BLOOD DAMAGE IN PROLONGED EXTRA-CORPOREAL CIRCULATION.

by

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It is not uncommon to see scientific investigation portrayed in books or on the screen in terms of the lonely secluded research worker, labouring diligently under primitive conditions, suddenly making a brilliant discovery and thereby winning fame and fortune. Fortunately this picture is fiction rather than fact. Successful scientific research today depends on the ability of a group of workers to pool their knowledge and skills in a concerted team effort. Science and Medicine have advanced so rapidly that this is the only economical and practical method of achieving worthwhile results.

The present study has been an example of such team-work, and represents the ultimate in co-operation between biochemists, allergists, surgeons and anatomists. Dr. D.R. Murphy, as Chief Surgeon of the Montreal Children's Hospital, has functioned as the "Team Leader". My association with him dates back to the days of Medical School, when for one summer I was engaged in work on the heart-lung machine. Continuing service under Dr. Murphy as an interne, Junior Resident in Surgery and finally as a Research Fellow has indeed been a privilege.

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Earle S. Wright

CHAPTER 1

INTRODUCTION AND HISTORICAL REVIEW

The first open-heart operation involving the use of an artificial heart-lung machine was performed by Gibbon(1954). However, this concept of artificial maintenance of the cardio-respiratory function had been considered over a century before. In 1812 Legallois stated "If one could substitute for the heart a kind of injection of arterial blood either natural or artificially made, one would succeed easily in maintaining alive indefinitely any part of the body whatsoever."

Commencing with the development of in vitro perfusion of isolated organs by physiologists such as Starling(1925), work in this field led gradually to the design of blood pumps and artificial oxygenators capable of supporting the entire circulation. Since Gibbon demonstrated that this extra-corporeal circuit could be used clinically the field has rapidly advanced, owing to the increasing understanding of the physiology of artificial circulation and the development of more efficient machines. At the present time extra-corporeal circulation is a widely used technique for open-heart surgery in all large medical centres. At the Montreal Children's Hospital approximately two hundred such operations have been performed over a five-year period.

Techniques and Apparatus Used for Extra-Corporeal Circulation

In the technique of extra-corporeal circulation venous blood is withdrawn from the patient by means of plastic catheters draining the superior and inferior vena cavae. This blood is then either pumped or drained by gravity into an artificial oxygenator from whence it is pumped back into the systemic arterial circulation. In addition to the

systemic venous blood there is also the coronary sinus blood and that flowing through a well developed bronchial collateral circulation which drains into the operative field. This additional blood is aspirated by means of a suction system and returned to the extra-corporeal circuit.

All donor blood collected for use in priming the machine is heparinized to the extent usually of 20 mgms. of heparin/500 ccs. of blood. Prior to the onset of cardio-pulmonary by-pass the patient is given heparin intra-venously in a dosage that varies from 1.5 to 3 mgms./Kgm. of body weight. Following the completion of by-pass the heparin is neutralized by either protamine or polybrene. Experience has shown that the heparin: protamine(polybrene) ratio should optimally be 1:1.5.

There are three basic types of oxygenators in use today(Fig.1.), Filming, Bubbling and Membrane.

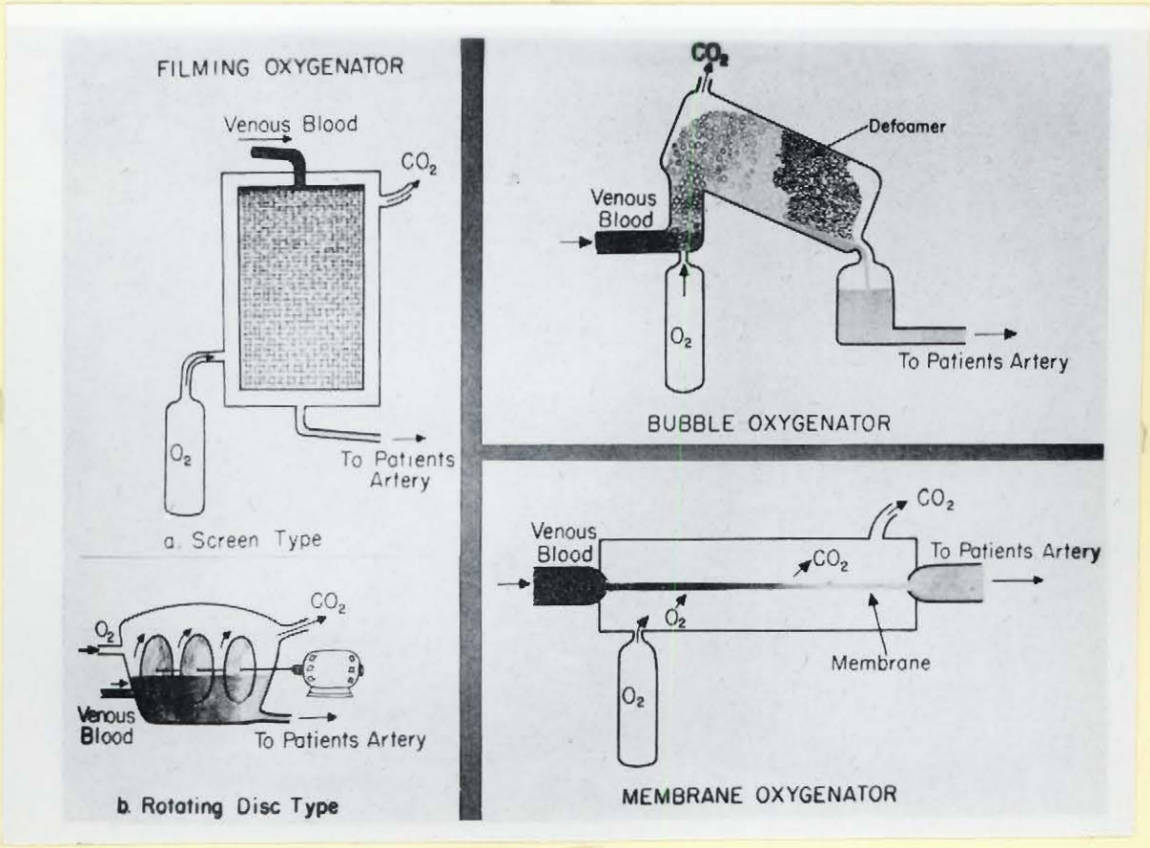
FILMING OXYGENATORS are of two designs. In the stationary-screen type developed by Gibbon blood flows down stainless steel screens in a thin film which is exposed to an oxygenating gas mixture. In the multiple-disc type the blood is lifted as a film on a series of rotating discs partially submerged in a pool of blood and exposed to the gas mixture.

In BUBBLE OXYGENATORS the blood is oxygenated and the carbon dioxide removed by the process of bubbling oxygen through columns of blood. Exposure to an anti-foaming agent is then required to disrupt the bubbles that have been produced.

The MEMBRANE OXYGENATOR is the most recent type to be developed and is in limited clinical use at a few centres. In this oxygenator blood is allowed to flow between plastic membranes such as teflon, which are permeable to the passage of gases. Oxygen is allowed to flow over the outside of these plastic envelopes. This type of oxygenator differs

FIG. 1.

Diagrammatic Sketch Showing Principles of the Types of Blood Oxygenators in Clinical Use(from Brown and Smith, Ann.Int.Med., 49:1035, 1958).


FILMING OXYGENATOR
Venous Blood
CO_2
O_2
To Patients Artery
a. Screen Type
CO_2
O_2
Venous Blood
To Patients Artery
b. Rotating Disc Type
CO_2
Defoamer
Venous Blood
O_2
To Patients Artery
BUBBLE OXYGENATOR
CO_2
Venous Blood
CO_2
To Patients Artery
O_2
Membrane
O_2
MEMBRANE OXYGENATOR

radically from those described above in that there is no direct exposure of blood to a gas interface and also there is no risk of air embolization.

The use of homologous and heterologous lungs has not proven to be feasible for artificial blood oxygenation. Waldhausen(1957), after an extensive study demonstrated rapid development of oedema and fibrin thrombosis in these donor lungs.

Various types of pumps have been designed to propel blood through the extra-corporeal circuit. The popular ROLLER PUMP compresses elastic tubing by means of rotating rollers. The SIGMAMOTOR unit utilizes compression by the peristaltic action of finger-like rods. Recently a diaphragm-type pump has been developed by Newman(1958), which is stated to produce minimal trauma to the blood. Pumps may be occlusive or non-occlusive and may be pulsatile or non-pulsatile.

Efforts are continually being made to make the apparatus for extra-corporeal circulation more efficient. One important aspect of this work is the problem of the relatively large amounts of fresh heparinized blood required to prime the machine. This imposes a great strain on the facilities of the Hospital Blood Bank, especially when a large number of these operations is scheduled. Continued research designed to reduce or eliminate this portion of blood trapped inside the machine is of obvious clinical importance. Other considerations in the design and construction of apparatus for extra-corporeal maintenance of circulation are ease of cleaning and sterilization, disposability of equipment for the handling of blood, and the production of minimal trauma upon the blood passing through it.

Review of Experimental and Clinical Studies with Prolonged Perfusion

In the great majority of heart operations involving the use of extra-corporeal circulation the heart and lungs are seldom by-passed for periods exceeding one hour. It is becoming increasingly apparent however, that there is a great need for the development of methods for long term perfusions, such as several hours, or even days. This would be of great value in the therapy of conditions such as intractable heart failure, acute myocardial infarction, pulmonary insufficiency and even for temporary support following extensive cardiac surgery. If patients in this category could be "tided over" the acute phase of their illness then it is reasonable to assume that many who at the present time have a poor prognosis could be salvaged.

A few limited clinical studies along these lines have already been carried out. As far back as 1952 Helmsworth discussed the use of partial cardio-pulmonary by-pass for the treatment of lung disease and described the use of a heart-lung machine in partial perfusion of a patient with cor pulmonale and a decreased respiratory membrane surface area. Blood was withdrawn from the femoral veins into the machine and then returned to the patient via the superior vena cava. The period of perfusion was 75 minutes. Helmsworth stated that the patient recieved striking relief from orthopnoea, dyspnoea and cyanosis.

The largest clinical series involving the use of prolonged partial by-pass was reported by Newman(1958) and his co-workers. A total of six patients were subjected to by-pass for periods of three to seven hours. Two patients were treated in an effort to relieve intractable heart failure from rheumatic heart disease, while four had profound hypotension secondary to massive myocardial infarction. All patients survived the actual perfusion. One patient was discharged home seven

weeks following perfusion, two survived for ten days, and another for thirty-six hours. In most cases perfusion was felt to benefit the patient's clinical condition. Newman fully realized that far more data was required before it would be possible to conclude that such patients whose survival rate was estimated at not over 15% could be salvaged by perfusion.

The longest clinical perfusion reported (Dickson 1959) was for a period of twenty-six hours in the treatment of a patient with intractable heart failure caused by amyloid disease. A closed extra-corporeal system was used in which blood was drained by gravity from the superior vena cava and pumped into the aorta. No oxygenator was incorporated into the circuit. A by-pass flow rate of approximately one litre per minute was employed. The patient did not appear to benefit in any way by this procedure.

It is now generally concluded that a great deal of experimental work will have to be carried out before large scale trials of long term cardio-pulmonary by-pass can be applied clinically. An increasing number of papers reporting work along these lines is now appearing in the literature. The prime prerequisite is the maintenance of a normal total or partial heart-lung by-pass for many hours under adequate metabolic conditions. In spite of reports showing that dogs may successfully tolerate partial by-pass for as long as fifty-two hours (Hamer 1959), most workers have found that morbidity and mortality parallel the duration of perfusion.

The largest series to be reported is that of Galletti and Salisbury(1959). In thirty dogs partial by-pass was carried out at a flow rate of 200-1200 ccs./minute for periods of three to five hours.

The mortality rate was 50%. Eight dogs died of ventricular fibrillation while seven had an irreversible bleeding tendency in the absence of heparin overdosage. Post-mortem examination revealed sub-endocardial and sub-epicardial haemorrhages as well as haemorrhages into the walls of the large veins. It was the opinion of these workers that protein denaturation caused by faulty oxygenator design may have been one of the factors responsible for the high mortality.

Fisher and Smyth(1959) have carried out an interesting study into the metabolic and biochemical changes that occur during prolonged partial perfusion. Six-hour perfusions were carried out on dogs using local procaine anaesthesia and employing disc or bubble oxygenators. Thirteen of the fifteen animals expired within fourteen hours of the conclusion of by-pass. No consistent pathology was noted on post-mortem examination and death appeared to be due to sudden cardio-vascular collapse. In general the animals tended to remain active and alert to the end of perfusion and several were able to walk back to their cages. Extensive haematological and biochemical studies revealed no significant changes except for an average increase of 110% in the serum inorganic phosphate. These workers concluded that this rise in phosphate may have represented a severe disturbance in the metabolic processes. It was also postulated that damage to serum proteins may have occurred.

Andersen and Hambraeus(1961) in a more recent paper also report failure of survival in dogs subjected to prolonged extra-corporeal circulation. In contrast to other series mentioned above, total body perfusion was effected using a general anaesthetic. A disc oxygenator was employed and cardio-pulmonary by-pass carried out for a period of four hours. Nine dogs were thus perfused and no long term survivals were reported. In the majority death occurred within two to eight

hours after perfusion as a result of rapid cardio-vascular collapse. No obvious cause of death was revealed by post-mortem examination except that the hearts of all animals showed rather diffuse sub-endocardial and sub-epicardial haemorrhages, with ecchymotic areas scattered over both ventricles. Serial physiological and biochemical observations were made before and during perfusion. The following changes were progressive and therefore appeared to be directly ascribable to the extra-corporeal circulation per se.

1. Moderate increase in lactic acid.
2. Slight decrease in pH.
3. Moderate decrease in potassium.
4. Marked increase in calcium.
5. Marked decrease in phosphorus.

In addition to attempts to obtain survivals following prolonged perfusion of normal dogs, experimental studies have also been carried out using animals in which heart failure was induced. In one report (Connolly 1958) acute cardiac failure was induced by constriction of the main pulmonary artery. When constriction was carried out to the point where slowing, irregularity, and cardiac dilatation occurred and asystole was eminent, partial perfusion was instituted using a vein to artery technique. In two cases 15% of the calculated resting cardiac output was by-passed for periods of eight and twelve hours. In these cases in spite of a constant fixed pulmonary artery constriction the cardiac output, systemic pressure, and ventricular contractions increased with the pump by-pass. More recent work by this group (Storli and Connolly 1960) has shown that in this series of dogs with acute heart failure partial by-pass increases the coronary blood flow. It was

postulated that mechanical support of the circulation produced its effect in two ways. Overloading of the right side of the heart was alleviated by removal of the excess blood proximal to the constricted pulmonary artery, and this blood was delivered under pressure to the arterial system raising the aortic pressure and increasing coronary flow and myocardial oxygenation.

Partial perfusion has also been used in the therapy of experimental pulmonary insufficiency(Krasna 1960). In this study dogs were made temporarily hypoxic by breathing gas mixtures containing graded amounts of oxygen. Prolonged partial perfusion was then instituted, using a veno-venous technique, blood being drained from the femoral vein and returned via the jugular vein. In six dogs thus studied there was an improvement in the arterial oxygen saturation as a result of perfusion ranging from 20% to 40% over the control values. It was felt that this technique would be of clinical value in the treatment of hypoxic disorders of pulmonary origin such as fulminating pneumonia and hyaline membrane disease.

In looking over the work that has been done clinically and experimentally in the field of prolonged extra-corporeal circulation certain points are brought out.

1. Consistent survivals cannot be obtained experimentally in dogs using the techniques and apparatus available at present.
2. The cause of death in these animals has not yet been definitely determined. Sudden cardio-vascular collapse at some period following what seems to be a successful perfusion appears to be the most frequent pattern. Post-mortem findings have revealed the occurrence of cardiac

haemorrhage in many instances. Biochemical and haematological studies have been largely non-contributory.

3. Although this method promises to be of the greatest importance clinically, much further research remains to be done before proper evaluation is possible.

Complications of Extra-Corporeal Circulation

It may be appropriate at this time to consider briefly some of the known complications of extra-corporeal circulation. These have usually been described as occurring following open-heart surgery for periods seldom longer than one to two hours. It may be expected, therefore, that many of the physiological derangements produced by these relatively short perfusions would be intensified during long term by-pass. A list of such complications comprises:

1. Metabolic acidosis
2. Bleeding problems
3. Central nervous system derangements
4. Decreased urinary function
5. Respiratory complications
6. Post-perfusion syndrome
7. Unexplained death.

Metabolic Acidosis.

It has been conclusively shown that this is engendered by the insufficient delivery of oxygen to the tissues on the basis of either poor oxygenation of blood or low perfusion flow rates(Pontius 1958 and McGoon 1960). This produces anaerobic glycolysis leading to the accumulation of acid metabolites such as lactic and pyruvic acids(Paneth 1957).

By ensuring adequate oxygenation and increasing the flow rates to basal levels the incidence of metabolic acidosis during extra-corporeal circulation has been markedly reduced.

Bleeding Problems.

In spite of adequate neutralization of heparin with protamine or polybrene several workers have reported post-operative bleeding (Osborn 1956 and Perkins 1958). Several components of the clotting mechanism have been found to be changed following cardio-pulmonary by-pass.

1. Loss of Platelets.

Platelets are routinely decreased in number during extra-corporeal circulation. Rarely, however, are values of below 50,000/mm^3 reported even with prolonged perfusions. Haemorrhage cannot be solely on the basis of thrombocytopenia, since the clinical signs such as positive Rumpel-Leede tests, are never seen(Brown 1958). The thrombocytopenia is due to

a. direct trauma

b. deposition on foreign surfaces

c. activation of the coagulation mechanism as at points of turbulence.

2. Decreased Fibrinogen Levels.

Osborn(1956) reported loss of fibrinogen during extra-corporeal circulation to a degree sufficient to cause haemorrhage. The exact cause of this was not determined but may have been due to inadequate heparinization or direct denaturation by excessive trauma. More recent experiments (Perkins 1958,and Penick 1958) report negligible loss of

fibrinogen during perfusion.

3. Activation of the Fibrinolytic System.

Von Kaulla and Swan(1957, 1958) observed some degree of fibrinolysis post-operatively in all patients undergoing perfusion, using a sensitive coagulographic technique. This was seldom prolonged or severe, but caused some fatalities.

4. Reduction of Anti-Hemophiliac Globulin Activity.

A decrease in anti-hemophiliac globulin has been stated to be associated with extra-corporeal circulation(Hoeksema 1959). This decrease was related to the duration of perfusion and the degree of hemolysis. Activation of plasma Christmas factor was also found. It was postulated that these changes reflected intravascular acceleration of clotting. Clinically it appeared that restoration of AHG activity was associated with more effective haemostasis.

It must be concluded therefore that in spite of the great deal of work that has already been carried out in this field the true etiology of the bleeding tendency with extra-corporeal circulation is still not clear. Clowes(1960) has proposed that blood is slowly clotting in the extra-corporeal system despite measures to counteract this and that the phenomenon may be caused by activation of a "prothrombin precursor" or a "prothrombin converter". This gradual onset of clotting is presumed to lead to secondary deficiencies of the coagulation factors.

Central Nervous System Derangements.

These vary all the way from transient loss of consciousness to deep coma and death. Malony(1961) has estimated the incidence to be as high as 6%. Various embolic phenomena have been implicated. Particulate emboli such as fibrin, antifoam, and fat have been found following

post-mortem examination. The use of Bubble oxygenators has also been shown to lead to air embolization(Clowes 1960).

Decreased Renal Function.

Several well controlled studies have shown that renal function during extra-corporeal circulation is impaired over and above the reduction caused by the surgical procedure. Senning(1960) compared the renal blood flow and glomerular filtration rate in dogs subjected to sham operations and those with extra-corporeal circulation. It was concluded that even at high flow rates there was some reduction in renal function. Frick(1960) has suggested that the cause of the decreased renal function may be the action of serotonin released from damaged platelets in causing renal vaso-constriction and an anti-diuretic effect.

Respiratory Complications.

Dodrill(1957) reported an incidence as high as 20%. He described a syndrome termed "Alveolar Collapse" characterized by:

1. various degrees of cyanosis and hypercapnea
2. scattered areas of alveolar collapse
3. fragmentation of alveolar elastic tissue.

Muller(1958) found pulmonary oedema and haemorrhages in the lungs of patients subjected to by-pass. Kolff(1958) has described a number of circumstances that may lead to post-operative pulmonary complications. These include:

1. pre-existing pulmonary vascular disease
2. Oxygen intoxication and lung dessication
3. excessive amounts of intra-venous fluid
4. temporary overloading of the pulmonary circulation with blood on the basis of
    a. forward overfilling when the heart is closed

while the coronary venous return pours into the right side of the heart,

b. filling of the pulmonary vasculature through collateral channels from the systemic circulation or a patent ductus arteriosus,

c. excessive accumulation of blood because of impeded venous return (e.g. in fibrillation) or mitral valvular stenosis. Many of these problems could be avoided by venting the left side of the heart with a catheter placed in the left atrium. Oxygen intoxication and lung dessication may be prevented by leaving the lungs partially inflated with a mixture of equal parts of helium and oxygen(Patrick 1957).

Post-Perfusion Syndrome.

This has recently been described by Kreel(1960). It comprises:

1. late anemia
2. albuminuria and microscopic haematuria
3. pyrexia and leucocytosis in the absence of overt infection.

Sub-haemolytic damage to erythrocytes during by-pass and undetected septic foci have been implicated. This syndrome is self-limiting and without any long term sequelae.

Unexplained Death.

Sudden death may occur following what appears to be a successful by-pass with post-mortem examination failing to reveal the cause of death. In recent years with the development of better techniques and apparatus, fewer such cases are being recorded.

In addition to the readily observed complications and physiological changes described above, extra-corporeal circulation may lead to less overt biochemical changes. This aspect of the problem is only now receiving the attention it warrants. Perfused patients have been shown to exhibit an elevation of serum glutamic oxalacetic transaminase(Crafoord 1957, and Snyder 1958) to greater concentrations than is found in non-perfused cardiac operations. This may be related to some degree of hepatic damage, since the extraction rate and the disappearance of Bromsulphalein has been found to be depressed during and after periods of cardio-pulmonary by-pass(Senning 1957).

The concept of "anoxic metabolism" occurring during extra-corporeal circulation has been proposed(Hyman 1959). The potentials of tissues during perfusion was observed by a carefully insulated high-resistance volt meter and a platinum-iridium wire. A drop in the potential of the liver was observed during, or more often after the termination of the perfusion. In some cases this drop was as much as 300 mV. in spite of high flow rates and adequate blood oxygenation in the machine. The potentials of the kidney, myocardium and blood remained stable, while that of the skeletal muscle showed a slight rise. Hypotension was ruled out as a possible cause of this fall in liver oxidizing potential since no such decrease was observed in dogs bled to a blood pressure of 35 mm. Hg. Hyman felt that possibly the rate of activity of the anaerobic metabolism was increased and oxygen uptake was impaired. He further postulated that damage to the respiratory enzyme cytochrome oxidase, which is a sensitive lipoprotein, was the responsible factor, and questioned the relationship of this oxidase damage to capillary anoxia and bleeding.

OUTLINE OF RESEARCH PROJECT.

From the above discussion it may be appreciated that there is yet a great deal of work to be carried out on the problems associated with prolonged extra-corporeal circulation. One aspect of this field which requires further elucidation is the question of the damage done to the blood components by the machine itself. As Clowes(1960) states, "The problem is no longer maintenance of flow but rather how long it can be carried out because of blood changes caused by the machines". Blood damage may well be responsible for the failure of many animals to survive prolonged cardio-pulmonary by-pass, owing to adverse effects on the circulation and on the functions of various organs of the body.

A survey of the literature showed that some work has been carried out on damage to cellular components. There was, however, an almost complete absence of any information concerning changes in the non-cellular blood constituents, such as the protein and lipid systems. The ensuing chapters will describe the research that has been carried out into the nature and severity of damage to blood cells, lipids and proteins.

CHAPTER 2.

PROTEIN DENATURATION IN EXTRA-CORPOREAL CIRCULATION.

One important aspect of the problem of blood trauma in prolonged extra-corporeal circulation is the possibility of denaturation of the plasma proteins by the artificial oxygenator. It is reasonable to assume that adverse effects upon the body mechanisms could result from severe degrees of blood protein damage. For example, denaturation of the coagulation factors such as anti-haemophiliac globulin could seriously impair the clotting mechanism. For this reason an effort has been made to determine the presence and severity of protein damage produced by the circulation of plasma through the heart-lung machine.

The Normal Structure of Proteins.

In recent years our knowledge of protein structure has increased considerably. Much of the pioneer work has been carried out by Linderstrom-Lang(1952). He considers a protein to have a primary, secondary and tertiary structure. The primary structure simply refers to the particular sequence of amino acid building blocks in the polypeptide chain. The secondary structure is the configuration of the polypeptide chains resulting from hydrogen bonding between the peptide N-H and C=O groups. The alpha helix arrangement is considered to be one such type of secondary structure. Finally, the tertiary structure is the pattern in which the secondary configurations are packed together in the native protein. This tertiary structure gives each protein molecule its individual shape, and is stabilized by interactions between side chains of amino acid residues and the formation of cross linkages

such as disulphide bonds.

A General Consideration of Protein Denaturation.

Protein chemists still differ on the question of an acceptable definition of the term "denaturation", and at the present time there is no universal agreement as to the precise meaning of the word.

Denaturation of a protein involves a change in the way the polypeptide chains are arranged within the protein molecule. Neurath (1944) defined protein denaturation as "Any non-proteolytic modification of the unique structure of a native protein, giving rise to definite changes in chemical, physical or biological properties." This definition excludes processes that result in the hydrolysis of peptide bonds. A more recent definition (Kauzmann 1959) states that the term denaturation denotes "a process in which the spatial arrangement of the polypeptide chains within the molecule is changed from that typical of the native protein to a more disordered arrangement." This definition permits the term to be used in conjunction with configurational changes that may accompany the rupture of primary chemical bonds in the protein molecule such as disulphide linkages. Although there is general agreement that marked hydrolysis of the peptide bonds cannot be considered as a form of denaturation, purists still disagree as to whether the rupture of a few such bonds warrants exclusion from the process of denaturation. Kauzmann concludes that at our present level of knowledge it is of more value to focus attention on the process of denaturation rather than on attempts to characterize what may well be an undefinable state.

It is generally agreed therefore, that when a protein is denatured the secondary and tertiary molecular structure is disrupted and the polypeptide chain is unfolded.

Agents That May Produce Denaturation.

A wide variety of factors can produce protein denaturation. The more important can be classified as follows.

1. Physical agents
    a. heat
    b. pressure
    c. irradiation
    d. sound waves
    e. surface forces.
2. Chemical agents
    a. organic solvents such as alcohol and acetone
    b. organic solutes such as urea and guanidine
    c. excess of hydrogen or hydroxyl ions.
3. Biological agents such as enzymes.

In most cases the exact mechanism by which these widely differing agents produce denaturation is completely unknown.

Surface Denaturation.

Owing to its pertinence to the experimental work to be described the question of surface denaturation will now be discussed in some detail.

It has long been recognized that when proteins are spread on to an aqueous phase or in an interface, denaturation occurs due to the unfolding of the molecule into partially or completely extended polypeptide chains. Ascheron(1870) found that a tough skin of protein was produced around oil droplets suspended in a solution of egg albumin. Devaux(1903) however was the first to actually spread proteins at the air:water interface. He further observed that these protein films could be collapsed to form insoluble fibers producing the so called

"Devaux Effect".

Surface denaturation of proteins was first extensively studied by the Chinese workers Wu and Ling(1927). They shook solutions of proteins including egg albumin and oxyhaemoglobin in air and found an irreversible loss of solubility. Important pioneer work was also carried out by Bull(1938). He clearly demonstrated the phenomenon of surface denaturation of proteins using a rotating porcelain drum dipping into an egg albumin solution. Denatured insoluble protein was produced at the solution:air surface and removed by a small amount of cotton wool placed in the container. The amount of egg albumin denatured per unit area of surface was found to be a function of the rate of creation of new surface.

Optical methods and later direct measurement(Neurath and Bull 1938) of the thickness of spread protein films have shown that these are only 8 - 10A^{o} thick, which is about the average length of an amino acid residue. Since the globular protein molecules in solution are about 40 - 80A^{o} in diameter it is obvious that intra-molecular unfolding to monomolecular layers is a feature of denaturation by surface forces.

At an air:water interface the orientation of the polypeptide monolayers appears to be similar to films of fatty acids with polar groups anchored in the aqueous phase and with the non-polar groups exposed to the air.

Surface denaturation was at first believed to be the most severe form obtainable. However it does not always entirely disrupt the secondary and tertiary protein configurations. Proteins that are first denatured by heat or ultraviolet irradiation exhibit larger limiting areas than films spread from native proteins(Kaplan and Fraser 1953). It appears likely therefore that these denaturing agents rupture bonds

which are not broken by spreading at the air:water interface.

Physiological Differences Between Native and Denatured Protein.

Several important physiological differences exist between native and denatured proteins. A careful survey of the literature has shown that denatured proteins show three types of biological derangement.

1. Rapid destruction of denatured protein.
2. Toxicity.
3. Impaired biological activity.

Denatured proteins are more rapidly destroyed.

Freeman(1959) injected rabbits at birth with human albumin until tolerance was developed and the animals failed to react to this protein. Labelled human albumin was then injected intra-venously into the adult rabbits and the half-life measured by whole body counting. Deliberately denatured albumin was eliminated more rapidly than normal protein over a period of a week or more. Freeman also perfused the liver of rats with labelled human albumin using both native and heat-denatured specimens. The denatured protein was retained by the liver to the extent of 75% in fifteen minutes, whereas the corresponding value for the unaltered albumin was only 1.5%.

Thorbecke(1960) has shown that the reticulo-endothelial system is the site of this rapid uptake and destruction of the denatured protein. Bovine serum albumin was denatured by various techniques including treatment with heat, guanidine and urea. These modified preparations were then

injected intra-venously after being labelled with radio-active iodine. The fate of these denatured proteins was determined by a study of the comparative speed of elimination from the blood, and of their distribution in various organs. The extent to which these preparations were taken up by the reticulo-endothelial system was assessed by a determination of the degree of interference by competition with the phagocytosis of carbon particles. In general the denatured protein preparations showed a rapid disappearance from the blood, paralleled by a high liver uptake and considerable interference with the phagocytosis of carbon. It was felt that various factors such as molecular aggregation, changed surface charge and diminished solubility at the pH of the blood plasma were responsible for the greater affinity of the reticulo-endothelial system for these denatured proteins. No correlation was found between uptake by the reticulo-endothelial system and the degree of antigenicity of the protein preparations.

An interesting concept of the adverse effects of a rapid uptake of denatured protein by the reticulo-endothelial system has been proposed by Suchinsky(1960). He suggests that these denatured proteins of high molecular weight may produce reticulo-endothelial blockade with an unfavourable effect on the reaction of this system to the shock situation.

Direct Toxic effect.

Depre(1952) has demonstrated a toxic effect produced by denatured protein. Sterile horse serum was denatured by heating at 85°C for thirty minutes. The serum was then cooled, diluted and injected into the ventral lymphatic sacs of frogs

in a dose of 0.1 ml./gm. of body weight. Control experiments were carried out by the injection of non-heated serum. Denatured serum produced a syndrome characterized by

a. generalized oedema
b. congestion and distention of the gastro-intestinal tract
c. a bleeding tendency particularly marked in the mucous membranes.

Death ensued within a period of one to four days. The control animals on the other hand showed no ill effects. Dialysis experiments showed that the toxic action was associated with the protein fraction of the serum.

Impaired biological activity.

Denatured proteins are usually biologically inactive. A well known example is the heat-inactivation of enzymes, complement and viruses. This inactivation however does not always accompany denaturation. Insulin retains full activity in spite of treatment with urea or spreading as a monomolecular layer, while lysozyme and ribonuclease are markedly heat resistant. Denaturation also increases the susceptibility of a protein to attack by proteolytic enzymes(Kauzmann 1959).

At the present time there is still some uncertainty concerning the effects of denaturation on the immunological properties of proteins, although a great deal of work has been done in an effort to elucidate some of the controversial points. MacPherson and Heidelberger(1945) have investigated the serological properties of egg albumin denatured by acid, alkali and heating. Significant differences from native egg albumin were demonstrated.

In general, ten times as much denatured egg albumin as the homologous native protein was required to give comparable precipitation in the precipitin test.

Di Jeso(1959) using an immuno-electrophoretic technique, concluded that aggregation following thermal denaturation of human blood serum led to a loss of antigenic properties. Pre-treatment of the serum with sodium mono-iodoacetate prevented the loss of antigenic properties, presumably by stabilizing the protein against the effect of heat.

Rothen(1947) studied the relationship of surface denaturation to changes in immunological properties. Protein monolayers of $10A^{o}$ were produced and it was found that these films retained the ability to combine with specific antisera.

Erickson and Neurath(1943) have concluded that immunological activity is largely independent of native protein structure and resides in structures that are among the last to be affected by denaturation. It is possible of course that various proteins differ in the changes in serological properties caused by denaturation. Heidelberger(1954) has shown that in contrast to the situation with ovalbumin, denatured serum albumin is rather similar immunologically to native serum albumin.

In this field it is essential to distinguish clearly between "antigenicity" and "specificity" which are really two distinct parameters of immunological activity. Evidence derived from denaturation of well defined proteins by a variety of agents such as urea, guanidine, heat and

surface forces suggests that the specificity is independent of a particular internal configuration, whereas antigenic activity appears to be reduced by processes which cause profound changes in protein structure.

It is obvious that vigorous and precise investigation in this field is still required.

Experimental Methods Available for Assessing Denaturation.

Kauzmann(1959) has extensively reviewed the various methods available for the study of protein denaturation. These are based on properties of the protein molecule which depend on the configuration of the polypeptide chains. One class of property, called the "shape property", depends on the overall molecular shape and is not affected by the spatial relationships between any particular atoms in the molecule. The second class, termed the "short range property" is related to changes in the immediate vicinity of individual groups in the molecule. Shape properties include

1. Hydrodynamic properties
    a. viscosity increment
    b. flow birefringence.
2. Radiation scattering
    a. light scattering
    b. small angle x-ray scattering.
3. Surface Properties
    a. surface dipole moment
    b. area of a solid film.

4. Diffusion through membranes with controlled pore size.

5. Electron microscopy.

Short range properties include

1. Thermodynamic properties

    a. solubility and phase distribution

    b. energy and heat capacity

2. Optical properties

    a. optical rotation and dispersion

    b. ultraviolet absorbtion

    c. wide angle x-ray diffraction

3. Surface phenomena

    a. spreadability

    b. surface viscosity

4. Chemical properties

    a. specific group reactivity

    b. electrophoretic mobility

    c. biological activity

    d. immuno-chemical properties

    e. binding of small molecules and dyes.

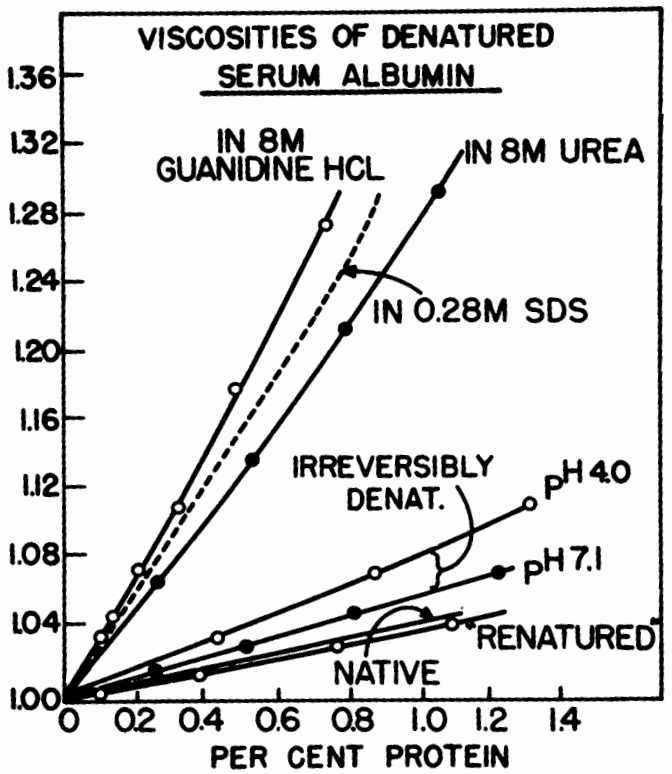
Several of these properties will now be discussed in greater detail since they provided the basis for methods for the assessment of protein denaturation in the experimental work which will be later described.

Viscosity increment.

Evidence suggests that changes in viscosity during denaturation result from changes in molecular shape. With globular proteins such as the serum albumins and globulins, various denaturants, such as heat, urea and detergents, all produce a large increase in specific viscosity(Fig. 2.).

FIG. 2.

(From Putnam, The Proteins, Vol. 1(B).



Relative viscosities of serum albumin plotted against the protein concentration in weight per cent. The curves refer to, in order of decreasing slopes, the denatured serum albumin in 8 *M* guanidine hydrochloride, in 0.28 *M* sodium dodecyl sulfate, and in 8 *M* urea, the irreversibly denatured protein at pH 4.0 and pH 7.1, the native serum albumin, and the "reversibly" denatured ("renatured") serum albumin. (Figure redrawn.)

It is now generally agreed that the increased frictional resistance of denatured proteins reflected as a viscosity increment is due to greater anisometry resulting from unfolding of the polypeptide chains. Such factors as increased hydration and aggregation are no longer thought to play any part in the viscosity changes.

Light scattering and turbidity.

Denaturation of proteins frequently leads to increased turbidity. The visible increase in opacity caused by heating a protein solution is a well known phenomenon.

When a beam of light passes through a colloidal solution it undergoes three possible fates.

1. A portion of the light is transmitted.
2. Some of the light may be absorbed, the so-called "consumptive absorption".
3. The rest of the light beam is scattered as a result of local inhomogeneities of the medium.

Since proteins are colourless and do not absorb light selectively in the visible region of the spectrum, any decrease in light transmission noted with solutions of denatured proteins must be due to increased light scattering. The mechanism of light scattering and its multiple ramifications are outside the scope of this work. It is sufficient to note that scattering depends on electronic oscillations set up in the molecules by the rapidly alternating electric field of the light wave. The intensity of this scattered radiation increases with molecular size and electric polarizability.

When a protein is denatured several structural changes may follow, each of which can influence light scattering.

1. Molecular shape changes. Denaturation leads to unfolding

of the polypeptide chains and increased molecular asymmetry.

2. Molecular aggregation may occur as a secondary process. When serum albumin is heated at neutral pH to 70°C. aggregation occurs(Pedersen 1931), with an increase in the molecular weight of about four times. Presumably denaturation results in rupture of intra-molecular bonds so that many unsatisfied bonding points result. Therefore, when denatured molecules come into contact with each other some of these ruptured bonds reform in an intermolecular manner, leading to a state of aggregation. If this process continues precipitation or gel formation may occur, with decreased solubility particularly at the iso-electric point.

3. Protein-protein complexes. When a mixture of different proteins is heated together various complexes may be formed. For example, when serum albumin and globulin are mixed and heated appreciable amounts of a new component are produced. This new component has an electrophoretic mobility that is intermediate between that of the albumin and the globulin(Van der Scheer 1941).

The intensity of light scattered at various angles from a monochromatic light beam passing through a solution may be measured by means of specially designed photometers. However, for the purpose of comparing the turbidity of protein solutions it is sufficient to measure the light transmission or optical density directly, using a standard spectrophotometer.

Ultraviolet light absorption.

The electronic absorbtion spectra of functional groups in organic molecules may be altered by changes in environmental factors.

These changes are manifested in both the shape of the absorption bands and their positions. Protein absorbs light in the ultraviolet region of the spectrum, the maximum absorption occurring in the 2750-2850A° range. Absorption peaks in this region are due to tryptophan, tyrosine and phenylalanine residues.

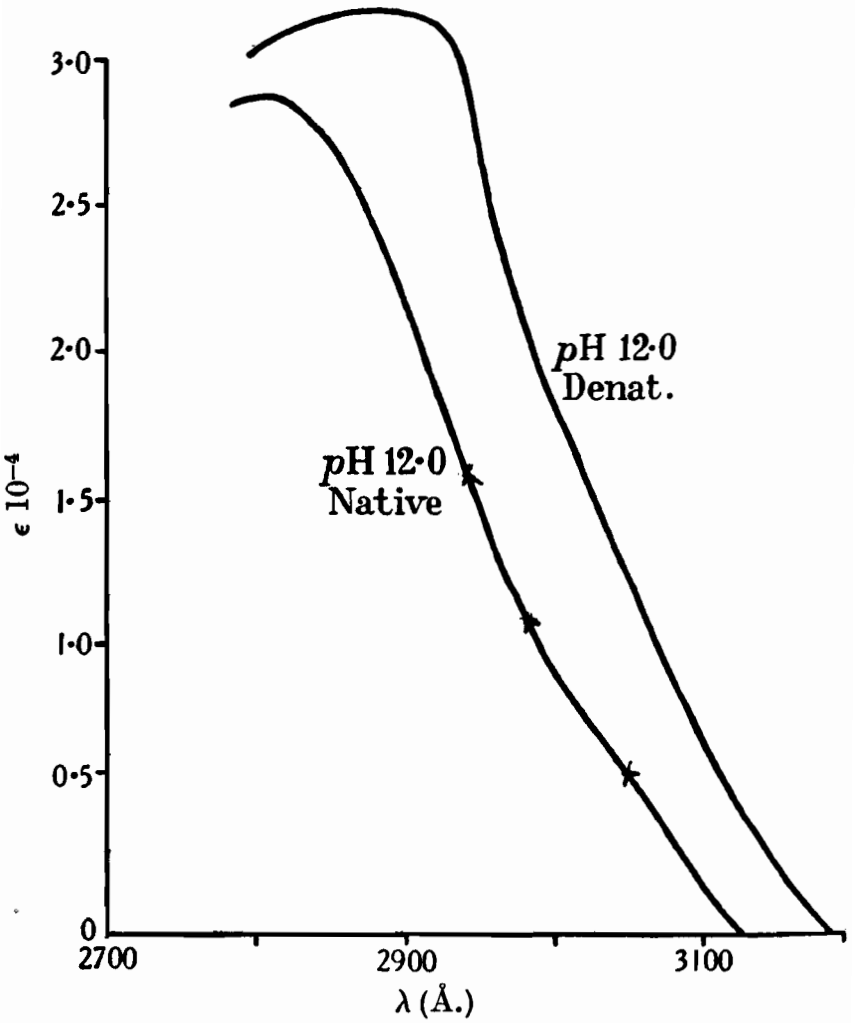
The effects of denaturation on ultraviolet absorption has received a great deal of study. Crammer and Neuberger(1943) found that denaturation of egg albumin by various agents such as alkalis, acids, heat and urea caused a definite change in the absorption spectrum characterized by increased intensity and a shift to longer wave lengths (Fig. 3.). The effect of increasing the pH in particular was given careful attention. It was found that there was little change until a pH of 13 was reached, when the characteristic differences suddenly appeared. These changes in ultraviolet absorption were attributed to increased ionization of phenolic groups, the reasoning being that these groups are bound in the native protein in a type of linkage which is broken in denaturation.

Barron(1952) demonstrated that x-irradiation of serum albumin produced an increase in the intensity of ultraviolet absorption(Fig. 4.). A similar change was caused by treatment of the protein with ultraviolet irradiation, alkalis and heat. In addition, alkalis produced a shift of the peak to a longer wave length. The changes in the ultraviolet spectrum paralleled a viscosity increase in the denatured protein.

In a more recent paper Deutsch and Morton(1961) studied the changes in ultraviolet absorption produced by the denaturation of ovalbumin. Heated egg albumin was found to have a markedly increased absorption in the region of 2500-2550A° and a less marked increase at 3050-3100A°. In addition to the increased ultraviolet absorption

FIG. 3.

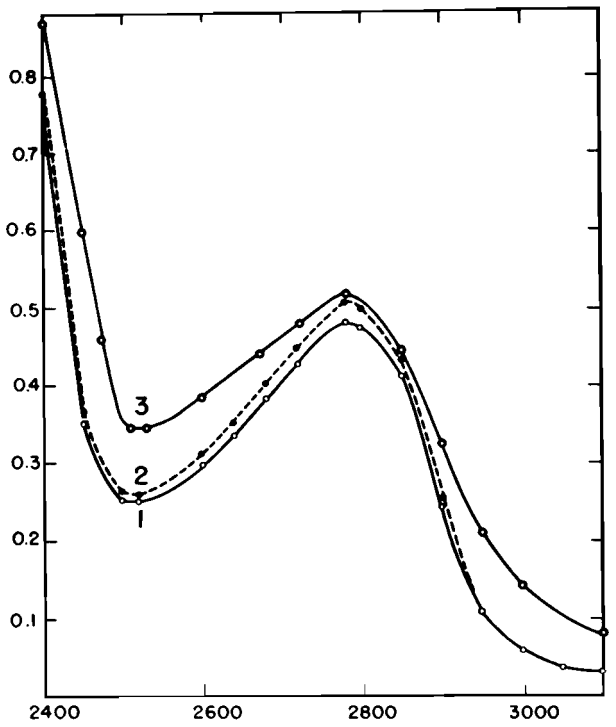
(From Crammer and Neuberger, Biochem.J., 37:302, 1943.)



Absorption spectrum of native and denatured egg albumin at *p*H 12.

FIG. 4.

(From Barron and Finkelstein, Archiv.Biochem., 41:212, 1952.)



Effect of x-rays on serum albumin, 200,000 r. X-Irradiation of "dry" plasma albumin and of albumin dissolved in water (0.07%). X-ray dose for "dry" albumin, 200,000 r; for 0.07% albumin, 150,000 r. 1, control; 2, "dry" albumin x-ray irradiated; 3, 0.07% albumin x-ray irradiated. Abscissa, wavelength in angstrom units. Ordinate, optical density.

there was also a slight shift in the peak of the curve from 2780 to 2750A°. Other proteins, such as lysozyme, $_{B}$lactoglobulin and myogen showed a similar change. Morton(1960) had previously obtained corresponding results for human gamma globulin subjected to x-irradiation. It was postulated that denaturation caused the formation of thiazole derivatives showing a strong ultraviolet absorption.

Glazer(1957) has also demonstrated a shift of the absorption maxima of denatured proteins to a shorter wave length. This has been referred to as the "denaturation blue shift". It is not as yet clear why these findings differ from the results of Crammer and Neuberger, where the shift was to longer wave lengths.

Various hypotheses have been advanced to explain these changes in spectral shifts and absorption intensity of denatured proteins. These include various considerations such as hydrogen bonding, vicinal charges, ion-dipole bonding and the formation of thiazole derivatives. Further research in this field will undoubtedly elucidate the basic mechanisms involved.

Specific group reactivity.

One of the oldest known properties of denatured proteins is the increased reactivity of certain groups as compared to a native protein. The protein sulfhydryl group in particular has been extensively studied and a great deal of information has accumulated concerning the altered reactivity of this group.

As far back as 1911 Arnold demonstrated that the denaturation of egg white was accompanied by the appearance of chemical groupings which reacted with nitroprusside. No trace of these groups was found in the native egg albumin. This work was later confirmed by Harris(1923) who treated egg albumin solutions with alcohol, mechanical shaking and

ultraviolet irradiation. These agents all led to the production of protein coagulation and the appearance of nitroprusside reactive groups.

It is now known that three types of sulfhydryl residues exist in proteins. Hellerman(1943) has proposed the following classification.

1. Freely reacting -SH groups.

   These are present in the native protein and are surface situated. They react readily with sulfhydryl reagents such as nitroprusside and ferricyanide.

2. Sluggish -SH groups.

   These are less available for structural reasons. They do not react with nitroprusside or ferricyanide but are accessible to stronger reagents such as p-chloromercuribenzoate.

3. Masked -SH groups.

   These will only react with reagents after the protein has undergone denaturation.

The number of measurable -SH groups depends on the particular protein and on the denaturing agent. Liver nucleoproteins contain only freely reacting groups, whereas all the -SH groups of serum albumin are masked in the native state. Not all denaturing agents produce the same increase in chemical reactivity. Guanidine and Duponol liberate nearly all the -SH groups of egg albumin, urea liberates 80% of the -SH groups, while with heat and surface denaturation only 50% are detected(Putnam 1953).

The measurement of -SH group activity does not appear to be as sensitive a test for denaturation as some of the other available techniques. Stanley(1939) has shown that the infective power of the tobacco mosaic virus is quite rapidly destroyed with loss of its birefringent properties, at concentrations of urea and guanidine that are too low to liberate

appreciable amounts of -SH groups. More recently Jirgensons(1952) has demonstrated that serum albumin may be denatured with a change in viscosity and optical rotation in the absence of any increase in -SH reactivity.

Under certain conditions there may be a decreased number of reactive -SH groups following denaturation. Goldblum(1954) using an amperometric titration method studied the effect of heat, irradiation and repeated freezing and thawing on the -SH reactivity of human serum. In all cases a decreased reactivity was found. Heating at 38°C. for one hour led to a decrease of 3% while at 55°C. the drop amounted to 14%. It was felt that oxidation of the -SH groups rendered unstable by denaturation was the responsible factor. Tsen and Tappel(1960) state that the -SH groups of denatured ovalbumin are readily oxidized by molecular oxygen.

Owing to the ease and specificity of measurement -SH groups have been most prominent in the literature on denaturation. It is now well established, however, that protein denaturation leads to the liberation of other reactive groups also. Some proteins such as egg albumin and serum albumin give little or no reaction for disulfide groups in the native state. After various denaturation processes the proteins give positive reactions for these groups(Mirsky 1936). Available evidence also suggests that whereas some phenol and indole groups may be evident in native proteins, the number is increased when the protein is denatured(Miller 1942).

The lack of reactivity of certain groups of native proteins may be explicable as follows. The groups may actually exist within the protein in the same form as those found in the free amino acids, but the rigid steric configuration of the native form of the molecule renders these groups inaccessible to their specific reagents. When the protein

is denatured the molecule is unfolded and the reactive groups become exposed and made more readily available to their reagents.

Electrophoresis.

A complex relationship exists between protein structure and electrophoretic mobility. Multiple factors such as ion binding, titration curve properties, molecular shape and ionic strength appear to be involved. This technique provides a useful tool for the detection of heterogeneity after denaturation.

In general it has been found that only small changes in electrochemical properties are observed when proteins are denatured despite the liberation of large numbers of reactive groups or large alterations in molecular kinetic properties. Although denaturation may cause marked changes in molecular homogeneity the corresponding changes in electrochemical homogeneity may be quite small. In fact, protein mixtures may undergo an increase in electrical homogeneity following denaturation. Heating serum at 65°C. (Van der Scheer 1941) and subjecting it to ultraviolet irradiation(Davis 1942) both cause the progressive formation of a new electrophoretic component at the expense of the albumin and globulin. This denatured component exhibits the mean mobility of the native serum. Whereas the heating of individual solutions of casein and gamma globulin produces no appreciable change in electrophoretic properties, heat treatment of mixtures of these proteins leads to a change in the electrophoretic pattern. This may well be on the basis of protein-protein interaction(Krejci 1942). The formation of a new electrophoretic component with a mobility between those of $alpha_2$ and beta-globulin occurs when serum is exposed to acetic acid at pH 3(Perlmann 1949).

Immunochemical properties.

The effect of denaturation on the serological properties of proteins has already been discussed. From a review of the literature it was concluded that whereas these altered proteins usually have decreased antigenic activity, immunological specificity is unchanged.

EXPERIMENTAL WORK.

The Hypothesis.

In most of the oxygenators in clinical use at the present time, thin films of blood are exposed to a gas or the gas is allowed to bubble through columns of blood. In both instances there is the creation of a blood:gas interface and therefore surface denaturation of plasma proteins would be envisioned. Theoretically surface denaturation should not occur in a membrane oxygenator where a direct blood:gas interface is absent. Experiments were designed to test the validity of this hypothesis.

Methods and Material.

The two types of oxygenators that were used for the experimental work are illustrated in Figs. 5 and 6. The Screen Oxygenator is manufactured by the Mark Co., Randolph, Mass., and this machine, as well as a newer modification are employed for open-heart surgery by the Cardiovascular Unit of the Montreal Children's Hospital. The Membrane Oxygenator was constructed according to the specifications of Pierce (1960). One-half mil. Teflon was selected as being the most suitable membrane on the basis of its gas diffusion properties and durability. For standardization the amount of membrane used was the same as the surface area of the blood filming screens in the Mark Oxygenator. In

FIG. 5.

Screen Oxygenator with Roller-Type Pumps

(Mark Co., Randolph, Mass.)

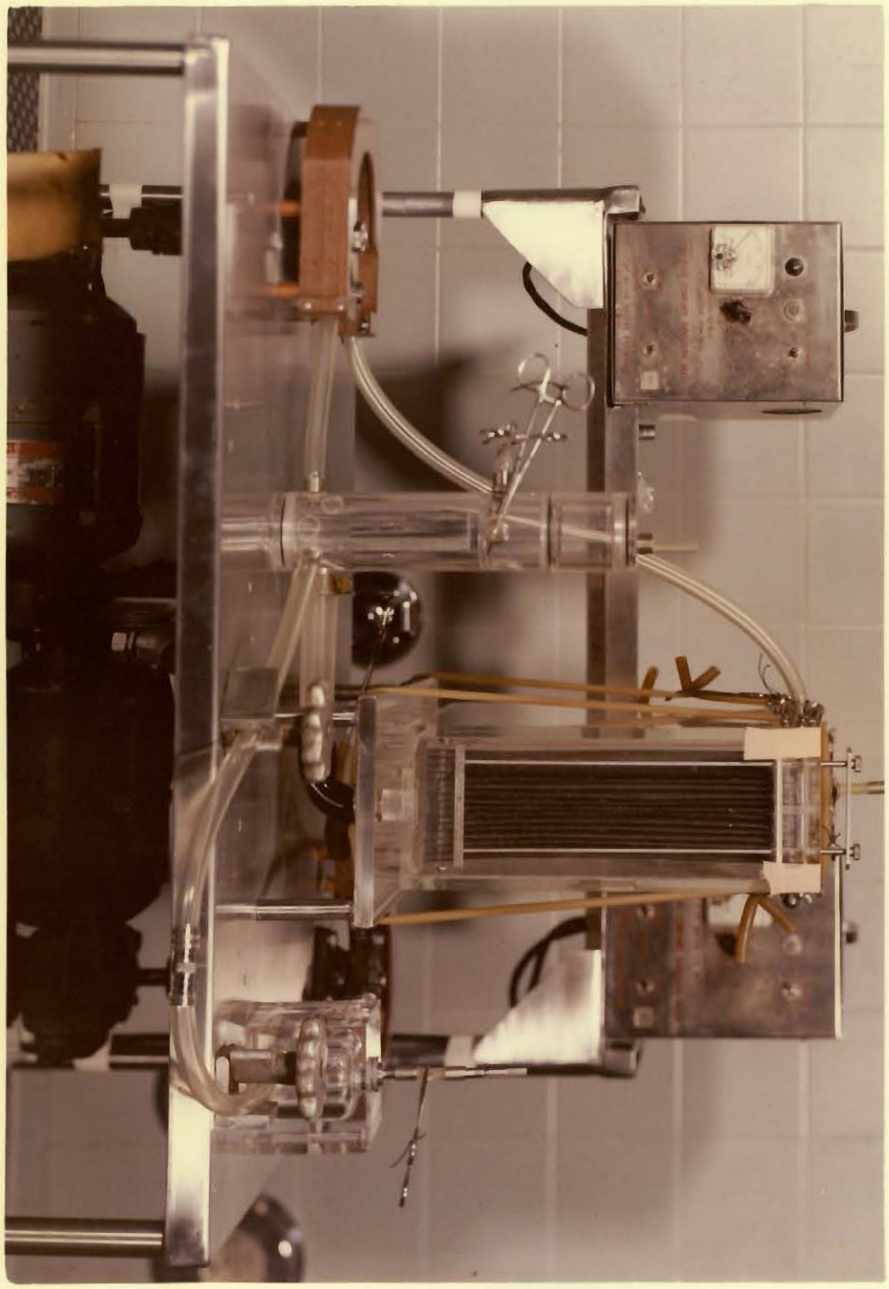
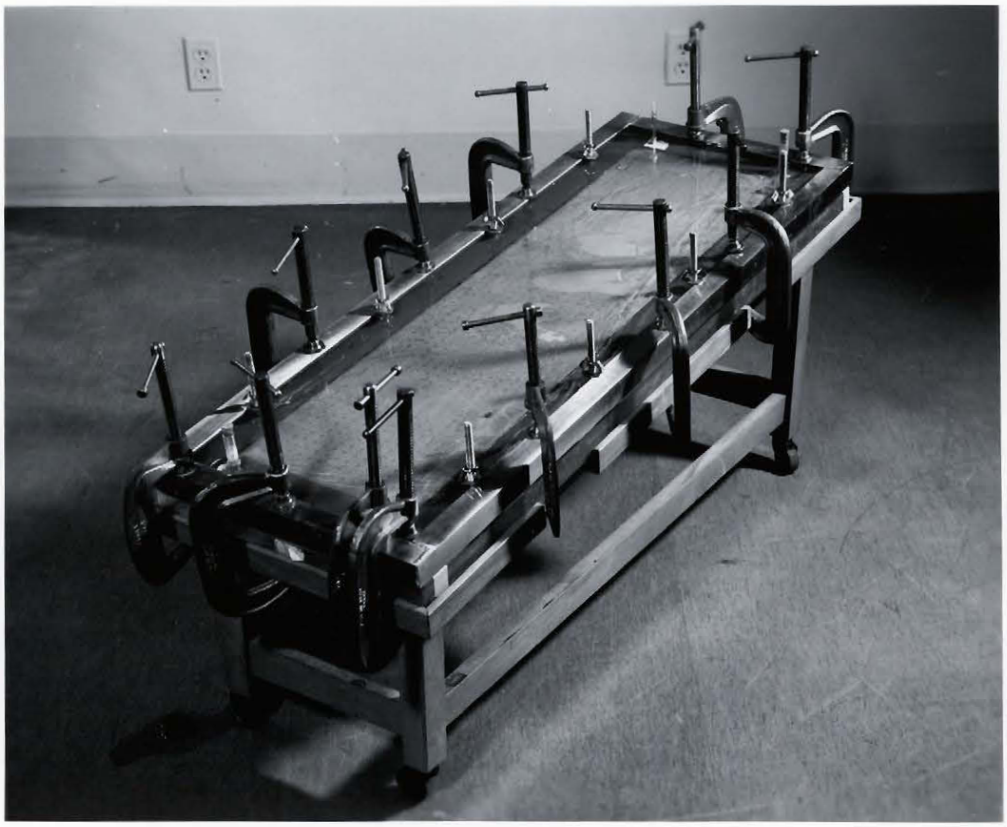


FIG. 6.

Experimental Membrane Oxygenator.



addition the same pumps and similar lengths of Tygon tubing were employed with each oxygenator. 100% oxygen was used for gas exchange and approximately 1200 ccs. of blood or plasma was used for each experiment.

All experiments consisted of recirculation of blood or plasma in a closed circuit. It was felt that since the effect of the machine only was to be assessed the incorporation of an experimental animal into the system would introduce an unknown factor.

Donor blood was obtained from healthy mongrel dogs. The femoral artery was cannulated and the animals exsanguinated into plastic bags* of 500 cc. capacity containing 2000 units of heparin in 30 ccs. of isotonic saline. Light nembutal anaesthesia was routinely employed. For the early experiments whole blood was used for recirculation, however it was soon realized that the hemolysis produced seriously interfered with the biochemical and biophysical studies and a change was made to the use of pooled plasma. This plasma was obtained by high speed centrifugation of the donor blood. The facilities of the Blood Bank of the Montreal Children's Hospital were made available for this work.

A total of ten plasma recirculation experiments were carried out. These were sub-divided as follows.

TABLE 1.

Plan of Experiments on Protein Denaturation

Number of Experiments	Code Numbers	Oxygenator Employed	Period of Recirculation
6	S10 a-f	screen	10 hours
1	S40	screen	40 hours
3	M10 a-c	membrane	10 hours

* Donated by Abbott Laboratories Ltd., Montreal.

Results.

Six methods were used for the study of protein changes.

1. Viscosity
2. Turbidity
3. Ultraviolet light absorption
4. -SH group reactivity
5. Electrophoresis
    a. paper
    b. starch gel
6. Immunochemistry(agar gel diffusion).

Viscosity.

An Ostwald capillary viscosometer was used for all measurements. The water discharge time of this instrument was 59.6 secs. The temperature was kept constant at $25^{\circ} \pm 0.01^{\circ}$C. Five mls. of undiluted plasma was used(after centrifugation at 3000 rpm. for 30 minutes) for each run, and four readings were taken for each sample. The results are reported in terms of the viscosity number, $\frac{\frac{n\ plasma}{n\ water} - 1}{concentration(gms.\%)}$.

Use of the viscosity number, where the concentration is taken into account, permits a comparison to be made between all the experiments in spite of varying protein concentrations of the plasma. For each individual experiment the plasma protein concentration of the control and recirculated plasma was found to be identical as measured by a standard Biuret method.

TABLE 2.

Viscosity Changes in Recirculated Plasma

Experiment Number	Viscosity No. of Control	Viscosity No. of Recirculated Sample	% Increase in Viscosity No.
S10 c	0.097	0.114	17.7
S10 d	0.115	0.122	6.0
S10 e	0.129	0.137	6.4
S10 f	0.129	0.142	10.0
S40	0.105	0.123	17.1
M10 a	0.101	0.106	5.0
M10 b	0.073	0.077	5.5
M10 c	0.075	0.078	2.4

Conclusions.

1. Screen Oxygenator recirculation leads to a definite, although small, increase in viscosity.
2. There is no apparent correlation with the period of recirculation.
3. Membrane Oxygenator recirculation also leads to an increased viscosity. This increment is somewhat smaller than that noted with the Screen Oxygenator.

Turbidity.

All samples were optically clear but were nevertheless centrifuged at 3000 rpm. for 30 minutes prior to the measurement of light transmission. 2 ccs. of undiluted plasma was used and duplicate readings taken at 620 m/u

in a Beckmann DU Spectrophotometer. A water blank was used as a measure of 100% transmission.

TABLE 3.

Turbidity Changes in Recirculated Plasma

Experiment Number	Tc % Transmission of Control	Tr % Transmission of Recirculated Plasma	$\frac{Tc-Tr}{Tc}$ x100
S10 a	83.2	74.2	10.8
S10 b	89.3	84.5	5.4
S10 c	79.4	71.0	10.6
S10 d	82.0	80.5	1.8
S10 e	84.5	83.2	3.8
S10 f	86.2	83.2	3.5
S40	81.3	77.8	4.3
M10 a	86.5	83.0	4.0
M10 b	47.5	46.9	1.3
M10 c	40.7	40.7	1.0

Conclusions.

1. Plasma turbidity is minimally increased by recirculation through the Screen Oxygenator.
2. There is no correlation between the increased turbidity and the period of recirculation.
3. Recirculation through the Membrane Oxygenator also produced a turbidity increase. This is less than that seen with the Screen Oxygenator.

The effect of ether extraction on the turbidity of the recirculated plasma was tested by shaking 2 cc. of plasma with an equal volume of anhydrous ether, followed by centrifugation at 2000 rpm. for five minutes, and removal of the ether layer. Repeated measurements of light transmission showed no decrease in turbidity. It was concluded therefore that changes in the lipid phase of the plasma were not responsible for the increased turbidity.

Ultraviolet spectrophotography.

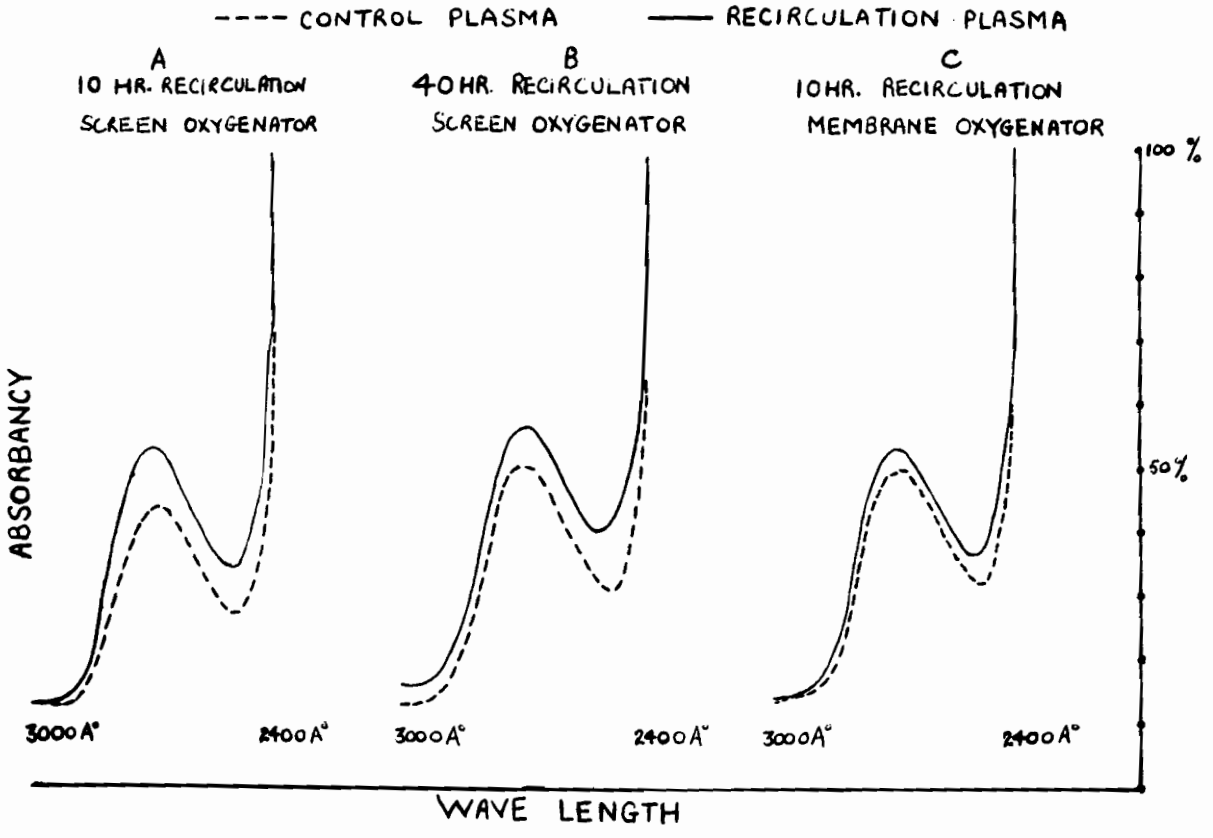
Plasma was diluted 1:2000 with phosphate buffer at a pH of 7.3. A Beckmann DU recording Spectrophotometer was used for measurement of the absorption spectrum over the 2400-3000A° range. The results are shown in Fig. 7. The curves "A" and "C" are typical of the six experiments using the Screen Oxygenator, and the three using the Membrane Oxygenator.

In all cases the recirculated samples show an increased intensity of absorption, most pronounced at 2700A° and 2500A°, as well as a slight shift of the peak to longer wave lengths. These results are similar to the spectrum of denaturation shown in Figs. 3 and 4. Although some of the increased absorption may have resulted from increased light scattering in the recirculated plasma, the greater increase in intensity at two particular wave lengths plus a peak shift to longer wave lengths clearly shows that protein structural changes have occurred. There is little difference between "A" and "B", therefore these changes do not appear to be progressive with time. The spectrum of the sample exposed to the Membrane Oxygenator is essentially similar to those used in the Screen Oxygenator, but the increase in absorption intensity is not as pronounced. Since the increase in turbidity of this particular sample

FIG. 7.

Ultraviolet Absorption Spectra of

Control and Recirculated Plasma.


---- CONTROL PLASMA
—— RECIRCULATION PLASMA
A
10 HR. RECIRCULATION
SCREEN OXYGENATOR
B
40 HR. RECIRCULATION
SCREEN OXYGENATOR
C
10 HR. RECIRCULATION
MEMBRANE OXYGENATOR
100 %
50 %
ABSORBANCY
3000 A°
2400 A°
3000 A°
2400 A°
3000 A°
2400 A°
WAVE LENGTH

was of the same order as that of the sample subjected to Screen Oxygenator recirculation for forty hours a difference in light scattering cannot explain this smaller rise in the absorption.

It has to be concluded that these changes in the ultraviolet absorption curves are proof that proteins are denatured when plasma is recirculated for ten-hour periods through both types of oxygenators. The degree of denaturation, however, appears to be smaller with the use of the Membrane Oxygenator.

-SH Group reactivity.

A large number of analytic methods are available for the determination of sulfhydryl concentration. This multiplicity of techniques is perhaps the best indication of the difficulties that have been encountered and no single method has been shown to be superior to all others. The procedures available include the use of oxidizing, mercaptide-forming and alkylating agents.

The analytical method used for this work is that of Alexander (1958). This is based on the combination of an alkylating agent N-ethylmaleimide(NEM) with the protein -SH groups. The decrease in absorption at 300 mμ that occurs when NEM combines with thiols is used as a means of following the reaction. Details of the procedure are given in Alexander's paper.

TABLE 4.

Changes in -SH Concentration in Recirculated Plasma

Experiment Number	Conc. -SH(µM%) of Control	-SH Conc.(µM%) of Recirculated Plasma
S10 a	38.7	38.7
S10 b	18.1	26.1
S10 c	36.1	51.5
S10 d	31.6	31.6
S10 e	49.0	50.3
M10 a	41.3	29.0
M10 b	48.4	35.5
M10 c	106.0	117.4

It is obvious that there are no consistent findings and therefore no conclusions can be made in relation to the question of plasma protein denaturation. In view of the difficulties of accurate measurement and of the fact that -SH reactivity is one of the less sensitive methods for assessing protein changes, these results are not unexpected.

Electrophoresis.

Standard paper electrophoresis was carried out on control plasma as well as the plasma recirculated through the two types of oxygenators. A Spinco Model R cell was used with barbital buffer of pH 8.6 and Whatmann 3 mm. filter paper. 5 ma. per cell was applied for eighteen hours at room temperature following which the paper strips were stained with bromophenol blue and scanned in a recording Densitometer.

TABLE 5

Protein Fraction	S10 d		S10 e		S40	
	A	B	A	B	A	B
Albumin	56.3	51.3	46.6	39.6	38.1	44.0
$Alpha_1$ Globulin	3.7	3.7	2.8	3.5	3.6	4.1
$Alpha_2$ Globulin	10.4	9.6	10.6	11.5	12.4	10.8
Beta-Globulin	21.1	29.5	27.2	32.0	32.3	31.2
Gamma-Globulin	8.5	5.9	12.9	13.3	13.6	9.9
Protein Fraction	**M10 a**		**M10 b**		**M10 c**	
	A	B	A	B	A	B
Albumin	52.0	51.2	60.7	63.0	54.4	57.3
$Alpha_1$ Globulin	3.8	3.3	2.6	2.2	3.8	3.0
$Alpha_2$ Globulin	11.1	11.7	6.5	6.0	10.4	7.5
Beta-Globulin	24.7	25.8	12.5	10.0	10.7	13.1
Gamma-Globulin	9.0	8.1	17.7	18.7	20.7	19.0

A = Control Sample B = Sample after recirculation

Table 5 shows the percent of the individual protein fractions of the control and recirculated plasma. Although slight differences were always noted, there was no consistent pattern or trend. Recirculation also did not result in the appearance of any new electrophoretic components.

In addition to standard paper electrophoresis, starch gel electrophoresis was carried out on samples obtained from four experiments with the Screen Oxygenator. Starch gel electrophoresis allows the differentiation of a far greater number of protein fractions. As many as thirty components may be demonstrated in human plasma. The main points in this technique are as follows. A suspension of "Starch Hydrolysed" (Connaught Laboratories, Toronto) in dilute borate buffer is heated until a homogenous liquid is obtained. The viscous liquid is poured into suitable trays and allowed to cool. Samples to be studied are inserted (Continued on next page)

into slots in the gel and electric contact made to the ends of the gel by means of filter paper soaked in borate buffer. A potential gradient is applied for about twelve hours, following which the gel is removed from the tray, sliced longitudinally and stained for protein with amido-black. Figure 8 shows the results. The only difference between the control and the recirculated samples appeared to be an increased mobility of the slow-moving alpha-globulin. This fraction has been found to be extremely heat-labile, and to have a large molecular weight(Brown 1954). The increased mobility of this sub-fraction in the recirculated plasma may represent a molecular dissociation resulting from surface denaturation. It is of some interest to note that a number of workers have postulated that macroglobulins represent polymeric proteins capable of ready dissociation to monomeric units(Franklin 1957 and Pedersen 1945).

Immunochemistry.

The immunochemical properties of the control and the recirculated plasma have been studied by means of the double diffusion gel technique described by Ouchterlony(1949). This method makes use of antigen and antibody diffusion in an agar gel medium. A shallow layer of the agar is allowed to solidify in a Petri dish, following which antigen and antibody are poured into wells left in the agar. The antigen and antibody diffuse to meet each other and at this junction a narrow band of precipitate is formed. Each antigen-antibody system gives a separate band. The morphological characteristics of these linear precipitation bands permit detection of the minimal number of reacting components and the determination by comparison of the identity or non-identity of any two antigens or antibodies, as well as their relative concentrations. This is illustrated in Fig. 9.

FIG. 8.

Starch Gel Electrophoresis.

a. Recirculated)
b. Control) $S10_c$

c. Recirculated)
d. Control) $S10_d$

FIG. 9.

Precipitation lines obtained when two antigenic preparations diffuse in a gel towards one preparation of antibodies.

A. Both antigen preparations are identical.

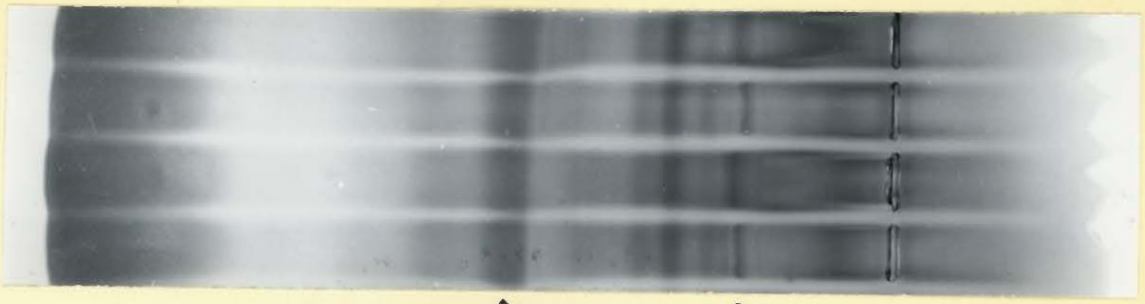
B. One of the antigens(X) is present in a different concentrations.

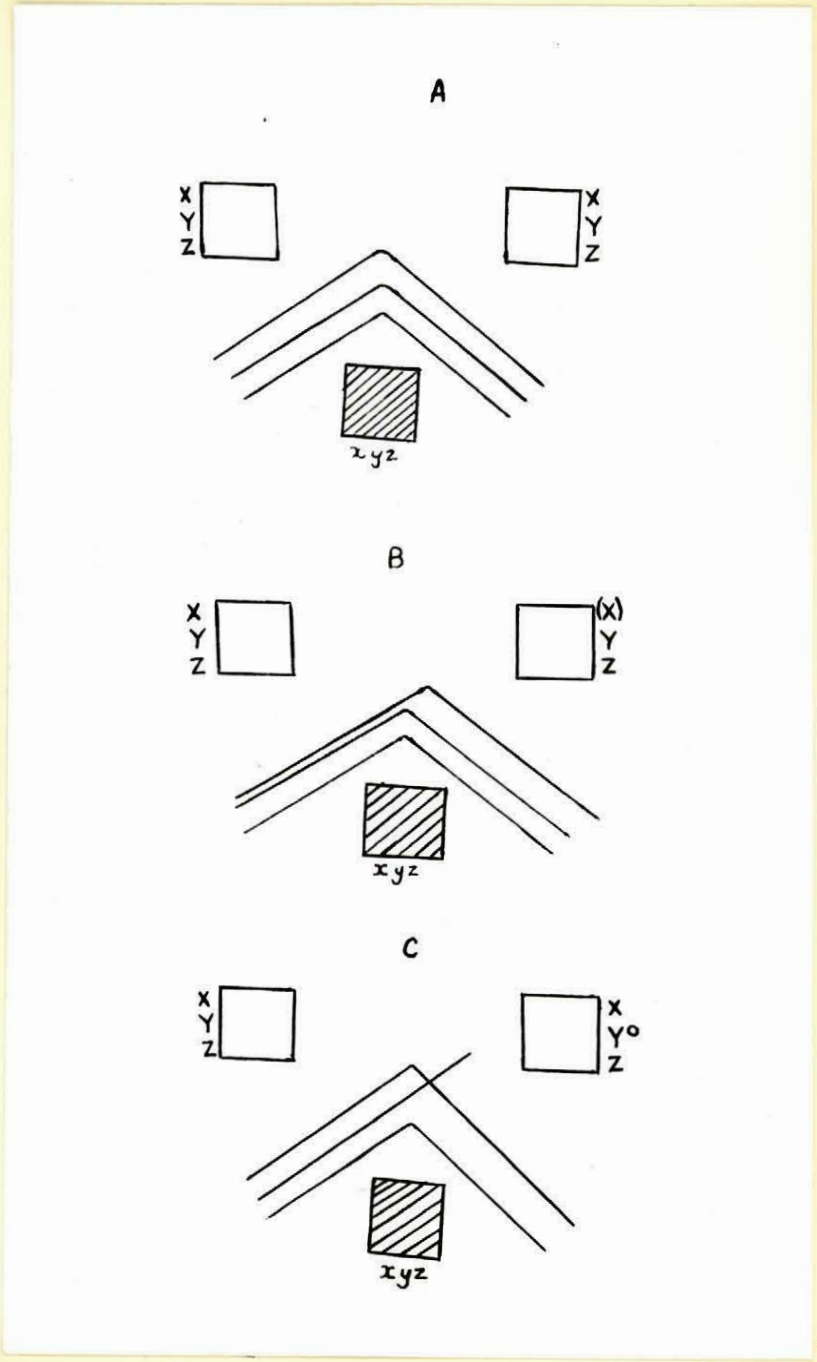
C. One antigen(Y) is absent from one of the preparations.

XYZ - antigens

xyz - antibodies

(From Ouchterlony, Lancet, 256:347, 1949)


a
b
c
d.
ALB.
β-GLOBULIN
S α₂-GLOBULIN


A
X
Y
Z
X
Y
Z
xyz
B
X
Y
Z
(X)
Y
Z
xyz
C
X
Y
Z
X
Y°
Z
xyz

In view of the fact that the globulin fraction of the plasma is more sensitive to surface denaturation than the albumin(Denstedt, personal communication, and Doty 1951) and in order to reduce the number of antigenic systems, the globulin fraction only has been studied. Rabbit anti-canine globulin serum was obtained(Nutritional Biochemical Corporation, Cleveland, Ohio) and allowed to diffuse against the control and recirculated plasma(shown as B and A respectively in the illustrations). The plates were incubated at 37°C. for forty-eight hours, at which time the precipitation bands were maximally developed. The reaction was then terminated by saline dialysis and fixation with 10% formalin, following which the plates were photographed(Figs. 10, 11, 12). In addition to this three-well technique, direct comparison of the Screen and Membrane Oxygenator samples was carried out on the same plate(Fig. 13).

It is seen that there is no profound difference in the agar diffusion patterns between the various samples. Four well-defined precipitation bands were usually identified. No lines of non-identity were present since the corresponding band on each side showed complete fusion. A close scrutiny of Fig. 10 shows that the line of fusion of the most forward band is displaced to the side of the control plasma(B), and the band on this side is at a greater distance from the antiserum. This is evidence of an increased concentration of one particular antigen in the recirculated plasma. From the electrophoretic analysis shown in Table 5 this is most likely to be the beta-globulin. A similar picture is seen in Fig. 13, where this precipitation band of the Screen Oxygenator recirculation sample is again displaced.

It must be concluded that no change in immunological properties as demonstrated by the Ouchterlony technique occurs when plasma is re-circulated through either the Screen or Membrane Oxygenator for prolonged

FIG. 10.

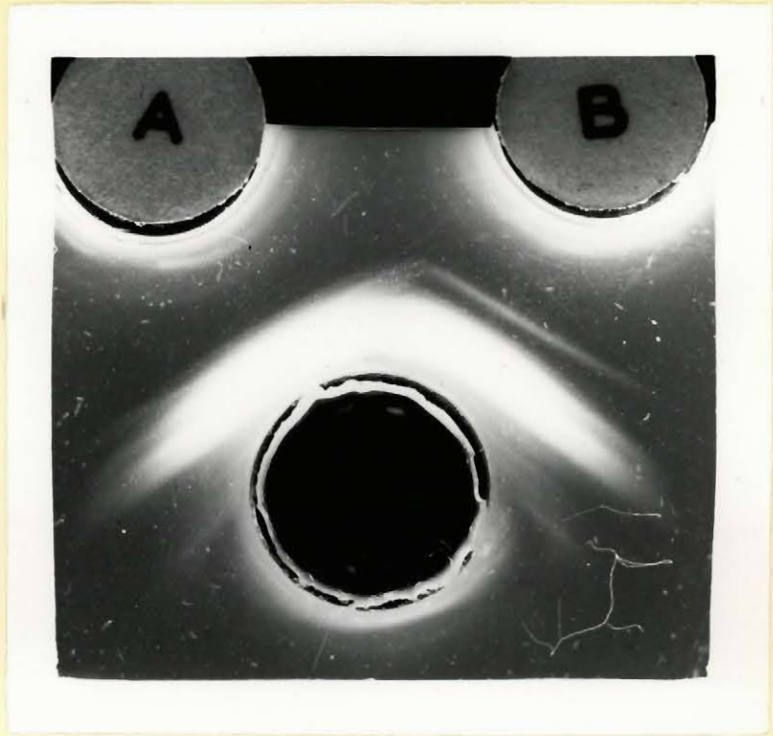
Experiment No. S10 d.

Agar gel diffusion of control plasma(B) and plasma recirculated through Screen Oxygenator for 10 hours (A) against anti-canine globulin.

FIG. 11.

Experiment No. S40.

Agaf gel diffusion of control(B) and plasma recirculated through Screen Oxygenator for 40 hours(A) against anti-canine globulin.


A
B

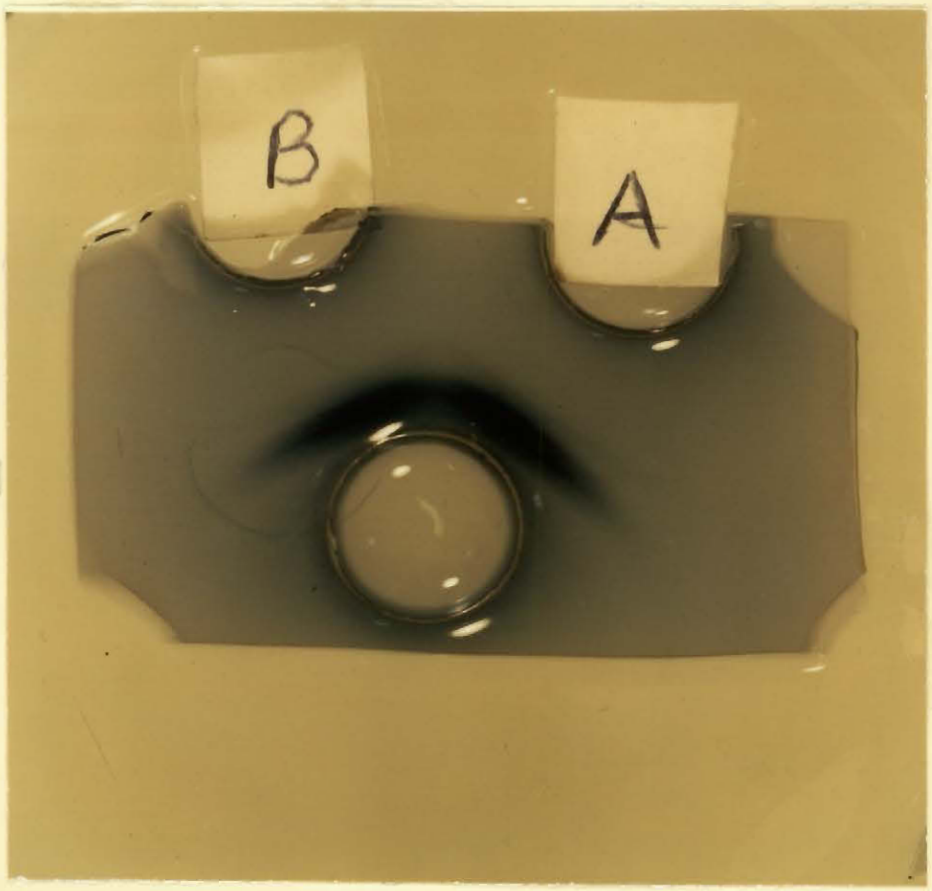

B
A

FIG. 12.

Experiment No. M10 a.

Agar gel diffusion of control plasma(B) and plasma recirculated through Membrane Oxygenator for 10 hours(A) against anti-canine globulin.

FIG. 13.

Agar gel diffusion of control plasma and plasma recirculated through Screen and Membrane Oxygenators against anti-canine globulin.

S - Control

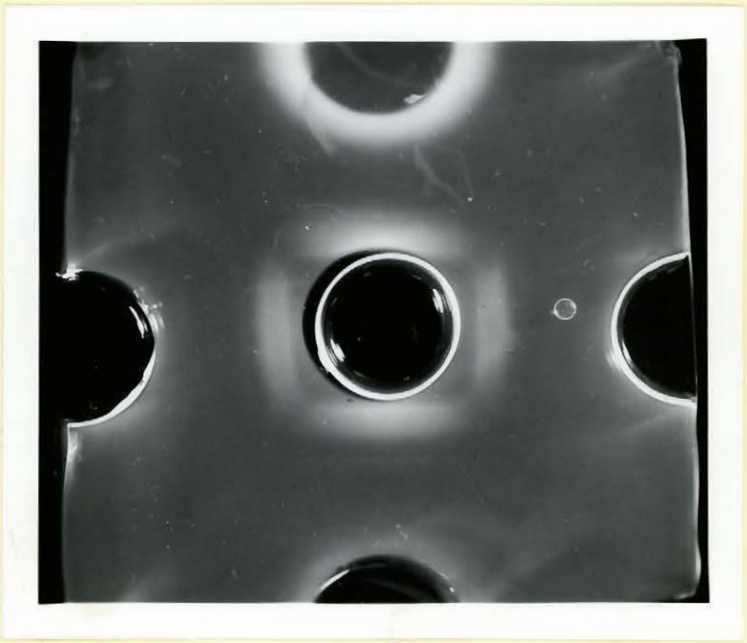
S_1 - Recirculated in Screen Oxygenator for 10 hours.

M - Control

M_1 - Recirculated in Membrane Oxygenator for 10 hours.

Antiserum in centre well.


B
A


S1
M
M1
S

periods of time.

Discussion of Results.

The results recorded above show that protein denaturation occurs when plasma is recirculated through both the Screen and the Membrane Oxygenator for periods of ten hours. This is clear from measurements of the viscosity, turbidity, ultraviolet spectrum and starch gel electrophoretic patterns. The data on changes in sulfhydryl reactivity cannot be satisfactorily interpreted while no changes in immunological properties have been observed with the agar gel diffusion method.

Having established the fact that denaturation occurs it is of some importance to assess the severity of this change. This is obviously quite difficult, since although protein denaturation is not an all-or-none phenomenon, it is not as yet possible to estimate with accuracy the degree of structural modification the protein molecule has undergone. About all that can be done is to compare the results obtained with the findings when proteins have been strongly denatured with agents such as heat, detergents and guanidine.

The increased viscosity of denatured proteins has been extensively studied by Jirgensons(1952) and the increments have been reported in terms of viscosity numbers. This allows direct comparison with the results of this study. Bovine native albumin and gamma-globulin were denatured with guanidine and detergents. The viscosity measurements are given below.

Bovine Native Albumin

	Viscosity Number	% Increase in Viscosity No.
In H_2O at pH 5	0.033	
Treated with 1.5M Guanidine	0.051	54.5
" " 2.5M "	0.150	355
" " 5.0M "	0.229	594
" " 0.5% sodium lauryl sulfate	0.134	306

Bovine Native Gamma-Globulin

In H_2O at pH 5	0.030	
Treated with 1.0M Guanidine	0.151	70
" " 2.5M "	0.177	490
" " 0.5% sodium lauryl sulfate	0.136	353

Even a low concentration of guanidine produced a 54.5% and a 70% increase in the viscosity numbers of denatured albumin and globulin respectively, whereas recirculation of plasma through the Screen Oxygenator for periods of up to forty hours led to an increase of only 17.1%.

The effect of guanidine on whole canine plasma has been assessed. This plasma was denatured by treatment with 2M guanidine hydrochloride for a period of two hours. Two such experiments were carried out and it was found that the percent increase in the viscosity number of the denatured plasma was approximately 90% and 103%.

It is well known that protein denaturation, particularly when effected by heat, leads to an increase in the turbidity. Murray(1953) has shown that heating of plasma at 60°C. for four hours leads to a marked opacity and the formation of a precipitate. Lee(1961) reported

a marked increase in the turbidity of plasma recirculated through various types of oxygenators for periods of ten hours. The most pronounced increase in turbidity occurred with the Bubble Oxygenator, where light transmission was reduced to about 10% of the control value after one hour of recirculation. Disc and Screen Oxygenators led to a 60% decrease in light transmission over a period of ten hours. Lee further demonstrated that most of this increased turbidity was the result of alterations in the lipid phase of the plasma, since extraction with ether restored light transmission to 60-80% of the control values.

The present study certainly fails to confirm this large increase in turbidity with plasma recirculation. The maximum decrease in light transmission was only 10.8%. This slight increase in the turbidity was not related to any lipid alterations since there was no change in the light transmission following ether extraction.

The electrophoretic properties of recirculated plasma have also been studied by Lee. Comparison of the control and recirculated samples using paper electrophoresis showed certain characteristic changes.

1. Condensation of components in the beta-globulin region to form an abnormal band.
2. A decrease in the albumin peak by about 10% with a corresponding rise in the alpha 2, beta and gamma-globulins. These changes were found to increase progressively with increasing time of recirculation.

The results of the present study with paper electrophoresis again do not confirm these findings. No condensation occurs in the beta-globulin region, and there are no consistent changes in any protein sub-fractions. However, using the technique of starch gel electrophoresis

an altered mobility of the S alpha$_2$-globulin has been detected, the implications of which have already been discussed. Comparable work with the Membrane Oxygenator samples was not carried out, so it is not known if this phenomenon is peculiar to the Screen Oxygenator.

The immunochemical studies show that the degree of surface denaturation of plasma proteins produced by recirculation through the oxygenators is not sufficient to lead to any changes in immunological properties.

In conclusion the following points may be noted.

1. Surface denaturation of proteins occurs when plasma is recirculated through both the Screen and the Membrane Oxygenators for periods of ten hours.
2. The degree of denaturation is minimal. It is far less than that produced by treatment of proteins with agents such as guanidine, detergents or heat. This is not surprising in view of the fact that surface forces have been shown to be one of the milder types of denaturants.
3. The fact that some degree of denaturation occurs with the Membrane Oxygenator is unexpected in view of the absence of a direct blood-gas interface. Lee(1961) has theorized that if the membrane is a non-wettable type with a low dielectric constant, such as is the case with Teflon, surface polarizing forces at the plastic-blood interface may denature proteins as at an oil-water interface. It is further stated that the ideal membrane should be hydrophilic with a high dielectric constant. The present study has shown that this hypothesis is entirely valid.
4. The slight degree of denaturation produced by recirculation of

plasma through a Screen Oxygenator should not preclude the use of this machine for prolonged extra-corporeal circulation.

CHAPTER 3.

Lipid Changes in Extra-Corporeal Circulation.

Several recent reports have focussed attention on the problem of fat embolization and a disturbed lipid metabolism as a result of extra-corporeal circulation. The earliest observations were made by Owens(1959, 1960) who found that many patients dying after cardio-pulmonary by-pass showed widespread fat emboli on post mortem examination. A thorough experimental study was carried out in which dogs were subjected to total perfusion with various types of oxygenators or given transfusions of blood that had been recirculated through these various machines. The results of Owens' experimental work can be summarized as follows.

1. Fat emboli were demonstrated in the various organs of the dogs perfused for two hours with the Screen or Bubble Oxygenator.
2. No fat emboli were demonstrated when a Membrane Oxygenator was used.
3. The passage of blood through the mechanical apparatus was responsible for the formation of these emboli, since they were widespread in the organs of dogs who received exchange transfusions of recirculated blood.
4. The oxygenator itself was the cause of this fat embolization since the transfusion of blood that had recirculated through a circuit from which the oxygenator had been removed did not lead to the appearance of fat emboli.
5. The addition of a non-ionic surface active agent, Pluronic F68, to the blood prior to extra-corporeal circulation led to a marked reduction in the severity of fat embolization.

Owens concluded that fat emboli were formed as a result of the coalesence of certain lipid elements of the blood, due to disruption of their surface tension following direct contact of the blood with the oxygenating gas. Pluronic F68 was presumed to exert a stabilizing action by means of its surface activity.

More recently Lee(1961) has stated that fat emboli were seen in 100% of patients subjected to cardio-pulmonary by-pass, and demonstrated that recirculation of plasma through Bubble, Screen and Disc Oxygenators for a period of ten hours led to the formation of chylomicron aggregates and free lipid globules up to 30 μ in size. Transfusion of dogs with this recirculated plasma produced widespread lipid embolization.

The work of Cossman and Kittle(1960) has clearly shown that a deranged lipid metabolism results from cardio-pulmonary by-pass. Measurements of serum lipase and urinary fat were carried out in a series of patients undergoing various surgical procedures. Nineteen were subjected to thoracotomy using extra-corporeal circulation, and a similar group had thoracotomy alone. The degree of lipasemia and lipuria was significantly higher in the first group. Both these factors may have been a reflection of the presence of fat emboli.

In the present study an attempt has been made to study the source of these lipid changes in extra-corporeal circulation. The work to be described can be divided into three sub-divisions.

1. The development of a satisfactory technique for the staining of fat globules directly in plasma smears.
2. The study of qualitative and quantitative changes in the lipids of blood and plasma recirculated for long periods through the Screen and Membrane Oxygenators.

3. A study of fat globulemia in clinical cases undergoing open-heart surgery with extra-corporeal circulation. This was carried out in an attempt to determine whether other factors than the passage of blood through the extra-corporeal circuit could account for these lipid alterations.

Development of a fat staining technique.

For the direct visualization of fat globules in the blood a suitable staining method was desirable. A survey of the literature showed that relatively few techniques had been developed. Peltier(1954) used a water soluble flurochrome, Phosphin 3R, for staining free fat globules in the blood. The advantage of this method was that being water soluble the dye did not dissolve out the smaller lipid particles. However, flourescent microscopy was required. Another water soluble fat stain, Nile blue, was therefore selected for trial, and a satisfactory staining technique was developed.

Bone marrow obtained by sternal puncture of cadavers was suspended in plasma and centrifuged at 2000 rpm. for 10 minutes. An applicator stick inserted to a depth of 1mm. below the meniscus was used to transfer a series of drops of plasma to a clear glass slide. One drop of 1% aqueous Nile blue was then added to each wet plasma smear. When dry, the slide was examined under the low power of an ordinary light microscope. Fat globules were demonstrated as bright orange spheres against a blue background of excess stain.

Qualitative and Quantitative Lipid Changes with Blood and Plasma Recirculation.

Using the above method of detecting fat globules, a series of experiments was designed to see whether fat globules were formed as the result of prolonged recirculation of blood and plasma through the Screen and Membrane Oxygenators. An additional study was carried out on the effect of Pluronic F68. These in vitro recirculation experiments were organized as follows.

1. Recirculation of whole blood through the Screen Oxygenator for three hours - five experiments.
2. Recirculation of whole blood through the Screen Oxygenator for three hours with Pluronic F68 at a concentration of 0.6 mgm./cc. of donor blood, added prior to recirculation- five experiments.
3. Recirculation of plasma through the Screen Oxygenator for ten hours - three experiments.
4. Recirculation of plasma through the Membrane Oxygenator for ten hours - three experiments.

Six smears were stained for each plasma sample. The incidence of fat globulemia has been graded as follows.

0 = no fat globules observed larger than 7 μ per low power field

\+ = one to two globules observed per low power field

++ = two to five " " " " " "

+++ = five to ten " " " " " "

++++ = ten to twenty " " " " " "

+++++ = over twenty " " " " " "

The results are shown in Table 6, and may be summarized as follows.

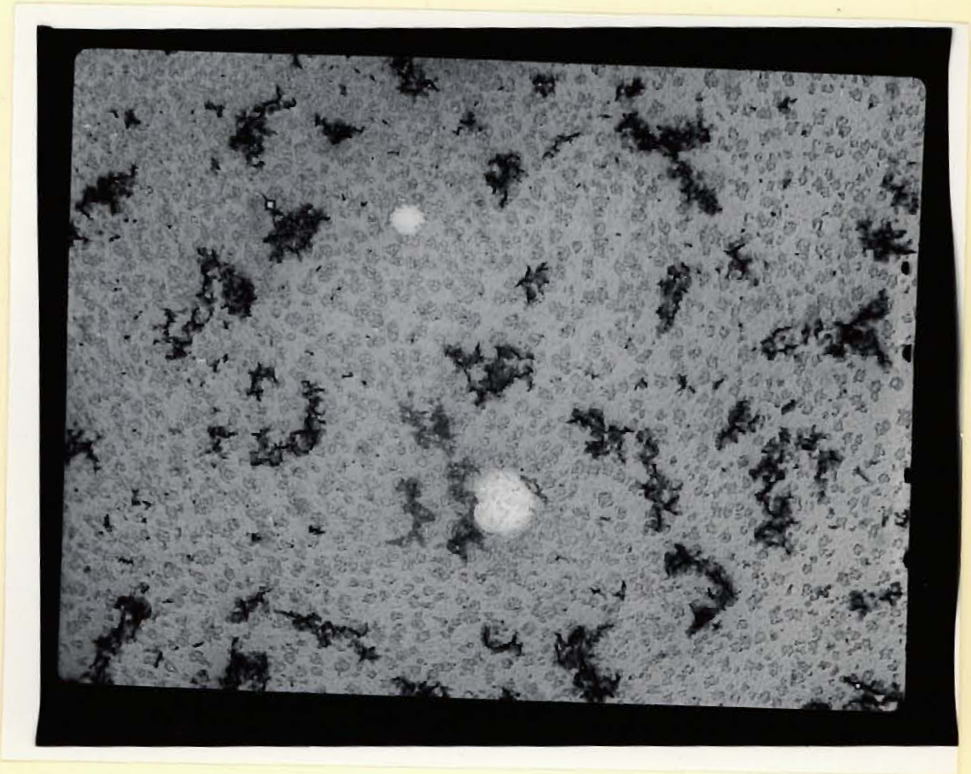
1. Fat globules are formed by the recirculation of blood and plasma through a Screen Oxygenator(Fig.14). These globules may be up to 40 μ in size. They appear as early as five minutes after the start of the experiment, and the degree of globulemia increases progressively with time.
2. The degree of fat globulemia is greater using whole blood rather than plasma.
3. Recirculation of plasma through a Membrane Oxygenator does not lead to the formation of any lipid globules.
4. Globule formation is completely prevented by the addition of a non-ionic surface agent to the pooled donor blood.

Quantitative studies of the total plasma lipids before and following recirculation were also carried out. Total lipids were measured using the procedure of Folch(1957), based on chloroform-methanol extraction and direct gravimetric analysis. The results are listed in Table 7, and from a study of these it may be concluded that

1. recirculation of whole blood through the Screen Oxygenator leads to an increase of the total plasma lipids, which averaged 36.7% for a four-hour recirculation
2. there is no progressive increase in total plasma lipids with longer perfusion times
3. total plasma lipids do not increase when plasma is substituted for whole blood, and the lipid increase must therefore be related to cellular breakdown.

FIG. 14.

Photomicrograph of stained plasma smear after 10-hour recirculation of blood in Screen Oxygenator. Two fat globules are shown, the larger measuring 40 μ.



Fat Globulemia in Open-Heart Surgery.

Having established the fact that fat globules up to 40 μ in size could be formed as a result of the repeated passage of blood and plasma through the Screen Oxygenator, a further series of experiments was carried out on clinical cases subjected to open-heart surgery using extra-corporeal circulation. A study of this nature was felt to be necessary in determining whether other factors than the oxygenating process were involved in the phenomenon of fat globulemia. A series of ten consecutive cases was selected for this study. With five of these patients a Disc Oxygenator was used, and with the other five a Screen Oxygenator was employed. Samples for fat staining were selected as follows.

1. Control specimens from the pooled donor blood in the oxygenator.
2. After ten minutes of recirculation prior to the onset of by-pass.
3. Ten minutes after commencing by-pass.
4. At the termination of by-pass.

The results of this experiment are shown in Table 8 and Figs. 15 and 16.

It is obvious that fat globulemia occurs in the majority of patients subjected to extra-corporeal circulation. The degree of globulemia is not progressive with time, and much larger globules are seen than were visualized during the in vitro recirculation experiments. Globules of up to 100 μ were regularly seen in blood samples taken from the machine during by-pass. It appeared likely therefore that other factors than the oxygenator itself were involved.

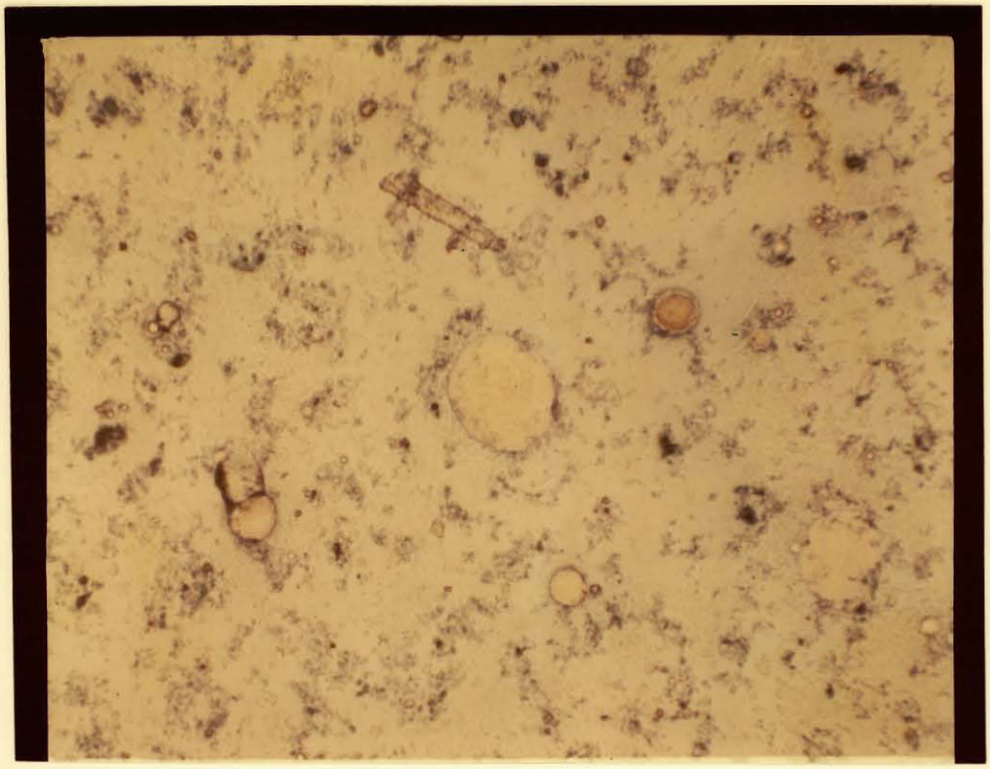
During the course of these operations involving cardio-pulmonary by-pass it was noted that pooled blood lying in the mediastinum

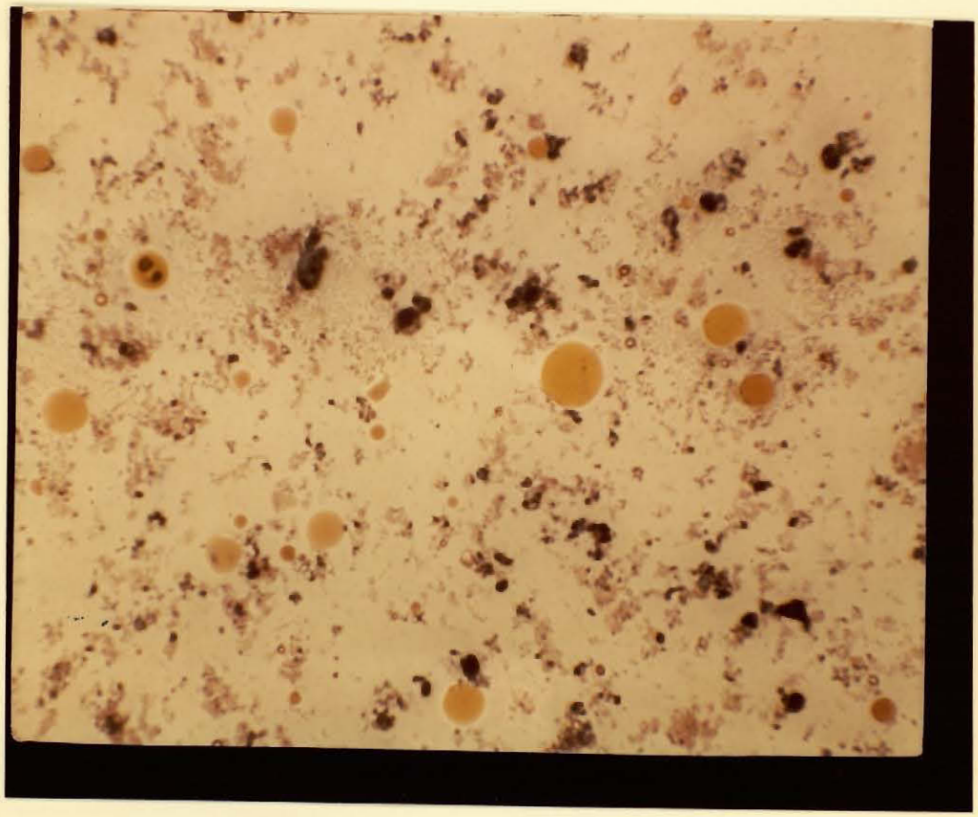
FIG. 15.

Photomicrograph (x160) of stained plasma smear of sample taken from Screen Oxygenator after 90-minute perfusion.(Patient No.1, Table 8) Largest fat globule measured 100 μ.

FIG. 16.

Photomicrograph (x80) of stained plasma smear of sample taken from Disc Oxygenator after 65-minute perfusion.(Patient No.10, Table 8)





was usually aspirated through the coronary suction system back into the extra-corporeal circuit. It was suspected that free fat globules released from mediastinal fat depots by trauma, could be present in this extra-cardiac blood pool. A second series of clinical studies was therefore carried out, and in addition to the samples listed above, for the first series, an additional specimen was taken from the pooled mediastinal blood. In five patients this blood was aspirated into the extra-corporeal circuit, and in the other five the coronary suction system was not employed for any extra-cardiac aspiration. The results are shown in Table 9 and Figs. 17 and 18.

In all cases the samples of blood taken from the pooled mediastinal blood contained massive amounts of fat with some samples having globules as large as 500 μ. In addition it was noted that the incidence and severity of fat globulemia during by-pass was far greater in those cases in which the blood in the mediastinum was aspirated into the extra-corporeal circuit.

Discussion.

A number of points brought out by the above results require further discussion. These are:

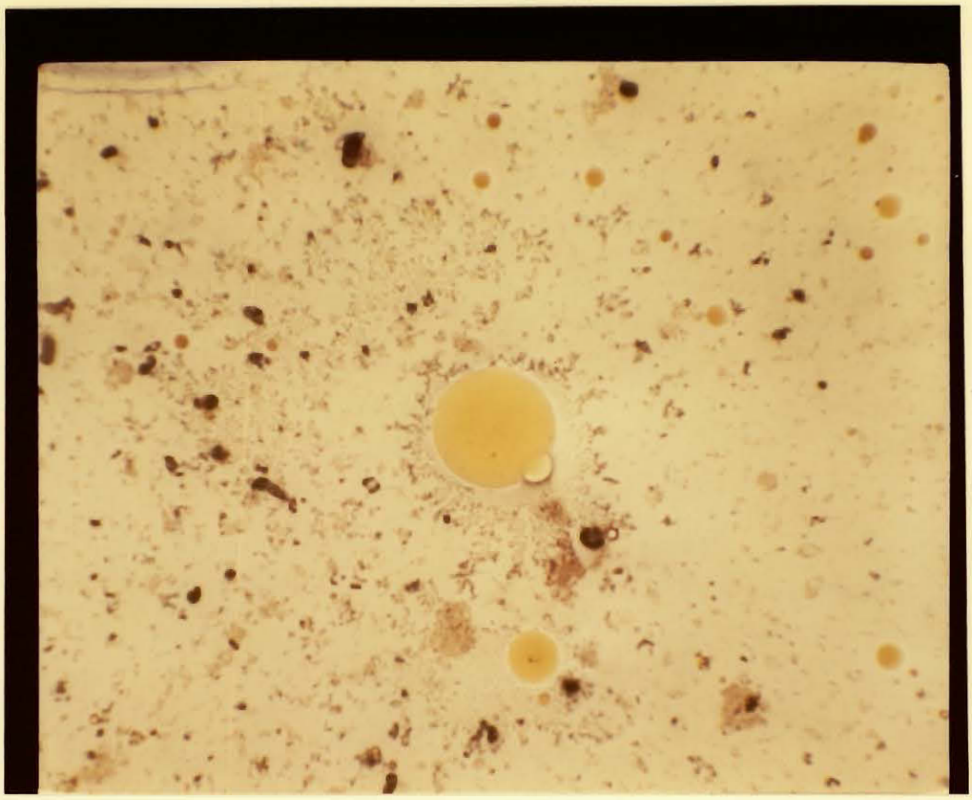
1. The mechanisms of formation of fat globules by recirculation of blood and plasma in the Screen Oxygenator.
2. The cause of the rise in total plasma lipid following blood recirculation and the observation that the severity of globulemia is greater when blood rather than plasma is recirculated.
3. The protective action of Pluronic F68.

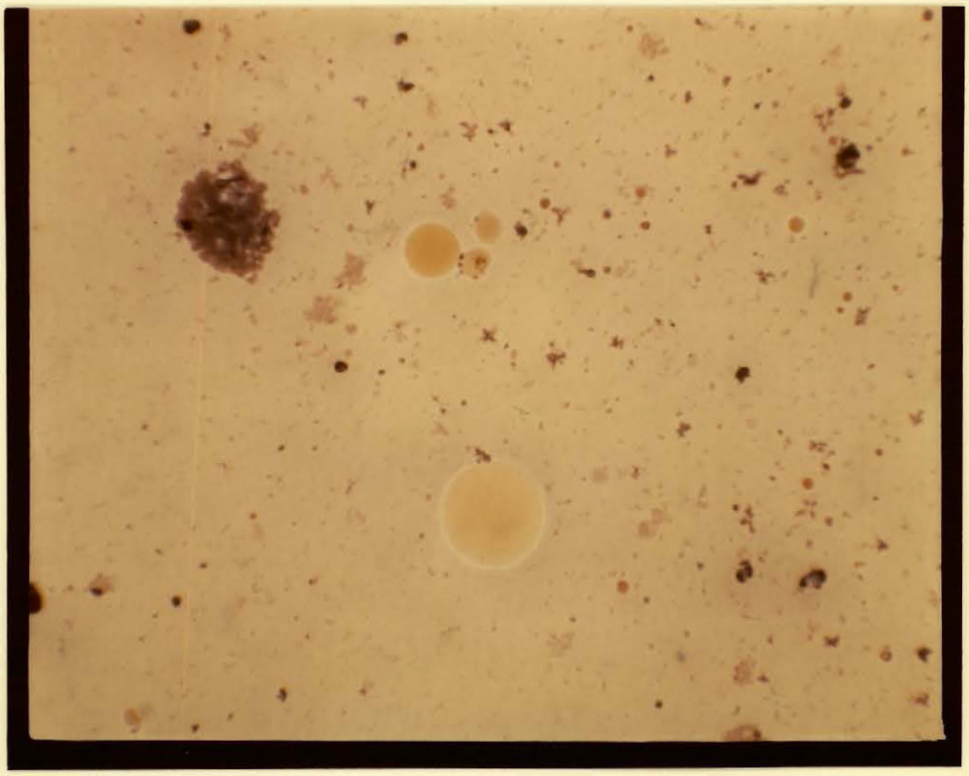
FIG. 17.

Photomicrograph (x80) of stained plasma smear from pooled mediastinal blood.(Patient No.11, Table 9) Large fat globule seen measured 200 μ.

FIG. 18.

Photomicrograph (x80) of stained plasma smear from pooled mediastinal blood. Largest fat globule seen measured 150 μ.





In order to suggest possible mechanisms responsible for the phenomenon of fat globulemia it is useful to approach the problem by an application of what is already known about colloidal systems. Although the complexities of this field are outside the scope of this discussion, a review of the basic concepts in so far as emulsions and their stability are concerned is required, in view of the fact that the blood chylomicrons are an ideal example of a stable biological emulsion.

An emulsion may be regarded as a system of two immiscible liquids in which the dispersoid is distributed as small drops throughout the dispersive medium. The tendency to form a stable emulsion depends on two main factors.

1. The size of the dispersoid droplets.

   A colloidal emulsion is arbitrarily defined as one in which the dispersed particle size is in the range of 1/u to 0.001/u. Particles larger than 1/u will not form emulsions, while particles smaller than 0.001/u are regarded as being in a true solution. This small size of the droplets contributes appreciably to the emulsion stability, since the normal tendency to settle out by gravity is much reduced.

2. The Presence of an emulsifying agent.

   These agents will stabilize emulsions even when present in small amounts. A number of properties are responsible for stabilizing action, and any particular emulsifying agent may possess one or more of these properties.

   a. Surface activity. A surface active agent is one that has the ability to reduce surface tension. The introduction of a large number of small droplets into another liquid greatly increases the surface area of the dispersing liquid. This process of surface expansion (continued on next page)

requires energy. A reduction in surface tension markedly reduces this energy requirement.

b. Net surface charge. Whenever an emulsifying agent is added to an emulsion it collects at all available surfaces. Thus its concentration is high not only on the surface of the solution but also on the surfaces of the dispersoid particles, forming an outer coating. If the emulsifying agent possesses a net electric charge the colloidal particles repel each other and aggregation is prevented.

c. Solvation and hydration. Many stabilizing agents are attracted to the dispersing medium. This property is usually considered in relationship to water which is the most common medium in biological emulsions. The emulsifying agent by means of dipole linkages and hydrogen bonds is able to bind molecules of water, and since water molecules are also loosely interlinked with each other, the protective agent itself becomes interlinked with the aqueous phase and particle aggregation is impaired.

It is surprising to find from a review of the literature that the chylomicron emulsion system of the blood has received little attention from colloid chemists. In contrast a considerable body of information exists concerning the colloidal properties of milk, which may be regarded as a closely related lipid emulsion. It has been shown that the stability of this emulsion system is due to a protective protein film adsorbed on the surface of the fat globules. The protein of this stabilizing film has been separated, and on the basis of its physico-chemical properties

identified as a gamma-globulin(Brunner 1953).

All proteins carry a net surface charge(except at the isoelectric point), and in addition bind water molecules. These two properties are responsible for the stabilizing action of the protein film, and the tendency of the fat globules to aggregate is prevented.

The stability of the lipid emulsion of milk or cream can be reduced by measures designed to alter or remove the protein films. In the formation of butter by churning, the mechanical agitation leads to surface denaturation of the stabilizing proteins. These denatured proteins undergo a loss of bound water, and decreased solubility. A tendency to flocculate develops, facilitating fat agglomeration(Jirgensons 1958). Another viewpoint concerning the coalesence of fat globules during churning is expressed by Dean(1948) who feels that the air surfaces introduced by the churning process compete for the protein, and the lipid emulsion is broken because the protein is preferentially adsorbed on the air bubbles. Regardless of the exact mechanism concerned, it is clear that alteration or denaturation of the protein film surrounding the lipid particles causes a marked loss of stability of the emulsion system.

Chylomicrons, like the fat globules of milk, have been shown to have an outer protecting film of proteins(Ludlum 1931) which is responsible for the stability of the chylomicron emulsion. Ludlum has demonstrated that reagents such as alkalis, acids and alcohol(which precipitate proteins or otherwise alter their properties) are effective in producing chylomicron coalesence. Fat globule formation by recirculation through the Screen Oxygenator may be theoretically outlined as follows.

It has been shown by Lee(1961) (continued on next page)

and confirmed by work described in Chapter 2, that plasma proteins undergo denaturation by surface forces during recirculation through various oxygenators. This denaturation may reduce the effectiveness as stabilizers of the proteins coating the chylomicrons, owing to changes such as impaired water binding, altered surface charge and decreased solubility. Partial loss of this stabilizing action leads to chylomicron aggregation, whereas complete loss leads to removal of the protein envelope and actual coalesence of the lipid residue to give large fat globules.

The fact that fat globulemia is more pronounced with blood rather than plasma recirculation may be related to the increment in total plasma lipids following whole blood recirculation. Owens(1960, and personal communication) found that an increase in total plasma lipids occurred when various gases were bubbled through columns of blood. The average increase was 6%. This increase in lipid concentration must be related to the deterioration or breakdown of the cellular components of the blood, and the consequent release of free lipids into the plasma. It is also interesting to observe that the rise after ten hours of recirculation is no greater than it is after four hours. This must mean that the lipid release is already maximal after a period of four hours. Platelets have been found to be particularly rich in lipid(Troup 1961), the concentration being 17% of its dry weight, whereas the corresponding figure for the erythrocytes is only 1.3%. Platelet lipid has been estimated to be 100 µgm./cc. of whole blood. Approximately 75% of this is made up of phospholipids such as lecithin and phosphatidyl ethanolamine. Leucocytes have been found to be rich in lipoid materials particularly in the granules of the neutrophils and eosinophils(Rebuck 1947).

It is reasonable to assume then that the increase in total plasma lipids seen with blood recirculation must be based on cellular damage. This increase may be related to the higher incidence of fat globulemia in recirculated blood. Two possible mechanisms are suggested.

1. Approximately 75% of the cellular lipids in the blood are phospholipids, particularly lecithin(Troup 1961). It has been demonstrated by Corrau and Lewis(1924) that while lecithin stabilizes water-in-oil emulsions and cholesterol promotes the oil-in-water type, the inversion point of systems containing both agents corresponds to a lecithin: cholesterol ratio of 1:1. Since this is the approximate ratio found in serum the possibility exists that a high lecithin ratio may tend to reverse and break the chylomicron emulsion system.
2. The released lipids may be bound to the protein films surrounding the chylomicron particles. This adsorption may lead to a loss of water of hydration and thus a decreased stability.

It now remains to consider the protective action of Pluronic F68 against the formation of fat globules. This substance which is a polyoxyethyleneoxypropylene polymer, is a non-ionic surface active agent. It is extremely soluble in water, has no hemolytic effect and is completely non-toxic, and has found extensive use as a stabilizer for clinical fat emulsions such as Lipumul. The interaction of proteins with anionic and cationic detergents has been a subject of considerable investigation. It has been shown that the usual result of such interaction is the denaturation of the protein with the usual changes in physicochemical properties(Putnam 1948). Until recently, however, the interaction

of non-ionic detergents with proteins had not been completely investigated, and it was assumed generally that they do not interact with proteins. Dowben(1961) has found that non-ionic detergents,such as the polyoxy-ethylene polymer Triton X-100, do indeed interact with proteins. No gross structural change suggestive of denaturation occurred in the protein, but instead there was increased hydration associated with binding of the detergent, and the envelope of structured water around the protein was reinforced and enlarged.

It is clear then that Pluronic F68 could act to protect the stability of the chylomicrons in two ways.

1. By its ability as a surface active agent to lower the surface tension.
2. By being bound to the protein film on the chylomicrons it could stabilize and extend the protective water envelope which acts as an inhibiting factor against particle aggregation.

The importance of fat globulemia lies in its relationship to fat embolism. Certainly fat globules of a size up to 500 μ as seen in the blood from the heart-lung machine must be regarded with suspicion. The studies reported above show that recirculation of blood through an oxygenator with a blood-gas interface leads to the formation of large fat globules. Another important cause of globulemia, which has been hitherto unrecognized, is the aspiration of pooled mediastinal blood containing free fat into the extra-corporeal circuit. The prevention of fat globulemia therefore rests on the addition of a stabilizer such as Pluronic F68 to the pooled donor blood prior to the onset of by-pass and the restricted use of the coronary suction system outside the heart. These two measures should reduce the hazard of fat embolism during prolonged extra-corporeal circulation.

TABLE 6

FAT GLOBULEMIA IN RECIRCULATION EXPERIMENTS

A. Recirculation of Blood Through Screen Oxygenator

Experiment Number	Control	5 Minutes	15 Minutes	30 Minutes	1 Hour	3 Hours
1.	0	0	0	+	++	++
2.	0	+	+	+	+	++
3.	0	0	0	0	+	+++
4.	0	0	0	0	0	++
5.	0	++	++	++	++	+++

B. Recirculation of Blood Through Screen Oxygenator; Pluronic F68 Added to Pooled Donor Blood (0.6 mg./cc.)

Experiment Number	Control	5 Minutes	15 Minutes	30 Minutes	1 Hour	3 Hours
1.	0	0	0	0	0	0
2.	0	0	0	0	0	0
3.	0	0	0	0	0	0
4.	0	0	0	0	0	0
5.	0	0	0	0	0	0

C. Recirculation of Plasma Through Screen Oxygenator

Experiment Number	Control	5 Mins.	15 Mins.	30 Mins.	1 Hour	3 Hours	10 Hours
1.	0	+	+	+	+	+	+
2.	0	0	0	0	+	+	+
3.	0	0	0	+	+	+	++

D. Recirculation of Plasma Through Membrane Oxygenator

Experiment Number	Control	5 Mins.	15 Mins.	30 Mins.	1 Hour	3 Hours	10 Hours
1.	0	0	0	0	0	0	0
2.	0	0	0	0	0	0	0
3.	0	0	0	0	0	0	0

TABLE 7

TOTAL LIPID CONCENTRATION IN CONTROL AND RECIRCULATED SAMPLES

A. Recirculation of Whole Blood Through Screen Oxygenator for 4 Hours

Experiment Number	Total Lipids(mgms.%) in Control Sample	Total Lipids(Mgms.%) in Recirculated Sample	% Increase in Total Lipids
1.	175	365	108
2.	347	449	29.4
3.	410	426	3.9
4.	330	444	34.6
5.	576	620	7.6
		Average % Increase:	36.7

B. Recirculation of Whole Blood Through Screen Oxygenator for 10 Hours

Experiment Number	Total Lipids(mgms.%) in Control Sample	Total Lipids(mgms.%) in Recirculated Sample	% Increase in Total Lipids
1.	387	439	13.4
2.	550	753	42.0
3.	303	468	54.3
4.	656	726	11.0
		Average % Increase:	30.0

C. Recirculation of Plasma Through Screen Oxygenator for 10 Hours

Experiment Number	Total Lipids(mgms.%) in Control Sample	Total Lipids(mgms.%) in Recirculated Sample	% Increase in Total Lipids
1.	560	545	-2.7
2.	365	373	2.2
3.	346	415	20.0
4.	615	627	2.0
5.	479	471	-1.7
6.	410	405	-1.2
7.	806	813	0.9
8.	366	360	-1.7
		Average % Increase:	2.2

TABLE 8

FAT GLOBULEMIA IN CLINICAL OPERATIONS USING EXTRA-CORPOREAL CIRCULATION

Patient	Diagnosis	Incision	Duration of By-pass	Oxygenator Used	Fat Globules in Samples 1	2	3	4
1.	Tetralogy of Fallot	Median Sternotomy	90 mins.	Screen	0	0	++++	++++
2.	Mitral Stenosis	Left Thoracotomy	40 mins.	Disc	0	0	++	+
3.	Pulmonic Stenosis ASD	Median Sternotomy	60 mins.	Disc	0	0	++++	++
4.	ASD Me	Median Sternotomy	30 mins.	Screen	0	0	+	0
5.	VSD	Median Sternotomy	60 mins.	Disc	0	0	0	0
6.	Transposition of Great Vessels	Median Sternotomy	35 mins.	Screen	0	0	++	++
7.	VSD ASD	Median Sternotomy	47 mins.	Screen	0	0	0	0
8.	VSD ASD Pulmonic Stenosis	Median Sternotomy	127 mins.	Screen	0	0	++	0
9.	Aortic Stenosis	Median Sternotomy	60 mins.	Disc	0	0	+++	+
10.	Aortic Insufficiency	Median Sternotomy	65 mins.	Disc	0	+	++	0

Samples: 1 - Control
2 - 10 mins. recirculation before by-pass
3 - 10 mins. after commencing by-pass
4 - Termination of by-pass

Grading of fat globules:
Grade 0 - No fat globules observed larger than 7 u.
\+ - 1-2 " " " per low power field
++ - 2-5 " " " " " " "
+++ - 5-10" " " " " " "
++++ - 10-20 " " " " " "
+++++ - Over 20

TABLE 9

FAT GLOBULEMIA IN CLINICAL OPERATIONS USING EXTRA-CORPOREAL CIRCULATION WITH AND WITHOUT ASPIRATION OF MEDIASTINAL BLOOD

A. Coronary Suction Not Used Outside Heart

Patient	Diagnosis	Incision	Duration of By-pass	Oxygenator Used	Fat Globules in Samples 1	2	3	4	Mediastinal
11.	Pulmonic Stenosis	Median Sternotomy	20 mins.	Screen	0	0	0	0	+++++
12.	Tetralogy of Fallot	Median Sternotomy	70 mins.	Screen	0	0	0	0	+++++
13.	ASD Secundum	Right Thoracotomy	44 mins.	Disc	0	0	+++	0	+++++
14.	ASD Secundum	Right Thoracotomy	34 mins.	Disc	0	0	0	0	+++++
15.	Aortic Stenosis	Median Sternotomy	81 mins.	Disc	0	0	0	0	+++++

B. Coronary Suction Used Outside Heart

Patient	Diagnosis	Incision	Duration of By-pass	Oxygenator Used	Fat Globules in Samples 1	2	3	4	Mediastinal
16.	Aortic Stenosis	Median Sternotomy	90 mins.	Disc	0	0	0	0	+++++
17.	VSD Patent Ductus	Median Sternotomy	45 mins.	Screen	0	+	0	+	+++++
18.	VSD	Median Sternotomy	90 mins.	Screen	0	0	*	+	+++++
19.	Aortic Stenosis	Median Sternotomy	99 mins.	Disc	0	0	0	+++	+++++
20.	ASD Primum	Right Thoracotomy	67 mins.	Screen	0	0	+	0	+++++

CHAPTER 4

CELL DAMAGE IN PROLONGED BLOOD RECIRCULATION

In contrast to the non-cellular components of the blood, a great deal of work has already been carried out on cell damage during extra-corporeal circulation and it is known that all three types of cells, platelets, leucocytes and erythrocytes,are affected.

As would be expected, the most severe damage is done to the platelets. Thrombocytopenia occurs as a result of deposition on foreign surfaces, direct mechanical trauma, and activation of the coagulation mechanism at points of turbulence(Perkins 1958, Osborn 1956). The major drop in platelet counts occurs within five minutes, and from then on the rate of destruction is quite slow(Kirby 1958). Using a Bubble Oxygenator Penick(1958) found that platelets dropped to 52% of the pre-perfusion level within five minutes. In spite of platelet values as low as $50,000/mm^3$, clinical signs of thrombocytopenia, such as a positive Rumpel-Leede test,have not been reported(Brown 1958).

Leucocytes have also been shown to be damaged by the extra-corporeal circulation. Melrose(1953) found that with partial by-pass at a flow rate of 750-1000 ml. per minute continued for one hour the leucocyte count dropped from $8,200/mm^3$ to $4,800/mm^3$. The mature granulocytes were the most severely affected. Melrose felt that this leucopenia could constitute a problem in long-term perfusions. However, several subsequent studies have shown that this leucopenia during perfusion is followed by a rapid rise to normal levels, or even by leucocytosis(Brown 1958, Gerbode 1958).

A rise in the free plasma hemoglobin is always seen with extra-corporeal circulation, regardless of the type of oxygenator or pumping

system used. Most of this damage is produced by the mechanical pumps, although the shearing force of gas bubbles is an additional factor in the Bubble Oxygenator(Merendino 1957). In addition to the immediate erythrocyte destruction that occurs during the by-pass, it has been found that the surviving cells have a decreased life span. This has been shown by Brown and Smith(1958) using radio-active chromium tagging. A delayed post-operative anaemia has been described by Kreel(1960) as being part of the so-called "Post-perfusion syndrome".

A limited study of cell damage during prolonged recirculation of blood has been carried out. In view of the extensive literature available concerning platelet destruction no work was done in this field. One aspect of the problem of cell damage that has hitherto received little attention is the release of substances with pharmacological activity from damaged cells. It is known that platelets contain serotonin, white cells have significant amounts of histamine, while the erythrocytes contain substances such as catecholamines, adenosine triphosphate and potassium. A preliminary study into this field has been carried out by measuring changes in the adenosine triphosphate, total catecholamines and potassium of the plasma following blood recirculation.

Methods and Materials.

The Screen Oxygenator was employed for all experiments. 1200 ccs. of fresh heparinized canine blood was used for recirculation and 100% oxygen was employed for blood oxygenation. In five cases the experiment was continued for ten hours, whereas in the other five the period of recirculation was only four hours. The following factors were measured.

1. Free plasma hemoglobin.

   This was estimated with the benzidine spectrophotometric method as modified by Hanks(1960).

2. Plasma adenosine triphosphate.

   A micro method using a pyrophosphatase prepared from the potato was employed, the details of which are described in the paper of Rehell(1952).

3. Plasma inorganic potassium.

   This was determined by standard flame photometry.

4. Total plasma catecholamines.

   All estimations were carried out by the Anaesthesia Department of the Montreal Neurological Institute, through the courtesy of Dr. R.A. Millar. A fluorimetric trihydroxy-indole method was used, the details of which have been fully described by Millar and Benfey(1958).

5. White cell counts.

   In the first eight experiments counts were done on control and terminal recirculation samples. In the remaining two experiments serial counts were carried out in order to assess the relationship between leucocyte breakdown and duration of recirculation. All counts were done in duplicate.

Results.

The results are shown in Tables 10, 11 and 12, and may be summarized as follows.

1. Increase in free plasma hemoglobin. This was progressive with the time of recirculation.

2. Marked rise in the plasma ATP levels.
3. Minimal increase in total catecholamines.
4. Minimal increase in plasma potassium.
5. A decrease in the leucocyte count averaging 50% over a period of four hours. The rate of destruction was maximal in the first five minutes.

Discussion.

Provided that an accurate method of measurement is used, then erythrocyte damage is a function of the increase in free plasma hemoglobin. Red cell damage caused by the extra-corporeal circuit cannot be properly assessed when a patient or experimental animal is placed in the circuit, since an appreciable amount of the plasma hemoglobin is cleared by the subject. This point has been emphasized by Ferbers and Kirklin(1958) who showed that after one hour of recirculation the plasma hemoglobin was approximately ten times the value when an animal was incorporated into the circuit. The mechanisms for the removal of free hemoglobin from the plasma include transformation in the blood to methaemoglobin, absorption by the reticulo-endothelial system, and renal excretion.

The highest value for the free plasma hemoglobin in the present series was 396 mgms.% after a ten-hour recirculation. Harrison(1947) has shown that in dogs levels of 3700 to 8000mgms.% are required to produce renal damage. The Screen Oxygenator may therefore be used without fear of renal damage on the basis of excessive plasma hemoglobin, for periods of up to ten hours, and probably much longer. Only a few scattered reports are available concerning the degree of hemolysis produced by recirculation for longer than ten hours. Taylor(1959) using a Membrane Oxygenator with diaphragmatic pumps found that the

average increase in plasma hemoglobin was 3 mgm./100 ml./hr. This worker has suggested that for prolonged perfusions the rise in plasma hemoglobin may be controlled by the periodic replacement of small amounts of the recirculating blood with fresh donor blood.

Of far greater importance than the accumulation of plasma hemoglobin may be the release from the red cells of other substances that possess pharmacological activity. It can be seen from Table II that plasma normally does not contain measurable amounts of ATP, but significant amounts of this agent appear following a ten-hour period of blood recirculation. The amount of ATP released appears to correlate with the degree of hemolysis. This is to be expected, since although small amounts of ATP are known to be present in the platelets, the major part of the blood cell ATP is present in the erythrocytes(Born 1956).

The pharmacological effects of ATP are well known. It leads to peripheral vasodilation, hypotension, bradycardia, impaired conduction with heart block, an increased respiratory volume, and decreased renal blood flow(Davies 1951). The deleterious effects of hemolysed blood have been recognized for many years. Phemister and Handy(1927) attributed this to the release of ATP from damaged cells. Deyrup(1951) has shown that quantities of adenosine derivatives from about 3 - 18% of the erythrocytes hemolysed in a given sample of blood produce marked hypotensive states. Shock has also been recognized as a possible complication after the transfusion of hemolysed blood(Chambliss1950). Disturbances in blood clotting have been attributed to hemolysis. Fogelman(1953) found an increased bleeding time when high degrees of hemolysis were produced. This is of interest since Libber(1955) using duck blood has shown that ATP exerts an anti-coagulant effect, partly

by the decalcification of plasma, and partly by forming a bond with thromboplastin.

It is of obvious clinical importance to estimate whether the amount of ATP liberated during extra-corporeal circulation could have any marked pharmacological activity. Davies(1951) studied the circulatory and respiratory effects of ATP in man. The main findings were a moderate fall in blood pressure, a decreased renal flow, and peripheral vasodilation. The average dose used was a single intravenous injection of 40 mgms. of the sodium salt of ATP dissolved in 50 ccs. of sterile saline and administered at a rate of 5 ccs./minute. Assuming that patients of average size were used with a blood volume of 5.5 litres, this corresponds to a blood concentration of less than 1 mgm.%. Concentrations of ATP as high as 16 mgm.% could have profound effects. It should be realized however that the body is capable of destroying ATP in the plasma by means of an enzyme, and for this reason stored hemolysed blood has little ATP activity(Phemister and Handy, 1927). This question cannot be settled without further experimental work, but the theory that released ATP may be a cause of some of the complications of extra-corporeal circulation must be regarded with an open mind. This idea has already been proposed by Sarajans and Frick(1959, 1960). These workers carried out simulated extra-corporeal circulation in dogs and rabbits by inserting a long section of polyethylene tubing from the femoral artery to the external jugular vein. The inside of the tube was scratched with steel wool to simulate the blood trauma caused by an artificial heart-lung machine. ATP levels measured on the average 1.9 mgms.% after a period of 90 minutes of shunting in the dog. A concurrent rise in plasma serotonin was also noted and attributed to platelet breakdown. It was concluded that released ATP and serotonin

could explain some of the pathological effects of extra-corporeal circulation such as hypotension, cyanosis, and a delay in the recovery from barbiturate anaesthesia.

In contrast to the marked rise in ATP there is only a minimal increase in the total catecholamines. These substances, which include adrenaline, nor-adrenaline and dopamine, are liberated from the adrenal medulla as well as from sympathetic nerve endings. Only adrenaline and nor-adrenalin are carried in the blood. Small amounts are carried free in the plasma, but most of the blood catecholamines are present in the red cells and platelets(Weil-Malherbe, 1954). The total catecholamine level in human plasma has been estimated as 1 mcgm./litre(Millar, personal communication), but the value apparently depends on the analytical techniques employed, hence the wide variations reported in the literature. The relationship of plasma catecholamine levels to open-heart surgery has been investigated. Conn and Millar(1960) noted a hypertensive phase immediately following circulatory occlusion in hypothermic anaesthesia for cardiovascular surgery. A close relationship was noted between the sudden increase in systemic blood pressure and the abrupt rise in plasma catecholamines. Total catecholamine levels of up to 18 mcgms./litre were obtained during the occlusion period. Hypoxia was felt to be the major stimulus in the release of these substances. Woods(1957) demonstrated an elevated catecholamine level in plasma following low flow rate perfusions. No substantial variations from control values were noted when optimal flow rates were used. Simply circulating fresh whole blood from donor dogs through the pump-oxygenator substantially increased the plasma concentrations of catecholamines. Agitation was apparently not the sole explanation, since when blood was vigorously shaken in a mechanical mixer these changes in catecholamine

levels were not obtained. This is certainly difficult to explain. Unfortunately Woods did not publish the actual figures obtained, so no comparison with the present study can be made.

In the human a dose of 2.5 mcgm./Kgm. of adrenalin, given intravenously, is required to cause any increase in the blood pressure. This effect is transient owing to rapid destruction of the adrenalin by an enzyme, amine oxidase(Lovatt, Evans, 1945). The small increase in catecholamines that has been seen cannot therefore be of any clinical significance.

Plasma potassium concentration has been estimated in three cases, and a small increase has been shown to occur following recirculation. This is due to the release of potassium from the erythrocytes. Leveen(1960) has recently drawn attention to the danger of potassium intoxication following massive transfusions of outdated bank blood containing large amounts of potassium. In a careful study of 157 cases of cardiac arrest, the transfusion of hyperkalemic blood was either a main or contributing factor in approximately 44%. An intensive investigation was carried out in dogs and the following points were established.

1. Animals with haemorrhagic hypotension were very susceptible to hyperkalemia. There was a marked inability of these animals to handle an increased potassium load. This was attributed to:
    a. decreased renal function,
    b. increased glycogenolysis from adrenergic stimulation with the release of glucose and potassium from the liver,
    c. generalized cellular release of potassium and simultaneous uptake of sodium.

2. The effect of potassium is potentiated by hypothermia owing to increased myocardial irritability.
3. Citrated blood also potentiates the action of potassium by temporary reduction of the ionized calcium.
4. The deleterious effect of hyperkalemia can be counteracted by the administration of digitalis.
5. Samples of venous blood do not give the true potassium picture, since "central" hyperkalemia may occur in the presence of normal peripheral blood levels. Large amounts of potassium released from the liver directly into the Inferior Vena Cava may lead to myocardial hyperkalemia. The peripheral venous blood has the opportunity of equilibrating with the extra-vascular space.

Extra-corporeal perfusions are usually done in the presence of:

1. increasing amounts of red-cell damage and therefore increased plasma potassium,
2. moderate or profound hypothermia,
3. some degree of hypotension,
4. increased blood levels of catecholamines.

It is reasonable to assume therefore that these procedures could be attended by hyperkalemic manifestations, such as cardiac arrest or difficulty in restarting a deliberately arrested heart. The highest plasma level of potassium seen was 5.72 meq. after a period of ten hours of recirculation. It is to be expected that higher levels would have occurred with the use of human blood, since the dog erythrocyte is a "sodium cell" containing approximately equal amounts of sodium and potassium (Rush 1961). Electrocardiographic changes usually occur when the serum

potassium exceeds 6.5 to 7.4 meq./litre(Pearson 1955). Although there are no reports concerning a hyperkalemic effect from erythrocyte damage during extra-corporeal circulation this may well become a problem if perfusion is so prolonged that toxic levels of potassium accumulate in the plasma.

Erythrocyte damage may have other pathological effects in addition to those discussed above. As far back as 1914, Barratt and Yorke noted that lethal hemodynamic alterations occurred when washed red cell stroma was injected into the circulation. Troup(1958) has shown that thromboplastin-like materials are present in the red cells in large amounts, and has stated that these materials, if released, may lead to depletion of the coagulation factors. Troup feels that the hitherto unexplained bleeding tendencies seen in certain hemolytic conditions may be a result of this mechanism.

The decreased leucocyte count is of itself not considered to be of any great importance, since a persistent leucopenia has never been reported following clinical perfusions. Nyhus(1958) however has reasoned that the breakdown of white cells during extra-corporeal circulation may release an excessive amount of histamine into the circulation,and suggests that the so-called sudden death syndrome following cardio-pulmonary by-pass may be regarded as part of a general histamine-like response. It is established that the blood histamine is almost entirely located in the leucocytes, and can be released into the plasma by blood trauma. Simply shaking rabbit blood in a tube will release significant amounts of histamine into the plasma(Emmelin 1945). It is safe to assume that most of the intracellular histamine of the blood will be set free during extra-corporeal circulation. At the present time it is not possible to say whether

sufficient amounts would be released to exert a pharmacological effect.

In addition to histamine the leucocytes contain significant amounts of proteolytic enzymes such as pepsin, cathepsin and trypsin, as well as the mucopolysaccharase lysozyme. Tullis(1958) has shown that these enzymes may be released without any outward changes in number or appearance of the leucocytes. A single pipetting of a blood sample into a non-siliconized pipette will lead to a considerable release of lysozyme, so that the trauma of extra-corporeal circulation would be expected to liberate the major part of this particular enzyme. The released lysozyme may attack mucoproteins in the capillary walls, disrupting their integrity and causing haemorrhage.

Although platelet damage and serotonin release have not been included in this present work, mention should be made of references in the recent literature concerning possible pharmacological effects produced by released serotonin. This substance, which is transported in the blood by the platelets, causes peripheral vasodilation, pulmonary vaso-constriction and potentiation of the effect of barbiturates. Sarajas and Frick, whose work has already been mentioned in respect to ATP, found that there was a rise in serotonin levels of the plasma during 90 minutes of simulated extra-corporeal circulation, from a level in the controls of 0.025 mcgm./ml. to 0.045 mcgm./ml. These workers feel that serotonin is responsible for renal vaso-constriction and a reduced renal blood flow, as well as hypotension and a delay in recovery from anaesthesia. Clowes(1960) has stated that the mechanism of serotonin release from damaged platelets may play a part in the pulmonary congestion and peri-bronchial haemorrhages that follow those perfusions in which other factors such as pulmonary venous hypertension may be eliminated as a cause.

SUMMARY AND CONCLUSIONS

An investigation of the blood damage that occurs during prolonged recirculation has been carried out and both non-cellular and cellular components have been studied. The effect of a direct blood-gas interface on protein and lipid alterations has been determined by comparable studies with Screen and Membrane Oxygenators.

Plasma protein denaturation has been found to be produced by both types of oxygenators, although the degree of this structural modification is greater with the Screen Oxygenator. In view of the fact that identical lengths of tubing and the same pumps were used, and since an equal surface area of Screen and Membrane was exposed to the blood, this difference has to be attributed to the effect of a direct blood:gas interface. The extent of these protein alterations is quite minimal, and should not limit the use of the Screen Oxygenator for prolonged clinical perfusions.

Exposure of blood films to an air interface leads to the formation of fat globules of up to 40/u in size. There is a decided advantage in the use of the Membrane Oxygenator, since fat globules were not seen with this machine. These fat globules are thought to result from an altered stability of the chylomicron emulsion system. The differences between the two types of Oxygenators in the production of fat globulemia may be related to differences in the degree of denaturation of the protein films which stabilize the chylomicrons. Pluronic F68, a non-ionic detergent, has proven to be completely effective in the prevention of chylomicron coalesence. A hitherto unrecognized source of fat globules during clinical cardio-pulmonary by-pass has been discovered. Pooled blood in the mediastinum, which was usually aspirated into the extra-

corporeal system, has been shown to contain large numbers of fat globules, some of which measure 500 μ in diameter. On the basis of these findings, the routine addition of Pluronic F68 to the pooled donor blood, and the avoidance of aspiration of mediastinal blood into the machine appear to be essential if the danger of fat embolism is to be avoided.

As could be expected, erythrocytes and leucocytes are destroyed by long periods of recirculation in the Screen Oxygenator. The actual loss of cells is not high enough to be of great clinical significance. Of more importance, however, may be the release from damaged cells of substances which possess powerful pharmacological activity. A preliminary study has been made into the changes in plasma concentrations of adenosine triphosphate, total catecholamines, and potassium. The finding of relatively large amounts of adenosine triphosphate in the plasma following recirculation for long periods of time may be of great clinical significance. In the writer's opinion, the release of pharmacological agents from damaged blood cells is at present the unknown factor in long term perfusions, and vigorous investigation should be directed towards an elucidation of this problem.

TABLE 10

EFFECT OF BLOOD RECIRCULATION ON PLASMA HEMOGLOBIN
(All Values Reported in mgms. %.)

Experiment Number	4 Hours Recirculation		Expt. No.	10 Hours Recirculation	
	Control	After Recirc.		Control	After Recirc.
1.	10	72	6.	5	104
2.	9	81	7.	27	243
3.	20	100	8.	27	396
4.	15	77	9.	16	220
5.	9	36	10.	9	198
Average Increase 60.6 mgm%			Average Increase 211.4 mgm%		

TABLE 11

EFFECT OF BLOOD RECIRCULATION ON PLASMA ATP, TOTAL CATECHOLAMINES AND POTASSIUM

Expt. No.	ATP Mgms. %		CATECHOLAMINES Mcgms./litre		POTASSIUM Meq./litre	
	Control	After Recirc.	Control	After Recirc.	Control	After Recirc.
6.	0	7.5	0.140	2.000	-	-
7.	0	9.0	0.441	0.724	-	-
8.	0	16.2	0.000	0.626	4.72	5.72
9.	0	7.8	0.190	0.854	4.6	5.5
10.	0	10.5	0.200	0.353	4.1	4.7

TABLE 12

CHANGES IN TOTAL LEUCOCYTE COUNTS WITH BLOOD RECIRCULATION

Expt. No.	Control	After Recirculation	% Decrease	Length of Perfusion
1.	4,600	3,400	25	4 Hours
2.	10,250	4,950	52	4 "
3.	5,450	2,950	46	4 "
4.	8,250	4,050	51	4 "
5.	19,800	7,550	62	4 "
6.	13,680	10,400	24	10 "
7.	13,100	8,300	37	10 "
8.	5,550	3,200	40	10 "

	Experiment 9.	Experiment 10.
Control	7,750	3,725
5 mins.	6,850	2,700
15 mins.	6,850	1,950
30 mins.	6,400	1,700
1 hour	6,300	1,550
4 hours	4,300	1,425
10 hours	4,000	1,200

BIBLIOGRAPHY

Alexander, N.M., Analytical Chem., 30:1292, 1958.

Andersen, M.N. and Hambraeus, G., Annals of Surgery, 153:592, 1961.

Arnold, V., Z. physiol. Chem., 70:300, 1911.

Ascheron, E., Arch.Anat.Physiol., 1840, 44.

Barratt, J.O.W. and Yorke, W., Brit.Med.J., 1:235, 1914.

Barron, E.S.G. and Finkelstein, P., Archiv.Biochem., 41:212, 1952.

Born, G., Biochem.J., 62:331, 1956.

Brown, R.K., Baker, W.H., Peterkovsky, A., and Kaufman, D.L., J.Am. Chem.Soc., 76:4244, 1954.

Brown, I.W., and Smith, W.W., Ann.Int.Med., 49:1035, 1958.

Brunner, J.R., Duncan, C.W. and Trout, G.M., Food Research, 18:454, 1953.

Bull, H.B., J.Biol.Chem., 123:17, 1938.

Bull, H.B., J.Biol.Chem., 133:39, 1940.

Chambliss, J.R., Demming, J., Wells, K., Cline, W.W. and Eckstein, R.W., Am.J.Physiol., 163:545, 1950.

Clowes, G.H.A., Physiol.Revs., 40:826, 1960.

Conn, A.W. and Millar, R.A., Can.Anaes.Soc.J., 7:443, 1960.

Connoly, J.E., Bacaner, J.E., Brons, M.B., Lowenstein, J.M. and Storli, E., Surgery, 44:255, 1958.

Corrau, A.J.W., and Lewis, W.C., Biochem.J., 18:1364, 1924.

Cossman, F.P., Adler, F., Kittle, C.F., and Peltier, L.F., J.Thoracic Surg. 40:430, 1960.

Crafoord, C., Norberg, B. and Senning, A., Acta Chir.Scand., 112:220, 1957.

Crammer, J.L. and Neuberger, A., Biochem.J., 37:302, 1943.

Davies, D.F., Gropper, A.L., Schroeder, H.A., Circulation, 3;543, 1951.

Davis, B.D., Hollaender, A., and Greenstein, J.P., J.Biol.Chem., 146:663, 1942.

Dean, R.B., in Modern Colloids, Toronto, D.Van Nostrand Co., 1949, p.239.

Depre, C., Arch.Int.Pharm.Ther., 92:71, 1952.

Deutsch, H.F. and Morton, J.I., Arch.Biochem.Biophys., 93:654, 1961.

Devaux, A., Prob.verb.soc.des sciences, phy. et nat., Bordeaux, Nov.1903.

Deyrup, I.J., Am.J.Physiol., 167:749, 1951.

Dickson, J.F., Dow, J.W. and Hamer, N.A.J., Trans.Am.Soc.Artificial Internal Organs, 5:210, 1959.

Di Jeso, F., Boll.Soc.Ital.Biol.Sper., 35:2138, 1959.

Dodrill, F.D., in Extra-Corporeal Circulation, J.G. Allen(ed.), Springfield, Ill., Thomas, 1958, p.327.

Doty, P., and Edsall, J.T., Advances in Protein Chemistry, 6:108, 1951.

Dowben, R.M., and Koehler, W.R., Archiv.Biochem.Biophys., 93:496, 1961.

Edsall, J.T., in The Proteins, Vol. 1,(B) p. 602

Emmelin, N., Acta Physiol.Scand., 11(Suppl. 34): 1, 1945.

Erickson, J.O. and Neurath, H., J.Expt.Med., 78:1, 1943.

Ferbers, E.W. and Kirklin, J.W., J.Thoracic Surg., 36:23, 1958.

Fisher, J.H. and Smyth, B.T., Surgical Forum, 10:604, 1959.

Fogelman, K.L., Robins, K.L., Hennington, R. and Stapp, W.F., Surgical Forum, 3:467, 1953.

Folch, S., J.Biol.Chem., 226:497, 1957.

Fox, S.W. and Foster, J.F., in Introduction to Protein Chemistry, N.Y., John Wiley & Sons, 1957.

Franklin, E.C. and Kunkel, H.G., J.Immunol., 78:11, 1957.

Freeman, T., Clin.Chim.Acta, 4:788, 1959.

Frick, M.H., Nature, 187:609, 1960.

Galletti, P.M., and Salisbury, P.F., Trans.Am.Soc.Artificial Internal Organs, 5:210, 1959.

Gerbode, F., Melrose, D.G., Norman, A., Osborn, J.J., Perkins, H.A. and Baer, D.M., Lancet, 2:284, 1958.

Gibbon, J.H., Minn.Med., 37:171, 1954.

Glazer, A.N., McKenzie, H.A., and Wake, R.G., Nature, 180:1286, 1957.

Boldblum, R.W., Piper, W.N., Olsen, C.J., J.Invest.Derm., 22:373, 1954.

Goldfarb, A.R., Science, 114:177, 1951.

Greenstein, J.P., J.Biol.Chem., 136:795, 1940.

Hamer, N.A.J., Dickson, J.F. and Dow, J.W., J.Thoracic Surg., 37:190, 1959.

Hanks, G.E., Cassell, M., Rayr, N. and Chaplin, H., J.Lab. & Clin.Med., 56:486, 1960.

Harris, L.J., Proc.Roy.Soc.B., 94:426, 1923.

Harrison, H.E., Bunting, H., Ordway, N.K. and Albrink, W.S., J.Expt.Med., 86:339, 1947.

Heidelberger, M., in Serological Approaches to Studies of Protein Structure and Metabolism, W.H. Cole(ed.), New Brunswick, N.J., Rutgers University Press, 1954, p.10.

Hellerman, L,, Chinard, F.P. and Deitz, V.R., J.Biol.Chem., 147:443, 1943.

Helmsworth, J.A., Clark, L.C., Kaplan, S., Sherman, R.T. and Largen, T.L., J.Am.Med.Assoc., 150:451, 1952.

Hoeksema, T.F., Mustard, J.F. and Mustard, W.T., Trans.Am.Soc.Artificial Internal Organs, 5:225, 1959.

Hyman, E.S., Trans.Am.Soc.Artificial Internal Organs, 5:257, 1959.

Jirgensons, B., Arch.Biochem.Biophys., 41:342, 1952.

Jirgensons, B., in Organic Colloids, Amsterdam, Elsevier Pub.Co., 1958, p.593.

Kaplan, J.G. and Fraser, M.J., Nature, 171:559, 1953.

Kauzmann, N.W., Adv.Protein Chem., 14:1-63, 1959.

Kirby, C.K., Discussion on Blood Changes in Extra-Corporeal Circulation, J.G. Allen(ed.), Springfield, Ill., Thomas, 1958, p.253.

Kraemer, E.O. and Lansing, W.D., J.Physiol.Chem., 39:153, 1935.

Kolff, W.J., Effler, D.B., Groves, L.K., Hughes, C.R. and McCormack, L.J., Cleveland Clinic Quarterly, 25:65, 1958.

Kolthoff, I.M., Anastasi, A., Stricks, W., Tan, B.H. and Deshmoka, J.Am.Chem.Soc., 79:5102, 1957.

Krasna, I.H., Steinfeld, L., Dreel, I. and Baronofsky, I.D., Surgical Forum, 11:218, 1960.

Kreel, I., Zaroff, L.I., Frankel, A., Canter, J.W., and Baronofsky, I.D., J.Mt.Sinai Hosp.(New York), 27:47, 1960.

Krejci, L.E., Jennings, R.K. and Smith, L.D., J.Immunol., 45:111, 1942.

Laskowski, M. and Scheraga, H.A.,, J.Am.Chem.Soc., 78:5793, 1953.

Lee, W.H., Krumhaar, D., Fonkalsrud, E., Schjeide, O.A., Maloney, J.V., Surgery, 50:29, 1961.

Legallois, J.J.C., in Experiences sur le principe de la vie, cited in by Dubbleman, 1948.

Leveen, H.H., Pasternack, H.S., Lustrin, I., Shapiro, R.B., Becker, E. and Helft, A.E., J.Am.Med.Assn., 173:770, 1960.

Linderstrom-Lang, K., in Lane Medical Lectures: Proteins and Enzymes, Stanford, Calif., Stanford University Press, 1952, p.58.

Lovatt Evans, C., in Principles of Human Physiology, London, J.&A. Churchill Ltd., 1945, p.1028.

Ludlum, S.D., Taft, A.E., Nugent, R.L., J.Physical Chem., 35:269, 1931.

Maloney, J.V., (1961) cited by Lee et al, Surgery, 50:29, 1961.

Melrose, D.G., Bassett, J.W., Beaconsfield, P., Gaaber, I.G. and Shackman, R., Brit.Med.J., 2:62, 1953.

Merendino, K.A., Quintow, W.E., Thomas, G.I., Jesseph, J.E., Herron, P.W., Tremblay, R.E., Maguire, R., and Vetto, R.R., Surgery, 42:996,1957.

Millar, R.A. and Benfey, B.G., Brit.J.Anaesth., 30:158, 1958.

Miller, G.L., J.Biol.Chem., 146:345, 1942.

Mirsky, A.E. and Anson, M.L., J.Gen.Physiol., 19:427, 1936.

Morton, J.I., Int.J.Radiol.Biol., 2:45, 1960.

Muller, W.H., Littlefield, J.B. and Damman, J.F., in Extra-Corporeal Circulation, J.G.Allen(ed.), Springfield, Ill., Thomas, 1958, p.336.

Murray, R. and Ratner, F., Proc.Soc.Expt.Biol.Med., 83:554, 1953.

MacPherson, C.F.C., and Heidelberger, M., J.Am.Chem.Soc., 67:585, 1945.

McGoon, D.C., Moffitt, E.A., Theyle, R.A. and Kirklin, J.W., J.Thoracic Surg., 39: 275, 1960.

Neurath, H., and Bull, H.B., Chem.Revs., 23:391, 1938.

Neurath, H., Greenstein, J.P., Putnam, F.W. and Erickson, J.O., Chem.Revs., 34:157, 1944.

Neurath, H. and Putnam, F.W., J.Biol.Chem., 160:397, 1945.

Newman, M.M., Trans.Am.Soc.Artificial Internal Organs, 4:76, 1958.

Newman, M.M., Stuckey, J.H., and Dennis, C., Paper presented at the Conference on Blood Problems in Extra-corporeal Circulation and Massive Transfusions, National Academy of the Sciences, Washington, D.C., 1958, p.56.

Nyhus, L., in Extra-Corporeal Circulation, Springfield, Ill,, Thomas, 1958, p.266

Osborn, J.J., MacKenzie, R., Shaw, A., Perkins, H., Hurt, R. and Gerbode, F., Surgical Forum, 6:96, 1956.

Ouchterlony, O., Lancet, 256:346, 1949.

Ouchterlony, O., Paper presented at the VIth International Congress for Microbiology, Rome, September 1953.

Owens, G., Adams, J.E., McElhannon, F.M. and Youngblood, R.W., J.Appl.Physiol., 14:947, 1959.

Owens, G., Phornphiboul, R. and Younger, R., Clinical Research, 3:59, 1960.

Owens, G., Adams, J.E. and Scott, H.W., J.Appl.Physiol., 15:999, 1960.

Paneth, M., Sellers, R., Gott, V.L., Weirich, W.L., Allen, R., Read, R.C. and Lillehei, C.W., J.Thoracic Surg., 34:570, 1957.

Patrick, R.T., Editorial, "Extracorporeal Circulation in Man," Anaesth.,18:643, 1957.

Pearson, C.M. and O'Meara, M.P., Ann.Int.Med., 43:601, 1955.

Pedersen, K.O., Nature, 128:150, 1931.

Pierce, E.C., J.Thoracic Surg., 39:438, 1960.

Peltier, L.F., Surgery, 36:198, 1954.

Penick, G.D., Averette, H.E., Peters, R.M. and Brinkhous, K.M., Thromb.Diathesis Haemorrhagica, 2:218, 1958.

Perkins, H.A., Osborn, J.J. and Gerbode, F., "Problems in Coagulation", in Extracorporeal Circulation, Springfield, Ill., Thomas, 1958, p.253.

Perlmann, G.E. and Kaufmann, D., J.Biol.Chem., 179:133, 1949.

Phemister, D.B., and Handy, J., J.Physiol., 64:155, 1927.

Pontius, R.G., Watkins, E., Manheim, B.S., Allen, R.G., Savage, L.R. and Gross, R.E., Surgical Forum, 8:393, 1958.

Putnam, F.W., in The Proteins, H.Neurath and K.Bailey(eds.), N.Y., Academic Press, Vol. 1(B), p.834.

Rebuck, J.W., Am.J.Clin.Path., 17:614, 1947.

Rehell, B., Forsander, O., and Raiha, C.E., Scand.J.Clin.& Lab.Invest., 4:211, 1952.

Rothen, A., Advances in Protein Chem., 3:123, 1947.

Rush, B.F., Wilder, R.J., Fishbein, R., Ravitch, M.K., Surgery, 50:40, 1961.

Sarajas, H.S.S., Kristoffersson, R. and Frick, M.H., Nature, 184:1127, 1959.

Senning, A., Andres, J., Bornstein, P., Norberg, B., and Andersen, M., Ann.Surg., 151:63, 1960.

Snyder, D.D., Barnard, C.N., Varco, R.L. and Lillehei, C.W., Surgery, 44:1083, 1958.

Stanley, W.M. and Lauffer, M.A., Science, 89:345. 1939.

Starling, E.H. and Verney, E.B., Proc.Roy.Soc.B., 97:321, 1925.

Storli, E.A., Connoly, J.E., Bacaner, M.B. and Bruns, D.L., Surgical Forum, 11:214, 1960.

Suchinsky, R.T., J.Clin.Invest., 39:1953, 1960.

Taylor, H.P., Kolff, W.J., Sindelar, P.S. and Cahill, J.J., Am.J.Obstet.Gynecol., 77:1295, 1959.

Thorbecke, G.J., Maurer, P.H. and Benacerraf, B., Brit.J.Expt.Path., 41:190, 1960.

Troup, S.B., and Reed, C.F., J.Clin.Invest., 37:937, 1958.

Troup, S.B., Reed, C.F., Marinetti, G.V. and Swisher, S.N., in "The Blood Platelets. International Symposium." S.A.Johnson(ed.), Boston, Little, Brown & Co., 1961, p.267.

Tsen, C.C. and Tappel, A.L., Can.J.Biochem & Physiol., 38:539, 1960.

Tullis, J.L., Paper read at Conference on Blood Problems in Extracorporeal Circulation and Massive Transfusions, National Academy of the Sciences, Washington, D.C., 1958, p.79,

Van der Scheer, J., Wyckoff, R.W.G. and Clarke, F.L., J.Immunol., 40:39, 1941.

Von Kaulla, K. and Swan, H., Circulation, 16:899, 1957.

Von Kaulla, K. and Swan, H., J.Thoracic Surg., 36:519, 1958.

Waldhausen, J.A., Webb, R.C., Spencer, F.C. and Bahnson, H.T., Surgery, 42:726, 1957.

Weil-Malherbe, H. and Bone, A.D., Nature, 174:557, 1954.

Woods, E.F., Richardson, J.A., Lee, W.H., Ashmore, J.D. and Parker, E.F., Circulation, 16:955, 1957.

Wu, H., and Ling, S.M., Chinese J.Physiol., 1:407, 1927.