

The copper/zinc ratio was much lower than normal in all the samples analyzed including the old material. There has been a suggestion that low copper/zinc ratios may play a role in heart diseases. Zinc and copper play a vital role in lipid metabolism. Copper is involved in enzymes that control collagen cross-linking and elastin formation. These materials are important in maintaining strong but flexible cardiovascular muscles. Deficiency in copper could weaken the walls of the blood vessels making them more
susceptible to injury. It is believed that atherosclerosis is initiated by injury to blood vessels that leads to clotting of the blood and trapping of the lipids in the clot (264). It may be that the copper and the zinc enzymes functions properly only when the copper/zinc is close to 1.

Copper and zinc are components of superoxide dismutase, which is an enzyme that prevents oxidation of lipids by the superoxide radical (268). Recent studies have shown that superoxide radical can damage the membrane by peroxidation leading to increased blood coagulation and formation of atherosclerosis (268, 263).

Hyperlipemic individuals have higher levels of peroxidation products and their blood is more susceptible to coagulating agents (263, 268, 270, 273). It may be that these people lack effective antioxidant mechanisms.

The Ca/Mg ratio was also higher than in the normal blood in all the samples analyzed, and sometimes as high as 50. Calcium and magnesium play a complimentary role in the body. Magnesium is believed to be natures' calcium blocker preventing calcium entry when it is not necessary (135). Increased calcium ions above the physiological levels may prevent the blocking action of magnesium and may lead to increased entry of calcium into the cell. Calcium overload decouples the excitation-contraction of the heart leading to heart attack.

Calcuta and cholesterol are required in the initial step that leads to blood coagulation (123). Factors that regulate levels of these chemicals may be at fault, leading to increased deposition of calcium (125). It has been shown that factors that affect calcium metabolism also affect lipid metabolism. For example, hypothyroidism increases calcium blood levels and it has been shown to increase the lipid levels (274).
There may be metabolic changes that alter the regulation mechanism of cholesterol. Recent studies have shown that oxidation of membrane lipids change the membrane permeability to lipids by exposing polar groups of the membrane bilayer (278). Blockage of cholesterol entry into the liver could result in increased synthesis of cholesterol by the liver since entry of the cholesterol into the liver is believed to initiate the mechanism that cuts down its synthesis in the livers (59). This could also result in accumulation of the insoluble cholesterol in the blood vessels. Recent studies have shown that lack of the cholesterol receptor at the membrane could result in cholesterol accumulation in the body(196, 279).

It is possible that people with high levels of cholesterol have some genetic predisposition due to altered gene that regulate cholesterol levels. It is believed that the HDL cholesterol increases membrane permeability while the LDL decreases the fluidity (196). Oxidation of these lipoproteins has been shown to decrease the membrane permeability. This could lead to accumulation of lipids in the blood but it also increases calcium influx into the cell(196). Oxidation of the lipids not only decreases the permeability of the membrane but it also increases the platelet aggregation (196).

Therefore, the main problem may not be the dietary cholesterol but it may due to metabolic changes that increase the free radicals that damage the membrane leading to the deposition of lipids. It is well known that most heart diseases are increasingly observed at old age. It could be that at this age the enzymes that control oxidation do not function properly. Our study has shown that the ratio of copper/zinc was much less than one. It may be that superoxide dismutase requires that the ratio of these metals be 1 in order for it to work properly. Also, increased levels of calcium compared to that of magnesium could have contributed to the problem of heart diseases.
Our results show that there is a necessity for a study that compares total and ratios of metals in the blood of heart patients with that of normal individuals matched both in age and diet to try to find out the role of metals in heart diseases.

Studies have shown that exercise reduces levels of cholesterol and also the risk of heart diseases. It is believed that exercise increases the production of HDL as well as prostacyclin which is an anticoagulant. It may be that the Maasai and the Eskimos, who live on high cholesterol diet are protected by their physical activity. It may also be that communities with low incidence of heart disease may be protected by their physical and emotional well being.

It has been shown that prolonged emotions especially those that display extreme anger or impatience leads to development of heart disease. Emotions alter the release of catecholamines that control nerve impulses as well as calcium influx into the cell (125).

L. CONCLUSION

The result of our analyses showed that there was significant difference between the lipid and metal composition in the newly deposited plaque and the plaque from the material that has been deposited since childhood. There was increased deposition of metals in the new deposits indicating possible metabolic changes as the disease progresses. There were also significant changes in metal ratios. Further research is needed to find the role of metal and especially metal ratios in heart diseases. Also, a more controlled study in necessary to find out if the individual variation observed in lipid and metal composition was due to diet, or to the disease. Patients matched in age, diet and treatment may give a clear answer same patient.
PART II

EFFECT OF ACID RAIN AND HUMIC SUBSTANCES ON ALUMINUM TOXICITY
CHAPTER 1

EFFECT OF ACID RAIN AND HUMIC SUBSTANCES ON ALUMINUM TOXICITY

A. GENERAL INTRODUCTION

1 Acid Rain

Acid rain is a problem that has resulted in environmental destruction as well as economical impact in the industrialized countries and some developing countries. Ancient buildings and historical monuments have been destroyed by acid rain (280, 281). Eutrophication of lakes, rivers and the disappearance of forests in USA, Canada, and Europe is believed to be a result of acid rain (282-285). It is important to find the actual role of acid rain and to find economically feasible ways to reduce its impact in the environment.

The role of acid rain in the destruction of the environment is still very controversial, because the impact of acid rain on the environment is influenced by environmental conditions. The total amount of acid rain falling on an area can vary with time, depending on rate of emission of acid sources, wind velocity, and direction, sunlight, presence of photo-oxidants, and buffering agents (286). The problem is compounded by the fact that there are organic acids produced naturally which may play a role in environmental destruction (287-292). Also different areas receiving the same amount of acid rain may not be affected in the same way (293). However, despite these problems, there is clear evidence that areas that produce sulphur and nitrogen oxides, which are precursors of acid rain, have more destruction by the environment than areas receiving little emissions of these pollutants.

113
Sources of Acid Rain

Normal rain has a pH of 5.6 due to dissolved carbon dioxide. In some parts of the United States, and Scandinavian countries, the rainfall has a pH range of 4.0-4.2 and sometimes it may be as low as 2.8, (294, 295), which is very acidic.

Acid rain is produced when sulphur and nitrogen oxides in the atmosphere interact with oxidants such as ozone, hydrogen, peroxide, etc, in the presence of sunlight to form sulfates and nitrates respectively. These sulfates and nitrates interact with water vapor to form the respective acids as shown in the simplified equations below.

\[
\begin{align*}
SO_2 + O_3 & \xrightleftharpoons{\text{sunlight}} SO_3 + O_2 \quad (1) \\
SO_3 + H_2O & \xrightleftharpoons{\text{(vapor)}} H_2SO_4 \quad (2) \\
NO_2 + O_3 & \xrightleftharpoons{\text{sunlight}} NO_3^- + O_2 \quad (3) \\
NO_3^- + H_2O & \xrightleftharpoons{} HNO_3 + OH^- \quad (4)
\end{align*}
\]

These acids may be dry deposited on surfaces on which they fall, if the atmospheric moisture is too low to form rain drops. Dry deposition of the acids can result in acid concentration on these surfaces, which can quickly be washed out by initial rain, producing acidic surges or flushes into an otherwise stable environment. This happens when the oxides and acids are deposited on snow and ice. When the ice or snow which has accumulated acids over a long period of winter time melts, it releases water that may be five to ten times more acidic than the rest of snow. These sudden acidic surges can have deleterious effect on plants and aquatic organisms (286).
b Effects of Acid Rain on Lakes and Rivers

When acid rain falls on lakes and rivers, that are not well buffered, it lowers the pH of these bodies of water. Most aquatic organisms are affected if pH of water goes below 5. Studies have shown that lakes and rivers with pH below 5 due to acid rain have declining populations of aquatic organisms including fish. Species diversity is also destroyed (284-287, 293). Only species that are able to adapt to acidic conditions survive. Aquatic plants are also affected by acid rain (281, 290, 294, 295, 297).

Toxic metals leached from soils by acid rain magnify the effect of acid on aquatic organisms (298, 299).

c The Effects of Acid Rain On Soil and Plants

Acid rain does not only affect rivers and lakes, but it also affects soil and trees (285, 300-304). Thin soil with bed rock low in calcium and other buffering ions, is most easily affected. The acid rain increases soil acidity which alters soil microorganisms, soil chemistry and reduces breakdown of decomposition of plant and animal material due to removal of bacteria that aid in breakdown of organic material (289-292). It also changes the soil moisture and increases dissolution and wash out of mineral nutrients, reducing soil fertility (305-307).

Acid rain not only leaches out nutrients, but also influences chemical exchange processes that affect uptake of nutrients. For example H\(^+\) may be exchanged for mineral nutrients such as Na\(^+\), K\(^+\), Mg\(^{2+}\), Ca\(^{2+}\), etc, resulting in poor plant growth (308, 286). Acid rain also dissolves toxic metals which poison plant roots causing the plant to die. For example, aluminum is released from the soil as shown below;
Clay mineral \( \xrightarrow{pH < 6.5} \) altered clay mineral +Al(OH)\(_3\)

Al(OH)\(_3\) \( \xrightarrow{} \) [(Al\(_7\)(OH)\(_{17}\))\(^{4+}\)]

The acid may affect the plant directly by reducing the foliage (309-312).

If the soil pH goes below 4, destruction to trees becomes severe and reforestation is not possible because seedlings will not grow in acidic soils (286, 313, 314).

d Effect of Acid Rain on Man and Buildings

Both dry and wet deposition of acid rain have been implicated in the deterioration of ancient buildings, especially those built on sandstone, limestone and marble (319).

Dry deposition of sulphur oxide on buildings results in sulphur oxide reacting with marble in presence of moisture to form soluble calcium salts, that wash down. As water evaporates, these salts form crystals that expand increasing cracking of the building (316-318, 306).

Acid rain damages cultural heritage by destroying fine arts and crafts. The economic impact can be enormous. Restoration of the damaged buildings can run into many millions of dollars. Fishermen can lose their livelihood and people a food source when fish populations decline.

Acid rain may have health effects in man, which may be direct or indirect. Indirect effect can be experienced by the release of toxic metals, such as cadmium, mercury, aluminum, etc., from acidified soils to the ground water used for drinking. These toxicants may be taken up by plants and animals. Therefore, the toxicant can get into the human body through contaminated foods and drinks.
Breathing air saturated with fine droplets saturated with acid rain can cause damage to the lungs, etc. Studies with both humans and animals have shown that exposure to acidic fumes increased viscosity of the lung mucous. This affects various function of the lungs. \( H^+ \) reacts with endothelial tissue resulting in increased permeability and other changes that reduce lung function, such as reduced particle clearance by the bronchial tracheal, or changes in the rate of respiration etc. (320-322).

Epidemiological studies have shown that people living in areas affected by acid rain have more respiratory illness, more circulatory disorders and more cancers than people living in less polluted areas (286).

The smog and haze produced by acid oxides and other pollutants, destroy the aesthetic beauty and reduce visibility.

2 Role of Aluminum in Acid Rain Problem

Most aquatic organisms and plants can survive at \( 4 < \text{pH} < 5 \) without any serious damage, if other toxic substances are not present. It is believed that the major effect of acid rain is due to the release of toxic metals from their stable, unreactive complexes in the soil, to states that are more toxic to plants and animals. (280-281, 294, 323, 328). One of the toxic metals released by acid rain is aluminum (309, 312). Studies have shown that aluminum is toxic to plants and aquatic organisms, especially fish at low pH (280-281).

a Aluminum In The Environment

Aluminum is one of the most abundant metals in the earth's crust, constituting 8.8% by mass (331). It is a major constituent of soils and clays. Aluminum concentration in the soils range from 10,000 to 300,000 mg/kg (331). Most of the aluminum in the soil is bound to \( \text{SiO}_2 \), but it also occurs in other aluminum minerals, in mixed oxides with other
metals, and metal halides etc. Aluminum is not easily released from its complexes at normal soil pH. It is therefore believed that aluminum does not constitute a health hazard to man or animals under natural conditions.

b Aluminum in Plants

Most plants contain about 15-20 ppm of aluminum on dry basis, but some species of plants, especially herbaceous trees, are reported to contain 200 ppm Al on dry basis. Some, especially the ferns may accumulate 3,000 to 4,000 ppm of aluminum (332).

c Aluminum Toxicity to Plants

Some of the plants that accumulate aluminum, have internal mechanism of detoxifying by immobilizing it following the formation of stable complexes. Some plants are also able to tolerate high concentration of aluminum by exuding chelating agents that complex it, preventing its entrance into the plant (323, 334, 335).

Only a few plants are able to tolerate high concentrations of aluminum. Studies have shown that aluminum is toxic to plants, with the roots being affected the most (334, 335). The toxic effect of aluminum to plants is believed to be due to interference in absorption of essential elements such as Ca\(^{2+}\), Mg\(^{2+}\), N, P, Na\(^{+}\), K\(^{+}\), etc, or replacement of these elements in important enzymes and hormones. Aluminum is believed to reduce the activity of Mg\(^{2+}\) dependent ATPase (334). It also alters membrane permeability by interacting with polar regions of the phospholipids (334,335).

3 Aluminum in Water

The concentration of aluminum in the lakes and rivers is variable. It depends on the types of soil and bed rock through which the river flows, and the acidity of the rainfall, in the region that the river flows. Sea water contains about 0.01 ppm of aluminum, while in
rivers and lakes it ranges from 0.003 to 9,000 ppm (336,337). The high values are found in very acidic lakes and rivers with no (or very little) buffering agent. The effect of the acid rain on the solution of aluminum has been studied extensively and has been shown to vary with time and pH as shown below:[286]

\[
\begin{align*}
\text{Al}_7(\text{OH})_{17}^{4+} + \text{H}_2\text{SO}_4 & \xrightleftharpoons{\text{acid rain}} 7\text{[AlSO}_4]^+ + \text{H}_2\text{O} \\
7\text{[AlSO}_4]^+ & \xrightleftharpoons{} 7\text{Al}^{3+} (\text{aq}) + 7\text{SO}_4^{2-}
\end{align*}
\]

a. Aluminum Aqueous Chemistry

In order to understand aluminum toxicity it is important to understand its chemistry in aqueous solution. Aluminum aqueous chemistry is very complex, and dynamic. It is influenced by many factors. These factors include the concentration, the length of time Al has been in contact with water, pH and other species present.

At pH < 5, aluminum exists as mononuclear octahedral hexahydrate, Al(OH)\textsubscript{6}\textsuperscript{3+}. This hexahydrate can disproportionate water in a series of reactions to give different species as shown below: [333]

\[
\begin{align*}
\text{Al(OH)}_2^{3+} + \text{H}_2\text{O} & \xrightleftharpoons{} [\text{Al(OH)}_5\text{OH}]^{2+} + \text{H}_2\text{O} \\
\text{Al}^{3+} + \text{H}_2\text{O} & \xrightleftharpoons{} (\text{AlOH})^{2+} + 2\text{H}^+ \\
(\text{AlOH})^{2+} + \text{H}_2\text{O} & \xrightleftharpoons{} \text{Al(OH)}_2^+ + \text{H}^+ \\
\text{Al(OH)}_2^+ + 2\text{H}_2\text{O} & \xrightleftharpoons{} \text{Al(OH)}_4^- + 2\text{H}^+
\end{align*}
\]
The $\text{Al(OH)}_4^-$ species forms in more basic solutions.

Aluminum forms stable soluble complexes with oxygen donor groups and reacts with fluoride, phosphates, citrates and sulfates to form stable soluble complexes.

Therefore, presence of inorganic ions and oxygenated organic compounds in water can alter aluminum chemistry and toxicity.

b Aluminum Toxicity to Aquatic Organisms

The toxicity of aluminum to aquatic organisms is dependent on pH, the species of the organism and the form of aluminum.

According to Smith and Hem (341), when a hydroxide is added to a solution of aluminum salt, the concentration of monomeric aluminum, $\text{Al}^{3+}$, $\text{Al(OH)}_2^+$, $\text{Al(OH}_2^+ \text{)}^+$ and $\text{Al(OH)}_4^-$ stabilize within 24 hours while polymeric aluminum hydroxide is slowly converted to larger units (340).

An organism in contact with freshly prepared solutions may be exposed to different toxic species from an organism exposed to aged solutions, where the concentration of monomeric species may be much lower and intermediate polymeric species may be absent (340). Monomeric aluminum species are believed to be more toxic than polymeric species.

Aluminum complexes with phosphates, sulfates and forms six different complexes with fluoride, but not with chloride. The stability of complexes is pH dependent, [173] and the toxicity of different complexes is different. The toxicity also depends on species of the organism.

Studies showed that 18 ppm of $\text{AlCl}_3$ killed shrimp (Penaeus setiferus) in 2 - 21 days at pH 5.5 - 6.8 while the same concentration at the same pH did not affect oysters.
(Oshrea, Virginica) for the same time duration. 18 ppm AlCl$_3$ killed speckled trout (cynoscion nebitosus) in 7 days at pH 5.5 - 6.7. (336). Injections of 13 mg Al/kg body weight to frogs did not affect these animals. 22 mg/l of aluminum immobilized daphnia in Lake Erie water within 20 hours. 36 ppm AlCl$_3$ killed shrimp within 2 hours at pH 4.7-4.8 (336).

Studies in Europe, Canada, and the United States have shown that areas affected by acid rain, especially where the pH is below 5, have lost most of fish species and the diversity of other species have declined (32, 336-345). These areas were shown to also have high concentration of aluminum.

Studies in our lab and elsewhere have shown that aluminum toxicity to fish is dependent on aluminum concentration, the pH and presence of other chemical species (350, 310-313).

The mode of aluminum toxicity to fish is not understood. Some believe that aluminum blocks fish gills suffocating the fish (351, 353), while others believe that aluminum interferes with absorption of essential elements such as magnesium, iron, and calcium (352), by competing for the same binding sites.

B. ALUMINUM TOXICITY TO MAN

1 Aluminum and Alzheimer's Disease

It is suspected that aluminum plays a role in the development of some form of Alzheimer's disease in humans (354-359). Alzheimer's disease is a progressive neurological disease which results in mental retardation and senility as a result of loss of cognitive functions, which involve memory, orientation, abstraction, and ability to learn new tasks (360). The disease results in neurofibrillary tangles. The disease was first described by Alois Alzheimer (1907). (361) The neurofibrillary tangle accumulate dense
fibrous strands within the penkaryl cytoplasm (352). Alzheimer's disease is also associated with senile plaques seen in certain regions of the brain specially the gray matter areas. (360) These plaques were shown to contain aluminum. (362)

Studies with animals showed degeneration of neurofibrillary following direct exposure of aluminum salts to the central nervous system. (363-364) X-ray diffraction and high resolution electron microscope studies have shown that aluminum silicate is present in the neurofibrillary tangles of Alzheimer's patients, and that these tangles consist of large numbers of pairs of filamentous proteins wound in helical fashions (356-365).

2 Symptoms of Aluminum Toxicity

People exposed to high concentration of aluminum develop skin lesions, gastrointestinal disturbances, impairment of locomotor behavior etc.

Chronic exposure to high concentrations of soluble monomeric or inorganic aluminum salts leads to fatal neurological syndrome - encephalopathy crippling bone condition - osteomalacia, pulmonary fibrosis, anemia, growth retardation and renal failure (355, 366-376).

3 Mode of Aluminum Toxicity

The way aluminum causes the body to malfunction is not well understood, but it is believed that aluminum interferes with absorption of magnesium phosphorus, calcium or iron in the intestinal mucosa (331). It may affect the membrane permeability by changing the chemical environment of the lipids as a result of binding directly to the polar regions of the phospholipids or indirectly binding to membrane proteins. (377-378, 333) It is believed to inhibit Mg$^{2+}$ dependent K$^+$ stimulated AtPase in plasma membrane. (377, 409) It forms stable Al-ATP complexes or inactivates the enzyme. (371-377, 334, 333, 380).
Aluminum ATP complex binds ten times more strongly to hexokinase than does the ATP-Mg complex. It is believed that aluminum replaces Mg$^{2+}$ in vital enzymes, where Al$^{3+}$ is not as easily exchanged with other cations, as Mg$^{2+}$ and therefore interferes with enzyme functions.(381).

It is also believed to interfere with the absorption of iron, and to replace it in its binding sites, especially where transferrin is involved (381). Aluminum also binds epinephrine and related catecholamines at all pH's. It binds ten times more strongly to the catecholamines moiety than Mg$^{2+}$ (333, 381), preventing the neurotransmitters from binding to receptor sites. Aluminum is found in the nuclei of neural cells and it is believed that aluminum binds to DNA (382), (383) but the mechanism by which Al$^{3+}$ binds to DNA is not known. However, it is suspected that aluminum binds to phosphoproteins resulting in interruption of protein phosphorylation - dephosphorylation reactions that provide regulatory mechanisms in the body cells (333, 384, 385).

4 Sources of Aluminum

Aluminum can get into the body through food and drinks. Tea leaves may accumulate as much as up to 30 mg/aluminum. Baking powders, antacids, anti-perspirant, buffered aspirin and deodorant also contain significant amounts of aluminum. Al$_2$(SO$_4$)$_3$ is used in tanning as a mordant. Aluminum salts are added to frozen strawberries, cherries processed cheeses and beer for improving appearance (333). All these substances can increase the amount of ingested aluminum. Most of aluminum salts are insoluble and are not easily absorbed through the intestine, but studies with humans showed that the presence of organic chelating agents such as citrates and diols (333, 386, 387) can increase absorption of aluminum.
124

C METHODS OF DETERMINATION OF ALUMINUM IN BIOLOGICAL MATERIAL

1 Introduction

It is difficult to measure trace amounts of aluminum, especially since there is a possibility of contamination due to enormous abundance of this element in the environment. The difficulty of determining aluminum was emphasized by Correlis and Schulyser. They looked at data published since 1974 on aluminum determinations in serum (or plasma) and urine of normal volunteers. They found wide range of variations. For example, they reported normal serum aluminum concentration ranged from 2 ug/l to 420 ug/l (388).

Improvement in sample collection and storage has reduced sample contamination. This improvement coupled with development of sensitive instrumentation capable of detection in nanogram levels, has increased the determination of aluminum in biological systems.

The methods commonly used for determination of aluminum include atomic absorption, atomic emission, neutron activation, X-ray fluorescence, colorimetry, polarography as well as physical and chemical methods.

a Determination of Aluminum Using Atomic Absorption and Atomic Emission

Total aluminum can be determined directly by use of either flame or graphite furnace atomic absorption. Flame techniques have been used to analyze aluminum in biological specimens such as brain, cerebrospinal fluids, (381) food, urine, faeces, (384, 389) and heart muscles (331, 390).
Flame methods use nitrous oxide-acetylene to increase sensitivity, but can only detect concentration of 0.1 mg/l and above (391, 392). The graphite furnace can be used to increase sensitivity, but this requires addition of matrix modifiers to reduce carboxide formation during atomization. (389) Use of pyrolytically coated graphite tubes minimizes reactions between carbon and the sample. The analytical sensitivity using graphite furnaces for aluminum is 2 ug/l.

Aluminum can be measured by emission spectrometry using either a nitrous oxide-acetylene flame or argon plasma, but these methods are not sensitive enough to detect the low levels of aluminum in serum (333). The detection problem can be eliminated by using inductively coupled plasma. Allain and Mauras (390) used ICP to determine aluminum in biological fluids and in water. The detection limit with this method is 0.4 ug/l, with a linear range form 0 - 200 ug/l. Alkali and alkaline earth metals interfere with aluminum analysis using ICP emission. Use of the standard addition technique, can eliminate interference.

**b Analysis of Aluminum Using Neutron Activation**

Analysis of aluminum in biological samples using neutron activation involve irradiating the sample with neutrons or charged particles, making the aluminum radioactive. \( ^{27}\text{Al} + n, \ 28\text{Al} \). \(^{28}\text{Al} \) has a half life of 2.24 minutes and can be quantitatively and qualitatively determined by its 1.779 MeV gamma ray emission. (363, 333).

Normally, the sample containing aluminum is irradiated for 5 minutes, at 3.1 x \( 10^{11} \) n/cm\(^2\) -s. The sample is then counted for 100 seconds beginning one minute after sample is removed from the reactor. The detection limit is 0.045 mg/L.
c  Colorimetric analysis

i  Ferroin Method

Aluminum in water can be determined colorimetrically by reacting with Ferron (8-hydroxy-7iodo-5-quinoline sulfonic acid) to produce a blue solution (393), which can be quantified colorimetrically. The detection limit using this method is 50 ug/l. The complex formed between aluminum and ferron has a maximum absorption at 370 nm. Fe$^{3+}$ interferes but this can be avoided by reducing the iron with hydroxyammonium chloride and complexing it with orthophenanthroline.

James and coworkers (394) modified this method to determine labile and total aluminum. Total aluminum was measured by digesting the sample with nitric acid. 8-hydroxy quinoline in an acetate buffer was added to the samples and shaken vigorously. The complex and unreacted dye was then extracted into butyl acetate and absorbance at 395 nm was measured. This gave the concentration of labile aluminum. The nonlabile aluminum complexed by fluorine, carbon, silicon and hydroxide in the sample was calculated by subtracting the concentration of labile aluminum from total aluminum for each sample.

ii  Eriochrome Cyanine Method

Solochrome cyanine R, a triphenylmethane also known as Eriochrome cyanine R, reacts with aluminum to produce a red aluminum complex. Aluminum has been measured by first adding ascorbic acid to the sample to eliminate interference from iron and manganese. The sample was buffered at pH 6.0 before 15 ml dye was added. The color of the aluminum dye complex fades within 15 minutes and therefore the absorbance was measured at 535 nm immediately after adding the dye (395, 352). The detection limit for this method is 20 to 300 ug/l.
Aluminon Method

Aluminum reacts with aluminon to give a deep red color (396). Aluminon is an ammonium salt of aurintricarboxylic acid, a triphenyl methane dye. The test is carried at pH 4 and is specific for monomeric aluminum ions. Polymeric aluminum species that can be easily converted to monomeric species can also be detected. The detection limit is approximately 0.02 mg/l.

Samples are boiled for fifteen minutes before the color intensity is read at 525 nm. Beer's Law is not followed over much of the concentration range and therefore a calibration curve must be prepared for each batch of aluminum to compare known and test samples. Fluoride, polyphosphate, and iron interfere with this method and must to removed or masked for accurate results can be obtained.
CHAPTER 2

EFFECT OF pH AND ALUMINUM ON FISH

A INTRODUCTION

Studies in our lab had shown that aluminum is toxic to golden shiners (Nontemiganus cryostoleucas). The study also showed that the aluminum toxicity was dependent on Al concentration and pH. At pH of 4.5, fish were shown to live for more than fifteen days in absence of aluminum, while in the presence of 10 ppm of aluminum, all fish died within 24 hours at the same pH. At pH of 5 and above, higher concentrations of aluminum were required to produce the same effect (350).

It is well known that the toxicity of a metal is influenced by the form of the metal, as well as the presence of other complex forming species. (333) Aluminum forms complex species depending on the pH of the solution, the age of the solution, and the presence of other substances. At pH below five, monomeric species of aluminum are predominant, i.e., Al(OH)²⁺, Al(OH)³⁺, Al(H₂O)⁶³⁺. At pH between 5 and 9 aluminum hydroxide and polymeric species predominate. However, aluminum is able to form polymeric species at very low pH upon standing for some time. These changes can affect the toxicity of aluminum.

1 Purpose of the Research

The purpose of this research was to study the effect of aluminum on tropical fish, to find the effect of different anions on aluminum toxicity, to find the effect of aging aluminum solution on fish, and to find the organ(s) that accumulate aluminum.
2 Reasons for Study

Most toxicity studies have used fish from temperate climates and not much research work has been done with tropical fish, especially those of commercial value. Most studies have used aluminum sulfate as the toxicant. Not much work has been done with other aluminum salts. It is known that a significant amount of nitric acid is present in acid rain (397). Aluminum nitrate and chloride form $\text{Al(H}_2\text{O)}_6^{3+}$ in aqueous media, while sulfate forms sulfato complexes i.e. $\text{Al(H}_2\text{O)}_5\text{SO}_4^+$ (333). Therefore, the presence of chloride and/or nitrate ions could alter the aluminum toxicity, since the hexa-aquo aluminum ion is believed to be the most toxic. Different salts have varying solubilities in water and this could also have an effect on aluminum toxicity. Therefore this study was also designed to find the effects of $\text{Cl}^-$, $\text{NO}_3^-$, and $\text{SO}_4^-$.

Tilapia Mosambica was used for this study. This fish was chosen for the study because it is a common fish in East Africa and it is widely used for food in areas around lake Victoria. This study was carried out in Nairobi as a result of a grant from Kenyatta University.

3 Experimental

Equipment, fish tank, fish food, oxygen meter, pH meter, thermometer.

4 Experimental procedure

Healthy four month old Tilapia fingerlings, matched both in size and age, were used for this study. They were obtained from the Fisheries Department in Kisumu, Sagana, and Nairobi.

Forty liter fish aquaria were used. They were placed in partially enclosed structure, open to the atmosphere, but away from direct sunlight. Tap water, aged by standing in
buckets for over four days was used to fill the aquaria. The fish were then acclimatized to the testing environment for one week before the study was started.

Initially, the fish were kept for two days without feeding. They were then fed twice daily using fish food provided by the fisheries department. Temperature, dissolved oxygen, and the pH of the tanks were monitored twice daily, using Cole-Palmer thermometer, Markson portable oxygen analyzer, and a portable Cole-Palmer pH meter. A third of the water in each tank was removed after every two days and an equal amount of aged water was added to each tank. This was done to prevent build up of toxic waste material from fish.

After one week of acclimatization, fifteen fish were transferred to each tank containing twenty eight liters of tap water at controlled pH. The pH in each tank was adjusted to the desired value by adding either 0.05M H\textsubscript{2}SO\textsubscript{4} or 0.05M NaOH. After the pH adjustment, the tanks were allowed to stand for one day for equilibration. The oxygen concentration, temperature, and pH were measured before fish were added in each aquaria. The fish were observed for forty eight hours before feeding was started. The oxygen, temperature, and pH were monitored closely to check any sudden or large deviation that could affect the results. The pH was maintained as constant as possible by adding either 0.05M NaOH or 0.05M H\textsubscript{2}SO\textsubscript{4}. A control aquaria contained fifteen fish in twenty eight liters of aged tap water without any pH adjustment.

In order to find the effect of aluminum on Tilapia at varying pH, Several fish tanks containing varying concentrations of aluminum as aluminum sulfate, were set up. The pH was adjusted to the desired value in each tank before the aquaria were allowed to stand for one day. Equal numbers of fish were added to each tank, and the fish were observed for two days before feeding was started. The control tank contained only fish, without any pH adjustment or aluminum. Other variables were monitored as before. Every two days, one
third of water in each tank was removed and replaced with a fresh solution containing the necessary adjustments. All solutions were made from aged tap water.

5 Result and Discussion

The results are shown in tables 14-14a. They show that the fish can survive for a long time at pH below 5 without any observable deleterious effect in the absence of aluminum. The fish kept below pH 5, were less active and showed less avoidance to being caught with a fish net. Aluminum sulfate solution produced a cloudy precipitate within a few minutes of dissolving, at pH of 5. The results showed that Tilapia fingerlings can withstand relatively high concentration of aluminum sulfate at pH of 5 and above. However, as the pH decreased below 5, the fish became increasingly more sensitive to the presence of even low concentrations of aluminum. 10 ppm aluminum killed all the fish in less than 24 hours at pH of 4.5. At pH 5, fish were shown to survive for more than 10 days in presence of 50 ppm aluminum as sulfate.

6 Conclusion

The results show that the tropical fish are affected by aluminum specially at pH below 5. The results show the synergistic effect of aluminum and acidity.

B. EFFECT OF ANIONS ON ALUMINUM TOXICITY TO SHINERS AND TILAPIA MOZAMBICA

1 Introduction

The toxicity of a metal is dependent not only on the concentration, but also on the species of the metal. For example, inorganic mercury is less toxic than organic mercury. Cr$^{3+}$ is necessary for glucose metabolism while Cr$^{6+}$ is carcinogenic. Aluminum forms both monomeric and polymeric species in water. The rate of formation of these
### TABLE 14

**EFFECT OF pH ALONE ON FOUR MONTH OLD TILAPIA FINGERLINGS**

<table>
<thead>
<tr>
<th>pH</th>
<th>Length of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.9  (control)</td>
<td>more than 15 days</td>
</tr>
<tr>
<td>5</td>
<td>more than 15 days</td>
</tr>
<tr>
<td>4.5</td>
<td>more than 15 days</td>
</tr>
<tr>
<td>4</td>
<td>more than 10 days</td>
</tr>
<tr>
<td>3.2</td>
<td>all fish dead in 2 hours</td>
</tr>
</tbody>
</table>

### TABLE 14 a

**EFFECT OF pH AND ALUMINUM ON FOUR MONTH OLD TILAPIA FINGERLINGS**

<table>
<thead>
<tr>
<th>pH</th>
<th>Al (ppm)</th>
<th>Length of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>20</td>
<td>greater than 10 days</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>greater than 10 days</td>
</tr>
<tr>
<td>4.5</td>
<td>20</td>
<td>all dead in 3 days</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>Half dead in 4 hours. All dead in 20 hours</td>
</tr>
</tbody>
</table>

**Aluminum salt**

- $\text{Al}_2(\text{SO}_4)_3 \cdot 16\text{H}_2\text{O}$
- $\text{Al}_2(\text{SO}_4)_3 \cdot 16\text{H}_2\text{O}$

**NOTE:** The actual length of survival of the fish was not determined because of time restrictions. However, it was greater than the number of days indicated.
compounds is influenced by presence of other anions and cations. Also, different aluminum salts have different solubilities in water and these differences could affect the toxicity of aluminum to aquatic organisms. Therefore, this study was initiated to find the effect of Cl\textsuperscript{−}, NO\textsubscript{3}\textsuperscript{−} and SO\textsubscript{4}\textsuperscript{2−} on aluminum toxicity to fish under varying acidic conditions.

2 Experimental Procedure

Healthy four month old Tilapia fingerlings were used for this study. These fish were acclimatized to the laboratory conditions for a week before the experiment was started. Temperature, pH, and oxygen concentration were monitored as previously described. Several fish tanks containing different aluminum salts at varying concentrations were set up. The solutions were adjusted to the desired pH and then allowed to stand for about a day to equilibrate.

The pH was readjusted as previously described. Equal numbers of fish were added to each tank and the fish were observed for two days before feeding was started. pH and Al were not adjusted in the control tank which contained only fish in aged tap water. Other variables were monitored as before.

The aquaria used for the shiners were equipped with air pumps and filtration systems. Oxygen concentration, pH and temperature were monitored and maintained at appropriate levels. Figure (25) show the results.

3 Results and Discussions

The results show that chloride and nitrate ions increased the aluminum toxicity at pH 5, with the chloride ion being more toxic than the nitrate. The sulfate was shown to less toxic than both chloride and nitrate at pH 5. At pH below 5, all the anions produced the same toxic effect on fish in presence of aluminum ions but the rate of death was higher in presence of chloride and nitrate ions than the sulfate. The results also showed that the
Fig. 25 Effect of Anions and Aging on Aluminum toxicity To Fish
shinners were more sensitive to aluminum ions than Tilapia at pH 5. The fact that at pH 4.5 the different anions are equally toxic indicates that the anions themselves may not be toxic but the increased toxicity may be due to the fact that the aluminum chloride and nitrate are more soluble at pH 5 than aluminum sulfate. The increased aluminum ions as results of higher solubility may have increased the toxicity.

4 Conclusion

The result show that Nitrate anions and chloride anions in the water can alter aluminum toxicity due to differences in solubilities or complex formation.

C EFFECT OF AGING OF ALUMINUM SOLUTION ON ALUMINUM TOXICITY TO FISH

1. Introduction

Studies showed that even at low pH, aluminum is capable of forming complex species upon standing (399), so that the monomeric species concentration may decrease with time. Thus, the aging of the solution could alter the concentration of the toxic species giving varying results. Fish in their natural environment are exposed to freshly formed aluminum ions as well as aged aluminum solution. It is therefore necessary to understand factors that could influence toxic effects.

2 The Purpose of This Study

The aim of this study is to find out the effect of varying the length of time aluminum solutions stands before fish are added.
3 Experimental Procedure

Several aquaria containing 28 liters of 20ppm aluminum chloride were set up. The pH was adjusted to 5 and the solution was allowed to stand for 4 hours. The pH was then readjusted before the 20 fish were added. The temperature, oxygen concentration, and pH were maintained as constant as was possible. The experiment was repeated but the aluminum solution was allowed to stand for differing amount of time.

4 Results

The result show that the freshly prepared aluminum solution is more toxic to fish than solution allowed to stand for several days. Aging of the aluminum solution reduces the mortality rate of the shiners, especially when sulfate and nitrate were used.

5 Discussion of the Results

The result indicate that a surge of highly acidic rain especially after a long dry spell could result in increased fish kills due to freshly released aluminum ions. This could also explain increased fish kill during the melting of highly acidic snow.

6 Conclusion

Age of aluminum solution does aluminum toxicity by altering the concentration of the toxic
D EFFECT OF LEAF EXTRACT ON ALUMINUM TOXICITY TO FISH

1. Introduction

The purpose of this study was to find out if the leaves can be used to reduce the aluminum toxicity, (1) when the pH is below 5, and (2) when the leaves are added to water at low pH but no further attempt was made to maintain this low pH. Attempts were made to find out if adding leaves themselves mitigated the toxic effect of aluminum.

2 Experimental

Equipment 10 gallon aquaria, oxygen analyzer, pH meter,
thermometer

Reagent; aluminum chloride, aluminum sulphate, sodium
hydroxide sulphuric acid, fish food live shiners (Notropis)

3 Experimental Procedure

The shiners were obtained locally. The fish were acclimatized in the laboratory conditions for two weeks before the studies were started. During the acclimatization period the fish were fed tropical fish food. The temperature pH and oxygen concentration were kept as constant as possible.

a Control Tanks.

Several 10 gallon tanks were set up. There were 3 set of control tanks. One set of control tank contained 20 fish in aged water without pH adjustment. The second controls contained two sets of fish (20 each) in 7 gallons of water at pH 4.5 and 5 respectively. The
third group contained two sets of 20 fish in 7 gallons of water that had 4 liters of leaf extract at pH 4.5 and at pH 5 respectively.

The experimental group contained three sets of two tanks. In the first set, 4 gallons of leaf extract (pecan) were added to each tank to make a total of 7 gallons of aged water containing 20 ppm of aluminum. The pH of the water was adjusted to 4.5 and 5, respectively. 0.05 M sulphuric acid or 0.05 M sodium hydroxide were used to adjust the pH. The solutions were allowed to equilibrate for 24 hours before 20 fish (notropis) obtained locally were added. The experiment was repeated with all the leaves used for this study (pine, oak, sweetgum willow, and cypress).

In other experiments, several sets of tanks were used. 7 gallons of 20 ppm aluminum solution was added to each tank. The water was adjusted to pH 4.5 and pH 5.0, respectively. 10 grams of dried leaf extract were added and the solution was allowed to stand for 24 hours before fish were added to each tank. There was no further attempt to readjust the pH. The fish were observed for 48 hours. During this period, the fish were not fed any food. Temperature, pH and oxygen concentration were maintained as constant as possible. The surviving fish after 48 hours were fed fish food twice daily. Another set of tanks were set up. 27 grams of dried pecan leaves were added to 7 gallons of 20 ppm aluminum solution at pH 4.5. The solution was allowed to stand for 24 hours before 20 fish were added to each tank. The experiment was repeated with 27 grams of a mixture of dried leaves (pecan, willow, alligator pine, and oak) and also with pine alone.

4 Results and Discussion

When the pH was maintained at 4.5, the presence of leaf extract did not mitigate the toxic effect of aluminum. At this pH, 20 ppm of aluminum killed all the fish within 24 hours. In the tanks containing leaf extracts and aluminum at pH 4.5, more than half the fish were dead in 4 hours, while more than half were dead in 12 hours in aluminum alone.
None of the fish in the control tanks died in 48 hours. In the control tank containing leaf extract at pH 4.5, there was increased mortality compared to the other control. About 60 mls of 0.1M sulphuric acid had to be added in this tank in order to maintain the pH at 4.5. The actual amount of acid added varied depending on the type of leaf extract. Also, there was much frothing indicating that the extracts were interacting with the acid added.

When the leaf extracts were added to 20 ppm aluminum at pH 4.5, but no further pH adjustment was done, all the fish were alive after 48 hours. The fish in this tank survived for more than 2 weeks. The pH of the water in this tank had gone from 4.5 to pH 6.2 and above depending on the types of leaves. Pine leaves soaked for three weeks did not increase the pH or improve the survival of the fish. However, when the pine leaves were soaked for more than 2 years, the extract increased the pH above 5, and there was a reduction in the fish mortality.

At pH 5.0, the presence of leaf extract reduced the fish mortality with and without pH adjustment. The reduction depended on the type of leaves. In the presence of alligator leaf extract, more than 90% of the fish were alive after 48 hours. The reduction in death rate was found to follow the order; alligator leaves > cypress > duckweed > willow > sweetgum > oak > pine.

The results showed that the dried leaves that increased the pH above 5.5 also reduced the fish mortality. Pine leaves increased the mortality of fish in the presence of aluminum. Results are shown in fig 26-27.

5 Conclusion

The results show that decayed leaves extract can be used to mitigate the toxic effect of aluminum at low pH by increasing the buffering capacity of the water and also complexing the aluminum. Leaves that maintain the water above pH 5 can also reduce the
FIG. 26  EFFECT OF LEAF EXTRACTS ON ALUMINUM TOXICITY TO FISH

AT pH5  3 Weeks soaking  20 ppm Al
FIG 27 EFFECT OF LEAF EXTRACT ON ALUMINUM TOXICITY TO FISH.
toxicity. The order of reducing the toxicity follows that of the increment in buffering capacity. Pine leaves caused the least reduction in fish mortality, while alligator leaves had the highest reduction of mortality.

E. UPTAKE AND ACCUMULATION OF ALUMINUM BY FISH

1. Introduction

Studies in Dr. Robinson's lab have shown that aluminum is toxic to fish (350). The mode of aluminum toxicity to fish is not well understood. Some believe that aluminum blocks the gills suffocating the fish (353). Others believe that aluminum interferes with absorption of important minerals such as K⁺, Ca²⁺ and Mg²⁺, or replaces these metals in the fish, affecting vital processes (381).

2. Purpose of Study

This study was designed to find out if aluminum is taken up by the body and the organ(s) that accumulate it.

3. Experimental

Equipment: Fish tanks, fish food, oxygen meter, pH meter, graphite AA, aluminum salts.

4. Experimental Conditions

Three 10 gallon aquaria, equipped with an undergravel filtration system were filled with about 7 gallons of aged tap water. The water in two of the tanks was adjusted to pH 4.5. One tank contained 5ppm aluminum as aluminum sulphate and the second tank contained only water at pH 4.5. The third tank contained water at ordinary pH of 8.0. About 15 shiners were added to each tank and the fish were observed for 96 hours.
Temperature, oxygen content, and pH were maintained as constant as possible during the testing. As soon as any fish died, they were rinsed thoroughly with deionized water to remove any aluminum adhering to the fish. The fish were allowed to air dry before weighing. The fish from each tank were then placed in acid cleaned beakers. The fish were acid digested using 70% analytical grade nitric acid. The resulting solution was made to the mark of 10ml volumetric flask using deionized water.

Aluminum concentrations were determined by injecting 10ul samples into a graphite furnace AA. The optimum instrument conditions were found to be:

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry</td>
<td>150°C</td>
</tr>
<tr>
<td>Char</td>
<td>1500°C</td>
</tr>
<tr>
<td>Atomize</td>
<td>2600°C</td>
</tr>
</tbody>
</table>

Standard concentrations were injected under the same conditions a calibration curve obtained, and used to determine the concentration of the aluminum in the whole fish. Gills were removed from some of the fish and rinsed with deionized water, weighted and then acid digested as above. Aluminum concentration in the whole fish and the gills was determined.

5 Results and Discussion

The concentration of aluminum in the fish organs is shown in table 15. The results show that there is very little aluminum in the whole body and the gill of unexposed fish to aluminum. Acute exposure of fish to aluminum solution leads to preferential accumulation of the aluminum in the gills. This indicates that aluminum kills the fish by causing suffocation.
<table>
<thead>
<tr>
<th>Organ</th>
<th>Average Conc.(mg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unexposed whole fish</td>
<td>0.187± 0.062</td>
</tr>
<tr>
<td>unexposed fish gills</td>
<td>0.083 ±0.018</td>
</tr>
<tr>
<td>exposed whole fish</td>
<td>0.78 ±0.028</td>
</tr>
<tr>
<td>exposed fish gills</td>
<td>11.66 ±0.24</td>
</tr>
</tbody>
</table>

**TABLE 15**

CONCENTRATION OF ALUMINUM IN EXPOSED AND UNEXPOSED FISH
Conclusion

From this study we can conclude that the aluminum appears to kill the fish by blocking the gills, preventing normal respiration.

EFFECT OF ALUMINUM AND LEAF EXTRACT ON FISH GILLS

Introduction

Our study showed that acute exposure of fish to aluminum at pH 4.5 resulted in fish death within a few hours. The study also showed that aluminum was taken up by the fish and most of it accumulated in the gills. Very little aluminum was found in the rest of the body. Our study showed that leaf extract interacted chemically with aluminum.

The purpose of this study was to find out if there were any changes in the gills after acute exposure to aluminum, and whether the presence of leaf extract altered the effect of aluminum on the gills.

Experimental

Equipment; 10 gallon fish aquaria. Aged tap water, air pump and filtration system.

Reagent; Aluminum sulphate, pH meter, oxygen analyzer, live fish, fixation solution, fish food.

Experimental Procedure

Fish were obtained locally. They were acclimatized to the laboratory conditions for two weeks before the experiment was started. During the acclimatization period, the fish were fed twice daily using tropical fish food.
Several 10 gallon aquaria were set up. Two tanks contained 7 gallons of 10 ppm aluminum sulphate at pH 4.5. Another contained 7 gallons of 10ppm aluminum solution and 10g of dried leaf extract. The extract was obtained by soaking leaves in deionized water for three weeks, filtering the resulting solution and then evaporating the water under nitrogen. The resulting extract was placed in a desiccator to dry. The leaf extract was added to the aluminum solution and acid was added until pH of 4.5 was reached. The next tank contained 7 gallons of 10ppm aluminum at pH 4.5. 10g of leaf extract was added to this solution but no further pH adjustment were done. The control tanks contained 7 gallons of aged tap water at pH 4.5 and the other contained 7 gallons at the aged normal tap water pH of 8.0. The tanks were allowed to equilibrate for one day before 20 shiners (Notropis) were added to each tank. Temperature, oxygen concentration, were maintained at constant levels during the testing. The fish were observed for 48 hours. During this period, fish were not fed.

When the fish showed signs of severe distress indicating imminent death, they were euthanized and immediately placed in preserving solution to prevent deterioration. The preserving solution was provided to us by the pathology department of the veterinary school of medicine at LSU.

The fish body and gills were observed under a microscope with the help of a veterinary pathologist.

c Results

The results showed that fish exposed to 10.0ppm aluminum alone and aluminum plus leaf extract at pH 4.5 died within a few hours. In the presence of leaf extract a considerable amount acid had to be added to maintain a pH of 4.5.
Acute exposure of fish to pH 4.5 in the absence of aluminum did not affect the fish gills at all. The gills looked normal under a microscope. Fish exposed to 10 ppm of aluminum plus leaf extract at pH 4.5 showed severe congestion of the gills, with much mucous and swelling of the lamelle.

Fish exposed to 10 ppm aluminum alone at pH 4.5 showed moderate to severe congestion, mild blunting of the gills, moderate proliferation of epithelial in intercellular spaces and also mild lamelle edema.

When leaf extract alone was added to the water but the pH was maintained at pH 4.5, there was a slight proliferation of the epithelial in the intercellular spaces.

When the leaf extract was added to 10 ppm aluminum solution at pH 4.5 but no further pH adjustment was done, the fish did not die. When these fish were euthanized and then observed under a microscope, they were found to be normal. The gills were normal. The water in the tank containing aluminum plus leaf extract without any further pH adjustment had showed a pH increment from 4.5 to 6.2. The pictures of gills exposed to aluminum and those not exposed are shown in figs 28-33.

**Discussion**

The result show that aluminum destroys the gill membrane and the gill structure of the fish. Presence of leaf extract at pH 4.5 does not mitigate the toxic effect of aluminum. However, the results show that the leaf extract can mitigate the toxic effect of aluminum by increasing the pH of water to levels where aluminum is not toxic.
FIG. 28 MICROSCOPIC PICTURE OF FISH GILLS EXPOSED TO 10 ppm ALUMINUM AT pH 4.5 AND LEAF EXTRACT.
FIG. 29  MICROSCOPIC PICTURE OF FISH GILLS EXPOSED TO 10 ppm ALUMINUM AT pH 4.5.
FIG. 39 MICROSCOPIC PICTURE OF FISH GILLS EXPOSED TO NORMAL WATER pH (8).
FIG. 31 MICROSCOPIC PICTURE OF FISH GILLS EXPOSED TO LEAF WATER ALONE AT pH 4.5
FIG 32 MICROSCOPIC PICTURE OF FISH GILLS EXPOSED TO ALUMINUM AND LEAF EXTRACT AT pH 5.0

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FIG. 33 MICRISCOPIC PICTURE OF FISH GILLS EXPOSED TO 10ppm ALUMINUM AT pH 4.5 AND LEAF EXTRACT WITHOUT pH READJUSTMENT.
e Conclusion

The results show that presence of aluminum at low pH results in destruction of the gills in the fish. Results also show exposure of fish to pH 4.5 alone does not harm the fish.

G EFFECT OF CATIONS ON ALUMINUM TOXICITY TO FISH

1. INTRODUCTION

Toxicity of a metal can be influenced by many factors. These factors may include temperature, pH, concentration, the species of the metal, and presence of other metal ions. The presence of other chemicals could have a synergistic toxic effect, or could reduce the toxicity of the metal in question.

Studies have shown that bodies of water with low buffering capacity are affected more by acid rain. Liming has been used to increase the pH had the buffering capacity of lakes. Buffering capacity is the ability to maintain a constant pH on addition of acid or base.

Although liming does prevent acidification, it has not completely eliminated the effect of acid rain (445). Trees continue to die in areas affected by acid rain despite liming (446). Continuous liming is necessary to maintain high pH. The long term effect of adding Ca$^{2+}$ is not well known. Liming is sometimes cumbersome in areas with high elevation.

Not much work has been done to find the effect of increased Ca$^{2+}$ concentration to the aquatic organisms especially when the pH is low. It is known that high calcium concentration can interfere with the absorption of essential metals such as zinc, iron, and magnesium. Calcium and magnesium are believed to follow the same absorption pathways.
Increasing calcium without the subsequent addition of magnesium could result in magnesium deficiency.

It is believed that a side effect of acid rain is the release of toxic metals from the soils. These toxic metals are believed to interfere with absorption of essential metals such as potassium, calcium, magnesium and iron (333).

Our studies have shown that aluminum is toxic to fish at pH 5 and below. It is believed that aluminum toxicity is due to its interference with the absorption of calcium, magnesium, and iron (333). It has been shown that aluminum has a high affinity for phosphate group (333) and may complex with the phosphate group of the membrane phospholipids. This may change membrane permeability.

It was felt necessary to find the effect of metals on aluminum toxicity. The purpose of this study therefore was to find out if the addition of other cations could mitigate the toxic effect of aluminum to fish.

2 Experimental Conditions

Reagent; 10 gallon aquaria, aluminum sulphate, ferric chloride, sodium hydrogen phosphate, calcium nitrate magnesium chloride, aged tap water, pH meters, oxygen meter, shiners (Notropis)

3 Experimental procedure

Several 10 gallon aquaria were set up as shown below in tables 16-17.

The experiment was repeated at pH 5. 20 fish were added to each tank. Temperature, pH, and oxygen concentration were maintained as constant as possible. The
### TABLE 16

**CONCENTRATION OF METALS IN THE TANKS (PPM) pH 4.5**

<table>
<thead>
<tr>
<th>Tank</th>
<th>Al</th>
<th>Fe</th>
<th>Mg</th>
<th>Ca</th>
<th>Na</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 17

**SURVIVAL OF FISH EXPOSED TO CATIONS AT pH 4.5**

<table>
<thead>
<tr>
<th>METAL CONCENTRATION (PPM)</th>
<th>SURVIVAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al 10 Mg Ca Fe Na P</td>
<td>more than 2 weeks</td>
</tr>
<tr>
<td>10 Mg 10 Ca Fe Na P</td>
<td>more than 2 weeks</td>
</tr>
<tr>
<td>20 Mg 20 Ca Fe Na P</td>
<td>all fish dead in 24 hours</td>
</tr>
<tr>
<td>20 Mg 20 Ca Fe Na P</td>
<td>all fish dead in 24 hours</td>
</tr>
<tr>
<td>10 Mg 10 Ca Fe Na P</td>
<td>all dead in 24 hours</td>
</tr>
<tr>
<td>10 Mg 10 Ca Fe Na P</td>
<td>more than two weeks</td>
</tr>
<tr>
<td>10 Mg 10 Ca Fe Na P</td>
<td>more than 2 weeks</td>
</tr>
</tbody>
</table>

Table 17-18 show effect of iron, magnesium calcium, and phosphorus on aluminum toxicity at pH 4.5.
fish were observed for 48 hours without feeding. After 48 hours, the fish were fed twice daily using tropical fish food. pH was adjusted twice daily or as necessary.

4 Results

The fish in the tanks containing aluminum alone, aluminum and calcium, aluminum + magnesium died within 24 hours at pH 4.5. At pH 5 the fish survived a little longer but there was no significant difference between the fish in the aluminum alone tank and those in presence of calcium or magnesium. A mixture of ferric chloride, and sodium hydrogen phosphate reduced the aluminum toxicity even when the pH was maintained at 4.5. Ferric chloride at equal concentration with aluminum also reduced the toxicity. Also, sodium hydrogen phosphate reduced aluminum toxicity.xic effect of aluminum to fish even at pH below 5. Magnesium and calcium did not mitigate the toxic effect of aluminum to fish although magnesium was shown to reduce aluminum toxicity to fish.

5 Discussion of Results

The results show that the presence of iron alone or in combination with sodium hydrogen phosphate mitigates the toxic effect of aluminum, while calcium or magnesium have no effect. It is possible that aluminum toxicity to fish may be due to its interference with the metabolism of iron and probably phosphate.

Our previous studies showed that aluminum destroys the membrane of the fish gills. Phospholipids play a major role in the structural integrity of the membrane. It is possible that the aluminum binds to the phosphate group of the membrane phospholipids, destroying its structure. Addition of phosphate ions could replace the membrane phosphate removed by the aluminum. It is also possible that iron may be more favorable. Addition of iron may prevent aluminum from binding at the membrane.
6 Conclusion

The presence of iron or phosphate could reduce the toxic effect of aluminum to fish even at pH below 5. Magnesium and calcium did not mitigate the toxic effect of aluminum to fish although magnesium was shown to reduce aluminum toxicity to fish.
CHAPTER 3

EFFECT OF pH AND ALUMINUM ON ROOT DEVELOPMENT IN
ALLIGATOR GRASS (Alternathera Philaxeroides)

A INTRODUCTION

There has been a decline in trees and forests in areas affected by acid rain (300-303). The cause of this decline is not well known but it is believed that acid rain may be playing a vital role (287, 288). The effect of acid rain depends on the pH and the species of the plant. Some plant can survive in acidic soils, while others die when placed in acidic soils. It is believed that acid rain affects the roots by either exchanging the H+ ions for important ions such as potassium, sodium, magnesium, and calcium (333), or it may release toxic metals such as aluminum from the soils and clay into the form which may destroy the roots (33, 400).

Aluminum is believed to interfere with root development by competing with essential elements such as magnesium, phosphorus, iron, and calcium (400-403). The toxic effect of aluminum is believed to depend on the pH, and the species of the plants. Some plants such as tea, are able to accumulate aluminum without any observable effects (333).

1 Purpose of Study

The main aim of this research was to study the effect of pH alone and pH with aluminum on roots development in alligator grass. Alligator grass was chosen for this study because it is an ubiquitous plant that grows in variety of conditions. It was also found to adapt very well to laboratory conditions. It was hoped that this study would
help us understand the actual role of aluminum and acidity in plant destruction, and it would help us find a way to reduce the effect of acid rain on plants.

2 Experimental

Equipment; plastic jars, pH meter.

Reagent; buffer solutions (4, 7, 10,) aluminum salts, (chloride, nitrate, and sulphate), plant food, deionized water, and tap water.

3 Experimental Procedure

Freshly cut alligator grass stems were placed in plastic containers containing plant media. The medium was prepared in the lab by adding salts of calcium, magnesium, sodium, potassium, iron and zinc to deionized water so that the concentration of each metal was about 5 ppm. The salts were added as either chloride, sulphate, nitrate or phosphate so that these non-metal elements were present in the medium. The containers with the plants were placed in a moisture and temperature controlled room for two weeks to allow acclimatization in the laboratory conditions.

After two weeks, healthy plants were selected for the study. Several containers were set up. The containers contained deionized water at varying pH. The control contained deionized water at normal pH of 5.8. The second set of experiment had several containers containing deionized water with varying concentration of aluminum at different pHs. The experiment was repeated using tap water instead of deionized water. A freshly cut plant stem was placed in each container. The plants were then placed in moisture and temperature controlled room for two week. During the observation period, the pH of the water was maintained as constant as possible by adding either 0.005 M. sulphuric acid or 0.005 M sodium hydroxide.
4 Results

The results obtained are shown in table 18. The results showed that plants placed in deionized water only, developed roots in three days even at pH as low as 4. However, at pH below 4, the plants did not develop roots. They started to wither within a few days. At pH below 5, presence of aluminum prevented the development of the roots. Presence of aluminum at pH between 5 and 8 did not have any effect on root development. The plants developed healthy roots even in presence of 10ppm aluminum at pH above 5. However, 1ppm aluminum prevented roots development at pH 4.5. Tap water and deionized water gave the same results at pH below 5 except that at concentration of 1ppm aluminum at pH 4.5 small roots developed but they looked unhealthy.

5 Conclusion

The results show that there is a synergistic toxic effect of aluminum and acidity. pH alone does not seem to have any observable toxic effect on alligator grass, except at very low pH.

B EFFECT OF ALUMINUM ON CHLOROPHYLL

1 Introduction

Chlorophyll is a substance that gives plants their green color. There are two major chlorophylls found in plants, i.e., chlorophyll a and b, with a ratio of 3:1 (344) Chlorophyll is involved in photosynthesis. It has magnesium at its center.
Table 18

EFFECT OF pH AND ALUMINUM ON ROOTS OF ALLIGATOR GRASS

<table>
<thead>
<tr>
<th>All (ppm)</th>
<th>pH</th>
<th>Effect on the plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.9</td>
<td>roots in three days</td>
</tr>
<tr>
<td>1</td>
<td>4.2</td>
<td>no roots</td>
</tr>
<tr>
<td>2</td>
<td>7.9</td>
<td>roots in three days</td>
</tr>
<tr>
<td>2</td>
<td>4.2</td>
<td>no roots</td>
</tr>
<tr>
<td>3</td>
<td>8.2</td>
<td>roots in three days</td>
</tr>
<tr>
<td>3</td>
<td>4.1</td>
<td>no roots</td>
</tr>
<tr>
<td>5</td>
<td>8.3</td>
<td>roots in three days</td>
</tr>
<tr>
<td>5</td>
<td>4.1</td>
<td>no roots</td>
</tr>
<tr>
<td>6</td>
<td>5.3</td>
<td>roots in three days</td>
</tr>
<tr>
<td>6</td>
<td>4.1</td>
<td>no roots</td>
</tr>
<tr>
<td>8</td>
<td>4.1</td>
<td>no roots</td>
</tr>
<tr>
<td>8</td>
<td>8.3</td>
<td>roots in three days</td>
</tr>
<tr>
<td>10</td>
<td>4.1</td>
<td>no roots</td>
</tr>
<tr>
<td>10</td>
<td>5.1</td>
<td>roots in three days</td>
</tr>
</tbody>
</table>
Magnesium plays a crucial role in trapping energy from the sun (344). Chlorophyll loses its function as energy trapper in the absence of magnesium, the central metal of the molecule.

When the chlorophyll is destroyed, the plant loses its green color and the color of the other pigments, such as xanthophylls, become more pronounced (344). When chlorophyll is degraded, especially when chlorophyllase is activated, the phytol group is lost. Production of hydrogen peroxide during decay, accelerates the degradation of the chlorophyll to a number of products.

In our previous studies it was shown that aluminum accumulates in the leaves of alligator grass (alternanthera phloxeroides). It was also noticed that the plants exposed to aluminum at pH below 5, turned yellow. Therefore, the purpose of this study was to find out if aluminum plays any role in the destruction of chlorophyll.

2 Experimental

a Equipment

1. Solvent delivery system; Perkin Elmer HPLC pump model series 2 equipped with two sinusoidal dual piston pump. Gradient elution with high pressure mixing was used.

2. Sample injection Rheodyne 7125 injector with 100 ml sample loop. A 100ml Hamilton syringe was used.

3. UV/VIS detector: Perkin Elmer variable wavelength (190-800nm) detector model L-C 75 with 8 ml flow cell.

4. Absorption spectrometer: Beckman D. B. spectrometer
5. Frame AA detector. Perkin Elmer atomic absorption spectrometer model 370A with thermospray nebulizer was used. Deuterium background corrector was used to eliminate background interference.

b Reagents

Analytical grade acetone, diethyl ether and distilled water was used.

Filter fiberglass filter, 0.45um teflon membrane filter.

3 Exposure of the Plants to the Aluminum

a Procedure

The plants were obtained from lakes around LSU. The stems were freshly cut and placed in a plant food medium. They were then placed in moisture and temperature controlled room for about two weeks to acclimatize them in the laboratory conditions. Healthy plants with luxurious healthy green leaves were selected for the study.

Several Nalgene containers with 100 mls of 5 ppm aluminum at pH 4.5 were set up. About 5 drops of plant food mixture were added to each container. The control contained only 100 mls of deionized water and 5 drops of the plant food at pH 4.5. Freshly cut alligator grass stems were placed in each container. The containers were placed in the moisture controlled experimental room for about a week.

4 Extraction of Chlorophyll from the Plants

The leaves were obtained from each plant, and were thoroughly rinsed with deionized water. The leaves were then dried between paper towels. About 1g of leaves from each container were ground in acetone using mortar and pestle. The resulting solution was filtered over fibreglass filter paper under suction. A little more acetone was
added to the leaves and the extraction process continued until all the green color had been extracted.

The filtrate was placed in a separating funnel containing about 50 mls of ether. The separating funnel was shaken vigorously. Deionized water was added to the funnel and the shaking was continued for about a minute. The different phases were allowed to separate before the aqueous layer was drained. The ether layer was washed several times with deionized water to remove polar compounds, such as humic substances. A pinch of sodium chloride was added to improve the separation when the aqueous layer was not clear.

Water was removed from the ether layer by adding anhydrous sodium sulfate. Ether was evaporated by blowing nitrogen into the container. The extract was redissolved in acetone, covered with aluminum foil to prevent light and air oxidation and was either analyzed immediately or was stored in the freezer until analyzed. HPLC, equipped with a thermospray flame atomic spectrometer as a metal specific detector, was used to separate and identify the chlorophylls.

Thermospray was introduced in our lab as an interphasing device for HPLC-flame AA by a previous researcher, to improve sensitivity. Interfacing HPLC with flame AA resulted in poor sensitivity and broadening of peaks. This was due to unmatched flow rates of the HPLC with that of the nebulized uptake (244). Various types of nebulizers were designed to improve the sensitivity of the HPLC-AA system. Although there was some improvement in sensitivity, it was not good enough for trace metal analysis. The major problem was due to the drop size and the fact that drying, ashing and atomization was done in the frame. To correct the problem, the eluent from the LC was passed through an electrically heated capillary tube (thermospray), which vaporized the sample into a fine mist which entered the base of the flame as dry sample. The sensitivity of the
flame was increased ten times with this devise (244). the HPLC was also equipped with a variable wavelength UV/VIS detector which was used to identify other compounds. Chromatographic condition used for separation of chlorophylls were those developed in our lab.(244).

C CHROMATOGRAPHIC CONDITIONS FOR ANALYSIS OF CHLOROPHYLLS EXTRACTED FROM ALLIGATOR GRASS.

1. COLUMN; Zorbax Rx SP/C-8 (4.1 mm x 15cm)

2. SOLVENT; A 50% methanol in water, B 5% Isopropyl alcohol methanol. Gradient 80% B to 1005 in 10 minutes linearly.

3. Solvent Flow Rate 1.5 mls/minute


5. UV/VIS detector wavelength 415nm

6. Flame AAS Mg at 285.2nm with slit width 0.7mm

1. Experimental Procedure

The chlorophyll extracts were filtered through 0.5mm Teflon membrane filter. 30ml of the filtered sample were then injected into the column. Flame atomic spectrometer equipped with a thermal spray developed in the lab was used to detect the metals. UV/VIS set at 415 nm was used to detect compounds separated from the chlorophyll extract. Gradient solution using a mixture 50:50 methanol:water as solvent A and 5:95 Isopropyl alcohol: methanol was followed.

The retention times of the chlorophylls were determined by the magnesium peaks. Presence of chlorophyll was confirmed by collecting the compound eluting at the
chlorophyll retention time and comparing the UV spectra with that of standard chlorophyll.

The chromatograms of chlorophyll extracted from plants exposed to aluminum were compared with the chromatograms of the plants not exposed to aluminum. Atomic absorption traces were used to test presence of magnesium and aluminum.

Chlorophyll eluting from the column was collected and divided into two portions. One portion was treated with aluminum as chloride and the other portion was untreated. The resulting solutions were run through the HPLC under the same conditions as the pure chlorophyll extract.

2 Results and Observations

The HPLC chromatograms of chlorophyll extracts are shown in figures 34-36. The chlorophyll extract from plants exposed to aluminum which had started to turn yellow, showed more peaks appearing earlier than was observed from pure chlorophyll extract. Also, an extra compound eluting much later than the chlorophyll was observed in aluminum treated plants. The leaves that did not change color had the same chromatogram as those not exposed to aluminum. When the pure chlorophyll was treated with solid aluminum chloride and resulting solution was run through the column, chlorophyll a and b peaks disappeared and the longer eluting compounds appeared at 12 minutes. The longer eluting compound did not contain magnesium but it was shown to contain aluminum. When pure chlorophyll a was treated with aluminum chloride its peak disappeared and the peak at 12 minutes appeared.

The AA traces of aluminum exposed chlorophyll showed that the magnesium peaks had shifted towards shorter retention times. The results show that the aluminum toxicity to plant may be due to its interference with the chlorophyll.
FIG. 34 MAGNESIUM AA TRACES OF CHLOROPHYL
FIG. 35  EFFECT OF AL⁺³ ON CHLOROPHYLL a
HPLC TRACES

RETENTION TIME IN MINUTE.
FIG. 36 CHROMATOGRAMS OF CHLOROPHYLL EXTRACT TREATED WITH ALUMINUM CHLORIDE.
3 Conclusion

Aluminum appears to interfere with chlorophyll especially chlorophyll a. This conclusion is supported by the fact that the chlorophyll peaks were reduced in aluminum treated plants and also by the fact that addition of some aluminum chloride to chlorophyll extract resulted in disappearance of the chlorophyll peak.

D EFFECT OF LEAF EXTRACT ON ALUMINUM TOXICITY TO ALLIGATOR PLANTS AT pH 4.5.

1 Introduction

Our previous studies showed that aluminum prevented root growth in alligator grass and that the presence of magnesium and or iron can mitigate the toxic effect of aluminum at pH below 4.5. Our studies also showed that leaf extract does complex aluminum.

The purpose of this study was to determine if leaf extract can counteract the toxic effect of aluminum at pH 4.5. This pH was chosen because aluminum was shown to be toxic to alligator plant below pH of 5. Above pH 5, aluminum was shown to be non-toxic.

2 Experimental

Equipment: plastic beakers, pH meter, volumetric flasks,

Reagent: aluminum chloride, plant medium containing 5 ppm of each (Ca, Mg, Iron, P, Zn, K, Na, N), Alligator grass stems, 3 week old leaf extracts, deionized water.
3 **Procedure**

The Alligator plants stems were obtained from lakes around LSU. The plants were placed into a plant medium containing 5 ppm each of the following metals Ca, Mg, Fe, P, Zn, K, Na and N. The plants were placed in a moisture controlled room for two weeks, for laboratory acclimatization.

The leaf water was obtained by soaking 270 gms of leaves in 27 liters of deionized water for three weeks. The resulting solution was filtered. Hundred mls of each extract containing 10 ppm of aluminum were placed into plastic containers. The pH was adjusted to 4.5 and the solution was allowed to stand for 24 hours. The pH was readjusted and a freshly cut alligator stems were placed in the containers. The experiment was repeated with all leaf extracts studied.

The control contained leaf extract alone at pH 4.5, deionized water at pH 4.5 and deionized water + aluminum at pH 4.5 and tap water + aluminum at pH 4.5. The plants were placed in moisture controlled room and were observed for about two weeks.

4 **Results**

The results showed that presence of leaf extract reduced the toxic effect of aluminum on roots of alligator grass even when, the pH was maintained at 4.5. However, pine and oak extracts did not reduce the toxic effect of aluminum. Analysis of the aluminum concentration in the containers, showed that the presence of the leaves decreased the uptake of aluminum by the plant. This was shown by the fact that higher concentrations of aluminum were found in the containers in which plants developed roots. There was less concentration of aluminum in the containers with pine and oak extracts indicating that more aluminum was taken up by the plant.
5 Discussion

Since aluminum was found to be toxic to alligator grass at pH 4.5, the results indicate that the leaf extract may be complexing aluminum preventing its entry into the plants.

6 Conclusion

The results showed that three weeks old leaf extracts can reduce aluminum toxicity to roots at pH 4.5 with the exception of pine and oak extracts which did not mitigate the toxic effect of aluminum to alligator roots even after 2 weeks. The plants found most effective in reducing aluminum toxicity were also those found to have high buffering capacity.

E EFFECT OF LEAF EXTRACTS ON ALUMINUM UPTAKE BY ALLIGATOR GRASS

1 Introduction

Our previous studies showed that alligator grass stems exposed to aluminum at pH 4.5 stopped growing roots. It was also found the the plants that had reduced root growth had increased accumulation of aluminum in the leaves. Our previous studies also showed that use of leaf water extract reduced aluminum toxicity to alligator grass.

Therefore the purpose of this study was to find out if the presence of leaf extracts reduced the aluminum toxicity to the plant by cutting down the uptake of aluminum.
2 Experimental

EQUIPMENT; Atomic absorption spectrometer
(Perkin Elmer model 403 equipped with a graphite furnace,
Plant containers.

REAGENT; analytical grade concentrated nitric acid, plants, plant
media.

3 Experimental Procedure

Healthy alligator grass plants that had been acclimatized in the laboratory
conditions, were used for the study. The plants were placed in plastic containers
containing 200 mls of water from the leaf extracts. The water contained 10ppm aluminum
at pH 4.5. The pH was maintained by adding either 0.05 M sulfuric acid or 0.05 M
sodium hydroxide. The plants were placed in the containers which were kept in a
moisture and temperature controlled room. The control plants were placed in containers
with either leaf water alone at pH 4.5, or tap water containing 10ppm aluminum at pH
4.5. The volume of water in all the containers was the same. The plants were observed
for about a month.

After a month, leaves were collected from each plant. They were rinsed with
deionized water, dried, weighed and then acid digested using analytical grade
concentrated nitric acid from Baker. The resulting solutions were made into known
volume, and the concentration of aluminum in each plant was determined using Perkin-
Elmer graphite atomic absorption. Instrumental conditions were described previously.

4 Results

The results are shown in Table 20.
TABLE 19

CONCENTRATION OF ALUMINUM IN ALLIGATOR GRASS LEAVES EXPOSED TO 10PPM ALUMINUM AND LEAF EXTRACTS AT pH 4.5

<table>
<thead>
<tr>
<th>Leaf extract</th>
<th>AL Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pine</td>
<td>25.0</td>
</tr>
<tr>
<td>Cypress</td>
<td>42.0</td>
</tr>
<tr>
<td>Alligator grass</td>
<td>24.0</td>
</tr>
<tr>
<td>Willow</td>
<td>18.0</td>
</tr>
<tr>
<td>Oak</td>
<td>22.0</td>
</tr>
<tr>
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The results show that plant exposed to aluminum alone at pH 4.5 had accumulated higher concentration of aluminum in their leaves than the plants exposed to aluminum and leaf extract. However, the concentration of aluminum in the leaves was not directly related to the reduction in toxicity. The presence of cypress leaves had the least reduction in aluminum uptake although it reduced aluminum toxicity. On the other hand, oak leaf extracts did not mitigate the toxic effect of aluminum, although they had reduced its uptake considerably.

The results indicate that the interaction of aluminum with leaf extract obtained after three weeks of soaking the leaves, resulted in formation of compounds which had different effects on the plants. It appears that the pine and the oak formed a compound or compounds that reduced the uptake of aluminum by the plant but was nonetheless toxic to the plant, while the cypress extract formed a compound that was easily uptaken by the plant but it was not toxic to the plant.

5 Conclusion

The result show that the interaction of the leaf extract with the aluminum is not simple. Different leaf extracts have different mode of action, or that the leaf extracts form different compounds with the aluminum and that these compounds have different rate of uptake by the plants and different toxicity.

F EFFECT OF CATIONS ON ALUMINUM TOXICITY TO ROOTS
IN ALLIGATOR GRASS

1 Introduction

The purpose of this study is to find out if the presence of iron, magnesium and/or calcium could mitigate the toxic effect of aluminum at pH 4.5. Our studies showed that
presence of aluminum interferes with root growth at pH below 5. At pH between 5 and 8 aluminum was shown to be less toxic to the plant.

2 Experimental

Equipment; Plastic containers, temperature and moisture controlled plant incubator.

Reagents; Deionized water, aluminum chloride, Ferric chloride, magnesium nitrate, calcium nitrate, plant food. Freshly cut alligator grass plants.

3 Experimental Procedure

Alligator grass stems were obtained from lakes around LSU. They were grown in the laboratory conditions for two weeks. During the acclimatization period, the plants were placed on a solution containing essential minerals.

Several containers with 100mls of deionized water with 10ppm aluminum and varying concentrations of cations were set up as shown below. The pH was maintained at 4.5.

Freshly cut healthy alligator grass stems were placed in each container and the plants were observed for a week.

4 Results

Plants in containers with deionized water alone with no pH adjustment developed roots within 3 days. Plants exposed to 10 ppm aluminum and a mixture of iron, calcium, and iron also developed roots on three days. Plants exposed to a mixture of aluminum, calcium and magnesium also developed roots in three days. The presence of iron alone and 10 ppm aluminum did not hinder root development. Presence of 10 ppm aluminum
and 20 ppm magnesium also did not interfere with plant growth or root development. The plants grown in 5 ppm magnesium and 10 ppm aluminum developed roots but the plant did not look as healthy as the others. Plants grown in 20 ppm calcium and the aluminum did not develop any roots.

The presence of calcium alone at 20 ppm resulted in poor roots growth, The plants grown in 5 ppm calcium grew healthy roots.

The presence of 10 ppm Al, 5ppm Ca and 5ppm Fe resulted in root development but there was some yellowing of the leaves.

5 Discussion

The results show that presence of magnesium, either alone or in combination with other metals reduced aluminum toxicity at pH 4.5. The presence of iron, alone or in combination with the other metals, also reduced aluminum toxicity, especially when the ionic concentration of these metals was equal or more than that of aluminum alone. Calcium did not reduce aluminum toxicity and its presence at 20 ppm resulted in yellowing to the leaves, even in the absence of aluminum.

The results show that calcium does not mitigate the toxic effect of aluminum at pH 4.5. They also indicate that in a nutrition deficient environment, increasing concentration of calcium ions could result in magnesium deficiency leading in yellowing of the plants.

The results indicate that liming without addition of magnesium could result in magnesium deficiency leading to yellowing of plants.

6 Conclusion

The result show that addition of iron and magnesium to areas affected by acid rain could mitigate its toxic effect of aluminum and acidity. Equal molar concentration of iron
seemed more effective, while magnesium at higher concentration than aluminum was most effective. The results indicate that the toxic effect of aluminum to plant at low pH may due to its interference with iron and/or magnesium. Presence of calcium alone seemed to have adverse effect on the roots development in alligator grass.
CHAPTER 4

STUDIES IN BUFFERING CAPACITIES AND CHEMICAL COMPOSITION OF DECAYED LEAVES

A INTRODUCTION

Industrialization has resulted in introduction of many substances in the environment. These chemicals have altered the chemistry of the atmosphere, soils, and bodies of water. Industrialization created the demand for fossil fuels which have resulted in emission of hydrocarbons, nitrogen oxides, and sulphur oxides. Interaction of these emitted chemicals with the sunlight and moisture in the atmosphere has resulted in the formation of acidic substances, which are deposited on the earth's surface by either wet deposition (acid rain) or dry deposition (409).

The effect of acidic deposition in an area depends on the chemical composition of the area. Lakes or forests with high buffering capacities are not affected much by acid rain (410). Buffering capacity is the ability of an ecosystem to maintain a constant pH, despite the addition of an acid or a base.

Soils high in limestone and silicate effectively neutralize the hydrogen ions from the acid rain and are able to maintain high pH, despite the acidic deposition(411). Thin soils especially those found covering granite or igneous rocks, lack the buffering material. They are slightly acidic and are not able to neutralize acid rain. The acidic deposition leads to leaching of essential nutrients such as calcium, magnesium and potassium. Toxic metals may also be leached from the soils.(311, 401).
Studies in NorthEastern USA have shown that areas with high organic content are not affected much by acidic deposition (412). It is believed that the decayed products in these areas buffer the effect of acid rain (409). The mechanism of this buffering is not well understood. However, it is believed that ion exchange processes are involved (409).

1 Sources of Organic Matter

The organic matter in lakes may come from decomposition of plants and organisms found in the lakes, or it may be transported into the lake by inflowing water, airborne litter or rain (413).

It is believed that airborne leaf litter falling onto lakes is between 200-500g dry leaves per meter of wooded shoreline (414).

2 Decomposition of Organic Matter

Although leaves continuously fall into lakes and rivers, they do not fill up with dead matter. Bacteria, fungi and protozoa found in these lakes effectively decompose all the material produced by the plants, within a relatively short time, i.e., within a year (416). The rate of decomposition depends on the type of plant (415).

3 Effect of the pH on the Rate of Decomposition

Studies have shown that lakes affected by acid rain accumulate a lot of undecomposed litter at the bottom of the lake (413). Investigation of these lakes showed that the microbial composition is changed by the acid At pH 4.0 fungi predominate, while at pH of 7.0 bacteria are the predominant species (409, 350). When the acid lakes were treated with lime, the organic litter started to decompose very rapidly, which indicated that low pH inhibits bacteria activities(415).
Investigations have shown that the breakdown of leaf litter depends not only on pH but also on the type of plant. Francis and coworkers (417) showed that the decomposition order for different leaves was red > maple > beach > sugar > maple > leather leaf > red spruce.

Our own investigation showed that after soaking leaves in deionized water, the rate of decomposition was alligator grass > duckweed > pecan = willow> cypress > sweet gum > oak > pine. Even after 3 years of soaking, most of the pine leaves were undecomposed.

4 Reasons for Study

It is believed that organic matter plays an important role in the buffering capacity of lakes and rivers (412, 417). However, not much work has been done to find the actual buffering capacities of decomposed leaf matter.

Studies have shown that Baton Rouge rain is quite acidic, especially after a long dry spell. The pH of the rain water was shown to go as low as 3.0 (418, 419). The lake and rivers around Baton Rouge maintain high pH, i.e. above 8, despite the acidic deposition (418, 419, 420).

Studies in our group showed that when the LSU lake was dredged, the pH went as low as 4.5 after it filled up with acid rain. However, the lake increased its pH to above 8 within two to three weeks(419). This indicated that the lake had high buffering capacity. One explanation of high buffering capacity is the presence of calcium or magnesium ions. However analysis of the metal composition in the lake did not show high concentration of magnesium and calcium to account for the high buffering capacity, (420). It was felt that the organic matter from plant found in this area might provide the answer. Therefore, the study was initiated to find out if decomposition products of leaves could increase the
buffering capacity of the water. It was hoped that the products found most effective in increasing the buffer capacity could be extracted and shaped to areas affected by the acid rain.

Liming has been introduced in areas affected by acid rain. However, it has been very unsatisfactory since continued liming is necessary to maintain high pH. Liming lakes at high elevation has also been cumbersome. The long term effect of increasing calcium ions are not known. Calcium and magnesium are believed to compete for the same absorption routes in plant roots and intestinal mucosa of animals (116). Increasing calcium concentration without subsequent increase in concentration of magnesium could result in negative magnesium balance that could have serious biological consequences.

It was proposed and our studies have shown that aluminum plays a major role in the toxic effect of acid rain (424). Our studies have shown that phosphate ion can reduce toxic effects of aluminum even at low pH. Increasing calcium ions could precipitate phosphates releasing aluminum ions. Studies in Europe have shown that trees continue to turn yellow in areas affected by the acid rain despite increasing the pH by liming.(425).

It is therefore necessary to find other material that may provide the buffering capacity. without creating other problems.

5 Experimental

a Equipment 10 gallon aquaria, Beckman Chem-mate pH meter.

Chemicals; distilled water, concentrated sulfuric acid, buffers (pH 4.0, 7.0, 10.0) obtained from Baker Company.
6 Experimental Procedure

Fallen leaves were collected in the Fall from around LSU. They were washed in deionized water, dried, and then weighed. The leaves studied were pine, pecan, sweetgum, oak, willow, duckweed, cypress, and alligator grass. 270 grams of leaves were soaked in 27 liters of deionized distilled water. The pH of the water was measured before and after addition of the leaves. The measurement of pH continued daily for a couple of months. The measurement of the buffering capacity of the water was started after the pH increased above 5. This was done by filtering 100 ml of the leaf water and titrating it to pH of 5.0 using 0.05 M sulfuric acid. A blank sample of distilled water was also titrated to eliminate the effect of dissolved carbon dioxide water.

The pH meter was standardized every day by using standard buffer solution at pH 4.0, 7.0 and 10.0.

The pH meter was rinsed with deionized water to constant pH before each measurement.

Figures 37-40 illustrate the results obtained. Tables No.23 also show daily changes in pH and buffer capacity.

a Alligator Grass

The results are shown in fig 38-39. Both stem and leaves from this plant were used since it was difficult to obtain enough leaves and also because the stem was as soft as the leaves. Within a few minutes of adding the leaves, the pH of the water increased from 5.7 to 6.5, and then dropped to about 6.0 within the first 3 days. It went up again very quickly and within two weeks it was above 7. In a month's time the pH of the water containing alligator grass had gone to 9, the highest pH reached for all the leaves studied.
Fig. 37 Effect of pine, cypress, and oak on the buffer capacity of deionized water.
CHANGES IN BUFFER CAPACITY WITH TIME

- Alligator
- Duckweed
- Willow

FIG. 38 EFFECT OF ALLIGATOR GRASS, DUCKWEED, AND WILLOW LEAVES ON THE BUFFERING CAPACITY OF DEIONIZED WATER.
FIG. 39  EFFECT OF ALLIGATOR GRASS, DUCKWEED AND WILLOW LEAVES ON pH OF DEIONIZED WATER
FIG. NC. 40. EFFECT OF OAK, PINE, AND CYPRess
pH OF DEIONIZED WATER.
The buffering capacity of the water with alligator grass also increased steadily with time. It very much followed the pH pattern of the leaves. The maximum buffering capacity was reached within a month, and it was 9 times higher than the next highest buffering capacity obtained from duckweed and willow, i.e., (40 mls vs 5 mls.)

**b Duckweed**

The initial pH dropped slightly in the first few days. It was then continued to increase steadily until the maximum pH 7.4, was reached in two weeks. The pH then dropped to 7.1, where it stayed for two weeks. The maximum pH reached was 7.6. The water containing duckweed had extremely foul smell and had to be destroyed after a month.

The buffer capacity of water containing the duckweed increased steadily and within two weeks a maximum of 5 mls was reached. It then dropped to 3.7, and remained there for about a week. It again started to go up slowly until the measurement was discontinued. The results are shown in fig.37, and 38.

**c Pecan**

The pH initially dropped from 5.8 to 5.6 and then steadily increased to 6.8 within 2 weeks. It remained at this pH for almost three weeks and then started to increase steadily until maximum pH of 7.5 was reached in two months.

The buffer capacity increased to 2.89 mls in the first two weeks decreased slightly and then increased again until maximum buffering capacity of 5 mls was reached after two months of soaking.
d  Cypress

The pH of cypress water was initially at 6.3, but it dropped to 5.2, where it remained for about a week before starting to increase until after it reached pH 7.5. It remained at this pH for the duration of study.

The buffer capacity followed a similar pattern. The maximum buffer capacity for cypress water was about 5 mls.

e  Willow

When the willow leaves were added to deionized water the pH immediately increased from 5.4 to 6.4. It then dropped to 6.0 within two days and then steadily increased until the maximum of pH 8.0 was reached in two months.

The buffer capacity followed the pH pattern, but the initial decrease was small. There was steady increase until a maximum of 8 mls was reached after two months.

f  Sweetgum

When sweetgum leaves were added to the water the pH immediately dropped from 5.4 to 4.7 and stayed at this pH for more than two weeks. After two weeks, it started to increase slowly until a maximum of 6.5 was reached in 3 months.

The buffer capacity was less than one for almost a month and then increased slowly until a maximum of 3 mls was reached after 3 months.

g  Pine

Initially the pH of pine water was at 5.4 but it dropped to 4.8 within a day. The pH of pine water remained below 5 for about 3 days. It then increased to pH 5.1, where it
remained for about a week. The pH then increased slowly to 6.2, remained at this pH for about a week, and slowly increased to pH 6.4, where it remained for more than three months. After six months of soaking, the maximum pH reached was 6.8.

The buffering capacity of pine water was almost zero for three months and even after six months of soaking, it remained less than one. It was concluded that decaying pine needles would not buffer acid rain but would actually acerbate the problem. This is an important observation when it is remembered that many lakes in the northern part of the country are surrounded by pine trees. Run off from fallen pine leaves would be expected to accentuate the effect of acid rain and contribute to the demise of the lakes.

**Oak**

The initial pH of 5.4 dropped to 4.7 and remained at this pH for about 10 days. It then continued to increase steadily until after a month when the pH of 6.9 was reached. The pH remained at 6.9 for another month and then increased to pH 7.1, where it remained for duration of the experiment.

The buffer capacity increased slowly until it reached 2.1 in about a month. It remained at this level for a week and slowly increased to a maximum of 2.25 mls where it remained for the duration of the study.

**Discussion**

**Effect of Leaves on pH of the Water**

The results show that addition of leaves to deionized water resulted in pH increase after an initial pH fall. The rate of pH increase was shown to be dependent on type of leaf and the length of time the leaves were in contact with water. The maximum pH reached
was also dependent on type of leaf and the length of contact time. The result are shown in fig.37-40.

The initial pH drop depended on type of leaf. Alligator grass, duckweed, willow and pecan leaves actually increased the pH of the water when they were initially soaked. Although the pH dropped after the initial increase, it still remained above 6 i.e., it was still higher than that of the pure deionized water. The leaves increased the pH of the water above 7 within two weeks.

The pine, sweetgum, and oak dropped the pH below that of the deionized water, and they maintained pH below 5 for more than a week. The pH increase in the water containing pine, oak and sweetgum was very slow and remained acidic for more than a month. For pine and sweetgum the pH remained below 7 for more than three months.

The rate of increase and the maximum pH reached was found to be in the following order; alligator grass > duckweed = cypress = willow > oak > sweetgum > Ppne had the lowest rate of pH increase and also the lowest maximum pH reached.

b Effect of Leaves on the Buffer Capacity

Our results showed that soaking dried leaves in deionized water increased the buffering capacity of the water. The increase in buffering capacity was dependent on the type of leaves and the length of time the leaves were in contact with water. The increase in buffering capacity followed the pH increase so that the maximum pH had the highest buffering capacity.

Alligator grass, duckweed, willow, and cypress reached the highest buffering capacity within a month. The alligator grass leaves increased the buffering capacity the most and these high buffering capacity was reached within a relatively short time.
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CHANGES IN pH AND BUFFERING CAPACITY OF LEAF WATER WITH TIME

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<td>7.6</td>
<td>3.2</td>
</tr>
<tr>
<td>40</td>
<td>8.9</td>
<td>36.5</td>
<td>7.1</td>
<td>3.1</td>
<td>7.6</td>
<td>3.2</td>
</tr>
</tbody>
</table>

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The oak, the pine and the sweetgum had the least buffering capacity. The buffering capacity of pine was so low that even after two years of soaking, it had not reached one ml. It is interesting to note that areas affected by acid rain have a lot of pine trees. These trees do not increase the buffering capacity. In fact they may contribute to the acid rain problem by decreasing the pH. The green leaves of this plant were also shown to decrease the pH.

The buffer capacity of the leaf water was found to have the following order: alligator grass >> willow > duckweed > cypress > pecan > sweetgum > oak > pine.

8 Conclusion

Different leaves have different effects on the pH and buffering capacity. Leaves that increase the pH of the water were shown to also increase its buffering capacity. The effectiveness of pH and buffering capacity increment does not only depend on the type of plant, but also on the length of time the leaves are in contact with water.

B USE OF UV/VIS AND CHROMATOGRAPHY TO STUDY THE COMPOUNDS OBTAINED WHEN LEAVES ARE SOAKED IN WATER

1 Introduction

Compounds containing atoms such as O, S, and N, with unpaired electrons or compounds with conjugated double bonds can absorb radiation in the UV or visible region and go to the excited state. When they return to the ground state or the unexcited state, they emit characteristic radiation. The wavelength of absorption depends on the number of pi electrons and the arrangement of the double bonds. Conjugated double bonds have pi electrons that are easily excited and therefore absorb radiation at longer wavelengths.
The absorption of radiation in the UV/VIS region can be used to determine the types of functional groups present in a compound, especially the presence of conjugated dienes. This study was therefore initiated to find out if the compounds extracted from different leaves were different or similar and to investigate the presence of chromophores in the extracts.

Our previous studies showed that the pH and buffering capacity of deionized water increased with the increase in the length of time the leaves were in contact with water and with the type of leaves. Pine leaves were shown to increase the pH and the buffering the least, while alligator grass increased the pH and the buffering capacity the most.

The purpose of this study was to find out if there was a significant difference in the compounds coming from the different leaves and also if there was a change in the number and the type of compounds coming after varying the length of time soaking the leaves in water. In addition, an attempt was made to find out if there was any change in the number of compounds coming out of leaves soaked in acidic water. (pH below 4.0)

**a Experimental**

**EQUIPMENT;**  HPLC system described earlier (Ion exchange column, Pump, UV/VIS ;detector, syringe, injection port) 10 gallon tanks, 0.45 nylon membrane filters.

**REAGENT;**  deionized water, dried leaves, Whatman filter papers

**b Procedure**

About 100 gms of dried pecan leaves were soaked in 10 liters of water. After one hour soaking, a small amount of water was removed and was filtered using 0.45 membrane filters. About 10 to 50 mls of the filtered solution was injected into the ion exchange
column. A mixture of sodium carbonate and bicarbonate was used as the mobile phase at a flow rate of 1 ml/minute. The compounds eluting from the column were detected using ether UV set at 254 nanometers, or conductometric detector. A portion of the leaf extract was scanned through a UV/VIS spectrometer from 700 nm to 200 nm. A portion of leaf water was obtained each day and the whole procedure was repeated for about two weeks. Some leaves were soaked in acidified water to find the effect of acidity on the compounds extracted from the leaves (pH below 3), and then their UV/VIS and chromatograms were obtained in the same way. Measurements were then taken once daily for about two weeks, the procedure was repeated with all the leaves studied.

Results

The chromatographic results are shown in fig. 41-42 and Table 26. All the UV spectra were similar. A sample UV spectra is shown on Fig 43. The UV spectra were featureless but increased absorption with a decrease in the wavelength was observed. Initially the leaf extracts absorbed only in the UV region but with the increased length of soaking there was increased absorption in the visible wavelength after only a few hours of soaking. Pine, oak and the sweetgum leaves took much longer for absorption in the visible region to be observed. The initial yellow color turned dark brown after several days of soaking but the depth of the color depended on the type of leaves and the length of time the leaves were in contact with the water. Pine and oak water remained yellow for much longer than the other leaves' water. The leaves soaked and maintained at pH below 4 remained yellow throughout the duration of the study.

Chromatographic results showed that there were a lot of compounds extracted from the leaves within an hour of soaking in the water. The retention times indicated that the compounds extracted from the different leaves were different, although there were some compounds having the same retention time. Duckweed, willow, cypress and alligator
FIG. 41 HPLC TRACES OF COMPOUNDS OBTAINED
AFTER ONE HOUR SOAKING
SOAKED LEAF EXTRACT
(CYPRESS)
FIG. 42 HPLC TRACES OF COMPOUNDS OBTAINED AFTER ONE HOUR SOAKING OF SWEETGUM LEAF EXTRACT
grass had a compound or compounds eluting at about 0.7 minutes. The other leaves did not seem to have this compound. After a few days of soaking, the number of observable compounds decreased and after about a week of soaking only two major peaks could be observed. When these two compounds were collected and their UV spectra taken, they were observed to absorb radiation in both the visible and the UV regions with increased absorption at shorter wavelength but no definite maximum.

For the leaves soaked in acidic water, the number of peaks did not seem to change much even after a months soaking. The water from leaves in acidic conditions, kept absorbed mostly in the UV region and there was very little absorbance in the visible region.

d Discussion

The results showed that the compounds extracted from the leaves were different. Compounds that come out of the leaves initially were either very unstable or they interacted with each other to form completely new compounds. The similar but featureless UV spectra of the leaf extracts indicated that the different leaves formed compounds that had similar chromophores. It also showed that compounds extracted from the leaves had a lot of conjugated groups. Keeping the leaves in acidic conditions prevented formation of UV and visible chromophores.

e Conclusion

Soaking leaves in water resulted in formation of compounds that absorbed radiation in the UV and the visible region. Maintaining the leaves in low acidic environment prevented formation of chromophores that absorb in the longer wavelength.

Table 26 shows the different compounds of leaf extract eluting from the HPLC column. HPLC Retention time of compounds obtained after one hour soakings shows that there are many compounds extracted from the leaves.
C A STUDY OF METAL COMPOSITION OF THE DIFFERENT LEAVES

1 Introduction

Previous studies showed that the soaking of leaves in deionized water resulted in an increase in the pH and the buffering capacity of the water. The pH and the buffering capacity increase depended on the type of leaves.

It is known that metals, especially alkaline earth metals, increase the pH and buffering capacity of bodies of water. In fact, lime has been used in areas suffering from the effect of acid rain to increase the buffering capacity of bodies of water in those areas. (432).

The purpose of this study was to try to see if there was a significant difference in the elemental composition of the different leaves that may account for the differences in the buffering capacity observed.

a Experimental

i Equipment; ICP Instrument

ii Reagents; Analytical grade conc. HNO₃, deionized water

standard solutions for the metals to be analyzed.
b. Experimental Procedure

The fallen leaves were washed in deionized water and then allowed to dry. The leaves were weighed and then ground into a powder and then acid digested using concentrated analytical grade nitric acid. The resulting solution was analyzed for metals using Jarrell-Ash ICP instrument. The concentration of the metals was determined by aspirating a standard mixture of metals with known concentration into the plasma.

c. Results

The results show that the concentration of the elements analyzed depended on type of leaves. The leaves that increased the buffering capacity on decaying had high levels of essential nutrients such as phosphorus, nitrogen, potassium, magnesium, sulphur and calcium. The leaves that had the least buffering capacity were found to have low levels of either nitrogen or phosphorus or both. Since all the leaves tested had varying concentrations of the different elements it is difficult of draw any definite conclusion on the role of these elements in the buffering of acidity. In fact alligator grass and pecan had the least amount of magnesium but the highest amount of calcium. All the other leaves had higher concentration of magnesium than calcium.

Pine leaves had the least amount of the iron, zinc, copper, sulphur, nitrogen, phosphorus, calcium, and manganese. Alligator grass leaves on the other hand had the highest concentration of nitrogen, phosphorus, potassium, calcium, zinc and copper and the least amount of aluminum. It was found that alligator grass leaves had the highest buffering capacity and increased the pH the most. The actual role of these elements in the buffering capacity is not well known. All that can be said from these results is that the leaves that were most effective in reducing the effect of acid rain also seem to have high levels of essential nutrients.
UV SPECTRA OF PECAN LEAF EXTRACT AFTER ONE HOUR SOAKING

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d Conclusion

The leaves with highest buffering capacity had relatively high levels of essential nutrient, but there was no correlation between the concentration of calcium and magnesium with buffering capacity.

D INTERACTION OF ALUMINUM AND DECAYED LEAF MATTER.

1 Introduction

High levels of aluminum have been observed in areas affected by acid rain (333). It is believed that the high levels of aluminum combined with low pH is responsible for fish kills in acidic lakes. Our previous studies showed synergistic toxic effects of aluminum and low pH.

Regions high in organic contents are not affected by acid rain (412,417). It is believed that organic matter in these areas sequesters aluminum in the sediment reducing its toxicity. High organic matter has been shown to reduce toxic effects of aluminum in the soils. (426). Our previous studies have shown that aluminum is toxic to fish and plants and toxic effects depend on pH. The study also showed that decayed leaf extract can increase the buffering capacity and the pH of water (350).

The objective of this study was to find out if leaf extract can interact chemically with aluminum, and if they could be used to reduce aluminum toxicity to aquatic organisms and plants.

2 Reason for Study

It is believed that organically bound aluminum is less toxic to both plants and animals (333). It was hoped that this study would show chemical interaction between
aluminum and leaf extract and it would lead to identification and extraction of active compounds to send in areas affected most by acid rain.

Chromatography, IR and solid NMR was used to study the interaction. Previous researchers in this group found solution NMR unsatisfactory, since it gave broad peaks at 0.5 ppm-4 ppm which were difficult to interpret (350). The addition of Aluminum did not show any significant change, in the spectrum. Chromatography was chosen because change resulting from any interaction would result in a change in peak height and or retention time of the interacting compound. Chromatographic methods have the advantage that the compound can be isolated and characterized using other analytical methods.

The compounds formed by leaves in contact with water were extremely polar. Therefore, reverse phase and ion exchange column were used. UV/VIS spectrophotometer and conductometric detectors were used. UV/VIS principles were discussed elsewhere. A brief discussion of conductometric detector will follow before discussion of experimental procedure.

3 Conductometric Detector

Conductometric detectors follow the principle of conduction of electric current by an electrolyte, across two electrodes between which an electric field is applied. If Ohms law is obeyed, the size of the current produced depends on the strength of the applied potential.

\[ V = IR \]  

where \( V = \text{voltage} \)  
\[ R = \text{resistance} \]  
\[ I = \text{current} \]
The conductance $G$ of a solution is expressed in terms of the solution electrolytic resistance expressed as follows:

$$G = \frac{1}{R} \quad 2$$

When the area and distance between electrodes are taken into account, then the specific conductance $K$ can be determined

$$k = G \frac{I}{A} \quad 1/A$$

where $l = \text{distance between electrodes}$

$A = \text{Area of electrodes}$

Since the major interest of conductometric analysis is measurement of the concentration of conducting species, equivalent conductance is usually measured since it takes into account concentration of ions in solutions. It is given by:

$$L = 1000K/C \quad 4$$

where $K = \text{specific conductance}$

$C = \text{concentration in equivalent per liter.}$

$L = \text{equivalent conductance}$

The conductance of solution can be expressed as $G = \frac{C}{1000K}$ where $K = 1/A = \text{cell constant}$. If cell dimensions are known, specific conductance can be calculated. Normally a solution of known specific conductance is measured and results obtained can be used to calculate the cell constant.
Experimental

Equipment: Ion exchange column Wesacan model 269-029 (25 cm 4.6 mm), Bondapack C18 10 u 15 cm 4.6 mm.

Conductometric meter, BM -2 conductometric meter by Dow chemical co. Physical research lab. UV/VIS detector Pump, 100 ml syringe, Injection port.

Experimental Procedure

A schematic diagram of chromatographic system used for the analysis is shown on fig 8.

The first step was to determine chromatographic conditions that would separate the aqueous compounds leached from the leaves.

A reverse phase column was tried first using methanol; water at various levels as the mobile phase. Also ion pairing agents were tried to see if separation could be improved.

All mobile phases tried are shown on table 24-25. In each case the mobile phase was allowed to move through the column at 1.0 mls per minute for about 1 hour before the samples were introduced. 10-50 ml samples were injected into the column, and the compounds eluting were detected using UV at various wavelengths. The reverse phase columns gave poor separation and ion exchange columns were then tried. When conductivity detector was used, mobile phase with low conductivity were tried. Potassium hydrogen phtalate and sodium benzoate at low concentration were tried. The give relatively good separation but since they absorb in UV region, they could only be used with UV detector. Sodium carbonate 24 mM and Sodium hydrogen carbonate 3mM mixture were
<table>
<thead>
<tr>
<th>MOBILE PHASES TRIED FOR SEPARATING COMPOUNDS IN LEAF EXTRACTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOBILE PHASES USED IN REVERSE PHASE COLUMNS</td>
</tr>
<tr>
<td>Water: methanol</td>
</tr>
<tr>
<td>Water: methanol + 5 mM heptane sulphonate</td>
</tr>
<tr>
<td>Water: ethanol + 5 mM tetraammonium iodide</td>
</tr>
<tr>
<td>sodium acetate + heptane sulphonate sodium salt</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Table 21</strong></td>
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<td></td>
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<td>MOBILE PHASES TRIED FOR SEPARATING COMPOUNDS IN LEAF EXTRACTS</td>
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<td>Water: methanol</td>
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<td>Water: methanol + 5 mM heptane sulphonate</td>
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<td>Water: ethanol + 5 mM tetraammonium iodide</td>
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<tr>
<td>sodium acetate + heptane sulphonate sodium salt</td>
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<td></td>
</tr>
<tr>
<td><strong>Table 22</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>MOBILE PHASES TRIED IN ION EXCHANGE COLUMNS</td>
</tr>
<tr>
<td>1  20 mM Tris buffer pH 8.2</td>
</tr>
<tr>
<td>2  20 mM Tris buffer + 500 mM NaCl pH 8.2</td>
</tr>
<tr>
<td>3  Benzoic acid 0.001 M</td>
</tr>
<tr>
<td>4  Potassium hydrogen phthalate 0.00005 pH 6.25</td>
</tr>
<tr>
<td>5  Mixture of NaHCO3 (3mM) and NaCO3 (24 mM)</td>
</tr>
<tr>
<td>6  Potassium Benzoate 0.0005 M</td>
</tr>
</tbody>
</table>

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## TABLE 23

HPLC RETENTION TIME OF COMPOUNDS OBTAINED AFTER 1 HOUR SOAKING

<table>
<thead>
<tr>
<th>Oak</th>
<th>Peak</th>
<th>Retention time</th>
<th>% area</th>
<th>Peak</th>
<th>Retention time</th>
<th>% area</th>
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<tr>
<td>2</td>
<td>1.62</td>
<td>3.2</td>
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<td>0.71</td>
<td>5.411</td>
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<tr>
<td>3</td>
<td>2.09</td>
<td>15.71</td>
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<td>1.1</td>
<td>7.02</td>
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<td>4</td>
<td>3.23</td>
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<td>2.76</td>
<td>150.356</td>
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<td></td>
<td>4</td>
<td>5.56</td>
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<td>4</td>
<td>11.32</td>
<td>33.1</td>
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<tr>
<th>Cypress</th>
<th>Peak</th>
<th>Retention time</th>
<th>% area</th>
<th>Peak</th>
<th>Retention time</th>
<th>% Area</th>
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</thead>
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<td>0.5</td>
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<td>18.86</td>
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<td>1.17</td>
<td>11.32</td>
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<td>3</td>
<td>1.6</td>
<td>6.27</td>
<td></td>
<td>3</td>
<td>1.84</td>
<td>0.85</td>
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<tr>
<td>4</td>
<td>2.09</td>
<td>30.635</td>
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<td>2.34</td>
<td>15.17</td>
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<td>10.52</td>
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<th>Sweetgum</th>
<th>Peak</th>
<th>Retention time</th>
<th>% area</th>
<th>Peak</th>
<th>Retention time</th>
<th>% area</th>
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<td>3.32</td>
<td>32.44</td>
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<td>4</td>
<td>19.235</td>
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<th>% area</th>
<th>Peak</th>
<th>Retention time</th>
<th>% area</th>
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<td>2</td>
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<td>0.81</td>
<td>5.23</td>
</tr>
<tr>
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<td>2.03</td>
<td>33.96</td>
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<td>3</td>
<td>1.03</td>
<td>0.65</td>
</tr>
<tr>
<td>4</td>
<td>3.35</td>
<td>6.07</td>
<td></td>
<td>4</td>
<td>1.26</td>
<td>20.85</td>
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<td>15.54</td>
<td></td>
<td>5</td>
<td>1.54</td>
<td>5.66</td>
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<table>
<thead>
<tr>
<th>Pine</th>
<th>Peak</th>
<th>Retention time</th>
<th>% area</th>
<th>Peak</th>
<th>Retention time</th>
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<tr>
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<td>2</td>
<td>0.81</td>
<td>5.23</td>
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<td>20.85</td>
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<td>5</td>
<td>15.54</td>
<td></td>
<td>5</td>
<td>1.54</td>
<td>5.66</td>
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<table>
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<tr>
<th>Willow</th>
<th>Peak</th>
<th>Retention time</th>
<th>% area</th>
<th>Peak</th>
<th>Retention time</th>
<th>% area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.72</td>
<td>2.13</td>
<td></td>
<td>1</td>
<td>0.72</td>
<td>2.13</td>
</tr>
<tr>
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<td>0.81</td>
<td>5.23</td>
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<td>2</td>
<td>0.81</td>
<td>5.23</td>
</tr>
<tr>
<td>3</td>
<td>1.03</td>
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<td>0.65</td>
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<td>1.26</td>
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<td>4</td>
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<td>5</td>
<td>1.54</td>
<td>5.66</td>
</tr>
</tbody>
</table>

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used also since both UV and conductometric detector could be used. The mobile phase used with ion exchange columns are listed in table 25. They were tried because they had been used before for separation of inorganic anions (237). We wanted to see if they could be used for organic anions.

Once chromatographic conditions were optimized, 10-50 ml of filtrate from leaf extract were injected into the column. Samples were obtained at intervals from 1 hr to two weeks of soaking the leaves. After filtering the samples, they were divided into two equal portions. One portion was treated with aluminium chloride and the other was treated with pure deionized water to minimize dilution effect. The solutions were centrifuged, and the supernatant liquid was injected into the column as before. The chromatogram of leaf extract with and without aluminum were compared.

Solutions that had been obtained from leaf extracts after soaking the leaves for varying length of time were run through the column to find out if there were significant changes in the number of compounds eluting as the length of soaking increased. Chromatographs of the extracts with and without aluminum were also obtained.

6 Results and Observation

The chromatograms of leaf extracts are shown in fig. 44-46. The results show that after one hour soaking, numerous peaks were observed indicating that numerous compounds were coming from leaves. As the length of time the leaves were in contact with water increased, the number of peaks observed decreased. In fact, after two weeks of soaking, only two major peaks were observed in most of the leaf extract. The decrease in number of peaks with time may indicate that most of the compounds initially coming out of the leaves were very unstable and volatilized easily, or that the compounds interacted with each other forming condensation products.
FIG 4.4 EFFECT OF ALUMINUM ON THE HEIGHT AND NUMBER OF PEAKS OBSERVED IN ALLIGATOR GRASS EXTRACT.
Fig. 45  EFFECT OF ALUMINUM ON THE HEIGHT AND NUMBER OF PEAKS IN PINE EXTRACT
FIG. 46. EFFECT OF TIME ON COMPOUNDS OBTAINED FROM PINE EXTRACT
<table>
<thead>
<tr>
<th>TYPE OF LEAF</th>
<th>N (ppm)</th>
<th>P</th>
<th>K</th>
<th>Ca</th>
<th>Mg</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cypress</td>
<td>0.00</td>
<td>0.10%</td>
<td>0.20%</td>
<td>1.30%</td>
<td>2.20%</td>
<td>73 ppm</td>
</tr>
<tr>
<td>Oak</td>
<td>0.012 ppm</td>
<td>378.5 ppm</td>
<td>557 ppm</td>
<td>1.50%</td>
<td>2.40%</td>
<td>62.5 ppm</td>
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<tr>
<td>Willow</td>
<td>0.00</td>
<td>0.10%</td>
<td>0.60%</td>
<td>1.40%</td>
<td>2.90%</td>
<td>61.5 ppm</td>
</tr>
<tr>
<td>Sweetgum</td>
<td>0.00</td>
<td>0.50%</td>
<td>0.30%</td>
<td>1.80%</td>
<td>7.00%</td>
<td>53 ppm</td>
</tr>
<tr>
<td>Pine</td>
<td>0.6 ppm</td>
<td>297 ppm</td>
<td>725 ppm</td>
<td>0.50%</td>
<td>1.80%</td>
<td>36 ppm</td>
</tr>
<tr>
<td>Sweetgum</td>
<td>0.00</td>
<td>0.40%</td>
<td>0.40%</td>
<td>2.50%</td>
<td>0.40%</td>
<td>- - -</td>
</tr>
<tr>
<td>Alligator</td>
<td>0.00</td>
<td>0.40%</td>
<td>4.60%</td>
<td>1.90%</td>
<td>0.60%</td>
<td>- - -</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TYPE OF LEAF</th>
<th>Mn ppm</th>
<th>Fe ppm</th>
<th>Zn ppm</th>
<th>Cu ppm</th>
<th>S ppm</th>
<th>Al ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cypress</td>
<td>80 ppm</td>
<td>305 ppm</td>
<td>29 ppm</td>
<td>4.8 ppm</td>
<td>0.20%</td>
<td>325 ppm</td>
</tr>
<tr>
<td>Oak</td>
<td>0.17%</td>
<td>315 ppm</td>
<td>31 ppm</td>
<td>5.3 ppm</td>
<td>0.20%</td>
<td>280 ppm</td>
</tr>
<tr>
<td>Willow</td>
<td>0.10%</td>
<td>185 ppm</td>
<td>290 ppm</td>
<td>4.5 ppm</td>
<td>0.20%</td>
<td>185 ppm</td>
</tr>
<tr>
<td>Sweetgum</td>
<td>820 ppm</td>
<td>290 ppm</td>
<td>95 ppm</td>
<td>5 ppm</td>
<td>0.20%</td>
<td>400 ppm</td>
</tr>
<tr>
<td>Pine</td>
<td>220 ppm</td>
<td>97 ppm</td>
<td>63 ppm</td>
<td>0.7 ppm</td>
<td>0.10%</td>
<td>105 ppm</td>
</tr>
<tr>
<td>Pecan</td>
<td>0.10%</td>
<td>72.5 ppm</td>
<td>98 ppm</td>
<td>12 ppm</td>
<td>0.80%</td>
<td>318 ppm</td>
</tr>
<tr>
<td>Alligator</td>
<td>155 ppm</td>
<td>145 ppm</td>
<td>190 ppm</td>
<td>30 ppm</td>
<td>0.20%</td>
<td>63 ppm</td>
</tr>
</tbody>
</table>

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It was also observed that addition of aluminum to leaf water after a few hours of soaking, did not seem to affect the compounds eluting, except for the leaf extracts that had started with an initial pH above 6. The alligator grass, duckweed, and pecan extracts obtained after one hour soaking showed some decrease in height of some peaks after addition of aluminum. A brown precipitate was observed when aluminum chloride was added to some of the leaf extract. The extracts that formed the precipitate also showed the greatest decrease in the peak height.

The amount of precipitate and the rate of formation of the precipitate depended on the type of leaf, and the length of time the leaves were in contact with the water. The alligator grass, the duckweed and the pecan extracts formed precipitate with aluminum almost immediately after only two weeks of soaking. Pine, sweetgum, and oak extracts showed little change upon addition of aluminum.

7 Conclusion

The results showed that the interaction of leaf extract with aluminum depended on type of leaves, and the length of time that the leaves had been in contact with water. The longer the time of soaking the greater the interaction. The leaves with high buffering capacity showed greater interaction than those with low buffering capacity.

E STUDY OF THE NATURE OF INTERACTION BETWEEN THE LEAF EXTRACT AND ALUMINUM

1 Introduction

Our previous chromatographic study of the leaf extracts showed that there was some interaction between the extracts and aluminum. However, the nature of this interaction is not known. It could be purely an adsorption process where the aluminum is
loosely attached to fine particles in solution causing aggregation and precipitation. There could also be a chemical bond formed between aluminum and the compound or compounds in the extracts.

The purpose of this study is to find out if there was a stoichiometric ratio between aluminum and the interacting compound.

a Experimental

Equipment; 10 gallon tanks, dried leaves, volumetric flasks Graphite AA equipped with a background correction, beakers
Reagents; concentrated nitric acid (analytical grade), deionized water, aluminum chloride.

b Experimental Procedure

370 gms of leaves were soaked in 25 liters of deionized water, and allowed to stand for 3 weeks. The resulting solution was filtered. Several 100mls portions were set up. Varying concentration of aluminum solution were added to the filtered leaf water. The solutions were allowed to stand for a day to allow equilibration. The solutions were then centrifuged. The resulting precipitate was washed several times with deionized water.

The precipitate was dried in a desiccator. Weighed, and digested using 70% nitric acid. The resulting solution was made to a known volume and the concentration of aluminum was determined using graphite atomic absorption. The instrument setting and conditions for analysis of aluminum were described elsewhere.

The results show that the amount of precipitate formed depended on the amount of aluminum solution added and the type of leaf. There was no consistent ratio between the amount of the aluminum and the weight of the precipitate. This makes it difficult to draw
any conclusion. The results however indicate that the observed interaction may be due to adsorption, or adsorption and chemical bonding may be taking place. The result show that alligator grass extract had more aluminum per gram of the precipitate than pine extract. The results are shown in Table 28.

\section*{Conclusion}

Non-Stoichiometric results were obtained indicating possibility of adsorption mechanism or both adsorption and chemical bonding.

\section*{F DISSOLUTION OF THE PRECIPITATE FORMED AFTER ADDITION OF ALUMINUM TO LEAF EXTRACT.}

\subsection*{a Procedure}

The precipitate formed after the addition of aluminum to leaf extract was found to be insoluble in water. Therefore the solubility in the following was investigated: Chloroform, carbon tetrachloride, tetrahydrofuran, dimethylsulfoxide, cyclohexane, methanol ethanol, dichloromethane. None of these solvents dissolved the precipitate. Dilute nitric acid and hydrochloric acid were also tried. There was a slight solubility but the bulk of the precipitate was insoluble. Then dilute sodium hydroxide was added to the precipitate and it dissolved readily.

\subsection*{b Result}

None of the organic solvent dissolved the precipitate, but sample was soluble in sodium hydroxide.
### TABLE 25

Concentration of Aluminum in Precipitate Formed after Exposure of Leaf Extract to Aluminum

**Alligator Grass**

<table>
<thead>
<tr>
<th>Amount of Al in ppm</th>
<th>Wt. of ppt (gms)</th>
<th>Conc. Al ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.035</td>
<td>0.049</td>
<td>21.12</td>
</tr>
<tr>
<td>1.674</td>
<td>0.0595</td>
<td>28.13</td>
</tr>
<tr>
<td>2.154</td>
<td>0.0885</td>
<td>24.34</td>
</tr>
<tr>
<td>7.515</td>
<td>0.0855</td>
<td>87.89</td>
</tr>
</tbody>
</table>

**PINE EXTRACT**

<table>
<thead>
<tr>
<th>Amount of Al in ppm</th>
<th>Wt. of ppt (gms)</th>
<th>Conc. Al ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.004</td>
<td>0.0283</td>
<td>0.141</td>
</tr>
<tr>
<td>0.0032</td>
<td>0.16</td>
<td>0.2</td>
</tr>
<tr>
<td>0.0027</td>
<td>0.0054</td>
<td>0.5</td>
</tr>
<tr>
<td>0.0029</td>
<td>0.0086</td>
<td>0.34</td>
</tr>
</tbody>
</table>
c Conclusion

Change in solubility after addition of aluminum to leaf extract indicated chemical bonding.

The lack of suitable solvent for the precipitate made it difficult to study it in solution. Methods that analyze solid material were tried. One observation was that the results indicated chemical bonding rather than adsorption.

G USE OF INFRARED SPECTROSCOPY TO DETERMINE THE NATURE OF INTERACTION BETWEEN LEAF EXTRACT

1 Introduction

IR Spectroscopy is a useful technique for qualitative identification of pure compounds. The functional groups present in a sample can be identified, since each individual compound has a spectra which is characteristic of that compound. Specific groups absorb radiation at a specific wavelength depending on the groups that are attached to it.

IR spectroscopy involves absorption of infrared radiation to go into excited vibration state. In order for a molecule to absorb radiation in the infrared region, there must be a changeable dipole movement. The absorption of IR radiation results in changes in vibrational energy of the molecule.

a Experimental

Perkins-Elmer 137 Infrared IR Instrument

Apparatus for making KBL Pellet Die
Reagent; KBr, and dried leaf extracts.

b Procedure

Leaves were soaked for three weeks and the extract was filtered. It was divided into two portions. In one portion aluminum was added and the precipitate formed was washed in deionized water and dried in the desiccator. The other portion was dried without anything being added to it. 0.3g of dried samples were mixed in a ball mill with about 250 mg. dry KBr. The mixture was placed in a 11 mm pellet die. Approximately 1500 psi pressure was applied to produce a clear pellet for IR study. The Infrared spectra of the pellet was taken from the 2.8 u to 15 u. The procedure was repeated for the other leaf extract.

c Results

The IR spectra of leaf extract with aluminum and without aluminum. The results show a relatively featureless spectra with majors broad peak between 2.9-3.4 microns indicating OH and CH stretchy bands and another peak between 5.8 -6.2 M indicating C=O stretch bands. The compound appears to be mostly aliphatic and perhaps carboxylic acid. Addition of aluminum to the leaf extract resulted in a reduction in OH peak and a peak appearing in region between 14 u and 16 u indicating metal M-C or metal M-O-C bonds. The results are shown in figures 47-50.

d Conclusion

The IR studies indicate that there is chemical interaction between the leaf extract and aluminum. The result of chromatographic, IR and florescence studies indicate that there is a chemical interaction between aluminum and leaf extract.
Fig. 47 IR Spectra Of Different Leaf Extracts.
ALLIGATOR EXTRACT WITH ALUMINUM

FIG. 48 IR SPECTRA OF ALLIGATOR GRASS TREATED WITH ALUMINUM.

IR SPECTRA OF EXTRACT ALLIGATOR GRASS ALONE

FIG. 49 IR SPECTRA OF ALLIGATOR GRASS WITHOUT ALUMINUM

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FIG. 50 IR SPECTRA OF PECAN EXTRACT TREATED WITH ALUMINUM
STUDY OF INTERACTION OF ALUMINUM WITH LEAF EXTRACT USING SOLID NMR

1 Introduction

NMR has not been used much for characterizing solvent samples because of line broadening caused by static dipolar interaction, which hides the characteristic sharp individual peaks. Also, line broadening can be caused by changes in chemical shift with the orientation of the molecule or change in the external magnetic field (430,431).

Recent developments have diminished the problems of line broadening. The newer methods use multiple high power rf pulse that decouples or saturates the surrounding molecules resonance frequencies. This saturation reduces the dipolar interactions.

It has been shown that spinning the solid sample at an angle of 54.7 degrees removes the differences in resonances resulting from different orientations relative to $H_0$ (effective magnetic field). At this angle (magic angle) the $H_0$ goes to zero according to the following equation,

$$H_0 = (3 \cos^2 \theta - 1)$$

$\cos^2 \theta = \text{cosine squared theta}$

Cross polarization coupled with magic angle spinning has enabled materials that would not customarily be soluble in solvents that are compatible with NMR, to be analyzed using this technique (430). Difficult materials such as soils and clays have been studied using this technique and useful information about soil structure has been obtained. Solid NMR has been used to study humic substances (430).
Al differs from proton in that it has nuclear quadrupole moments and therefore its relaxation process is dominated by nuclear quadrupole relaxation which results in line broadening because unpaired electrons creates fluctuating magnetic fields, which decreases the relaxation time giving broad lines. High frequency pulses have reduced the problem (391).

Solid NMR has been used to study the chemistry of aluminum in aqueous samples and the presence of complex polymeric species have been confirmed using the latest NMR techniques. For example the presence of dimers such as Al$_2$(OH)$_2$(H$_2$O)$_8$ and polymeric species of aqueous solution of AlCl$_3$ were demonstrated by the NMR work of Akitt and co-workers (432). The presence of sulphato complexes (Al(H$_2$O)$_5$SO$_4^+$ in sulfate containing solutions of aluminum salts were also shown by Akitt and co-workers through solution NMR (433,434).

The chemical shift range for aluminum compounds is approximately 450 ppm. Aluminum species with octahedral symmetry are found in the low frequencies range (-50 ppm to +20 ppm). Aluminum species with tetrahedral symmetry are found in the region (-25 ppm to 108 ppm). Aluminum alkyl compounds are found in the frequency range (+156 to +221) (350).

Our studies showed that the Al-leaf extract complex was insoluble in both the organic solvents and in water. This made it very difficult to study the characteristics of the complex using solution NMR. It was felt that solid state NMR may provide some clues as to the nature of the interaction between the leaf extract and aluminum.
a Experimental

EQUIPMENT, Bruker, WP-200 FT-NMR spectrometer

Broad band tunable probes with total frequency range 10-90 MHz. Aluminum frequency generated with a PTS-160 frequency synthesizer.

b Spectral Parameters

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>27Al</th>
</tr>
</thead>
<tbody>
<tr>
<td>spectrometer frequency</td>
<td>52.1 MHz</td>
</tr>
<tr>
<td>offset</td>
<td>-1507.9</td>
</tr>
<tr>
<td>number of scans</td>
<td>70,000</td>
</tr>
<tr>
<td>pulse width</td>
<td>3, micro seconds</td>
</tr>
<tr>
<td>size</td>
<td>50,000 Hz</td>
</tr>
</tbody>
</table>

c Procedure

Pecan leaves were soaked in deionized water for three weeks. The resulting solution was filtered and aluminum chloride was added. The solution was allowed to stand for a day to allow the reaction to complete. The solution was then centrifuged and the precipitate was rinsed several times with deionized water, and was allowed to dry in the dessicator. The dry precipitate was ground into a fine powder using mortar and pestle. The $^{27}$Al NMR of the powder was obtained a using the cross polarization/magic angle spin technique. 200 Mhz NMR instrument in the LSU Chemistry Building was used. The procedure was repeated with the alligator leaves extract, and a mixture of leaf extracts from sweetgum, willow, cypress and pine. The results obtained are shown on figures 51-53.
d. Results and Discussion

All the leaf extracts give a single peak at range of +20ppm to -50ppm, which was in the region of octahedral symmetry of aluminum. This indicates that the aluminum interacts with the leaf extract to form complexes.

e. Conclusion

Solid NMR spectra of aluminum-leaf extract complex indicated that the complex had an octahedral symmetry.

I UV FLUORESCENCE OF LEAF EXTRACT IN PRESENCE AND ABSENCE OF ALUMINUM

1. Introduction

When some substances absorb radiation and enter the excited state, they immediately return to the ground state by emitting radiation. Alternatively in some cases nonradiative methods may be more favorable ways to return to the ground state because they decrease the life time of the excited state (165). In this case the atom in excited state passes into the ground state, i.e., a stable lower energy electronic state, without emitting characteristic radiation.

In fluorescence, the rate of radiationless relaxation is slowed due to structural and environmental conditions. The molecule in the excited state converts to an excited vibrational state of lower energy and then returns to the ground state emitting radiation of longer wavelengths than would be expected for emission spectroscopy. Deactivation of the excited state may involve interaction and transfer of energy from excited electronic state to solvent or other molecules.
FIG. 51 SOLID NMR SPECTRA OF PECAN LEAF EXTRACT_ALUMINUM COMPLEX
FIG. 52  SOLID NMR OF ALUMINUM_LEAF EXTRACT MIXTURE

FIG. 53  SOLID NMR SPECTRA OF ALLIGATOR GRASS EXTRACT_ALUMINUM COMPLEX
Fluorescence is usually observed in compounds containing $n$ and $P$ electrons or compounds with low energy transition such as Aromatic or aliphatic compounds with highly conjugated double bond structures or alicyclic carbonyl structures fluorescence.

Fluorescence of a compound can be affected by a change in temperature, pH, polarity or a change in rigidity. (165). It has been shown that fluorescence of certain organic compounds can be increased by complexing with a metal (165, 412, 449, ). Fluorescence emission can also be affected by concentration. High concentration reduces fluorescence intensity due to self absorption (165).

a Purpose of Study

The purpose of this study is to observe any changes in the fluorescence of leaf extract in presence and absence of aluminum.

b Reason for Study

Our preliminary studies showed that shining UV light to the leaf extract in solution produced a green colour, indicating that the extracts were fluorescing. It has been shown that complexing of organic compounds with metals changes the fluorescence intensity (440). Therefore, we wanted to see if there was a change in fluorescence intensity indicating a chemical interaction between the leaf extract and aluminum.
c Experimental

**Equipment** - (Aminco model SPF - 125 Spectrofluorometer, excitation at 350 nm, maximum absorption at 450 nm.)

**Reagent** - leaf extracts in solution, aluminium chloride.

d Experimental Procedure

The leaves were soaked in deionized water for three weeks as explained earlier. The extracts were filtered and then divided into equal portions. In one portion, aluminum solution was added and, in the other, an equal amount of deionized water was added. In cases where a precipitate was formed on addition of aluminum, the solution was centrifuged and the supernatant liquid solution was used for the study. The fluorescence emissions of the samples were obtained within an hour of the addition of the aluminum. Dilute solutions were used for the study.

e. Results and Discussion

The fluorescence spectra of the leaf extracts are shown in figs 54-58. After the addition of aluminum to the leaf extract, there was an increased fluorescence emission intensity. The emission maxima are also shifted to shorter wavelengths. The maxima were shown to be in the same region as the fluorescence maxima of humic substances.

The increase in intensity together with a shift to shorter wavelength, indicated the presence of humic substances especially fulvic and humic acids (440).

Pine, sweetgum and oak after being in the water for about a month, showed the highest increase in intensity upon addition of aluminum. Willow, cypress, pecan, and alligator showed the least intensity increase upon addition of aluminum. The change in
FIG. 54  FLUORESCENCE EMISSION SPECTRA OF ALLIGATOR GRASS EXTRACT

FIG. 55  FLUORESCENCE EMISSION SPECTRA OF SWEET GUM EXTRACT

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FIG. 56  FLUORESCENCE EMISSION SPECTRA OF CYPRESS EXTRACT

FIG. 57  FLUORESCENCE EMISSION SPECTRA OF PINE EXTRACT.
FIG. 58 FLUORESCENCE EMISSION SPECTRA OF OAK EXTRACT
intensity and shift in the shorter wavelength indicate that there is a chemical bond between the leaf extract and aluminum.

Conclusion

The fluorescence spectra of the leaf extract indicate that there is a chemical interaction between the leaf extract and aluminum. The fluorescence emission maximum and the wavelength of emission resemble that of humic substances, especially fulvic and humic acids.

When leaf extract alone was added to the water but the pH was aluminum at low pH results in destruction of the gills in the fish. Results also show exposure of fish to pH 4.5 alone does not harm the fish.

J DETERMINATION OF THE PRESENCE OF HUMIC AND FULVIC ACIDS IN THE LEAF EXTRACTS BY SOLUBILITY

I Introduction

Although humic acids have been studied for the last 100 years their structure is not well known (435). Their complete characterization has been hampered by their insolubility in organic solvents. Oxidative degradation has been used to identify the functional groups present in humic acids. However, it is difficult to determine if the compounds obtained during oxidation are part of the original compound or whether they were formed during the degradation process.

Nondegradative methods such as IR, UV, and NMR are preferable to characterize the functional groups of in humic substances. Humic acids can be separated from the fulvic acid by acidification to pH 2 and below. Humic acids precipitate out at this pH while
fulvic acid remain in solution. Humic acids on the other hand are soluble only in basic media.

The purpose of this study is to investigate the presence of humic acids in the leaf extracts, by their solubility in acidic and basic medium.

2 Reason for the Study

Our previous studies of the leaf extracts using Fluorescence, UV, and IR gave results that indicated that the material may contain humic acid and/or fulvic acids. The UV and IR spectra were featureless. When the leaf extracts were exposed to UV light they give a green light, indicating that they contained groups that fluorescence.

Excitation of dilute solutions of these extracts at a wavelength of 320nm resulted in emission spectra with broad peaks between 400nm - 500nm which is in the region of fluorescence emission spectra of humic acids. Addition of aluminum to the extracts resulted in increase of the emission spectra whose bands also shifted to shorter wavelength. These results indicated that humic acids were present. Therefore, we wanted to see if we could isolate the humic acids from the other soluble material.

3 Experiments

EQUIPMENT; 10 gallon tank, balances, funnels, filter paper, beakers

REAGENTS; dilute hydrochloric acid, sodium hydroxide, deionized water, dried leaves.

4 Experimental Procedures

270 grams of washed and dried leaves were soaked in 25 liters of deionized water for three weeks. The resulting extract was filtered. The filtrate was treated with 0.5 M HCl until pH 2 was reached. The solution was allowed to stand for about a day in the
fridge. The solution was centrifuged to separate the precipitate formed. The precipitate was rinsed several times with dilute acid to remove soluble components. The precipitate formed was then dried in a desiccator. The dried precipitate was weighed and divided into two portions. One portion was dissolved in dilute sodium hydroxide for further testing. The procedure was repeated with all the leaf extracts.

5 Results

On acidification of the leaf extract a brown precipitate was formed. The amount of precipitate formed depends on the type of leaf. The actual amount of precipitate formed may vary with other environmental factors and the weights given are a rough estimate since the experiment was done in the lab under uncontrolled conditions. There was no attempt to control the moisture of the microbial population in the tanks. The main thrust of this experiment was to determine the presence of humic acids in the extracts.

The formation of precipitate that was insoluble in dilute acid but soluble in dilute sodium hydroxide indicated presence of humic acid. The UV spectra of the sodium hydroxide solution was featureless with a maximum around 220 nm. The weight of humic acid is shown in table 29.

6 Conclusion

The results indicate that the leaf extract may contain humic acids and the amounts of humic acid formed depends on the type of leaf species.
<table>
<thead>
<tr>
<th>Type of Leaf</th>
<th>Weight of Precipitate in g/270g of leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>cypress</td>
<td>7.18</td>
</tr>
<tr>
<td>sweetgum</td>
<td>14.98</td>
</tr>
<tr>
<td>Oak</td>
<td>6.23</td>
</tr>
<tr>
<td>Willow</td>
<td>22</td>
</tr>
<tr>
<td>Alligator grass</td>
<td>34</td>
</tr>
</tbody>
</table>
K DETERMINATION OF PRESENCE OF HUMIC AND FULVIC ACIDS IN THE LEAF EXTRACTS USING THE RATIOS OF ABSORBANCES AT 465 AND 665 NANOMETERS

1 Introduction

There are two major soluble humic substances found in lakes and rivers. These are fulvic acids and humic acids. These substances are very complex organic polymers, with variable molecular weights (440). They are polydisperse and very polar compounds, which has made their complete structural characterization very difficult, although a lot of money and time has been spent on characterization of these compounds. Despite their elusive structure, their functional groups and reactivity has been well studied, and they have been shown to play a major role in aquatic chemistry (447). They influence the bioavailability of both toxic and useful chemicals in the food chain (446, 447).

Humic and fulvic acids strongly absorb UV radiation, but their UV spectra is featureless. Their IR spectra is also featureless because of many functional groups that absorb in different wavelengths which makes their identification difficult. These humic substances fluoresce when excited. Microbially formed humic substances have more differentiated spectra and fluorescence more strongly than the aquatic humic substances, with their excitation maxima occurring at much lower wavelength (440). Lower molecular weight fractions are believed to have higher excitation and emission wavelength and intensities than high molecular weight substances. Temperature and pH are believed to affect the intensity and wavelength of emission.

Humic acids and fulvic acids can be characterized by their UV absorbances at 465 nanometers and at 665 nanometers. The ratio of absorbances at these wavelengths can identify the fulvic acid from the humic acids. The lower the ratio of absorbance at 465
can identify the fulvic acid from the humic acids. The lower the ratio of absorbance at 465 and at 665 (E465/E665), the higher the molecular weight of the humic substance. According to Chaudry (440), the humic acid have the absorbance ratio in the range between 3.8 to 5.8 while the fulvic acids have absorbances in the range between 7.6 to 11.5 and in dilute solutions the ratio is independent of concentration.

The purpose of this study is to confirm the presence of humic and fulvic acids in the leaf extract by finding the ratio of the absorbances of the leaf extracts at 465 and 665 nanometers.

a  Reason for Study

Our previous studies showed that decayed leaf extract can mitigate the toxic effect of acid rain and aluminum. The study also showed that the leaf extract fluoresced when exposed to UV light with the fluorescence spectra that resembled that of humic substances. Treatment of leaf extract with dilute acid to pH below 2 resulted in formation of a precipitate, indicating the presence of humic acids. Therefore, the purpose of this study was to confirm presence of humic and fulvic acids in the leaf extracts by their absorbances at 465 and 665 nm.

b  Experimental

Equipment; UV spectrometer Perkin Elmer UV/VIS model, Beckmann DB with cuvettes of 1cm path length, 10 gallon tanks.

Reagent; deionized water dried leaves

c  Experimental Procedure

Dried leaves were obtained from around LSU lakes. The leaves were washed in deionized water and allowed to dry. 270 grams of leaves were soaked in 27 liters of
deionized water and were allowed to stand for varying lengths of time. The resulting solution were filtered and then diluted with deionized water. The UV absorbances of the leaves were read at 465 and at 665 nanometers. The results obtained were used to calculate the absorbance ratios. The results are shown in tables 30-33.

The results of E4/E6 after 2 years of soaking are shown in table 30a. The ratios were calculated in the same way.

d Results and Discussion

The results showed that all of the leaf extracts except pine contain either fulvic acid or humic acids, or both. The pine leaf extracts did not seem to have any humic or fulvic acids, even after two years of soaking. The E4/E6 ratios obtained from the pine extract also indicated that there was less degree of condensation in the pine than in the other leaf extracts. Presence of humic acids depended on type of leaves and the length of soaking. The results also showed that the degree of condensation indicated by low E4/E6 ratio was also dependent on the type of leaves and the length of time the leaves have been in contact with water. Longer soaking resulted in formation of humic acids, while shorter soaking resulted mostly in fulvic acid. After only 3 weeks of soaking, the willow and the alligator grass had formed the humic acids, while the other leaves contained mostly fulvic acid. The leaves that were shown to increase the pH and the buffering capacity of water were also shown to easily form higher molecular weight fulvic and humic acids when in contact with water. The solution formed after a few hours of soaking with the exception of alligator willow and pecan leaves did not show any absorbances at 665 nanometers.

e Conclusions

The leaves that decompose easily to form humic and fulvic acids are more effective in increasing the buffering capacity of water and can be used of counter the toxic effect of
### TABLE 27

The E465/E665 absorbance ratios of leaf extract

<table>
<thead>
<tr>
<th>Leaf extract</th>
<th>E4</th>
<th>E6</th>
<th>E4/E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alligator grass (6m)</td>
<td>0.9</td>
<td>0.24</td>
<td>3.75</td>
</tr>
<tr>
<td>Pecan (6 month)</td>
<td>0.4</td>
<td>0.19</td>
<td>2.1</td>
</tr>
<tr>
<td>Willow (6 month)</td>
<td>0.95</td>
<td>0.45</td>
<td>2.1</td>
</tr>
<tr>
<td>Cypress (6 month)</td>
<td>0.64</td>
<td>0.085</td>
<td>7.53</td>
</tr>
<tr>
<td>Oak</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweetgum (6 month)</td>
<td>0.84</td>
<td>0.095</td>
<td>8.84</td>
</tr>
<tr>
<td>Pine (6 month)</td>
<td>0.26</td>
<td>0.01</td>
<td>26</td>
</tr>
</tbody>
</table>

27. a. Absorbances ratios after 3 weeks of soaking

<table>
<thead>
<tr>
<th></th>
<th>E4/E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 week pecan</td>
<td>0.645</td>
</tr>
<tr>
<td>3 week willow</td>
<td>0.68</td>
</tr>
</tbody>
</table>

27. b. The U V Absorbances Ratio (E465/E665) After 2 Years Soaking

<table>
<thead>
<tr>
<th>LEAF EXTRACT</th>
<th>E4/E6 RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>oak</td>
<td>5.6</td>
</tr>
<tr>
<td>Sweetgum</td>
<td>4.7</td>
</tr>
<tr>
<td>Alligator grass</td>
<td>3.2</td>
</tr>
<tr>
<td>Pecan</td>
<td>3.2</td>
</tr>
<tr>
<td>Pine</td>
<td>37.2</td>
</tr>
<tr>
<td>Willow</td>
<td>4</td>
</tr>
<tr>
<td>Cypress</td>
<td>2.3</td>
</tr>
</tbody>
</table>

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### TABLE 3
The UV absorbances of 6 months leaf extract in presence of aluminum

<table>
<thead>
<tr>
<th>Leaf Extract</th>
<th>E465</th>
<th>E665</th>
<th>E465/E665</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cypress</td>
<td>0.445</td>
<td>0.046</td>
<td>9.9</td>
</tr>
<tr>
<td>Willow</td>
<td>0.37</td>
<td>0.04</td>
<td>9.25</td>
</tr>
<tr>
<td>Pecan</td>
<td>0.375</td>
<td>0.03</td>
<td>12.5</td>
</tr>
<tr>
<td>Pine</td>
<td>0.04</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Alligator Grass</td>
<td>0.8</td>
<td>0.06</td>
<td>13.3</td>
</tr>
<tr>
<td>3 weeks pecan</td>
<td>0.64</td>
<td>0.05</td>
<td>12.8</td>
</tr>
<tr>
<td>3 weeks willow</td>
<td>0.60</td>
<td>0.07</td>
<td>8.6</td>
</tr>
<tr>
<td>6 weeks sweetgum</td>
<td>0.73</td>
<td>0.05</td>
<td>14.6</td>
</tr>
</tbody>
</table>

### TABLE 29
E4/E6 values in presence and absence of Al after 3 weeks

<table>
<thead>
<tr>
<th>Leaf Extract</th>
<th>Ratio of Absorbances</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
</tr>
<tr>
<td>Pecan</td>
<td>9.85</td>
</tr>
<tr>
<td>Cypress</td>
<td>7.54</td>
</tr>
<tr>
<td>Willow</td>
<td>2.1</td>
</tr>
<tr>
<td>Pine</td>
<td>26.0</td>
</tr>
<tr>
<td>Alligator Grass</td>
<td>3.75</td>
</tr>
<tr>
<td>Sweetgum</td>
<td>8.84</td>
</tr>
</tbody>
</table>

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### Table 30

UV Absorbances at 465 and 665 Nanometer of Leaf Extract Fractions Collected from Size Exclusion Column

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>FRACTION E4</th>
<th>E6</th>
<th>E4/E6</th>
<th>FRACTION</th>
<th>E4</th>
<th>E6</th>
<th>E4/E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.03</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>0.05</td>
<td>0.025</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0.03</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.13</td>
<td>0.03</td>
<td>4.3</td>
<td>3</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.395</td>
<td>0.045</td>
<td>7.8</td>
<td>4</td>
<td>0.03</td>
<td>0.045</td>
<td>6.7</td>
</tr>
<tr>
<td>5</td>
<td>0.07</td>
<td>0.025</td>
<td>2.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Two year oak after adding aluminum

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>E4</th>
<th>E6</th>
<th>E4/E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.05</td>
<td>0.025</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Two year pine after adding aluminum

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>E4</th>
<th>E6</th>
<th>E4/E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.03</td>
<td>0.045</td>
<td>6.7</td>
</tr>
<tr>
<td>2</td>
<td>0.23</td>
<td>0.035</td>
<td>6.6</td>
</tr>
<tr>
<td>3</td>
<td>0.43</td>
<td>0.035</td>
<td>12.3</td>
</tr>
<tr>
<td>4</td>
<td>0.145</td>
<td>0.035</td>
<td>123</td>
</tr>
<tr>
<td>5</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Six month willow treated with aluminum

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>E4</th>
<th>E6</th>
<th>E4/E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.005</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.005</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.15</td>
<td>0.045</td>
<td>3.3</td>
</tr>
<tr>
<td>4</td>
<td>0.075</td>
<td>0.006</td>
<td>12.5</td>
</tr>
<tr>
<td>5</td>
<td>0.05</td>
<td>0.01</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>0.06</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>0.055</td>
<td>0.02</td>
<td>2.75</td>
</tr>
</tbody>
</table>

Six month sweetgum treated with aluminum

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>E4</th>
<th>E6</th>
<th>E4/E6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.005</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0.005</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0.15</td>
<td>0.045</td>
<td>3.3</td>
</tr>
<tr>
<td>4</td>
<td>0.075</td>
<td>0.006</td>
<td>12.5</td>
</tr>
<tr>
<td>5</td>
<td>0.05</td>
<td>0.01</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>0.06</td>
<td>0.02</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>0.055</td>
<td>0.02</td>
<td>2.75</td>
</tr>
</tbody>
</table>
aluminum and acid rain. Reforestation of the areas affected by acid rain with trees whose leaves decompose easily to form humic acids may increase the recovery of these areas from the ravages of acid rain. The humic acid formed from these leaves could also trap the aluminum and other toxic metals and prevent them from being leached out of the soil and into the lakes and rivers.

EFFECT OF ALUMINUM ON THE E4/E6 RATIOS OF THE LEAF EXTRACT

Introduction

Our previous results showed that leaves in contact with water decompose to form humic substances. The type of humic substances formed depended on the type of leaves and the length of time the leaves were in contact with water. The studies also showed that the leaf extract can interact with aluminum reducing its toxicity to fish. The presence of humic substances was indicated by the ratio of absorbances at 465 and 665 nanometers.

The purpose of this study was to find the effect of aluminum on absorbance ratios of the leaf extracts.

Experimental Procedure

The equipment used were the same as described earlier. The leaves were soaked for six months. The extracts were filtered and aluminum chloride was added to each extract. The mixture was stirred and then allowed to stand for about six hours to allow enough time for the reaction to take place. The resulting solution was filtered to remove any precipitate formed. The absorbances of the solutions at 465 and 665 nanometers were taken and compared with absorbances in absence of aluminum.
b  Result

The results show that addition of aluminum to the leaf extracts resulted in the removal of humic acids from the solution leaving low molecular weight substances. It is also possible that aluminum helped in the breakdown of loosely bound groups resulting into smaller units, or that the complexation of aluminum with the leaf extract removed longer wavelength chromophores.

c  Conclusion

The results showed that aluminum removed the humic acids from the solution leaving lower molecular weight compounds in solution. This was indicated by increase in the E4/E6 ratio after addition of aluminum.

M USE OF GEL-PERMEATION TO DETERMINE THE RELATIVE MOLECULAR WEIGHT OF THE LEAF EXTRACT

1 Introduction

Although humic substances have been studied for more than a century, their structure or molecular weight is not known for sure. Some hypothetical structures have been suggested (399). The humic substances are polydisperse aggregate that have similar functional groups but varying molecular sizes (399). The structure may be influenced by the degree of humification, the source of the humic material, moisture, presence of microbial organisms, present of other chemicals and exposure to sunlight. As a result of these problems, there has been varying estimates of the molecular sizes of these substances.
Gel-permeation or size exclusion chromatography is a method of separating sample constituents by their sizes and shape, without any chemical interaction between the sample molecules and the column material or the mobile phase.

The gel-permeation column is made up of various particle size pores. As the sample molecules move down the column, the smaller molecules become trapped by the pores and therefore take along time to elute. The intermediate molecules are trapped by the larger pores only and they spend less time in the column compared to the small molecules. The larger molecules are not retained at all and they elute ahead of the mobile phase. Therefore, a sample containing varying molecular sizes can be resolved. The disadvantages of gel-permeation is that it is incapable of separating samples with close molecular sizes.

There are many column packing materials for gel-permeation. They include dextran (sephadex), semi-rigid cross linked polymers such as styrene-divinylbenzene, sulphonated polystyrene beads, and rigid-pore size controlled glass or silica gels (240-243).

Since there is no interaction between the sample and the mobile phase, any solvent that can dissolve the sample and does not attack the column material, can be used. However low viscosity solvents are preferable since they decrease the retention time of the sample molecules.

The molecular size of unknown sample can be determined by using standard markers whose molecular sizes are known.

**a Experimental**

**EQUIPMENT** 30 cm column, UV / VIS detector,
REAGENT Tris buffer, (20mM pH 7.65) sephadex packing material (G 25 in Tri-
Cl pH 7.65) aluminum salts ,leaf extract.

b Procedure

Sephadex (cross-linked dextran) was placed in 50 mM tris buffer and was allowed
to stand for a day. The slurry was then packed into the column. Glass wool was placed at
the bottom and at the top of the column to protect the column material from being eroded.
50 mls of 20 mM tris buffer were passed through the column. A small volume of the leaf
extract was placed on top of column and was eluted with 20mM tris buffer. Fractions were
collected every 10 minutes and the presence of the sample was detected by UV/VIS
absorbances at 465 and 665 nanometers.

1,10 phenanthroline ferrous sulphate salt (mw 962.54) was used as the standard.
Aluminum treated leaf extract and those not treated with the aluminum, were run through
the column. The results are shown in Table 29.

c Results

The results showed that each leaf extract contained a number of compounds with
varying molecular weights. The retention times were close to those of the standard. Some
eluted just before the 1, 10 phenanthroline and others eluted after. This indicated that the
molecular sized varied from around 500 to about 1000. Most of the leaf extract
components eluted within the first hour.

Treatment of the leaf extracts with aluminum seemed to remove the early eluting
compounds in alligator leaves, cypress, and willow, while it seemed to increase the early
eluting compounds in pine, oak.
d Conclusion

Soaking leaves for about three weeks results in formation of compounds whose molecular weights appear to be around 500 to 1000 daltons.

N SUMMARY OF RESULTS

1. There was synergistic toxic effect of acidity and aluminum. Both fish and alligator grass could survive for several weeks at pH between 4 and 5 in absence of aluminum, but presence of small amounts of ionic aluminum killed both fish and the plant. pH above 5, aluminum was found to be relatively non toxic.

2. Aging of aluminum solution reduced its toxicity. This may be due to formation of polymeric species.

3. Aluminum chloride and nitrate were found to be more toxic than the aluminum sulphate.

4. Presence of dried leaves in deionized water increased both the pH and the buffering capacity of the water. The increase depended on the species of leaves and the length of time the leaves were in contact with water. Alligator grass was the most effective in increasing the pH and the buffering capacity, while the pine leaves were the least effective.

5. Presence of decayed leaf water reduced the aluminum toxicity to fish and the alligator grass. The leaves extract found to be most effective in reducing the aluminum toxicity were those that increased the buffering capacity of the water the most.
6. When the pH of the water was maintained at 4.5, presence of the leaf extract did not mitigate the toxic effect of aluminum to fish.

7. When the leaf extract was added to aluminum solution at pH 4.5 but no further pH adjustment was done the aluminum toxicity was mitigated. The leaf extracts were able to increase the pH of the water from 4.5 to 6.2. The leaf extract reduced the toxicity of aluminum to alligator grass even when the pH was maintained at 4.5. Pine and oak leaves extract obtained after three weeks of soaking did not mitigate the toxic effect of aluminum.

8. Aluminum prevented root development in alligator grass and destroyed the gill epithelia in fish.

9. Elemental analysis of the leaves did not show significant differences in metal concentrations to account for the differences in buffering capacities observed, although the concentration of the essential elements were higher in the leaves that increased the buffering capacity the most than those that had the least effect. Pine leaves had the lowest concentration of the essential elements.

10. Studies of the leaf extract showed that the leaves that were most effective in reducing aluminum toxicity were those that decomposed easily to form fulvic and humic acids. The results indicated chemical interaction between the leaf extract and the aluminum.

11. It was shown that presence phosphorus and/or iron could mitigate the toxic effect of aluminum to fish even when the pH was maintained at 4.5 while presence iron or magnesium was shown to mitigate the toxic effect of aluminum to alligator grass at pH 4.5.
1. Aluminum accumulates mostly in gills upon acute exposure

2. Aluminum destroyed the gill epithelial

3. Aluminum interfered with the Chlorophyllis in plants.

4. The decomposition products of leaves were observed to mitigate the toxic effect of aluminum to plants roots at pH 4.5.

5. Complexation and buffering effect may be involved.

6. Leaf extracts mitigate toxic effect of aluminum on fish at pH 5 but not when pH is maintained at 4.5.

7. The order of reducing toxicity was alligator grass>pecan>cypress> willow>sweet gum>oak> pine.

8. Leaves that increase the pH of water above 5 can also mitigate toxic effect.

9. The leaves found most effective in mitigating the toxic effect of aluminum were those which decomposed easily to form humic acids.

10. Magnesium and iron mitigated toxic effects of aluminum in plants, while iron and phosphorus mitigated toxic effect of aluminum in fish.

11. Calcium which is used to increase the buffering capacity, does not mitigate toxic effect at low pH. However, the presence of both calcium and magnesium mitigate toxic effect of aluminum to plants.
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VITA

Jane Igoki Murungi, a mother of four children, was born on December 1, 1949, in Meru, Kenya. She attended Mutindwa Primary school from 1956-1963. She joined Kaaga girls high school in 1964. In 1967, she took Cambridge school certificate examination, which she passed in the first division. She joined University of Dubuque in Dubuque, Iowa, and later transferred to Southern Illinois University. She graduated from Southern Illinois University with a bachelor of science degree in chemistry in 1973.

She returned to Kenya in 1974, where she taught in high school for one year before joining Kenyatta University College as a laboratory tutor. She was awarded a Fulbright scholarship to study chemistry at Louisiana State University in 1980. In 1981 she graduated from LSU with a masters' degree in analytical Chemistry. She returned to Kenya where she resumed her teaching at Kenyatta college.

She returned to LSU for further studies in August 1985. She is currently a candidate for a doctor of philosophy degree in analytical chemistry. Upon graduation, she plans to continue her teaching and research at Kenyatta University.
Candidate: Jane Igoki Murungi

Major Field: Chemistry

Title of Dissertation: Effect of Acid Rain and Humic Substances on Aluminum Toxicity. A Comparative Study of Chemical Composition of Old and Newly Deposited Plaque From Heart Patients.

Approved:

[Signature]
Major Professor and Chairman

[Signature]
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signature]

[Signature]

[Signature]

[Signature]

Date of Examination:

April 26, 1990