An Investigation of the Clot Deposition from a Flowing Blood Analogue by Means of Ultrasonic Imaging

by

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The work described in this thesis is the original work of the author and was carried out without the assistance of others, except where explicit credit is given in the text. It has not been submitted, in whole or in part, for any other degree at any University.
I dedicate this thesis to my parents,
Bill and Thelma,
as their love and support during all my endeavours
have been the key to my successfulness.
Numerous major inventions and discoveries made during the 20th century have required painstaking hours in a laboratory, large amounts of money and ingenious minds. However, something that requires none of these factors, yet would be of greater value than all that the 20th century boasts, has not been achieved... world-wide egalitarianism. Why is this so difficult to accomplish?
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Although defective heart valves are routinely replaced by either tissue or mechanical substitutes, neither alternative valve type is completely satisfactory. Tissue valves are subject to calcification and lipid deposition, while mechanical prostheses tend to cause thrombosis and hence require long-term anticoagulation therapy, which has undesirable side-effects. The thrombogenicity of mechanical heart valves, in particular, provided the motivation behind the work presented here.

Thrombogenicity of prostheses is currently assessed by collation of experimental results from animal trials. This technique has several drawbacks, such as its great expense and the uncertainties due to species-related differences of blood. The use of blood in vitro, as an alternative way to assess thrombogenicity, is neither practical nor convenient; blood is valuable and relatively scarce, and it often behaves differently in vivo than in vitro. An alternative to blood for use in thrombogenicity trials in vitro is therefore desirable. Such a blood substitute could be used to investigate the influence of the flow characteristics of valves on their clotting propensities. The work reported here was accordingly intended to clarify the relationship between deposition tendency and flow conditions (as distinct from surface chemistry), by use of a blood analogue fluid.

A milk preparation has been shown by several previous workers to have potential as a coagulable analogue fluid for the study of flow related thrombosis in vitro. Lewis found that a flowing milk mixture deposited clot in vitro in the neighbourhood of certain hydrodynamic disturbances particularly associated with thrombus formation in a similar flow of blood, and that the milk and blood deposits were similar in physical structure, both on a microscopic and gross scale. Christy used bodies of revolution in pulsed and steady milk mixture flows, in an attempt to identify the hydrodynamic causes of blood clotting in determinate and simple flow situations. The varying extent of deposition on the downstream-side of test-bodies of different shapes indicated stasis and some aspect of agitation in the vicinity of a surface as the concomitant conditions necessary for clot to occur on that surface.
The principal aim of the research reported here was to develop Christy's milk coagulation experiments further, by obtaining more information about deposit growth and hence to shed light, by inference, on mechanisms of flow-related thrombus formation. To this end, milk experiment reproducibility was improved, ways to eliminate bubble and wall clot formation were attempted, and the feasibility of ultrasonic imaging as a means for continuously measuring the thickness of deposited clot was assessed and subsequently employed.

The use of real-time ultrasonic imaging enabled wall clot deposition, and its influence on milk experiments, to be continuously monitored macroscopically. It may also provide a way for assessing various methods aimed at preventing wall clot formation. The prospective advantages of ultrasonic imaging were not fully realized by the arrangement adopted in these preliminary experiments, but it now appears that ultrasonic imaging could be of use for studying curd deposition on test-objects, with certain recommended modifications to the current arrangement.
Chapter 1

Introduction

1.1 The heart

The heart comprises two synchronous pulsatile pumps working side by side (figure 1.1); the right side pumps deoxygenated blood to the lungs while the left side pumps oxygenated blood to the body. There are four separate chambers, two receiving chambers, the atria, and two pumping chambers, the ventricles. Each receiving chamber is connected to a pumping chamber by a non-return valve, the right atrium and right ventricle by the tricuspid valve, the left atrium and left ventricle by the mitral valve. The two pumping chambers are connected to their respective arteries by one-way valves also; the right ventricle to the pulmonary artery by the pulmonary valve, the left ventricle to the aorta by the aortic valve\(^1\).

The blood is progressively deoxygenated as it circulates round the body. It then collects in the great veins, flows into the right atrium and through the tricuspid valve into the right ventricle. Contraction of this ventricle and closure of the tricuspid valve results in
Head and upper extremities

Figure 1.1: Simple representation of the heart and lungs\textsuperscript{[1][3]}.

blood being expelled through the pulmonary valve into the pulmonary artery. Carbon dioxide and oxygen are transferred from and to this blood, respectively, as it flows through the lungs. The reoxygenated blood then flows through four pulmonary veins into the left atrium. During diastole, the filling phase of the cardiac cycle, the blood flows through the left atrium and the mitral valve and into the left ventricle. The left atrium then contracts, near the end of this filling phase, to eject blood into the left ventricle. When the diastolic cycle is complete, ventricular systole occurs; the left ventricle abruptly contracts, the mitral valve closes and blood is ejected through the aortic valve, passing through the aorta and arterial tree\textsuperscript{[2][3]}. 
1.2 Heart valve defects

A normal adult heart on average beats about 40 million times a year without failure. At rest, the heart beats at a rate of approximately 70 beats/min, with a cardiac output close to 5 l/min, while during rigorous exercise, a normal heart may deliver up to 35 litres of blood in one minute at a heart rate of 180 beats/min[2]. Given the numerous demands on heart valves, both natural and artificial ones, it is not surprising that they may sometimes become defective.

In many cases, abnormalities of the heart valves cause trivial effects on the function of the heart and, provided certain precautions are taken, will not interfere with the person’s life. Occasionally, however, the effects may be more severe so that an undue strain is placed on the muscle of the heart. In such cases it may be necessary to operate on, or even replace, one or more valves. The replacement of diseased heart valves is not a rare operation; 51,000 artificial cardiac valves were implanted in patients in 1988 in the United States alone[4].

The two most common haemodynamic defects which natural heart valves can develop are called stenosis (narrowing of the valve orifice when open) and incompetence (failure of the valve to close completely on flow reversal allowing a regurgitant jet of blood to flow past the valve)[1][5][6]. These defects tend to affect mitral, aortic, tricuspid or pulmonary valves, in that order of incidence[1][5]. This order may be partially attributable to the larger systolic pressures experienced in the left ventricle. Obstruction of flow and inefficiency due to leakage result in increased strain and demand on the heart, causing the heart muscle to thicken. In time the supply of blood to this muscle will become insufficient to meet its increased demand. Angina, followed by death, awaits the sufferer unless treated.

There are many occasions when valve replacement is necessary. When the aortic valve is diseased severely enough to need surgery it always has to be replaced[7]; repair is never possible. However, repair of other damaged valves is sometimes appropriate, but conditions have to be absolutely right (i.e. the valve must not be too badly damaged
by disease) for a successful outcome. For this reason, if there is any doubt about the valve continuing to function properly in the long term, it will be replaced by a new one. It is then that the patient faces the risk of unappealing complications associated with current artificial heart valves.

1.3 Artificial cardiac valve requirements

The criteria for an ideal artificial heart valve are numerous. The substitute valve must not only fulfil the function of the natural valve but must also be compatible with both the blood and surrounding tissue. The main requirements of such a valve have been proposed\cite{8-11}; the prosthesis must:

(i) conform to the heart structure, the volume swept out by the occluder lying within the limits of the dimensions of the heart
(ii) offer minimum resistance to forward flow; have the maximum possible orifice size when the valve is open, to avoid the need for excessive pressure to maintain the cardiac output
(iii) allow minimal reverse flow; at the onset of reverse flow, the valve must close quickly and completely to prevent regurgitation
(iv) have low inertia, to minimize the work required of the weakened cardiac muscle in opening and closing the valve
(v) be surgically possible to insert; have ease of implantation and tolerance by surrounding tissues after healing
(vi) be sterile
(vii) cause no blood trauma; when open the valve must have a high flow rate with no turbulence, and allow no leakage during diastole when the valve is closed
(viii) be non-thrombogenic
(ix) be durable
(x) operate quietly
(xi) cost as little as possible.

There are two categories of valve that can be used to replace a diseased heart valve: mechanical (man-made, mechanical valves) and biological (valves made from human or...
specially treated animal tissue). Tissue valves currently in use are mainly heterografts, usually of porcine or bovine tissue, and some homografts. They mimic the cusp structure of the natural valves, performing as central flow valves. Mechanical valves, such as poppet, tilting-disc, and hinged-disc valves, have either peripheral or semi-central flow. Photographs of a few currently used heart valves of both categories are shown in figure 1.2. There are many unresolved problems associated in different degrees with both types of artificial valves, such as red cell destruction, thromboembolism, congestive heart failure, cardiomyopathy, infection, material fatigue, leakage and tearing of sewing suture.

1.4 The shortcomings of prosthetic heart valves

Since the 1960s, when replacement of diseased aortic and mitral valves with prosthetic devices began on a widespread basis, improved prosthesis design and increasing operative experience have reduced the morbidity and mortality associated with valve replacement. Valve replacement is, however, still palliative and not curative. A prosthetic valve has still to be designed which provides the ideal performance of the natural, healthy heart valve. Although many types of prosthetic valves are currently available, the complications and natural histories are similar for most of them. It is possible that every model of cardiac valve implant ever marketed has suffered some mechanical failures resulting from thrombosis, degradation, or structural fractures.

Haemolysis and thrombosis are of concern with all systems designed to support or replace organs of the circulatory system, and are the two major postoperative problems of prosthetic valve implantation. The high shear stresses associated with regurgitation or back flow can cause haemolysis (the breakdown of red cells) which, in turn, can lead to the necessary replacement of erythrocytes putting a strain on the kidneys and bone marrow. Haemolysis can also induce the release of clotting factors that enhance cell-wall adhesion\cite{12}, promoting thrombus formation. Thrombus deposition on the foreign surface of a blood-handling device, potentially the more severe problem of the two, can impair the device's function and has the potential to generate emboli, which can be fatal.
Figure 1.2: (a) and (b) are biological trileaflet valves; (c) and (d) are Starr-Edwards and Bjork-Shiley mechanical valves, respectively.
To quantify statistically the predicament of the recipients of heart valve prostheses, curves of survival rates are normally constructed. Due to numerous variables these plots can only be considered as approximate guides to expected prosthesis performance. Lytle et al. produced extensive long-term results that indicate the success rates one may expect. They considered primary aortic valve replacement only, employing either bioprostheses (tissue valves) or mechanical valves in a large group of patients (figure 1.3). As commonly found with increasing post-operative years, the number of surviving substitute valve recipients gradually decreases and of these survivors the number encountering no complications dwindles.

![Figure 1.3: Late survival and event-free survival for 1317 in-hospital survivors of isolated aortic valve replacement from 1972 to 1983](image)

Lytle et al. investigated the effects of a few variables on survival rate of prosthesis recipients. Figure 1.4 indicates that the age of the recipient is relevant to the post-operative survival rate, the younger recipients apparently having better success, though the success of the implant is strongly dependent on other factors also. Differences were

1 Among the many variables, apart from age, associated with survival of prosthesis recipients are preoperative NYHA (New York Heart Association) function classification, symptoms and heart disease history of the patient, year of operation, the valve model implanted, etc.
also found in the early and late survival of patients, when grouped into the categories of mechanical and bioprostheses, with and without anticoagulation therapy (warfarin) (figure 1.5). Shortcomings were demonstrated by both types of valve.

It is far less common for recipients of tissue valves to need anti-coagulants than for mechanical valve recipients. Without anticoagulation therapy, the number of patients with tissue valves that had event-free survival was much greater than that for mechanical valve recipients, who were found to have more strokes, myocardial infarctions, bleeding complications, and thromboembolic events. Figure 1.5 clearly indicates that the quality of mechanical valve performance drastically decreases with time, when anticoagulation therapy is not employed.

Figure 1.4: Survival for valve recipients of different ages\textsuperscript{[14]}. 
Most surgeons believe that failure to anticoagulate exposes the mechanical heart valve recipient to an unreasonable risk of clotting complications, and accordingly provide the patient with long-term anticoagulation therapy\textsuperscript{[15]}. However, this treatment is not trouble-free. The dose of anticoagulant has to be precise; inadequate anticoagulation could be fatal, as could excessive anticoagulation which can cause haemorrhaging and puts a strain on the liver and kidneys\textsuperscript{[16]}. Therefore, blood tests have to be made at regular intervals to ensure that the dosage is correct, and the patient must avoid, or at least be cautious when taking, drugs (such as aspirin, antibiotics, and alcohol) that can alter the effect of anticoagulants.

Whilst bioprostheses are less thrombogenic than mechanical heart valves, they too have shortcomings, such as deterioration and stiffening of leaflets, and are prone to complications requiring reoperation, more so as the number of post-operative years increases (figure 1.6)\textsuperscript{[14]}. Hence, only a small number of tissue-valve recipients are
reported as long-term successes. It is the opinion of most surgeons worldwide that implanted tissue valves are less durable after ten years and should be reserved for very elderly patients and for those patients considered inappropriate for the long-term anticoagulation therapy necessary for all mechanical heart valves\textsuperscript{[11]}.

![Hazard Estimate Diagram](image)

Figure 1.6: Hazard function curves for the first occurrence of reoperation and endocarditis (i.e. inflammation of the endocardium) according to prosthesis type\textsuperscript{[14]}.

1.5 Evaluation of prosthetic valve thrombogenicity

Thrombosis in flowing blood around mechanical prostheses can arise from both the materials of construction of the valve and the local haemodynamics\textsuperscript{[17]}. Artificial materials (e.g. pyrolytic and vitreous carbon) commonly used for mechanical valve construction are intrinsically less thrombogenic than the valves made from them, so the flow characteristics of these valves appear to be the aspect requiring more consideration.

While research has revealed that the flow fields created by today's mechanical valves are considerably different from those of natural valves, involving areas of high fluid velocity associated with turbulence and high shear rates, slow-moving separated regions and
stagnation point flows, the fluid mechanical correlates of thrombus formation are as yet not understood or even fully identified. Clotting may occur in vivo in regions of the flow field that are not considerably disturbed by the presence of the prosthesis, even when the implant is constructed of inert and relatively athrombogenic materials; also, regions of large fluid mechanical disruption may show no link with thrombus formation. It appears that the next step forward to developing a mechanical valve which can be successfully employed without anticoagulant therapy is to find a way to identify aspects of the flow through present-day valves that lead to thrombus formation and then remove these flow characteristics or minimize their effects in new valve designs.

The progress of designing devices more efficacious than current artificial heart valves has been hindered by the absence of a reliable non-clinical practical method for evaluating valve thrombogenicity. At present, the only avenue for evaluating mechanical valve designs with respect to their clotting propensity is by collation of experimental results from animal trials with different valves. Such methods are not only distasteful, costly and time-consuming, but are of questionable worth due to uncertain effects of species-related differences between man and animals, with respect to blood characteristics and physiology\[^{18}\].

If an in vitro method were devised for evaluating the influence of configurational changes on thrombogenicity, the use of blood would be neither practical nor convenient in such experiments. Blood is a valuable fluid which is difficult to work with, as it tends to clot indiscriminately on foreign surfaces, and in vivo it reacts differently to some substances than when in vitro\[^{16}\]. For example, it is not correct to assume that a substance which interferes with some aspect of the coagulation of shed blood will affect blood circulating in the mammal in the same way; the delicate protective balance of activating and inhibiting mechanisms which the intact mammal possesses is not present in shed blood. Considering blood transfusion as an illustration of this, it is found that the addition of calcium ions to the donor's (extracorporeal) blood, will clot it; however, the blood does not clot, once received, even though the recipient possesses more than enough calcium to clot the transfused blood. Laboratory tests involving
blood coagulation may not, therefore, give any indication of the situation in living vessels. An in vitro test may be best served by a blood analogue fluid.

Work by Jolles\cite{19} indicates milk is in many ways similar to blood in its coagulation behaviour, and other research by Lewis\cite{20}, Christy\cite{21} and Macleod\cite{22-24}, in this laboratory, has shown that a milk mixture, containing rennet and calcium chloride, is a promising coagulable blood analogue fluid. It has been attempted here to further this work with milk, with the ultimate goal of establishing a way to assess prosthetic heart valve thrombogenicity and to facilitate a systematic empirical approach to the design of valves which do not induce thrombosis. To this end the milk experiments conducted by Christy and Macleod have been repeated and attempts made to obtain more information about flow characteristics conducive to clotting.

This thesis provides a review of the thrombosis mechanism and ways in which it can be initiated, of experimental findings and their limitations regarding blood clotting in vivo and in vitro, and of work conducted using milk as a coagulable blood analogue fluid. A method for observing milk clot deposition as it occurs is assessed, the recommissioning of Christy's and Macleod's experiment is described, and an investigation of the origin of experimental problems and the mechanism of clotting, achieved principally by means of the development and commissioning of an ultrasound visualization technique, is presented.
Chapter 2

Blood and its Coagulation

There are two modes of blood coagulation: haemostasis, which occurs in stationary blood, and thrombosis, a distortion of the haemostatic process, involving the deposition of blood constituents from flowing blood. Haemostasis is the natural process of blood clotting by which the body inhibits bleeding from wounds, and can be regarded as the disturbance of the balance between two processes - coagulation and fibrinolysis[25]. Each of these reactions is strictly controlled by plasma protease inhibitors and in vivo clearance mechanisms. The major function of the blood coagulation system is the generation of insoluble fibrin at the site of injury or trauma in order to limit the amount of blood loss. When this aim has been achieved and tissue repair is under way, it is the function of the fibrinolytic pathway to remove deposits and restore the patency of the damaged blood vessel. General descriptions of blood, coagulation and fibrinolysis, and the factors affecting blood coagulation are provided in this chapter.
2 Blood and its Coagulation

2.1 Blood and the coagulation cascade

Blood is a viscous fluid suspension, consisting of deformable 'formed elements' suspended in a liquid medium called plasma. Red cells (erythrocytes), white cells (leukocytes) and platelets compose the formed elements and account for about 45% of blood's volume, while the remaining volume consists of plasma, an aqueous solution containing colloidal proteins and numerous low molecular weight organic and inorganic materials\cite{26,27}.

The proteins which participate in coagulation are termed factors and, for simplification, each has been assigned a Roman numeral\cite{28}. Table 2.1 provides a list of these factors, along with their respective concentration in plasma. Plasma contains all but one of the coagulation factors, tissue factor (III), and fibrinogen is the only coagulation protein of appreciable concentration in plasma.

Blood coagulation entails a multistep cascade of activations of protein factors\cite{2} that results in the polymerization of a polypeptide monomer (modified fibrinogen) into an insoluble gel called fibrin (figure 2.1). This complex series of enzymic reactions, in which a factor activated in one stage activates an inactive factor in the next stage, like a waterfall conversion, can be initiated either by the interaction of blood contacting with a foreign or non-endothelial surface (intrinsic pathway) or by damage to the vascular endothelium\cite{3} (extrinsic pathway). This leads to the production of thrombin, which catalyzes the conversion of fibrinogen to fibrin which then polymerizes into a mesh-like structure, in which blood platelets, erythrocytes and leukocytes are trapped\cite{32}.

In the intrinsic (contact activation) pathway, the plasma protein that is bound onto and activated by a foreign surface is called Hageman factor (factor XII). Activated factor XII in turn converts the zymogen factor XI to the enzyme XIa. The function

\footnote{Tissue factor (III) is readily available for initiating coagulation if vascular injury occurs\cite{29}.}

\footnote{Most of the factors exist as zymogens (factor precursors) and must be modified by hydrolytic cleavage to achieve full activity.}

\footnote{The endothelium is a continuous, single-cell-thick lining of all surfaces of the circulatory system that would otherwise be in direct contact with blood\cite{28,29}.}
Table 2.1: Blood coagulation factors\cite{25}

<table>
<thead>
<tr>
<th>Factor number</th>
<th>Common name</th>
<th>Conc\textsuperscript{a} in 1ml plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>fibrinogen</td>
<td>~3mg</td>
</tr>
<tr>
<td>II</td>
<td>prothrombin</td>
<td>~200\mu g</td>
</tr>
<tr>
<td>III</td>
<td>tissue factor</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>calcium ions</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>proaccelerin</td>
<td>?</td>
</tr>
<tr>
<td>VI</td>
<td>abandoned for Va</td>
<td>-</td>
</tr>
<tr>
<td>VII</td>
<td>proconvertin</td>
<td>~2\mu g</td>
</tr>
<tr>
<td>VIII</td>
<td>antihaemophilic factor</td>
<td>?</td>
</tr>
<tr>
<td>IX</td>
<td>Christmas factor</td>
<td>~3-4\mu g</td>
</tr>
<tr>
<td>X</td>
<td>Stuart factor</td>
<td>~6-8\mu g</td>
</tr>
<tr>
<td>XI</td>
<td>plasma thromboplastin antecedent</td>
<td>~7\mu g</td>
</tr>
<tr>
<td>XII</td>
<td>Hageman factor</td>
<td>~40\mu g</td>
</tr>
<tr>
<td>XIII</td>
<td>fibrin stabilizing factor</td>
<td>-</td>
</tr>
</tbody>
</table>

of activated factor XII is enhanced by high molecular weight kininogen (HMWK), which is also a cofactor required for the activation of factor XII by kallikrein\cite{33}. These contact-activated reactions are calcium independent, but some of the subsequent blood coagulation reactions (those inside the box of figure 2.1, and the activation of factor XIII\cite{34}), require phospholipids\cite{35} and Ca\textsuperscript{2+} for rapid reaction rates\textsuperscript{4}. It is likely that tissue factor provides the lipid for the extrinsic pathway, and platelets supply phospholipid for the intrinsic pathway\cite{28}\cite{37}. Factor XIa activates factor IX, and factor IXa catalyzes the conversion of factor X to Xa in the presence of activated factor VIII, calcium and negatively charged phospholipids.

Factor X can also be activated by the extrinsic pathway, by exposing blood to tissue factor III, calcium and factor VIIa. Factor VII does not express coagulant activity until bound to tissue factor (factor III), which is not normally present in the circulation and is released from the site of vascular injury, initiating the extrinsic system. The intrinsic and extrinsic pathways lead to a common pathway, where prothrombin is converted to thrombin, in the presence of factor Xa, calcium, phospholipid and factor Va. By a highly selective cleavage of two Arg-Gly peptide bonds from amongst 181 peptide bonds in human fibrinogen\cite{38}, thrombin converts soluble fibrinogen to fibrin, which initially

\textsuperscript{4}For example, the rate of conversion of prothrombin is five hundred times greater in the presence, as opposed to the absence, of phospholipid\cite{36}.
Figure 2.1: Cascade mechanism of blood coagulation. There are a variety of postulated coagulation cascades\cite{32}\cite{25}\cite{38}. The scheme above has a box enclosing reactions requiring phospholipid and Ca$^{2+}$; broken lines representing positive and negative feedback loops; thick lines denoting the action of enzymes on their specific substrates; and solid lines indicating activation pathways. HMWK = high molecular weight kininogen, the subscript ‘a’ indicates an activated substance, and * denotes an enzymatic component which requires more than one factor in its activation.

Initially is relatively unstable and susceptible to proteolytic degradation\textsuperscript{5}. Catalyzed by factor XIIIa, the fibrin monomers spontaneously polymerize into a crosslinked, insoluble fibrin network.

\textsuperscript{5}Thrombin not only proteolyzes fibrinogen, but plays other important roles in coagulation, including activating factor XIII (the fibrin-stabilizing factor), increasing the activity of factor V, and enhancing the aggregation of platelets and triggering their release reaction\cite{25}.
2.2 Platelets, haemolysis and thrombus growth

The sequence of deposition of blood components on a foreign surface has been experimentally determined and a thrombus growth mechanism postulated\[39]\[31]. All nonphysiological surfaces brought into contact with blood rapidly acquire a thickening film of protein, followed by platelet adsorption and possibly platelet aggregation, depending on the conditions (figure 2.2). Blood platelets arrive continuously, but hardly any cell adhesion occurs until the "conditioning" film of protein achieves a critical but non-equilibrium state\[31]. It has been suggested\[40]\ that if the strength of the stimulus (i.e. the concentration of activating species, which may be released through alteration or damage to platelets, red blood cells and/or connective tissue\[41]\[26]) and the number of platelets carried to the surface are sufficient\[42]\[43], the coagulation process will be triggered, resulting in the formation of platelet aggregates, which are strengthened by a mesh of fibrin. If either of these conditions is not met then aggregate growth stops, as illustrated in figure 2.2. The thrombus growth mechanism is considered in more detail in section 3.3.

![Figure 2.2: A postulated sequence of events leading to thrombus formation\[39].](image)

Platelets play a major role in the response of blood to the introduction of foreign materials, chemicals and infectious agents into the blood stream. In the normal circulation, the only structures which platelets normally encounter are red cells, white cells and the endothelial lining of blood vessel walls, so all other materials are foreign to
them. Human platelets have no tendency to adhere to blood vessel walls, to other blood cells or to each other, but have an ability to adhere to foreign surfaces, such as artificial heart valves and the subendothelium, and when exposed to a variety of chemical stimuli (including thrombin), will aggregate and release a number of substances which promote further platelet aggregation\cite{44,45}. Platelets possess intracellular storage granules, which contain different materials, such as fibrinogen, adenosine diphosphate (ADP) and serotonin\cite{46}. Secretion of these contents may accompany aggregation, which can promote additional platelets to aggregate and stimulate the coagulation process.

Haemolysis (the disintegration of erythrocytes) can also promote thrombus growth, depending on the severity of the haemolytic process\cite{47} and the concentration of the activating species released. If the components released from red blood cells, such as ADP, enter an area of stasis in the vicinity of a foreign surface, they may reach a concentration sufficient to result in platelet adhesion and activation of the coagulation process.

2.3 Fibrinolysis

Thrombogenesis is combatted or regulated by the endothelium, liver, inhibitors of activated coagulation factors and fibrinolysis\cite{45}. The protective mechanism considered here is fibrinolysis. As healing and regeneration of the vessel wall occur, the fibrin clot becomes redundant and must be removed to restore full patency to the damaged vessel. Mammalian blood contains an enzymatic system capable of dissolving blood clots, called the fibrinolytic system\cite{48}, which is dependent on the presence in plasma of a number of activators and inhibitors\cite{38,49} (figure 2.3). The active enzyme involved in fibrin hydrolysis, plasmin, is normally absent from flowing blood, and blood plasminogen activator, responsible for generating plasmin from its inactive precursor, plasminogen, occurs in very small quantities. Plasminogen activation may occur via an intrinsic pathway, possibly mediated by the components of the contact activation process, or via an extrinsic mechanism, involving plasminogen activators derived from the vessel wall. The activity of the fibrinolytic system is modulated by inhibitors that serve to regulate both the activation of plasminogen and the proteolytic effect of
plasmin, as depicted by the broken lines in figure 2.3.

Figure 2.3: Basic representation of the interaction of the coagulation and fibrinolytic systems[25].

While deficient fibrinolysis may result in delayed clearance of fibrin and thus an increased susceptibility to thrombosis, excessive fibrinolysis can cause a thrombus to be dislodged, with the resultant thromboembolus capable of inducing a stroke by arresting the flow in one of the small cranial arteries.

### 2.4 Factors affecting coagulation

The initiation of the intrinsic system of coagulation occurs when blood contacts any one of the artificial surfaces that have been tested to date[50]. In the search for a way to avoid unwanted thrombus formation, scientists have concentrated on three distinct origins of coagulation activation in the vicinity of foreign surfaces, first proposed as determinants of clotting more than a century ago and known as Virchow’s triad[51]: blood chemistry, the surface material and the flow dynamics (figure 2.4). The progress made regarding each of these factors is briefly discussed below.

#### 2.4.1 Biochemistry

The inability of mechanical valve recipients to survive free of thrombus-related events has led to the need for long-term employment of anticoagulants, such as warfarin
and heparin, which fail to offer completely satisfactory protection. Whilst heparin interferes with all stages of blood coagulation\cite{161}, enhances fibrinolysis, and influences the aggregation of platelets and the release of chemicals from them, it also puts a strain on the kidneys and liver\cite{154}, and if dosage is inaccurate, can itself be a cause of death\cite{155,25}. Accordingly, studies have been conducted to explore ways of replacing or improving the performance of anticoagulant drugs; for example, thrombolytic agents (e.g. streptokinase and urokinase), which supplement the role of therapeutic anticoagulants by enhancing fibrinolysis and thrombolysis\cite{56}, and agents which prevent development of the initial platelet thrombus\cite{46}.

### 2.4.2 Surface characteristics

When choosing a nonphysiological material for blood contact applications, the texture, surface charge and surface chemistry of the substrate should be scrutinized.

Since large pores may entrain cells and provide regions of stasis which can inhibit dilution of activating species, allowing reaction sequences to propagate, a surface that is smooth with respect to blood cells (i.e. having pores with diameter and depth of the order of 1\(\mu\)m or less) should be sought\cite{6,30}. Since gas-liquid interfaces have been found to initiate aggregation and fragmentation of protein molecules\cite{579}, and bubbles can result in flow disturbances, all surface irregularities should be thoroughly prewetted or degassed before contact with protein-containing solutions\cite{31}. Researchers\cite{39} tend to prime their equipment with saline, in order to prevent the formation of an air-blood

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Figure 2.4: Triad of sources of coagulation activation\cite{53}.
interface and the trapping of air bubbles. Degassing has also been shown to reduce protein adsorption, when tiny air nuclei get trapped in the surface roughness\[58\].

The charge that a surface possesses appears to affect but not solely determine the thrombogenicity of that surface. Gott and his colleagues\[59][60] have performed a large number of in vivo material biocompatibility tests in dogs, some of which included the use of vena caval rings constructed of charged materials\[61\] or materials with an induced charge\[62\]. They found that a methyl methacrylate ring dipped in graphite prior to testing was relatively free of clot after two hours in a canine vena cava, and this result was repeated when a small negative charge was applied to this ring; however, when this ring had an induced positive charge it completely thrombosed within one hour. Sawyer\[63\] has also found that positively charged surfaces are thrombogenic, and that materials which are homogeneously negative tend to be nonthrombogenic. Conversely, Gott et al.\[61\] have found that both electronegative and electropositive materials provide significant thromboresistance. It has been said that while all blood proteins and cells are electrophoretically negative, they tend not to be repelled by negative surfaces, as their surfaces consist of both positive and negative groups\[67a\]. Perhaps the thrombogenicity of a surface is only partly dependent on its charge, and this lack of agreement of results is due to other differences in surface chemistry.

The surface material reactivity determines to a large degree which blood components are adsorbed and how, and is thus relevant to thrombogenesis\[64\]. The sequence of events leading to thrombus deposition on a foreign surface (figure 2.2) begins with the adsorption of a protein layer\[31][39], which physically separates the foreign surface from blood flow. The nature of this layer determines, to some degree, whether or not the ensuing sequence of events lead to a thrombus\[53\]. When a protein solution contacts a foreign surface, the quantity of protein adsorbed, the nature of the bond and the changes that take place in the configuration of the bound protein molecule depend primarily on the nature of the foreign surface and the structure and concentration of the proteins in solution\[57b\]. Friedman et al.\[65\] concluded that the platelet deposition rate is not a strong function of the type of surface, but the morphology, reactivity and
aggregation potential of the first adherent platelets, which may be why the deposition rate and final extent of thrombus have been found by others[31][68] to differ on different materials. The explanation for these findings might be that protein factors in blood may be activated, if they are adsorbed by a surface, in such a way that their native configuration is altered[57a]. Inert surfaces, such as certain types of carbon[67], may adsorb proteins such that their structure is not distorted and/or a stable protein layer is achieved, resulting in the inhibition of platelet adhesion[58][53].

Generally, there are two ways to prepare materials with thromboresistant interfacial characteristics. The first is the biochemical approach where reagents, such as anticoagulants and platelet inhibitors, are immobilized on or slowly released from the surface of the material. The second method involves exploiting the physicochemical properties of microdomain-structured materials, such as block copolymers, to tailor protein adsorption patterns in a way that limits activation by alteration of adhering platelets[57a][64][69–72].

The controlled release or immobilization of pharmaceuticals at the surface of short-term implants has proved successful in preventing thrombogenesis[64][69] and provides a few advantages over conventional anticoagulant therapy, but has yet to be proven feasible for long-term implants in non-laminar blood flows. Immobilization of agents on polymer surfaces appears to be the more promising of the two biochemical approaches, as this method does not suffer drug depletion[69][73]. However, although some researchers[61] have found that coating artificial materials, with substances such as graphite-benzalkonium-heparin (GBH), has enabled a number of implants in laminar blood flow to be thrombus-free after 2 years in dogs, such coating has not allowed implants to maintain a thrombus-free surface in non-laminar flow conditions similar to those near present-day prosthetic heart valves. The biochemical approaches mentioned above do, however, appear capable of preventing the global reduction of blood clottability and the embolic events which are observed with present-day anticoagulant therapy.
Microdomain-structured materials, such as Biomer (segmented polyether polyurethane-urea), appear promising as athrombogenic materials. Kim and Okano have noted that the type and ratio of different proteins adsorbed on a surface are relevant to its thrombogenicity; polymers with a low ratio of adsorbed fibrinogen or γ-globulin to adsorbed albumin, tend to be blood tolerable. They also found that when contacting blood with a hydrophilic-hydrophobic microdomain surface, the adsorbed proteins form an organized structure corresponding to that of the surface microdomain; albumin adsorbs preferentially on the hydrophilic regions, while fibrinogen and γ-globulin adsorb on the hydrophobic areas. Okano et al. constructed block copolymers which exhibited a hydrophilic-hydrophobic type of microdomain structure, and found that an arterio-venous shunt with a surface consisting of alternate lamellar microdomains experienced no thrombus formation for the 3 weeks it was in a rabbit. They attributed this result to suppression of platelet adhesion and minimal morphological changes of adhered platelets. Long-term results of devices composed of these substances are eagerly awaited.

2.4.3 Haemodynamics

The nature, extent and occurrence of thrombus deposition are influenced by the haemodynamic conditions present. It is made apparent that the composition and rheology of fibrin deposits are dependent on local flow conditions when comparing clots and thrombi, which form in stagnant and flowing blood, respectively. When stagnant blood contacts a foreign surface, it solidifies into a dark red, gelatinous clot of blood components evenly distributed in a fine web of fibrin. When flowing blood is in contact with a foreign surface, white, firm and friable deposits are observed, which have nonhomogeneous section, consisting largely of platelets, fibrin and white cells. Kocha et al. discovered that the kinetics of blood protein adsorption onto foreign surfaces was different in systems which were unstirred to ones that had controlled stirring, both in rate of uptake and composition of the steady-state adsorbed layer. Given this and the finding by Kim and Okano, that the type and ratio of different proteins adsorbed by a surface influence that surface's thrombogenicity, it would be expected that the haemodynamics will indeed play a role in determining the tendency of a surface to be thrombogenic. There are numerous examples where blood-material
interactions have been greatly influenced by haemodynamic conditions. For example, increasing the flow rate in a clotting chamber, which results in a smaller mean fluid residence time and elevated shear stresses, has been shown to reduce deposit extent, but can also cause lysis of cells, releasing species which can trigger the coagulation mechanism. Introduction of a separated flow region has been shown to transform a flow obstruction composed of an otherwise athrombogenic material into one which is prone to thrombus formation[76].

The following chapter provides an overview of the fluid mechanics of blood and a review of some of the studies conducted by numerous investigators on the relation between thrombosis and flow conditions, outlining the results and limitations of this work.
Chapter 3

Thrombosis and Flow

Conditions

In the design and application of artificial implants, one of the most critical areas where fundamental knowledge is needed is the effect of fluid flow pattern on the incidence of thrombosis. Blood-material interactions are greatly influenced by the haemodynamic conditions. In particular, some of the causes of implant-induced thrombosis appear to be related to the nonphysiological flow characteristics of the implants themselves, as many artificial materials used for valve construction are essentially less thrombogenic than the valves made from them. Introduction of a valve occluder into an aortic flow field generally produces regions of highly accelerated flow in the annular region between the occluder and aortic walls, and these regions are associated with turbulence production and regions of stagnation and separated flows (figure 3.1).

This chapter provides a survey of the search for a relationship between haemodynamic conditions and thrombus formation, preceded by a general look at the fluid mechanics of blood and cardiovascular modelling.
Figure 3.1: Instantaneous streamline flow patterns for three fully open prosthetic heart valves: the Starr-Edwards caged-ball (figure 1.2(c)), Bjork-Shiley tilting disc (figure 1.2(d)) and the Kay-Shiley caged disc prostheses\cite{81}.

### 3.1 Mechanical properties of blood

Whole blood is a pseudoplastic, or shear-thinning, fluid with a density of about 1059 kg/m\(^3\)\cite{78} and a viscosity that is a function of shear stress, haematocrit (the percentage of the volume occupied by red cells relative to the total volume of blood), and plasma viscosity. Blood behaves in many circumstances as a fluid with shear-independent viscosity, but when the shear rate is reduced to less than 10 s\(^{-1}\) it exhibits a non-Newtonian behaviour largely due to red cell deformation and rotation and rouleaux (long chain) formation\cite{79}. 
Red blood cells are highly flexible, biconcave discoids which constitute 99% (vol.) of the cellular phase\(^1\) in whole blood\(^{[80]}\) and play a major role in the fluid mechanics of whole blood. Red cells have a tendency to aggregate side-by-side to form cylindrical stacks or arrays, termed rouleaux and illustrated in figure 3.2, and in a steady sheared flow the proportion of the cells aggregated into rouleaux at any instant diminishes with increasing shear-rate\(^{[26]}\). Red cells are seen to deform and spin, with the rate at which they do so also being dependent on the shear-rate. The transformation from a suspension of rouleaux to a suspension of individual red cells occurs over a range of shear rates of 0.1 s\(^{-1}\) to 10 s\(^{-1}\) and results in a rapid variation of blood viscosity\(^{[79]}\), as indicated in figure 3.3. At a shear rate of about 1 s\(^{-1}\) the cells bend as they spin, but as the shear-rate is increased they no longer spin and in pipe flow align themselves with the flow\(^{[26]}\). When the shear-rate rises above 100 s\(^{-1}\), the apparent viscosity approaches an asymptotic value of about 0.0033 kg/ms\(^{[82]}\) and blood displays Newtonian behaviour.

Both the level of red cell aggregation and the plasma viscosity are increased by increasing plasma fibrinogen levels, with fibrinogen acting as a bridging macromolecule between red cells\(^{[83]}\). Recipients of substitute mechanical heart valves have been found to experience increases in plasma fibrinogen concentration\(^{[84]}\) and are accordingly given thrombolytic therapy, which is said to ‘thin’ the blood as it lowers the fibrinogen concentration thus reducing plasma viscosity and red cell aggregation\(^{[85]}\).

### 3.2 Modelling of the circulatory system

Whilst only clinical application can offer proof of success of artificial heart valves, evaluation and comparison of new prosthetic valves prior to implantation in humans plays a vital role in their development and acceptance and can indicate the proper direction for improvement in the design of new valves. Ideally, such testing would be performed in an environment that accurately reproduces the clinical conditions that

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\(^1\)The concentration, diameter and thickness of red cells in whole blood are about \(5 \times 10^6/\text{mm}^3\), 8\(\mu\text{m}\) and 1–3 \(\mu\text{m}\), respectively, and these cells comprise about 45% of the blood's volume. White cells are roughly spherical and have a respective concentration and diameter in whole blood of about 4–11\(\times 10^9/\text{mm}^3\) and 7–22 \(\mu\text{m}\), constituting about 1% (by vol.) of blood. Platelets are rounded or oval, with a concentration, diameter and thickness of 250–500\(\times 10^3/\text{mm}^3\), 2–4 \(\mu\text{m}\) and 0.5 \(\mu\text{m}\), respectively, comprising less than 1% of the volume of blood\(^{[117]}\)\(^{[99]}\).
Figure 3.2: Red cell rouleaux formation on a microscope slide\textsuperscript{[86]}.

Figure 3.3: Variation of apparent viscosity of human blood with rate of shear, assuming the viscosity of plasma is $1.2 \times 10^{-3}$ Ns/m\textsuperscript{2}\textsuperscript{[87]}.
will be demanded of the implant, that permits controlled perturbation of each of the known variables, and that allows continuous analysis of the events associated with or caused by the prosthesis. Tests which do not mimic \textit{in vivo} conditions accurately cannot provide definitive information. Some of the factors that determine an implant's thromboresistance are its configuration, the haematologic and haemodynamic variables and its exposure time to blood\cite{88}. Tests that do not allow systematic control and alteration of each of these variables cannot be expected to produce precise information concerning the relative thromboresistance of an implant. At present, there is no testing technique that satisfies all the criteria of the ideal study method.

However, flow models have been devised that allow some information to be gained regarding implant performance. Current cardiovascular models can generally be classified into three categories: mathematical and computer models\cite{52}; physical models; and animal models. Both physical and animal models are briefly examined here, with a discussion of the fundamental aspects that should be considered when attempting to mimic the human circulatory system and of some of the difficulties encountered when attempting such modelling.

In order to determine the essential properties of a mock flow system, whether of \textit{in vivo} or \textit{in vitro} nature, it is necessary to consider the human circulatory system. The complexities of the cardiovascular system and blood make the construction of a model which mimics well the behaviour of the blood, the heart and its neighbouring passageways an ambitious goal. The characteristics of both blood and the cardiovascular system are quite different from those encountered in classical hydrodynamics; the unsteady flow of the non-homogeneous, complex fluid occurs in a system with distensible walls, frequent branchings, taper and curvature. The circulation of blood depends on the muscular and elastic powers of the heart and of the arteries. The overall elastic behaviour of a blood vessel is determined by its dimensions and the properties of its various constituent materials. About 70% of the walls of all blood vessels consist of water and the remainder of a composite of four different types of tissue: the smooth endothelial lining, which contacts blood; a smooth layer of elastic
fibres just behind the endothelial lining that provides elastic tension; collagen fibres, which produce a high resistance to distension when the vessel's size limit is approached; and smooth vascular muscles, which act to change the diameter of the vessel[2].

The design of a flow experiment with the intention of mimicking another system necessitates careful attention to the requirements of dynamic similitude[89][90]. Dynamic similitude means that all fluid forces acting at any point in the original flow system are in the same ratio as those acting at the corresponding points in the model. The concept of corresponding points implies that the original and model systems must be geometrically similar; and as the ratios of forces acting at a given point are dimensionless, it is evident that a further condition for dynamical similarity is that the non-dimensional numbers representing all relevant force ratios should be the same for model and original. One must establish the kind and range of phenomena to be studied and thus the appropriate values for the relevant non-dimensional parameters. Then one must consider how these values can be achieved and, if some are not attainable, what departures are least unacceptable. For incompressible steady flows in rigid ducts and without any free fluid surface, the Reynolds number (Re) is the only parameter that must be maintained constant for a prototype and a geometrically similar model to be comparable[89a]. For steady-state oscillatory rigid pipe flows[2], the additional important parameter is the frequency parameter (α). Re (= \( \frac{\bar{u}d}{\nu} \)) represents the ratio of inertial force to viscous force (where \( \bar{u} \) denotes the mean velocity of the blood flow in a vessel of diameter d, and \( \nu \) represents the kinematic viscosity of blood), and \( \alpha (= \frac{\bar{r} \sqrt{\frac{\omega}{\nu}}} {r} \) is the ratio of the oscillatory inertial force to viscous force (where \( r \) is the vessel radius, \( \omega = 2\pi f \), and \( f \) is the pulsatile frequency).
3.2.1 Physical models

Many researchers have devised physical models which consist of intricate mock circulation loops or systems with simple flow patterns, and have used flow measurement apparatus and blood analogue fluids, in order to determine the flow characteristics associated with prosthetic valves which are then used to predict valve performance in vivo. Generally, physical models may be classified into three categories: geometric models, which are scaled reproductions of the prototype; distorted models, where the model and the prototype may share only limited geometric resemblance; or dissimilar models that bear no geometric resemblance to the prototype.

One of the most difficult aspects when designing a physical model, is the selection of the parameters that must be included in the model. It is customary to begin with a simple model, as total comprehension of the limitations and assumptions incorporated in the design of the model is essential for proper interpretation of all the results. Though simple physical models provide easy accessibility for carrying out measurements or observations, they have limited versatility, particularly in the number of parameters that may be controlled. In addition, when constructing a simple model, the omission of features that are judged to have only secondary importance, such as distensibility of the walls, or aortic sinuses, may seriously distort the data.

A well-designed flow model, with similar boundary motions and wall compliance and impedance to that in the cardiovascular system, is required to simulate the pulsatile flow and pressure waves found in the cardiovascular system. For an adult human at rest, about 5 l/min of blood flows through the arteries at a pulsatile rate of about 70–80 beats/min, with the typical period of systole being 0.3 s and that of diastole 0.5 s. Using a positive-displacement pump in a mock flow loop, the operator can vary stroke volume, pulse rate and the length of the systolic and diastolic phases of the flow. However, even the use of moulded replicas of a human aorta and distensible tubing with directional reinforcements, in attempts to duplicate the shape, wall compliance and

\(^{2}\) Many different flow visualization techniques have been used, including hot-wire, hot-film and laser Doppler anemometry, and the use of dye injections.
anisotropy of the cardiovascular structure surrounding heart valves for *in vitro* testing, cannot at present provide the intricate and complex waveforms, haemodynamics and flexibility/rigidity variations occurring in the human heart. Such intricate models may provide a system bearing more resemblance to the physiological reality than a simple model, but how accurately these models duplicate the conditions *in vivo*, and thus how informative they can be, is questionable.

The use of blood *in vitro* is impractical for a number of reasons. Blood clots indiscriminately on foreign surfaces, so *in vitro* systems require that the blood be anticoagulated; however, this limits the usefulness of using animal blood as an analogue to human blood since the response of different animal species to anticoagulants, such as heparin and citrate, varies widely[^76]. Even if this problem were avoided, by using human blood in an *in vitro* model, extrapolation from the behaviour of surfaces *in vitro* to their thrombogenic behaviour *in vivo* is not a reliable way for predicting blood compatibility[^88]. The study of blood coagulation *in vitro*, in the absence of a vessel wall and of mechanisms that may operate at or through the vessel wall and in the presence of currently prescribed anticoagulant, all of which influence platelet behaviour[^99], can only provide approximations to coagulation *in vivo*. Many investigators use simple blood analogue fluids, as opposed to blood, for *in vitro* flow analysis tests of prosthetic heart valves. In many flow situations *in vivo*, such as in vessels of the size of the larger arteries and aorta, blood can be considered to be a homogeneous, Newtonian fluid[^100][^91] with an absolute viscosity of about 0.003–0.004 kg/ms. Generally, researchers simulate the viscosity and density of blood by using a transparent glycerine-water solution, allowing valve motion and the associated flow field to be studied optically.

Some investigators conduct steady, rather than pulsatile, flow experiments but the usefulness of these experiments is debatable. In their favour it has been pointed out that of the time for systole, the opening sequence of an aortic valve takes only 15 percent followed by a quasi-steady phase, which approximates to steady flow[^81], for 55 percent. If the region of interest lies in a vein, steady flow simulation may be acceptable since blood flow in veins has much less of a pulsatile nature than arterial flows. On
the other hand, while this suggests that steady flow experiments are useful, the fact that they can only provide incomplete information must be remembered; undesirable flow conditions arising due to pulsation will go undetected. For example, the question of whether or not the shedding or disruption of separated flow regions occurs will go unanswered, and the lack of a diastolic phase means that questions of the valve's ability to close and of phenomena such as regurgitant jets (associated with high shear stresses) will not be addressed. In addition, Roshcke and Harrison[102] found from their approximate calculations that the shear stresses which occur during the accelerating and decelerating flow phases, before and after the quasi-steady flow phase in pulsatile flow, are markedly greater than those measured in steady flow[81].

3.2.2 Animal models

In the absence of established, reliable in vitro methods for implant thrombogenicity assessment, an obvious avenue open to investigators is the experimental placement of implants in the intact animal cardiovascular system, which allows test materials and implants to be assessed under more clinically relevant situations than in vitro systems.

Certain anatomical and physiological similarities exist between human and numerous animal species which suggest that experimental results obtained in animal trials can be extrapolated to predict certain physiological and pathological phenomena in humans[103]. Animal trials are currently used to assess the adequacy of substitute heart valves for humans, usually by measuring the amount of thrombus present at some point in time after surgical placement, and have been used to study the flow dependence of thrombosis, using a wide variety of axi-symmetric test-bodies inserted into the large arteries or veins[104][105]. Of the numerous parameters that may be considered when selecting an animal model[2][106][107], such as species type, health, age, sex and size, experimental design and cost, the two basic criteria used in choosing an animal are the flow parameters termed the Reynolds number (Re) (evaluated for mean or maximum flow in the cardiac cycle) and the frequency parameter (α), defined earlier. Table 3.1 gives the mean values of the frequency parameter, evaluated at the aortic root in several mammalian species.
Generally, *ex vivo* experiments, where blood is drawn directly from the animal's vascular system into the experimental flow chamber, tend to be conducted for biocompatibility studies. Biocompatibility in such experiments is generally measured by platelet adhesion, quantified by means such as radiolabelling of platelets, and scanning electron microscopy\(^{108}\). While *ex vivo* experiments generally examine short-term interactions, *in vivo* studies allow long-term analysis of the impact of implants and their thrombogenicity.

<table>
<thead>
<tr>
<th>Species</th>
<th>Pulses/min</th>
<th>Radius (mm)</th>
<th>(\alpha) (fundamental)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse</td>
<td>600–730</td>
<td>0.3–0.1</td>
<td>1.19–1.74</td>
</tr>
<tr>
<td>cat</td>
<td>180</td>
<td>2.0</td>
<td>4.4</td>
</tr>
<tr>
<td>dog</td>
<td>72–125</td>
<td>5.5–6.0</td>
<td>8.27–10.68</td>
</tr>
<tr>
<td>man</td>
<td>55–72</td>
<td>10.8–11.1</td>
<td>13.5–16.7</td>
</tr>
</tbody>
</table>

Table 3.1: Mammalian characteristics\(^2\)

Whilst animal trials are currently the best technique available for predicting implant thrombogenicity in humans, they have numerous shortcomings. Not only are such experiments undesirable, regarding animal sacrifice, but there are also uncontrolled variables that seriously compromise their quantitative usefulness, including loss of thrombus by embolization and/or fibrinolysis\(^{109}\); differences in effects of surgery; timing of observations; biological variability from animal to animal\(^{104}\); and species-related variable haemodynamic characteristics of the test system. For example, the contact activation system varies between species, so the undesirable effects caused by an implant in humans could possibly be missed if certain animals are used. For instance, dogs, rabbits, seals and cows possess a contact activation system in which activation of factor XII is slower and the efficiency of plasma protease inhibitors is greater than that in humans. Thus the use of these animals could lead to erroneous conclusions concerning the efficacy and safety of the biomaterial in question\(^{60}\). The above limitations make it difficult to infer from the behaviour of a device in an animal its response in a human. Another shortcoming of animal trials is that since each *in vivo* study generally allows only one experimental observation period with little certainty that all system variables are controlled, statistical requirements demand performance of a relatively large number of experiments, the cost of which is considerable. *Ex vivo* methods can enable a more controlled dynamic picture of thrombosis and embolization.
to be made, but such experiments do not eliminate all of the shortcomings; for example, studies have shown that, as with in vivo trials, platelet deposition on biomaterials is highly dependent upon animal species\textsuperscript{[10]}\textsuperscript{[98]}.

Given the difficulties and major problems encountered using physical and animal models for implant assessment, it is natural to enquire whether a more fundamental approach to blood clotting effects might not prove more advantageous. Experimentation at a basic level to discern the relative roles of haemodynamic variables in thrombosis may be the way forward. By measuring the extent to which a prosthesis is associated with hydrodynamic factors predominant in clotting, it should in principle be possible to infer its likely propensity to cause clotting. However, the relationship between each of the flow phenomena associated with prosthetic valves and thrombosis is uncertain, as the ability to isolate each phenomenon has not yet been achieved. The remainder of this chapter is a discussion of what flow characteristics are believed to affect thrombosis, with reference to results obtained by researchers using physical and animal models.
3.3 Flow phenomena and thrombosis

3.3.1 General principles
When flowing blood contacts a foreign surface and thrombi form, their development involves adhesion and aggregation of platelets on the surface, which is promoted by the release of aggregation-inducing chemicals from both blood and endothelial cells, followed by the development of a fibrin-mesh between aggregates, which traps red cells. Although the flow conditions may not solely determine the degree to which activating species are released, they do appear to determine whether or not a thrombus develops following activation of the coagulation cascade\[^{60}\][\(^70\)][\(^40\)]. The flow characteristics which contribute to the interaction of cells with the vessel wall and prosthetic implants, and are thought to influence the formation of thrombi in circulating blood, include stasis, separated flow, surface contact duration, mass transfer rate, turbulence and shear stress level. These haemodynamic phenomena are frequently associated with each other and may occur simultaneously at sites which are found to be highly conducive to the deposition of platelet thrombi; consequently, their relative importance for thrombus formation has not yet been established. A review of some of the findings regarding the relationship between thrombosis and haemodynamic conditions is presented here.

A region of stasis, in which the local fluid residence time exceeds the mean fluid residence time, can occur adjacent to a step or inhomogeneity in a surface or distal to a sudden flow constriction or expansion. The fluid in such a region can exist as a ‘quiet pocket’ or as a closed vortex (recirculating fluid), depending upon the bulk flow conditions and size of the flow disturbance. Flow separation and recirculation can occur when a flowing fluid is suddenly accelerated and then immediately decelerated, as occurs when passing through an abrupt flow constriction (e.g. artificial heart valve); streamlines separate from the vessel wall slightly downstream of the constriction, and reattach further downstream. The contents of regions of stasis are mainly excluded from convective exchange with the mainstream.
Since branchings and bends of the human circulation are often associated with separated flow\[^{111}\] but not necessarily thrombi, it appears that certain conditions other than stasis must be satisfied at such sites for thrombi to form. Perhaps trauma, sufficient to damage blood or endothelial cells so that they release activating species, is necessary. In the presence of such trauma, regions of separated, recirculating flows adjacent to a foreign surface can promote aggregation, if platelets are trapped and activating species are secreted or trapped in these vortices. Prosthetic surfaces, which are adjacent to slow flow, stagnant zones or flow separation, have been linked to observed sites of thrombus in vivo and in vitro\[^{66}\][\[^{81}\] \[112-114\]. In the case of haemolytic mechanical heart valves, the platelet-activating chemicals released from lysed cells could be trapped and thoroughly mixed in nearby vortices, as regions of elevated shear are followed, almost invariably, by 'deadwater' zones. Diffusion between the bulk flow and these vortices is slow and so dilution is poor, hindering both the clearance of activated coagulation factors and their mixing with inhibitors\[^{115}\][\[^{116}\], possibly allowing the local concentration of aggregation-inducing species to rise above some threshold value (figure 2.2). In addition, vortices promote aggregation and deposition, by increasing the number of collisions between cells and between cells and the surface (i.e. increased blood-surface contact time)\[^{80\[111\]. The lifetime of the vortex and its supply of platelets and activating species may be the factors limiting the level of thrombosis incurred.

It appears that the combination of separated flow and high mass transfer rate may provide a site favourable to thrombus deposition, when the coagulation cascade has been triggered. Increased mass transfer to a foreign surface will increase the interaction of platelets with that surface, and if a region of stasis is present, which allows released activating species to increase in concentration, then the coagulation cascade is likely to escalate. Using electrochemical techniques and an electrolyte solution, Galanga and Lloyd\[^{117}\] studied the flow-induced mass transfer distribution in the vicinity of two prosthetic heart valves in vitro, and correlated their findings to thrombus growth in vivo. They found that large mass transfer coefficients occurred in the vicinity of flow impingements or regions of high shear stresses, and in regions of flow separation, upstream of and distal to the sewing ring as well as immediately downstream of the valves, where shear stresses would be low. In vivo studies\[^{118}\][\[^{119}\], with the same two
artificial heart valves, have revealed that the regions found by Galanga and Lloyd to consist of high mass transfer and low shear-stresses correspond to common sites of thrombi. The regions of both high mass transfer and high shear stresses may have been thrombus-free due to short blood-surface contact times and/or the adverse effect of high shear stresses on cell-cell and cell-surface bonds (figure 3.4).

Turbulence is another flow phenomenon which may influence thrombosis. It provides elevated shear stress levels and increased mass transfer rates, which can either inhibit or favour thrombosis according to the circumstances.

From in vitro and ex vivo studies it has been recognized that platelet-surface interactions are sensitive to the velocity of blood\(^{[98][120]}\), and that increasing shear rate can either inhibit or promote thrombus growth, depending upon the conditions present.

High flow rates and high values of shear at the surface of a boundary (short of that necessary to damage red cells) have been found to minimize the incidence of thrombosis\(^{[89]}\), while low flows have been associated, both clinically and experimentally, with early thrombosis\(^{[120]}\). For example, vascular implants are found to maintain patency more often when in arteries (high flows) than when in veins (low flows)\(^{[121]}\). An increase in flow rate reduces the blood-surface contact duration and increases the rate of supply and removal of cells adjacent to a surface, so unless the fluid is readily coagulable so that aggregation can occur on contact, such sites are unlikely to be favoured for deposition. High shear rates at the surface may also overcome the adhesive forces between blood elements and the surface, as mentioned above. In addition, high surface shears can inhibit coagulation by damaging the species involved in the clotting mechanism\(^{[122][123]}\) and fragmenting the fibrin network as it forms\(^{[124]}\).

In some circumstances high shear rates can promote thrombus buildup\(^{[17][125-127]}\). Subjecting red cells, platelets and the endothelial lining to shear stresses sufficient to cause damage, results in the release of aggregating agents into the plasma which can
Figure 3.4: Deposition of cells depends on the balance between adhesive, cohesive and shear forces[125].

render blood hypercoagulable (i.e. highly likely to coagulate). Further complicating matters, increasing the shear rate can increase the red cell collision frequency and tumbling, enhancing mixing and increasing the rate at which platelets diffuse to a surface and thrombosis occurs[128][129]. Therefore, no simple or universal relation exists between shear stress and thrombosis.

Both the magnitude and duration of the stresses to which cells are subjected (i.e. the 'shear exposure') influence the severity of cell damage. Haemolysis has been found to occur when erythrocytes are exposed to viscous shear stresses of over 4x10³ N/m² for 0.01 ms[131]; of 450 N/m² for 1 ms[132]; and above 150 N/m² for a 2 minute exposure time[133]. Wurzinger and Schmid-Schonbein[130] have found that the lysis rate of red blood cells and platelets subjected to high shear forces correlate in a linear fashion to the duration of exposure. Human platelets have a substantially lower tolerance to shearing than erythrocytes; the first signs of platelet lysis, based on lactic dehydrogenase (LDH) release, have been found to occur immediately at or above a 10–16.5 N/m² range of shear stress[134]. Wurzinger[17][135] found that a shear stress of 170 N/m², acting for

3 It follows that an increase in haematocrit would further improve platelet diffusivity, and this has been found to be the case experimentally[124].
7 ms, made available procoagulant phospholipids from platelets, which accelerate the clotting process. For longer exposure times, such as 113 ms, a shear stress of 57 N/m² was able to produce the same effect on platelets. A typical stress duration time for a blood cell experiencing a prosthetic valve flow field has been said to be of the order of 10 ms[81][136], but the actual time will depend on the specific valve flow field. While formed elements of the blood will pass through heart valves many times during their lifespan, such that their total shear exposure increases on every pass, the endothelium adjacent to a valve is continuously subjected to the heart valve flow field. Fry[128][137] has reported that exposure of the endothelium of the thoracic aorta of dogs to shear stresses greater than 37.9 ± 8.5 N/m², for as little as one hour, resulted in marked deterioration.

Due to the different equipment and fluid treatments used when assessing the critical shear exposure of blood cells, there is disagreement regarding the exact values of shear stress required to cause cell lysis[138]. Nevertheless, it is generally found that mechanical heart valves cause shear stresses sufficient to lyse cells.

3.3.2 Measurement of flow characteristics of prosthetic valves
Yoganathan et al.[98] conducted steady flow in vitro experiments, using a blood analogue fluid, a laser-Doppler anemometer (LDA) and a mock flow loop, in order to measure velocity profiles in the vicinity of five different types of aortic prostheses. At flow rates corresponding to peak systolic flow rates of about 2.5 and 5.5 l/min, the experimentally observed wall-shear stresses and turbulent-shear stresses were respectively about 100 N/m² and 10–100 N/m²; such stresses are capable of damaging the endothelial lining of the ascending aorta, cells adhered there and formed elements in the flow. Figliola and Mueller[81] conducted similar steady flow studies, and also found the shear stresses caused by four different types of prosthetic heart valves to be large enough to cause cellular damage, based on the results of haemolysis-shear tests carried out by others.

It must be realized that valve performance results obtained from steady flow in vitro studies cannot provide the investigator with a thorough idea of how a valve will perform...
more included to be shed or released some of the their contents in pulsatile flow. Since the
which may prove to be very important to thrombosis, is that close to where may be
distinguishable walls must be realized. One difference between steady and pulsatile flows,
correlations, where in vitro studies lack pulsatile flow phenomena, relate motion and
predict the velocities and shear stresses occurring in vivo. The limitations of such
Once again velocity measurements taken in steady flows in which have been used to

sites where thrombosis and lumen growth occurs at the vessel wall are more likely to occur.

blood cells occur and regions of vessel flow/swear are nearly the latter are
respectively. These results appear to indicate that when shear stress capable of inducing
in the major and minor out low region of 100 cm/s 70 cm/s and 20 cm/s 17 N/m²
are the same face of the disc, and average velocities and corresponding peak shear-stresses in
indicate the pressure of a stagnation zone approximately 20 mm wide across the

measured along the perimeter of the minor outflow region. The velocity measurements
and/or on the hinge mechanism (in the minor outflow region), and lumen overgrowth
observed in the depression of the saccular face (the downstream side of the disc) in
regions. With all three values implemented to varying degrees the thrombus formation was
peak systolic flow rate of about 4.5-5.5 liters/min. When the Bar-Jones-Shiley valve is open, the
were conducted in vivo with a steady flow of blood analog to fluid correlations to a

peak stress associated with this valve, and correlated these valve flow characteristics to
I.DA velocity measurements in the vicinity of a Bar-Jones-Shiley prostheses (native
I.DA velocity measurements with the Bar-Jones-Shiley prosthetic heart valve. They performed
overgrowth, associated with the Bar-Jones-Shiley prosthetic heart valve. They performed

Vogelstein et al. [112] have obtained results which appear to indicate a direct correlation

be larger.

with pulsatile flow in vivo, where an acceleration phase occurs, the shear stresses would
in pulsatile flow, as discussed in section 3.2.1, but it may be reasonable to assume that

3. Thrombosis and Flow Conditions
position of the reattachment point of the separated flow region depends on the flow rate, and so is likely to oscillate back and forth in pulsatile flow, the "activated" blood may be released into the circulation from the faltering vortex\cite{130}.

Given the above findings, mechanical heart valves of the types examined all appear to be traumatic to the blood flow, and concomitant formation of regions of stasis adjacent to such valves could be fatal. The observations reported below further elucidate the relation between flow and thrombosis.

### 3.3.3 Deposition experiments in specially designed blood flow systems

Gott and co-workers have conducted a large number of material compatibility tests with flowing blood and various in vivo screening techniques involving the insertion of "flags", "swords" and "rings" in the canine circulation\cite{59,62,139}. The most consistent of these techniques was found to be that in which a ring constructed of the material of interest and of dimensions 9 mm long, 8 mm o.d. and 7 mm i.d. is inserted in the canine vena cava. The reasons for this may be that the smooth tube not only protects the deposit within it from vessel wall movements but may also promote more controlled flow patterns.

The testing sequence adopted by Gott and his colleagues was to conduct a two-hour duration ring test and, if the material was promising with regard to its thromboresistance, a second test similar to the first procedure but for a two-week duration. Gott \textit{et al.}\cite{61}, however, found that they needed an even more severe screening test, as several materials enabled the ring to be thrombus-free for the duration of their two-week implantation tests. In order to categorize further these materials with respect to their thromboresistance, they introduced a flow disturbance in the middle of the ring in the form of an orifice plate or 1 mm wide circumferential "curbstone" (figure 3.5)\cite{60}.

They found that flow separation and recirculation occurring behind the "curbstone"

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\textsuperscript{4} The work reported here and conducted by Yoganathan \textit{et al.} and Figliola and Mueller included the study of the Starr-Edwards 1260 and 2320 (ball), Bjork-Shiley tilting disc, Smeloff-Cutter A-5, Cooley-Cutter A-25, and Kay-Shiley caged disc valves\cite{61,62,112}.
promoted thrombosis; non-ridged rings that remained free of thrombus after two weeks of vena caval placement were heavily thrombosed after 30 min, when a ridge of 0.6 mm height or greater was present. Rounding the sharp leading edge of the ridge was found to eliminate thrombosis when the ridge was 0.6 mm in height, but not when it was 1 mm high. These results indicate that thrombus formation is affected not only by the surface chemical properties but also by the flow conditions near the surface, and that a flow disturbance associated with flow separation and recirculation can promote thrombosis, when occurring adjacent to a foreign material, even when that material is highly thromboresistant.

Vorhauer and Tarnay\textsuperscript{[105],[140],[141]} conducted two sets of experiments in dogs, to evaluate the effect of prosthesis shape on thrombus extent. Nine models (illustrated in figure 3.6) consisting of a body of revolution of 7.6 mm diameter mounted on a support rod, both constructed of aluminium and coated with polyurethane, and extending from a thin-walled cylindrical stainless steel support sleeve, which fits closely inside the artery, were individually implanted in the canine descending aorta. In the first set of experiments, the weight of thrombus on the test-body was of interest, so the model was weighed before and after each 45 min experiment, with any coagulum adhered to the cylinder being removed prior to the final weighing. In the second set of experiments, the aim was to correlate the thrombus extent in the cylindrical steel support to the level of turbulence caused by the different test-bodies, so only the thrombus within the steel cylinder was weighed. In order to avoid the test-bodies themselves accumulating thrombus, which would alter their shape and thus the turbulence intensity they cause,
each implant was subjected to only 20 minutes of blood flow in the second set of experiments. 36 dogs were used for each set of experiments, with each dog having successive implantations of three different models before sacrifice. Each test-body shape was tested 12 times and the order in which they were implanted (i.e. first, second or third) equally distributed, in order to obtain a mean thrombus weight value and to allow comparison of the thrombus extent on the different test-bodies without interference due to changes in blood properties during the three experiments in each dog. The ordering of test-body shapes was found not to be of consequence, however, as the results of a
statistical cross-analysis indicated no significant correlation between thrombus weight and order of implantation for each test-object.

The results of the first set of experiments, using the test-bodies illustrated in figure 3.6(a), appear to indicate that implant thrombogenicity, as measured by deposition on the body itself, is related to the size of associated areas of stasis and turbulence; streamlined test-bodies that induce little turbulence and have small wakes, such as the teardrop, accumulated the least extent of thrombus.

In the second set of experiments, the long teardrop-shaped test-body once again proved to be the least thrombogenic profile, with regard to thrombus extent in the steel cylinder downstream; but for the other test-bodies there was no obvious explanation for the pattern of relationships found between the turbulence intensity and thrombus extent at the wall of the steel cylinder. Vorhauer found that the steel cylinder, without a test-object attached, accumulated a larger quantity of thrombus than when any of the test-objects were in place, with the sole exception of the test-body at the bottom of figure 3.6(b). The explanation for this result may be that the presence of a test-object generally inhibits thrombus formation by disturbing the region of stasis at the cylinder wall (i.e. the boundary layer), by redirecting flow towards this wall, but may, in the exceptional case, promote deposition, by increasing diffusion to, and cell collisions with, the cylinder wall.

The results of Vorhauer and Tarnay's experiments seem to imply that minimizing the size of areas of stasis and the level of disturbance adjacent to a foreign surface (i.e. by streamlining an object) will reduce the extent of thrombus formed, and perhaps that the effect on thrombus deposition of varying the turbulence level depends on the system conditions and the mechanism by which the turbulence acts.

To determine the effect of turbulence on thrombus extent, Smith et al.[142] conducted experiments in dogs. Two arteriovenous shunts were implanted in each dog from a femoral artery to the contralateral femoral vein (figure 3.7(a)). The shunts were
identical in shape, surface area and material, except that one shunt contained a tapered constriction, reducing the flow diameter from 4.8 mm to 1.6 mm, producing turbulent flow, while the flow in the other shunt was laminar. Electromagnetic flow meters were used to monitor the flow rate in both shunts. In order to maintain similar flow rates in both shunts, when the flow rate dropped in one shunt (due to thrombus deposition) the flow rate was decreased accordingly in the other shunt. Flow was stopped and both shunts removed, once the flow rate had diminished to a certain value; this procedure resulted in each shunt being subjected on average to 19 min of blood flow per test. While no gross deposits were found upstream, thrombi were observed downstream from both the turbulence generator and the laminar flow shunt, with the extent of thrombus being much greater in the former shunt.

![Diagram of the turbulent and laminar flow shunts](image)

Figure 3.7: Diagram of the turbulent and laminar flow shunts, used by (a) Smith et al.[142] and (b) Stein and Sabbah[143], through which blood flowed from a femoral artery (FA) to a contralateral femoral vein (FV).
Stein and Sabbah\textsuperscript{143} conducted experiments of a similar type to those of Smith et al.\textsuperscript{142}, but with a few differences: the turbulent and laminar flow shunts were of similar size but different design (figure 3.7(b)), the dogs used were larger, the mean flow rates and Reynolds numbers were lower, and each experiment lasted about 7, as opposed to 19, minutes. In addition to their canine studies, Stein and Sabbah used heparinized canine blood and a hot-film anemometer, in \textit{in vitro} flow studies, in order to determine the turbulence intensity within the turbulent flow shunt and correlate this to thrombus extent. They observed a marked increase in thrombosis when a flow disturbance was introduced to the laminar flow field, as did Smith and his colleagues, and found that increasing the Reynolds number in the turbulent flow shunt increased both the turbulence intensity and weight of thrombi within this shunt. The increase in thrombus extent, when going from the laminar to turbulent flow shunt, and when increasing the turbulence intensity, may have resulted from the concomitant increases in shear stresses, mass transfer of species to the wall, and the number of cell-cell and cell-wall collisions.

The stagnation point flow experiment (SPFE), developed by Dr. Harry Petschek, has been used to study thrombus formation both as a function of haemodynamics and material surface properties\textsuperscript{144,145}, as it provides a technique for directly observing the interaction of blood elements and thrombus formation on artificial surfaces\textsuperscript{146}. In this experiment, blood flows directly from the carotid artery of an anesthetized dog into a flow chamber (figure 3.8), where the first foreign surface it contacts is a 25 mm diameter microscope cover slip on which it impinges and assumes a radially symmetric flow pattern. The flow rate through the chamber is variable, and controlled by a withdrawal system. The blood flows radially outward, between the cover slip and the bottom of the chamber, into a collecting trough from which it is withdrawn at a steady rate and discarded. Experiments of up to 3 hours' duration are usually possible, during which the interaction of the cellular blood elements, fibrin formation and thrombosis on the coated cover slip are directly observed and recorded non-invasively, using dark-field illumination and cinematography. The shape of the test-surface (cover slip) and/or the blood velocity can be altered to facilitate haemodynamic studies. A more detailed description of the system and experimental procedure has been given elsewhere\textsuperscript{146,146}. 
Petschek and co-workers\cite{39,40,66,112,147,148} have conducted stagnation point flow experiments, during which they have observed white cells both flowing over and settling on the surface of the microscope cover slip, individual platelets depositing on this surface, the formation of platelet aggregates, red cell entrapment, and the flow disturbances caused by platelet aggregates forming on the cover slip. Their observations, made both during blood flow and of the stained cover slips, enabled them to construct diagrams of thrombus development (figures 3.9 and 3.10), where a number of phenomena occur in a definite time sequence, as found in part by others\cite{31,114}, some of which are dependent upon the flow and surface conditions.

Before reporting the results obtained from using the SPFE, it is worthwhile noting that the net rate of deposition of blood particles on a surface is dependent upon the rate at which the particles are brought to the surface, as well as other factors such as surface properties, adhesiveness of the particles and surface shear. The local velocity gradient determines the relative diffusion rates of species\footnote{Red blood cells tumble at a rate proportional to the velocity gradient, and this tumbling enhances platelet diffusion to the surface\cite{112,128}.} which may either inhibit or...
Protein layer in about 1/2 to 1 minute adsorbed to the surface and presumably denatured (no formed elements adhered to the surface)

Formation of platelet monlayer (Adherence rate related to the flow velocity and roughly independent of the surface)

Low Flow Velocity

High Flow Velocity

White cell circle (white cell attachment appears to be shear limited)

Symmetric thrombi

Platelet depletion

No further development of thrombus or changes in platelet morphology for up to 3 hours

OR

Thrombus formation and growth on local imperfections

Figure 3.9: Sequence of events observed when fresh canine blood impinges on a microscope cover slip[112].

Figure 3.10: Growth profile of a thrombus as a function of time (t); where \( t_1 < t_2 < t_3 < t_4 < t_5 \), \( W \) denotes the thrombus width, and \( r \) represents the distance from the stagnation point[39].
be essential to the growth process, and is indicative of the shearing stress on any surface element. In the case of a jet impinging perpendicularly on a surface, the shear stress at the central stagnation point is zero and rises radially outward, unlike the mass transfer coefficient which remains constant throughout this region\[^{149}\]. The following sequence of events have been found to occur on the surface of the cover slip in the SPFE.

Within a few seconds of first blood contact, all artificial materials acquire a rapidly thickening film of protein, but no formed elements adhere until about one minute after first blood contact, irrespective of the flow rate. At this point, arriving platelets begin to adhere to the surface, at a rate dependent on the flow velocity but roughly independent of the surface material (i.e. whether or not it is a thrombogenic surface is inconsequential), and a monolayer of platelets is formed. However, the morphological changes and aggregation potential of the first adherent platelets differ on different materials\[^{31}\]; subsequent deposition is dependent upon the surface properties of the test-material and the haemodynamics.

With high blood flow rates and a relatively nonthrombogenic cover slip surface, the uniform layer of platelets remains intact, adhered platelets experience no visible changes, and the only thrombi that form are wedge-shaped thrombi that originate at surface imperfections as small as 20 or 30 \(\mu\)m. The wedge-shaped thrombi have their apex pointing toward the stagnation point, grow in different directions at different rates (figure 3.10), and their shape, size and structure are dependent on local flow conditions\[^{40}\][\(^{112}\)]. The reason why growth of the wedge-shaped thrombi is predominantly in the downstream direction may be that the species released from the adhered platelet aggregates preferentially collect in the wake of the deposit.

On surfaces of moderate thromboreistance and/or at lower flow rates, white cells attach to the surface within a roughly circular region near the stagnation point. The centre of the "white cell circle" (WCC) lies at the stagnation point and its radius decreases with increasing flow rate. Since the blood velocity and surface shear increase with distance from the stagnation point and with increasing blood flow rate\[^{146}\], the mass
transfer coefficient is constant in the impingement zone\cite{149}, and leukocytes only adhere below certain shear rates (i.e. white cells attach to polyurethane for shear flows below 5.5/s, in the SPFE\cite{112}), it appears that the radius of the WCC is shear-limited\cite{113}. Sometimes, platelet aggregation within the WCC, and wedge-shaped thrombi inside or outside the WCC, are observed also. The explanation for white cells adhering in a circular pattern, but the other cells not doing so, is not known; perhaps it is because the white cell-surface bonds are more sensitive to shear and/or the white cells experience a greater force than red cells and platelets, since white cells are larger.

Under extremely low flow conditions and/or on relatively thrombogenic surfaces, white cells may adhere over the entire surface, as well as large, symmetric platelet aggregates or thrombi.

The SPFE can be used to determine relative haemocompatibility of different materials, by comparison of platelet population density, the diameter of the WCC, and/or the flow conditions below which extensive platelet aggregation routinely forms on the test-surface. This will not be discussed further, as the effect of haemodynamics on the development of thrombi is of most interest here and so is discussed below.

Assuming that the surface imperfections which initiate thrombi cause local platelet aggregation, and that the aggregated platelets release species which can cause further aggregation (section 2.2), it has been suggested that platelet aggregate growth occurs by an unstable diffusion-limited mechanism\cite{40}, involving diffusion of platelets and the chemical species responsible for aggregation. When a platelet encounters a critical concentration of activating species near the surface, it sticks to the surface and joins in the aggregation, releasing more of the species to activate other platelets. The growth of the aggregate can be limited only by a shortage in the release rate of the activating species or a shortage in the arrival rate of platelets (figure 2.2). This is in agreement with the growth pattern of wedge-shaped thrombi (figure 3.10).
The effect on thrombosis of introducing a separated flow region to the SPFE has been studied. The SPFE has been performed, using a cover slip with a circular cavity, such that the blood flows radially outward from the stagnation point and encounters a forward-facing step, which is 250 \( \mu \text{m} \) deep and at a radius of approximately 1 mm from the stagnation point\[148\]. The separated flow region, formed upstream of the step, was found to contain freely floating platelet aggregates, which were observed forming and growing (by collisions with other aggregates) and on some occasions embolizing. Presumably, the chemical reactions which cause platelets to aggregate were initiated by the agents released on interaction of plasma proteins and cells with the surface material, with subsequent mixing of the products throughout the volume of the separated region leading to the formation of freely floating aggregates. While extensive symmetric aggregation was observed only at the lowest flow in the normal SPFE, all experiments with a step present produced symmetric platelet aggregation immediately distal to the step, indicating the thrombus-promoting affect of flow separation. Goldsmith and Karino\[80\] have studied cell motions in annular vortices formed at the corner of a tubular expansion, using suspensions of human red cells and platelets. They too found that freely floating aggregates form in a vortex.

The results obtained by Petschek and his colleagues indicate that as the flow rate is reduced, the chemical properties of the artificial surface become more significant and thrombus formation is more likely (as is found when comparing the incidence of thrombosis on implants in arteries and veins\[121\]). Under high flow conditions, the success of an artificial surface may be more dependent upon the elimination of microscopic imperfections than upon the gross properties of the bulk material, provided that the material is not extremely thrombogenic. Although increasing the flow rate favours thrombus deposition, in that it increases the velocity gradient and consequently the diffusion rate of species, it also acts to increase the surface shear and decrease the blood-surface contact time, so that thrombus formation is less likely. In areas of low shear, such as those occurring adjacent to surface steps or at low flow rates, the blood-surface contact time is greater and thrombus deposition is more common, even though the rate of diffusion to the surface is lower.
Hladovec and Riha\cite{Hladovec1980} conducted experiments to simulate the development of a thrombus \textit{in vitro}. They recirculated citrated whole blood, platelet rich plasma (PRP) or platelet poor plasma (PPP) through a system comprising a 50 cm long tube, of 3 mm i.d., with a glass pipe of equal diameter housing a net at its outlet, a pressure transducer and manometer, and a peristaltic pump (figure 3.11). Both the entrance and exit of the tubing were placed in a siliconized glass cuvette, with a nylon net (mesh about 0.25 mm) fixed to the end of the tubing through which blood entered the cuvette. The small volume of fluid (about 7 ml) was circulated at 55 ml/min and 300 pulses/min, at 37°C or room temperature, with changes in the pressure drop (due to thrombus growth) being continuously recorded.

White thrombus formed on the downstream side of the net only (figure 3.11), at a rate which increased with increasing temperature and was unaffected by increases in mesh size (from 0.25 to 0.4 mm) and tube diameter (from 2.7 to 4.4 mm). The formed thrombus occupied less than 1% of the circulating fluid volume. The plot of pressure against time, denoting thrombus development and its resistance to flow, indicated the occurrence of three different phases: no noticeable change, followed by a gradual pressure rise and some aggregation, and ending with an abrupt increase in pressure corresponding to a mass of thrombus formed on the net. All three phases were observed, but were of different duration, when using whole blood, PRP and PPP. Although the
initial rate of thrombus growth was very small with PPP, relative to blood and PRP, all experiments did produce thrombus after about 60 to 70 s.

The trauma experienced by the fluid constituents, when contacting foreign surfaces and being subjected to the stresses caused by the net, is likely to be the cause of aggregation-inducing species being present in the flow system. The small regions of stasis, in the wake of each wire of the mesh, might be expected to trap some of these activating species, since these wakes are adjacent to sites likely to cause trauma. Activating species would also be released into, and would recirculate with, the bulk flow, but no caking of thrombi occurred on the upstream face of the net. Given the concept depicted in figure 2.2, regarding platelet stickiness and some threshold concentration of activating species, the explanation of thrombus forming on the downstream side of the net only, not on the upstream side of the net or in the bulk flow, might be that the concentration of activating species exceeds the threshold value in the wake of the mesh wires but not in the bulk flow. This would mean that platelet-platelet collisions would only result in significant aggregate growth in the wake of the net. Another relevant factor might be the shear stress level; while in the bulk flow the stresses may be degradative to aggregates, inhibiting their growth, the relatively low shear region behind the net is more favourable to aggregation. Lewis[20] proposed that if an increase in the size of the thrombus was accompanied by a larger wake region, then with time larger particles might be trapped in this region. This indeed appears to be the case, as Hladovec and Riha reported that the size of the particles composing the thrombus increased with the direction of the flow, as found in vivo. The shape of the pressure profiles and the duration of their three phases is as would be expected, if a dependence of thrombus growth on species concentration and wake region size did exist. The initial build-up of thrombus is slow, more so when less platelets are present to supply activating agents, possibly as a result of the dependence of thrombus growth on the concentration of activating species and platelets in, and the size of, the region of stasis. An increase in wake region, which appears to occur, would explain the abrupt increase in thrombus growth rate, as more species and collisions are able to occur in this region. These findings appear to illustrate that stasis, which by itself does not initiate thrombosis[151], is essential for thrombosis.
3.4 The next step

An in vitro method for determining the influence of configurational changes on thrombogenicity would be of great value to the heart valve designer. It is clear that there exists a complicated relationship between thrombosis and the haemodynamics, where more than one particular flow condition requires to be present for thrombus formation to succeed. The shear stress level, mass transfer rate and regions of stasis have all been shown to be influential, but their relative importance to thrombosis remains uncertain. It is also apparent that whilst in vitro testing of artificial heart valve configurations is an essential tool for refining a given design, and the only route to assessing the thrombogenicity of a valve is by collation of experimental results for different valves in sequences of animal trials, neither of these methods of testing, whether used separately or in conjunction, is completely satisfactory for assessing the adequacy or biocompatibility of implants intended for human use. Consequently, a nonthrombogenic mechanical heart valve has yet to be designed. In order to improve on the present methods of valve testing, one must continue to try different methods of analysis. Rather than trying to design a complicated protocol, which enables total assessment of the numerous requirements of a heart valve, it may be wise to target specific performance criteria and keep the tests simple, feasible and reproducible. It is with this in view that the idea was pursued of using a coagulable blood analogue fluid in in vitro experiments.

A coagulable blood analogue fluid, which coagulates in a similar way to blood but not as uncontrollably, could potentially be used to predict the thrombogenicity of heart valve prostheses. A model which employs such a fluid may also provide experimental insight into the effects of valve design modifications and the relative importance of different flow phenomena to thrombogenicity. In the cardiovascular flow system, disturbances are introduced by the motion of the heart, the valves, and the arterial wall which, in turn, affect the flow phenomena occurring. The inability to duplicate such aspects of this system means physical models may enable only comparison of valves but not prediction of their individual thrombogenicity. If the elevated shear stresses caused by the valve are capable of triggering blood coagulation but not the analogue fluid’s
coagulation mechanism, complementary tests for evaluating the potential of valve-induced haemolysis and initiation of thrombosis could be combined with tests using the coagulable blood analogue fluid to aid in the assessment of prosthetic valves. It is most likely that a combination of complementary tests will always be used to evaluate new heart valve prostheses, and hopefully progress will take a path away from distasteful animal experimentation.

Promising work has previously been conducted, whereby a coagulable blood analogue fluid has been used to identify flow characteristics likely to be conducive to thrombus formation. This work, as well as the analogy on which it is based, is described in the following chapter.
Milk Clotting and its Analogy with Blood Clotting

The use of blood in *in vitro* coagulation tests is associated with considerable inconvenience and is impractical for many reasons (section 3.2.1). While the use of a coagulable blood analogue fluid is unlikely to provide information regarding the effects of biochemistry and surface properties on thrombosis, such a fluid could provide a more convenient means of studying the hydrodynamic causes of thrombosis.

Lewis\textsuperscript{[20]} and Christy\textsuperscript{[21]} have noted the numerous criteria that a coagulable blood-analogue fluid should satisfy: it should be readily available, of reproducible properties and composition, inexpensive, safe to use, and as similar as possible to blood in respect of its essential clotting propensities and in the adhesiveness and rheology\textsuperscript{1} of the formed clot. The coagulation of blood is a complex cascade of reactions, probably too

\textsuperscript{1}Thrombus formation is a cumulative process whose later course is partially dependent on the rheology of the clot itself.
intricate to be mimicked exactly; however, for the assessment of hydrodynamic effects on thrombosis, the effects attributable to blood chemistry and surface chemistry do not necessarily have to be replicated. The cascade of reactions leading to coagulation can be initiated by damage to the endothelium lining and/or the presence of a foreign material. These two causes are always present when a mechanical heart valve has been implanted – the endothelium lining is damaged by the stitches which fix in place the sewing ring, and the valve provides the foreign surface. Therefore, the blood passing the valve may already be in a hypercoagulable state. Christy and Macleod[24] have suggested that if this is an accurate representation of what is occurring it may be sufficient for the analogue fluid to mimic only the final stage of blood coagulation, that is the action of thrombin on fibrinogen and the subsequent formation of a fibrin thrombus, not necessarily the whole cascade of reactions (of the intrinsic pathway) leading to the formation of thrombin.

Several substances undergo coagulation reactions: gelatin, myosin and albumin form gels under the action of heat, while other proteins, such as ovalbumin[151], procollagen[152] and casein[153] (found in milk) undergo enzymic coagulation under protease treatment. The analogue fluid considered here is milk.

Milk is a fluid which satisfies the above blood analogue fluid criteria and has many advantages over the use of blood in vitro. Milk is not only more convenient to handle than blood (with no infection hazard), but is also inexpensive, readily available and of reproducible clotting behaviour over an extended series of experiments. The amount of enzyme present in enzyme-induced milk coagulation can be controlled, whereas the relevant enzyme present in blood (prothrombin) is present as a precursor and varies in quantity from donor to donor. Jolles[19] has compared the clotting of milk and blood, indicating a number of similarities between enzymic coagulation of milk and the final stage of blood coagulation. Other researchers have found that heated milk flows, to which cheesemakers’ rennet and calcium chloride are added, show clotting behaviour and clot morphology resembling those for flowing blood, at three levels. On a microscopic scale, Petschek’s stagnation point flow experiment with milk[154] gave
deposition results that were remarkably similar to those obtained with blood\[39\]. On
a visible scale, the milk curd deposition observed in a system similar to Hladovec’s
net experiment\[20\] was similar to the in vitro thrombus formation observed with blood
by Hladovec and Riha\[150\]. On a gross scale, Bjork-Shiley and Starr-Edwards heart
valves were tested, in a specially designed heart-valve chamber, and the sites and
pattern of milk curd deposition were found to be similar to those reported for thrombus
deposition in vivo\[22\]. Subsequent experiments, performed by Christy\[21\], produced
results implicating stasis as a prerequisite for milk curd deposition, but indicating that
it was not sufficient by itself; agitation was suggested as a necessary concomitant flow
characteristic for clotting. Whilst these experiments have indicated the suitability of
milk as a blood analogue fluid, and have highlighted certain flow characteristics thought
to promote thrombus deposition, there are numerous questions still to be answered in
this field of work.

In this chapter milk and its clotting are described, with reference to its analogy to
blood and thrombosis, and previously conducted work with milk in the capacity of a
blood analogue fluid is reported. Since development of Christy’s work is the goal of my
research, the difficulties that arose during his use of coagulating milk are outlined,
and ideas of how to resolve them are discussed. This is followed by a review of
the dependence of milk coagulability and rennet activity on various factors, which
is intended to assist workers trying to achieve reproducible milk clotting.

4.1 Milk

About 4000 mammalian species secrete milk with characteristics specific to species
type and geographical position\[155\]. Bovine milk, namely cow’s milk, is the milk type
considered here.

Milk is an aqueous suspension containing both fat globules and protein-rich
microparticles\[156\], called micelles; these micelles are roughly spherical, have a diameter
between 20 and 600 nm\[157\], and contain a calcium caseinate-phosphate complex and
about 80% of the protein in milk. The composition of milk is approximately, by
weight, 87% water, 5% sugar, 4% fat, 3% casein and 0.4% other protein\textsuperscript{[158–160]}, with a calcium ion concentration of about 30 mM, of which about 27% occurs in the micelle\textsuperscript{[155]} (table 4.1). Whole casein is a heterogeneous group of phosphoproteins, with the major fractions designated \(\alpha\)-, \(\beta\)- and \(\gamma\)-caseins\textsuperscript{[161]}, and \(\alpha\)-casein is a complex containing \(\alpha_\text{\(\alpha_1\)}\), \(\kappa\)- and \(\lambda\)-caseins\textsuperscript{[162]}. The caseins have different numbers of phosphate groups: \(\alpha_{\text{\(\alpha_1\)}}\) and \(\alpha_{\text{\(\alpha_2\)}}\)-caseins have about 8 or 9; \(\beta\)-casein has about 5; and \(\kappa\)-casein generally has one phosphate group. The micelle contains two groups of casein components, one soluble and the other insoluble in the presence of \(\text{Ca}^{2+}\) at room temperature; the insoluble components are \(\alpha_{\text{\(\alpha_1\)}}\)-casein and \(\beta\)-casein, to which calcium ions bind strongly, and the soluble protein is \(\kappa\)-casein, which does not bind \(\text{Ca}^{2+}\) to the same degree as the other caseins\textsuperscript{[155]}. The amphiphilic \(\kappa\)-casein molecule contains 169 amino acid residues\textsuperscript{[19]}; the weakly positively charged N(aminoo)-terminal two-thirds (para-\(\kappa\)-casein) are hydrophobic, and the negatively charged C(carboxyl)-terminal third (\(\kappa\)-casein(glyco)peptide) is hydrophilic.

<table>
<thead>
<tr>
<th>Component</th>
<th>Content (g/100g micelles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha_{\text{(\alpha_1)}})-casein</td>
<td>35.6</td>
</tr>
<tr>
<td>(\alpha_{\text{(\alpha_2)}})-casein</td>
<td>9.9</td>
</tr>
<tr>
<td>(\beta)-casein</td>
<td>33.6</td>
</tr>
<tr>
<td>(\kappa)-casein</td>
<td>11.9</td>
</tr>
<tr>
<td>whole casein</td>
<td>93.3</td>
</tr>
<tr>
<td>calcium</td>
<td>2.87</td>
</tr>
<tr>
<td>inorganic phosphate ((\text{PO}_4))</td>
<td>2.89</td>
</tr>
<tr>
<td>total inorganic material</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Table 4.1: Average composition of casein micelles in cow's milk at room temperature\textsuperscript{[163–167]}

4.1.1 Micelle structure

There has been considerable debate about the structure of milk micelles, in particular the location of \(\kappa\)-casein. It is thought that the location of \(\kappa\)-casein in the micelles has a pronounced effect on clotting behaviour, since \(\kappa\)-casein acts as the main agent for preventing aggregation of the formed micelles and is the casein cleaved during the primary phase of enzymic coagulation (section 4.1.2).

\textsuperscript{2}An amphiphile is a molecule which possesses distinct, separate regions of hydrophilic and hydrophobic character.
A number of models of casein micelles have been proposed, which can be divided into three categories: coat-core models; internal structure models; and models in which the micelle is considered as an aggregate of submicelles.

In the coat-core model a core of $\alpha_s$- and $\beta$-casein is completely covered by $\kappa$-casein, and thus prevented from flocculation by calcium. It appears that $\kappa$-casein does indeed reside at or close to the micelle surface, as Dalglish\[168\] found little difference between the amount of $\kappa$-casein hydrolyzed by immobilized and soluble preparations of chymosin\[3\]. However, Ashoor et al.[169] obtained results that oppose the concept of a coat-core model. They added to the milk cross-linked papain, which cannot penetrate into the interior of the micelles, and found that all of the micellar caseins were hydrolyzed at nearly the same rate as molecularly dispersed sodium caseinate, indicating a mixed surface composition.

An internal structure model was proposed by Rose[170], in which the micelles were built of $\beta$-casein polymers to which $\alpha_s$- and $\kappa$-casein were attached by hydrophobic bonding and which were interconnected by CCP (colloidal calcium phosphate) bridges. Because of the dissociation of $\beta$-casein at low temperatures[171], such a model would imply that micelles disintegrate completely upon cooling, which is not observed.

Several investigators have postulated models consisting of casein submicelles. Pessen et al.[172] performed small-angle X-ray scattering on whole casein. They obtained results which indicate the micelle consists of two concentric regions of different electron density, such as might correspond to submicellar inhomogeneous particles containing hydrophobically stabilized inner cores existing within the colloidal micelles (figure 4.1). An interesting model is that by Schmidt[157], who proposed that a number of small subunits, casein submicelles, are linked together by $\text{Ca}_9(\text{PO}_4)_6$ to form micelles (figure 4.2). In this model, the submicelles consist of an average weight ratio of $\alpha_{s1}$-, $\alpha_{s2}$-, $\beta$- and $\kappa$-casein of $3 : 0.8 : 3 : 1$, except at low temperatures, where $\beta$-casein dissociates from the micelles. The submicelles may be built as shown in figure 4.2a, being

\[3\]This conclusion is based on the belief that diffusion of caseins within the micelle is not fast.
almost spherical and having a non-uniform number of κ-casein molecules. Schmidt and Both\cite{180} carried out electron microscopic studies on artificial micelle systems, showing that κ-casein is almost exclusively located at the micellar surface whereas α₁-casein and β-casein are scattered throughout the whole micelle including the surface. Dalgleish and his colleagues\cite{181} conducted light-scattering studies of milk and discovered that the casein micelles have α₂-caseins composing about half of their surface layer and half of their interior, while β-casein appears to be located almost exclusively in the interior of the micelle. They too found κ-casein to reside predominantly at the micelle surface, and additionally that the hydrophobic portion of κ-casein is just beneath the micelle surface, and is ~ 10 nm long, while the other half assumes an outer 'hair' position (figure 4.1).

![Figure 4.1: A proposed structure of the milk micelle.](image)

![Figure 4.2: Schematic representation of a submicelle (a), and a casein micelle (b) composed of submicelles.](image)
This end (or 'hair') of K-casein is cleaved off by the action of chymosin. The amino acids on the caseino(glyco)peptide are mostly negative and so this Phe-Met bond breakage reduces the micellar net negative charge (−18mV to −11mV, approximately).

Figure 4.3: \(\kappa\)-casein cleavage\(^{[155]}\).

The remarkable colloidal stability of the casein micelles is believed to be due primarily to electrostatic repulsion and steric stabilization\(^{[182]}\), and the two constituents: \(\kappa\)-casein and colloidal calcium phosphate (CCP). Micellar stability is at least partly governed by the fact that micelles possess an overall negative charge on their surface, causing them to repel one another and assume spaced out positions\(^{[183]}\). At the natural pH of fresh milk, pH 6.6, \(\kappa\)-casein has a −3.9 mV charge, \(\alpha_{\text{x}}\)-casein −20.0 mV, and \(\beta\)-casein a −12.8 mV charge\(^{[155]}\), and the native casein micelles have a high negative zeta-potential of about −18mV\(^{[184]}\)\(^{[185]}\). The hydrophilic C-terminals of \(\kappa\)-caseins act like hairs on the surface of the micelle\(^{[186]}\) (figure 4.1), tending to make it more difficult for micelles to come together, taking up space and carrying an appreciable part of the negative micelle surface charge. In addition, \(\kappa\)-casein is thought to give stability to the protein micelles in milk\(^{[187]}\), by interacting both with water and the hydrophobic \(\alpha_{\text{x}}\)-casein\(^{[19]}\); \(\kappa\)-casein is able to stabilize \(\alpha_{\text{x}}\)-casein against precipitation with calcium ions\(^{[162]}\)\(^{[187]}\). In spite of its low proportion, CCP plays a key role in maintaining the integrity of the casein micelles\(^{[188a]}\)\(^{[188]}\). The mechanism by which it does this is, however, uncertain\(^{[189a]}\)\(^{[189]}\).

When chymosin removes the stabilizing macropeptide tails of the \(\kappa\)-casein (figure 4.3), it reduces the steric effects and the micelle zeta-potential by 5–7 mV\(^{[183]}\)\(^{[184]}\) making aggregation more likely.

### 4.1.2 Milk clotting

The enzymic coagulation of milk involves \(\kappa\)-casein (the substrate on which the enzyme acts) and the proteolytic enzyme, chymosin, which is the predominant protease in the fourth stomach of the young calf and catalyzes this clotting reaction with a high
degree of specificity. The coagulation of milk by chymosin can be divided into primary, secondary and tertiary stages[190], as outlined below.

During the primary or enzymic stage, chymosin destabilizes the micelles by shaving off the \( \kappa \)-casein 'hair', producing a differently 'coated', more hydrophobic, micelle. Chymosin attacks the \( \kappa \)-casein[187], specifically cleaving its phenylalanine-methionine (Phe\textsubscript{105}-Met\textsubscript{106}) bond, with the amino acid groups adjacent to this bond apparently promoting the attack at this specific site[181][192]. This yields two peptides of differing properties: the hydrophilic, soluble caseinomacropeptide moiety (residues 106-169)[193], which will diffuse away from the micelle after \( \kappa \)-casein cleavage, and the para-\( \kappa \)-casein moiety (residues 1-105), which is strongly hydrophobic and remains with the micelle. The progressive hydrolysis of the \( \kappa \)-casein during the primary stage leads to the alteration of the properties of the casein micelles, such as rearrangement of hydrophobic sites, which increase in number and density on the surface of the casein micelles. The efficiency of micelle fusion appears to depend on the number of sites activated per micelle, which in turn will be proportional to the enzyme concentration. A certain extent of \( \kappa \)-casein proteolysis, about 80%[194], appears necessary before effective aggregation is possible. Each micelle is a large assembly of thousands of individual casein molecules, containing a few hundred or thousand \( \kappa \)-casein molecules, and the enzyme needs time to orientate itself onto the \( \kappa \)-casein molecule. This phase is therefore a relatively slow one, dependent on the chymosin concentration, and is termed the lag-phase, during which no solid particles are present.

The aggregative phase is the secondary or non-enzymic stage of the reaction, during which the formation of coagulum occurs as the destabilized casein micelles aggregate in the presence of calcium ions. \( \text{Ca}^{2+} \) ions accelerate this stage, probably because they help neutralize the already weakened micellar negative charge (section 4.4.5). The milk clot is made up of the three proteins para-\( \kappa \)-casein, \( \alpha \)-casein and \( \beta \)-casein. The secondary step is accompanied by an increase in the milk's turbidity and viscosity, with the probable formation of linear chains. Subsequently, linear casein chains start to cross-link and this leads to a sol-gel transition, where milk changes from a viscous fluid
to a viscoelastic solid. The curd becomes firmer as casein hydrolysis, more cross-links and structural rearrangements continue to occur\textsuperscript{[196]}. 

The tertiary stage\textsuperscript{[196]} involves processes such as syneresis (the expulsion of liquid by the curd) and non-specific proteolysis of the caseins in the curd.

### 4.1.3 Milk clotting time and reaction order

By the criterion of the visible appearance of clots in enzyme-treated milk, the rate of the milk clotting reaction, to a first approximation, is directly proportional to the concentration of clotting enzyme\textsuperscript{[197]}. However, the appearance of visible clots is the result of two different reactions, the enzymic attack on $\kappa$-casein and the aggregation of micelles, which are not separable\textsuperscript{[194]}, since aggregation is initiated before conversion of the $\kappa$-casein substrate by the proteolytic enzyme(s) is complete. The time required to complete these two phases ($t_{\text{clot}}$), when milk is renneted\textsuperscript{4}, is generally referred to as the rennet coagulation time (RCT) and can be represented by the two-term Holter relationship\textsuperscript{[198–201]}:

$$ t_{\text{clot}} \approx \frac{K}{C_{\text{enz}}} + t_2 \ldots (4.1) $$

where $C_{\text{enz}}$ is the rennet concentration, $t_2$ is the time between the end of the primary enzymatic reaction and the onset of the clotting and K is a constant. The first term on the right-hand side of this equation corresponds to the time taken for the primary phase of milk coagulation, and the second term represents the duration of the second phase (aggregation). This equation can be improved, if the enzymic stage is defined and if both the state of the system at which visual clotting is observed and the relationship between the enzymic and coagulation stages are established\textsuperscript{[190]}. Equations\textsuperscript{[202][203]} more complicated than equation 4.1 have been formulated, but how well these and equation 4.1 fit experimental results is determined by the system conditions (section 4.4.5). For example, a drop in pH, or a change in Ca\textsuperscript{2+} concentration, may alter the basic premise on which an equation is based.

\textsuperscript{4}Milk can be coagulated by the addition of cheesemakers' rennet, which contains chymosin (section B.2).
The two phases of milk coagulation have varying degrees of dependence on different system variables. The enzymic (lag) phase appears to be chemically limited, with factors such as the temperature and enzyme activity and concentration dictating the rate of micelle destabilization[2066a], while diffusion and Ca²⁺ concentration are very important in the aggregation phase[2068]. Therefore, system conditions will play a large part in determining which of the two stages will be the rate determining step (R.D.S.) and thus the apparent reaction order.

The activation of micelles is such a slow process that the degree of mixing need not be very large for the enzymic phase to be the R.D.S. To determine the effect of agitation at different stages during the coagulation of milk, a modified version of the Lee-White test was conducted by Christy[24][21]. The Lee-White test is a simple test that was previously devised for blood clotting analysis[207]; 1 ml of blood is placed in a 8 mm i.d. test-tube, which is then plugged and inverted every 30 s, and the end-point taken as the time when the blood no longer flows. Christy's tests also involved inversions every 30 s, but had one 30 s period chosen, for each test, during which the test-tube was shaken vigorously. Christy used a mixture composed of 5 ml of unpasteurized milk and 0.4% (vol.) of both cheesemakers' rennet⁵ and saturated CaCl₂ solution, at 33°C. No matter at what stage during these tests agitation was performed, the clotting time was always 2.5 minutes. Agitation during the lag-phase did not enhance clot formation. However, when agitation was carried out at the stage when particles first became visible to the naked eye, a preferential deposit of coagulum occurred at the wall of the test-tube whose adhesivity to the surface was greater than if no agitation had occurred. The interpretation of these observations may be that during the early enzymic stage there are no precipitated particles which agitation can induce to collide and the diffusion of enzyme to the micelle surface may not be the limiting aspect of this stage. On the other hand, in a region where the fluid has spent a time long enough to develop incipient particles, agitation aids clotting by increasing the collision rate[208] between activated micelles and promoting their intimate contact with the solid surface to which they bond. No reduction in clotting time is observed, either because this test

⁵Rennet contains the enzyme chymosin.
is performed in such a way that the clotting time is very approximate (solidification
being checked for only every 15 s) and/or because the second phase is so fast at this
temperature and Ca$^{2+}$ ion concentration that agitation, whilst enhancing collisions,
may not be able to make a marked difference in the clotting time.

4.1.4 Comparison of milk and blood, and their coagulation

The fluid mechanical properties of milk are substantially different to those of blood,
but this does not seem to be a significant factor in the comparison. Milk is known to
show a very slight increase in viscosity with falling shear rate$^{[209]}$, while the viscosity
of blood increases much more rapidly (figure 3.3). Even at shear rates which are
high enough to make blood behave in an approximately Newtonian manner, milk and
blood still possess several fluid mechanical differences. For example, whole blood and
milk have a density of approximately 1059 kg/m$^3$$^{[78]}$ and 1030 kg/m$^3$$^{[210]}$ at 25°C,
and a Newtonian viscosity of about 0.0033 kg/ms$^{[82]}$ and 0.0013 kg/ms$^{[212]}$ at 37°C,
respectively. In addition, non-Newtonian flow properties exist in blood due to red cell
spin and migration, which lead to increased diffusion rates$^{[213]}$; rennet causes a small
reduction in milk viscosity$^{[214]}$, which increases diffusion in milk; and the fat globules
and micelles in milk will affect its flow properties. Such fluid mechanical differences
between milk and blood do not appear to invalidate the inferences drawn from results
obtained using milk as a coagulable blood analogue fluid (section 4.2).

Although blood and milk are markedly different constitutionally (milk being a
suspension of micelles and fat globules which have little in common with platelets,
erthrocytes and leukocytes present in blood), it has been found that the enzymic
coagulation reaction of milk resembles the final stage of blood coagulation (figure 4.4).
Jolles$^{[19]}$ compared the structural aspects of the clotting of milk and blood, and showed
that although there are obvious differences between the two there is an overall similarity.
Both reactions occur as a result of the action of a proteolytic enzyme on a soluble protein
to yield an insoluble protein, which undergoes a second order polymerisation reaction
to form a fibrous mesh; and furthermore, the kinetics of the two reactions appear to
be the same$^{[215]}$. Both clotting reactions exhibit an induction period during which no
solid material is visible, and the duration of this lag-phase is inversely proportional to the enzyme concentration and reduced by an increase in temperature. The lag-phase of both clotting processes is followed by the appearance of particles which rapidly coagulate to form a structured clot, and the final stages, syneresis and fibrinolysis, both act to break down the formed clot.

The causes of the lag/induction phases in blood and milk coagulation may be different, although both are likely to be due in part to a difference in reaction order between the enzymic and polymeric reactions\[216\]. In blood, the formation of a cross-linked mesh appears to require the removal of both fibrinopeptides, FPA and FPB, from the fibrinogen substrate, with the removal of FPA occurring first\[217\][218]. In experiments in which the rate of release of these fibrinopeptides is measured, a delay is apparent before any FPB is released. The delay in the coagulation of milk appears to be due to a threshold value of the extent of $\kappa$-casein cleavage, below which micelles cannot aggregate\[168\][219], as several active sites on a micelle are needed before collisions will lead to coagulation\[215\][220].

Both thrombin and chymosin are proteases, which have very high specificity, causing limited proteolysis of specific linkages in the fibrinogen (Arg-Gly) or $\kappa$-casein (Phe-Met) molecule respectively, resulting in each case in the liberation of soluble acid peptides (fibrinopeptides or caseino(glyco)peptides) and an insoluble portion (fibrin or para-$\kappa$-casein) which polymerises, in the presence of Ca$^{2+}$.\[19\]
The amino-acid sequences surrounding the thrombin- and chymosin-sensitive linkages of milk \( \kappa \)-casein and human fibrinogen determine in part the susceptibility of these linkages to thrombin and chymosin hydrolysis\(^{[19][191][192][22]} \). The length\(^{[222]} \), composition and sequence\(^{[223]} \) of peptide substrates have been found to be important determinants of enzyme-substrate interaction prior to hydrolysis. In particular, there are indications\(^{[224]} \) that histidine residues of both \( \kappa \)-casein and human fibrinogen play an important part in the interactions between these two proteins and their respective proteases. Several long sequences in \( \kappa \)-casein, corresponding to 80% of the molecule, have 31–42% homology with the fibrinogen molecule\(^{[158]} \), indicating an additional commonality. That the two fluids are genetically related is supported by work done by Fiat et al.\(^{[225]} \), who found that there is an immunological cross-reactivity between bovine fibrinogen and bovine \( \kappa \)-casein.

The above mentioned similarities between milk and blood coagulation prompted experiments to compare the adhesion of clotting milk to surfaces and the behaviour of blood under similar circumstances. Some of these experiments are described in section 4.2, indicating the similarities between deposition from milk and blood flows.

### 4.2 Experiments demonstrating the milk-blood analogy

If milk is to serve as a blood analogue in clotting studies, where the location and growth characteristics of deposition are of interest, it must be capable of depositing clot in identical locations and in similar relative amounts to thrombus formed in vivo, under the same flow conditions. This implies either a clotting reaction kinetically similar to that of fibrin, if kinetics are dominant, or similar diffusive behaviour of clotting elements if the reaction is diffusion-limited. The formed clot should also adhere to surfaces and have rheological properties similar to those of thrombus.

#### 4.2.1 Clot adhesion

It appears that the adhesiveness, and thus the extent, of milk and blood deposits at a flow boundary, are partially dependent on the type of surface material. The stronger
the adhesive bond between the clot and the surface, the greater the potential for a large deposit to develop.

Berridge\[226]\[227] has examined the adhesion of milk coagulum to a number of different surfaces under similar conditions. To allow the enzymic phase of the rennet action to be completed prior to experiments, Berridge treated raw milk with 0.024% (vol.) rennet, at a temperature of 2–4 °C, 18 to 24 hours before each experiment. This cold renneted milk was pumped (at about 200–250 ml/min) via a heat exchanger (which elevated its temperature to 30°C) to a mixing cell where it was mixed with a flow of CaCl₂ (usually 1 ml/min, corresponding to approximately 0.45% (vol.)) sufficient to ensure immediate coagulation on the test-material\(^6\). A film of the milk mixture, on the point of clotting, flowed down a strip of material (usually 25×1.0×0.1 cm) suspended vertically by one end from a balance, producing adhered clots of varying weight. The weight of the strip, and any attached clot, was recorded at intervals until the clot fell off. A strip coated with cholesterol gave by far the best adhesion (a clot of 9 g was achieved after 2.5 hours, with no clot loss), whereas coating the strip with lecithin or viscose gave poor clot adhesion (reaching at most 1.5 g of clot before clot dislodgement). These results and those he obtained from other material tests led Berridge to conclude that the extent of curd adhesion could be reduced to very low levels, if not zero, when the surface is very hydrophilic and a compound of carbon, hydrogen and oxygen only, and a sink for Ca\(^{2+}\). Withdrawal of Ca\(^{2+}\) reduces the rate of the clotting reaction. The curd adhesion being more extensive when on hydrophobic surfaces, as opposed to hydrophilic surfaces, agrees with the known importance of hydrophobic interaction in clotting\[189a]\[195]. A hydrophobic surface might promote coagulation of micelles at the surface, because the tendency for milk to clot in the bulk, as opposed to at a surface, will depend on the interaction forces between the destabilized micelles themselves and that between these micelles and the surface. If the surface is more attractive to the micelles, then the micelles will adhere to the surface preferentially.

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\(^6\)Renneted milk at 4°C will remain a fluid for hours\[228\], but at 37°C will clot in less than a minute. If extra calcium is added clotting will take place in seconds.
The experiments of Gott\textsuperscript{[61][69]} (reported in section 3.3.3), in which rings of different materials were implanted in the venous circulation, and Berridge\textsuperscript{[227]}, with milk (reported above), illustrate the importance of surface chemistry in coagulation. The protective action of lecithin, when used to coat the surface of a milk test piece, is very similar to that of heparin coating a Gott ring. This similarity in the material-dependent nature of both coagulation reactions is perhaps attributable to the influence of material properties on protein adsorption, given that the initial stage in the adhesion of both thrombus and milk curd appears to be the adsorption of a protein layer on the surface.

Before a protein molecule can be adsorbed to a surface it must approach the surface closely enough to allow interaction between the molecular forces involved. This approach is governed by diffusion, so flow and/or stirring or agitation, as opposed to stasis, might be expected to enhance mass transfer and thus the deposition rate. Results have been obtained which imply flow\textsuperscript{[226]} and agitation\textsuperscript{[24]} increase the extent and adhesiveness of milk clot. Berridge\textsuperscript{[226]} found that renneted milk, which is about to clot and that is continuously passed over a surface, forms clot which has stronger adhesion than a clot formed in static milk. He stated, “adhesion in the system milk+rennet occurs most markedly when milk on the point of clotting is moved continuously over a surface”\textsuperscript{[227]}. This may be due to the increased mass transfer rate of micelles to an attractive surface, and enhanced collisions/fusion of deactivated micelles. The results of the modified Lee-White tests, conducted with milk by Christy\textsuperscript{[24]} and described in section 4.1.3, indicate that agitation can result in a tendency for solid-liquid boundary clot formation as opposed to coagulation of the bulk fluid, and improved clot adhesion.

\textbf{4.2.2 Clot rheology}

Scott Blair\textsuperscript{[209][229][230]} has shown that there is a general similarity between the setting process of bovine (and human) blood coagulated by plasma geloplatin, and of milk coagulated by chymosin, and between the rheological properties of the formed clots. He conducted experiments using 50 ml samples of blood or milk in a U-tube gelometer\textsuperscript{[209]}, with air pressures being applied alternately to each side of the column of setting fluid.
The changes in rigidity of coagulating milk and blood with time follow a similar pattern, as illustrated in figure 4.5. However, rigidity moduli (G) of even the very soft blood coagula were many times greater than those of a well-established milk gel. The formed coagulum is simpler for blood than for milk, thrombus behaving like a Maxwell body\(^7\) and milk curd as a Burgers body\(^8\)\[^{229,230}\]. Scott Blair found blood coagula to show extremely simple rheological behaviour, except in the earliest stages of coagulation when the blood gels resembled those of milk. Unlike milk gels, blood coagula gave very simple “creep” curves and almost immediate elastic recovery when the applied pressure was removed, whilst milk gels were found to show hysteresis and memory effects.

\[^7\]A Maxwell body is like a single elastic spring attached in series to a single viscous dashpot.
\[^8\]A Burgers body is like an elastic spring and a viscous dashpot attached in parallel, connected in series with a Maxwell body.
cause some variation in the behaviour of clot, especially in oscillating high shear flows; to compensate for this, the rheology of the clot deposited from milk could be controlled over a wide range by adjustment of added electrolyte concentration\(^{231}\) and temperature\(^{232}\) (section 4.4.5).

### 4.2.3 Milk experiments by Lewis and Christy

With the desire to establish an in vitro means for determining the effect of implant configuration changes on the clotting tendency of prototype devices, Lewis and Christy devised milk experiments with previously performed in vivo and in vitro blood experiments in mind. The milk mixture they used comprised heated unpasteurized milk and small amounts of commercial cheesemakers' (calf) rennet essence and calcium chloride. They concerned themselves with deposition patterns and not with the full sequence of reactions that lead to blood becoming hypercoagulable, starting the analogy at the stage where blood and milk have coagulating species present and are in the vicinity of a solid object. The assumption on which their work was based was that the presence of a heart valve or other foreign body will initiate the sequence of clotting reactions which lead to the blood becoming hypercoagulable and, since the lag-phase of blood is very short, any stasis or regurgitation could enable blood to remain in contact with the sewing ring and artificial valve for a time in excess of the lag-phase. Accordingly, having assumed that the blood is in a hypercoagulable state, which is likely if it is passing over a rigid, foreign material\(^{233}\), their intention was to determine under what hydrodynamic circumstances the activated milk and, by inference, blood will clot on that surface. The lag-phase of milk coagulation enabled milk flow experiments to be conducted, where bulk coagulation does not occur until the milk leaves the test-section. Thus, any clot found on a test-object in the flow is not due to bulk coagulation of the milk at that point. The experiments conducted by Lewis and Christy, their limitations and the implications of the results obtained are considered here.
Experiments by Lewis

To determine whether or not deposition from activated milk and blood is affected in similar ways by the same fluid mechanical phenomena, a series of experiments, executed previously with blood, were performed by Lewis\cite{20}\cite{22}\cite{154}: the Lee-White test; the stagnation point flow and net experiments; and a test for assessing heart valve thrombogenicity, based on hydrodynamics. His results indicate that the clotting behaviour and clot morphology (i.e. clot form and structure) of coagulating milk flows resemble published results for flowing blood, at three levels: microscopic, visible and gross.

Lewis's first milk test was the Lee-White test, which was briefly described in section 4.3.1. In these tests, Lewis used 5 ml of unpasteurized milk and 1% (vol.) of rennet and saturated aqueous CaCl$_2$, at room temperature, and found milk to behave in a manner apparently identical to blood, forming a stationary plug of clot after a definite time delay. Lewis also used the Lee-White test for the assay of clotting activity of rennet and milk, to check for inconsistencies prior to tests. It is noteworthy that these Lee-White tests provide only approximate clotting times, as 30 s between inversions is a relatively long time.

Lewis investigated deposition from a milk mixture flow at different times, temperatures and levels of shear stress, in a stagnation point flow chamber, similar to that used by Petschek\cite{79} with canine blood (described in section 3.3.3 and depicted in figure 3.8). The results of this work, which allowed comparison of the microscopic deposition of renneted milk impinging normally on a glass cover slip with the blood deposition studies by Petschek et al.\cite{66}\cite{114}, signify similitude of clotting behaviour for milk and blood. There was a striking resemblance between the microscopic appearance of the milk and blood deposits forming at and around the stagnation point, and between the sequence of events during these depositions. In medium and low shear flows, Lewis observed wedge-shaped deposits that, as with blood, appeared to be initiated at imperfections on the cover slip. Lewis also found, as did Petschek, that clots did not form at all in high shear flows.
Lewis constructed a replica of Hladovec and Riha's\textsuperscript{[150]} net apparatus, described in section 3.3.3 and illustrated in figure 3.11, to simulate the development of a thrombus, using milk. Ten ml of the milk mixture was circulated by a peristaltic pump at 75 ml/min, through a 50 cm long, 3 mm i.d. tube containing a net at its outlet. Remarkable similarities were found between the results obtained with blood and milk. Milk clot was found to form downstream of the net, and the pressure variation upstream of this net revealed three distinct phases of deposition similar to those observed with blood. Furthermore, microscopic examination of the sectioned milk coagulum formed revealed a similar structure to that reported for blood. The milk clot comprised a protein network in which the size of the trapped constituents (fat globules) increased with distance from the net, and calcium deposits were at its most downstream end. The time of clot formation was found to be unaffected by variation in mesh size and decreased with increasing temperature, as found by Hladovec and Riha with blood. The shearing effect of the flow was found not to dislodge clot from the net, indicating a well adhered clot. This is not surprising, given Berridge's observations\textsuperscript{[227]} (section 4.2.1) and Christy's findings\textsuperscript{[24]} that sufficient stasis and agitation will promote the formation of well adhered clot.

Lewis further extended the range of this experiment by assessing the effects of varying mesh material, milk temperature, and the rennet and CaCl\textsubscript{2} concentrations on clotting time and clot extent. Variation of the net material type did not appear to alter the clotting time, but did alter the final quantity of clot adhered to the mesh; this is in agreement with the findings of Berridge\textsuperscript{[227]}, that adhesion of coagulating casein differs on different surfaces. The relationships between clotting time and temperature, rennet concentration and CaCl\textsubscript{2} concentration are shown in figure 4.6, where the clotting times correspond to the time taken for a 30 mmHg increase in pressure drop to occur\textsuperscript{[234]}. The effect of fluctuations in rennet and CaCl\textsubscript{2} concentrations could be minimized in future experiments, by adopting reagent concentrations which lie on the plateau of the plots for these reagents; however, no temperature-independent region appears to exist, so no choice of experimental conditions can reduce the importance of temperature control.
Figure 4.6: Variation of milk clotting time with varying temperature, and rennet and calcium chloride concentration [20].

The results of the Lee-White test, together with those of the net and stagnation point flow experiments, indicated far reaching physical similarities between milk and blood deposition. These justified Lewis's next step, which was to investigate milk deposition on prosthetic heart valves using milk and an artificial heart chamber.

Unpasteurized milk was pumped at a fixed rate from a 140 litre reservoir to a degassing vessel, via a heat exchanger which elevated its temperature to between 36 and 43°C. The degassed milk was pumped to a constant head tank, from which it was drawn by the pulsations of a hydraulically driven diaphragm pump of an artificial heart system through the prosthetic valve(s) under test. These valves were housed in rigid, perspex heart chambers situated at the diaphragm pump's entrance and exit, respectively. The heart chambers had circular cross-section and the same hydraulic diameters as the respective flow areas adjacent to the mitral and aortic valves in the human heart. Upstream of this artificial heart system about 1% (vol.) of rennet and CaCl₂ were
continuously injected into the milk flow. The milk flow was at a rate in the range 1.5 to 3 l/min, giving experimental durations between 45 to 90 min, and had a pulsatile frequency of 60 to 70 beats/min. At the end of each experiment, the valves were removed for examination and their clot formations were photographically recorded.

The aim of the valve test was not to provide information on blood chemistry or surface chemistry effects but to determine whether or not valve sites prone to thrombus deposition could be identified by trials with milk more readily than by existing techniques. Lewis was unable to rank the valves relative to one another with respect to their thrombogenicity, due to obvious deposition variation resulting from the valves available being constructed of different materials. He did, however, obtain results that indicated the promise of renneted milk as a blood analogue fluid. Lewis found that the location and appearance of the clots forming on the valves with renneted milk were similar to thrombus found in clinical practice, and that the results were reproducible. Curd deposited on the struts and sewing ring of the Bjork-Shiley valve (figure 1.2(d)), which are locations prone to thrombus deposition in vivo, and sometimes a more generalised clot formed on the disc. Milk curd formed inside the sewing ring and on the struts, but not at all on the ball, of the Starr-Edwards prosthesis (figure 1.2(c)). These locations of curd deposition are common sites of thrombus in vivo, with the exception of the valve struts, which seem to accumulate very little thrombus in vivo. Lewis suggested that this difference might be due to the walls of the ventricle or aorta contacting the struts of the caged-ball valve, as the thrombus found on these cage struts in vivo, if any, is on the inside rather than outside of the cage.

Lewis also studied the effect of varying a few aspects of the system conditions. A reduction in the flow rate resulted in greater deposition on the valves (as is found with blood in vivo[121]); this may have been due to lower shear, greater fluid-surface contact time and/or the extended time interval from when milk is first renneted to when it contacts the valve. The earlier the milk is renneted, the more imminent clotting becomes. This was made obvious by the finding that all valves placed in the aortic position in the artificial heart chamber experienced much greater clot extent than when
in the upstream mitral position. An increase in rennet concentration, or slight increase in temperature (i.e. 2°C), were also found to increase clot extent, acting to reduce the clotting time.

The assumptions on which the milk-blood analogy experiments were based have proved to be acceptable, as the milk clotting results Lewis obtained from several experiments correlated well with the location and appearance of thrombus formed with blood under similar flow conditions. It is, therefore, apparent that renneted milk shows great potential as an analogue fluid for use in assessing flow-related implant thrombogenicity.

The only warnings that appear at this stage are the variations in appearance of milk deposits due to bubble impingement and that, in order to avoid misleading indications due to surface chemistry variations, comparisons of clot extent between different implants should be restricted to implants that are prepared in a similar way and are either constructed of or coated with similar materials.

**Experiments by Christy**

Given the promising milk clotting results obtained by Lewis, Christy\(^ {21}\) endeavoured to devise a milk experiment allowing identification of flow characteristics conducive to thrombus formation. A simple flow chamber and determinate, identifiable flow patterns offer at the outset a plausible way of attempting to differentiate the effects of different flow phenomena, so Christy did not develop a model designed to mimic the heart but rather developed an easily characterizable test-chamber for these pioneering experiments. Christy and Macleod\(^ {23}\)\(^ {24}\) constructed an apparatus (figure 4.7), which allowed them to examine curd deposition from steady and pulsatile milk flows on four simple bodies of revolution axially located in a cylindrical test-chamber, and compared their results with those obtained by Vorhauer and Tarnay\(^ {140}\) for blood deposition on similarly shaped test-bodies suspended in the canine descending aorta (section 3.3.3).

In the milk experiments, 15 to 30 gallons of fresh, unpasteurized milk was drawn by a peristaltic pump from milk containers through a plate heat exchanger, where it was
heated to 37°C, then through a degassing vessel containing ceramic balls, and into an elevated header vessel. The milk then flowed at 2 l/min to the 18” long, vertical test-section, either steadily under the influence of gravity or in a pulsatile manner (70 pulses/min) by means of a diaphragm pump. Immediately prior to the test-section, the milk stream was divided into two equal streams to allow continuous injection of 1% (vol.) cheesemakers’ rennet into one and saturated aqueous calcium chloride into the other stream. The two streams were then recombined near the entrance to the test-section, ensuring that the calcium chloride and rennet were well mixed with the milk, and a diffuser nozzle at the mouth of the test-chamber provided a gradual increase in the flow cross-section, from 10 mm to 32 mm. An axisymmetrical test-object was mounted axially in the test-chamber, fixed at its downstream end to a vertical 50 mm long stainless steel rod. The milk stream leaving the test-section was sent to a drain which was copiously flushed with water. The four test-bodies used in these studies were the teardrop, sphere, disc and upstream-apex cone depicted in figure 4.8, all of which were fabricated of P.V.C., with the exception of the sphere, which was nylon⁹. At the

⁹Christy apparently assumed that the effect on clot deposition of this compositional difference is
end of each milk experiment, which had a duration of about 35 min, the test-object was removed, photographed and weighed.

The mean residence time of the bulk milk mixture, on passing the test-object, was kept much less than the estimated lag-phase time for coagulation. The time required to achieve a non-flowing plug in a modified Lee-White test at 37°C (with inversions every 15 s), lay between 30 and 45 s. The residence time of the renneted milk prior to encountering the test-object, determined by Christy using water intermittently injected with dye, was 9 ± 3 s.

The main purpose of the milk clotting system devised by Christy and Macleod, with its rigid, cylindrical test-section, was not to mimic a heart but to assess the relationship between flow characteristics and deposition. However, if comparisons were to be made between deposition in this milk system and that occurring in vivo, dynamic similitude would be desired. Nerem has shown that the onset of turbulence in pulsatile flow depends on both the Reynolds number (Re) and the frequency parameter (α) (defined in section 3.2), and these quantities were also suggested by Schultz and others as the standard ratios for ensuring dynamic similarity in pulsatile flows. Thus, in order to correlate the clot formations occurring in the milk experiments described above to those expected in dog or man, the Re and α values of the respective systems should ideally be similar.

negligible. This assumption will be used here, for discussion purposes, but it is suggested that one should endeavour to use similar materials whenever possible.
Schultz[235] reported that for large, 20 kg dogs, the diameter (d) of the aorta in the
neighbourhood of the aortic arch is between 1.5–2 cm; the kinematic viscosity (\( \nu \)) is
4x10^{-6} m^2/s; the mean flow velocity (\( \bar{u} \)) is 0.125 m/s; and the heart beat rate is
between 150–200 pulses/min. Where \( \text{Re} = \frac{\pi d}{\nu} \) and \( \alpha = \frac{d}{2} \sqrt{\frac{\bar{u}}{\nu}} \), these values for a dog
 correspond to a \( \text{Re} = 470–625 \) and \( \alpha = 15–23 \). Vorhauer's test-bodies had a diameter
of 7.6 mm[140], so their Reynolds number, \( \text{Re}_{\text{test-body}} \), would have been about 240, in
dogs with the above cardiovascular characteristics.

The human adult aorta has a diameter of about 25–30 mm[3]; the viscosity and density
of blood (at 37°C) and the cardiac output of an average healthy adult at rest are
approximately 0.0033 kg/ms^3[82], 1054 kg/m^3[93] and 5 l/min[237], respectively; and
the pulse rate in man is about 55–100 pulses/min[235][2]. The above values for man
correspond to a \( \text{Re} = 1120–1340 \) and \( \alpha = 17–27 \).

The viscosity and density of unpasteurized milk are 0.0013 kg/ms (at 35°C)[212] and
1030 kg/m^3 (at 20°C)[210], respectively. Christy chose to use a test-chamber of 32 mm
i.d., a milk temperature of 37°C, and a milk flow rate and pulse rate of 2 l/min and
70 pulses/min, respectively. He also employed a systole : diastole ratio\(^{10}\) of 1 : 1,
to give a simple sinusoidal pulse and for ease of analysing the flow. These conditions
approximately correspond to\(^{11}\) a \( \text{Re}_{\text{pipe}} = 1051 \), \( \text{Re}_{\text{test-body}} = 328 \), and \( \alpha = 39 \).

The consequences of the values of Re (1051) and \( \alpha \) (39) in the milk flow being much
greater than those of the Re (470–625) and \( \alpha \) (15–23) of blood flow in dogs and different
to the values in man (Re = 1120–1340 and \( \alpha = 17–27 \)), are that comparisons of
results obtained from the milk experiments to results from in vivo studies of thrombus
deposition might not be appropriate. Increasing the Reynolds number may increase

\(^{10}\)Typically a systole : diastole ratio of 3 : 5 occurs in humans[27]; some investigators have conducted
in vitro studies using this ratio, and others have adopted values such as 3 : 4[39] and 1 : 2[80].

\(^{11}\)I have discovered that Christy published miscalculated values[241], writing that “the mean pipe
Reynolds number is 700, the mean Re based on the projected diameter of each test-object is 220, and
\( \alpha \) is 25”. The incorrect values of \( \text{Re}_{\text{pipe}} \) and \( \text{Re}_{\text{test-body}} \) were apparently due to the use of an incorrect
milk viscosity of 0.002 kg/ms and a slightly high value for the density of milk. The value of \( \alpha \) is 31.1,
when using the value 0.002 kg/ms for milk viscosity, so some additional mistake was also made by
Christy to obtain a value for \( \alpha \) of 25.
both the size of the flow disturbance caused by the test-bodies and the duration for which this disturbance lasts. Increasing the frequency parameter might increase the propagation of disturbances and make the flow less stable; however, Christy\textsuperscript{[24]} has found that varying \(\alpha\) by about 12\% appeared to have no influence on the overall structure or behaviour of the flow in dye experiments conducted using water in place of milk, indicating that the results of clotting experiments may not be very sensitive to variation of \(\alpha\).

Christy found that the coagulum deposited on the upstream face of each test-body varied more in extent from one experiment to the next, was generally more randomly distributed, and had a more coarse-grained spongy appearance than that deposited on each test-body's downstream side (figure 4.9). The deposit on the upstream face possibly arose for reasons not related to the shape of the object, perhaps due to bubbles adhering to this part of the test-body or impinging clot dislodged from the test-chamber wall further upstream. The downstream coagulum, however, indicated a more reproducible, less random deposition pattern. Accordingly, the character of the downstream deposit was the quantity further analysed.

Christy chose to compare his milk deposition results to Vorhauer's thrombus deposition results, which were obtained using similarly shaped test-bodies implanted in dogs (section 3.3.3). Figure 4.10 allows a comparison of the deposition results obtained by Vorhauer to those obtained by Christy, indicating that there are both differences and similarities between the two sets of results. In pulsatile flow milk experiments, Christy's ordering of the test-body shapes, based on the extent of deposit adhered to the test-objects, is the complete opposite of that based on the results of Vorhauer's work; in the steady milk flow experiments, however, a close similarity exists between the relative extents of canine thrombus and milk clot. With the exception of the teardrop, which was associated with the second largest extent of milk clot but the least canine thrombus, the order of clotting propensity for the test-bodies was the same for steady milk flows and canine blood flows.
Figure 4.9: Christy’s photographs [24] of the P.V.C. test-objects after a milk experiment: (a) & (e) feature the teardrop; (b) & (f) are of the sphere; the cone is in (c) & (g); and (d) & (h) are of the disc, where the left column corresponds to steady flow tests and the right column to pulsatile flow studies.
Figure 4.10: Ordering of test-body shapes, based on the weights of thrombus or clot formed. Vorhauer's results correspond to test-object thrombus weights, while Christy's clot weights are those of clot recorded on the downstream-side of the test-bodies. Approximate residence times of vortices trapped downstream of each test-body in the milk experiment apparatus are also shown.

The differences between the ordering of test-body shapes, with regard to clot extent, could have occurred for a number of reasons:

1. The Re and $\alpha$ were different in the milk experiments and the dogs, as indicated above, but how different and to what effect cannot be ascertained. Vorhauer did not measure the kinematic viscosity, flow rate or pulse rate of the canine blood, and these values can be very different from one dog to the next. For instance, Darke quoted a mean canine blood flow rate of 3 l/min and pulse rate of 70–140/min for a resting dog of about 20 kg weight; based on the aortic diameter used in the above calculations for a dog, these values correspond to $\bar{u} = 0.2$ m/s, mean $\text{Re}_{\text{pipe}} = 910$, $\text{Re}_{\text{test-body}} = 385$, and $\alpha = 10–19$, which are very different from the values for dogs calculated earlier.

2. It is possible that the extent and shape of clot differed due to the different extents of occlusion caused by the test-bodies used by Vorhauer and Christy. Vorhauer's test-bodies occluded about half of the cross-sectional flow area in the canine aorta, whereas in Christy's milk experiments only $\frac{1}{10}$th of the flow area was occluded. The greater local flow acceleration due to the test-body in the canine studies would cause greater shear stresses and strongly influence the wake region's size and turbulence intensity. Given the large size of test-objects used by Vorhauer, they may even have contacted the wall of the aorta.
3. Christy noted that this large degree of occlusion (resulting in a high pressure drop), and the distensibility of the aortic wall, may have had such a damping effect on the flow in the canine descending aorta, that this flow may have been largely steady with only a small oscillatory component\(^{[24]}\). This would explain the deposition similarities between the canine experiments and steady, as opposed to pulsatile, flow milk studies.

Although the values of Re and \( \alpha \) used by Christy do not lie directly in the range expected to occur in man or dog, his results do shed light on the effect of implant configurational changes and flow phenomena on deposit extent.

Large differences in the extent of coagulum deposited in the test-body wake regions occurred on changing the flow regime from steady to pulsatile. Such differences are presumably due to changes in local fluid residence times, levels of agitation and sizes of wake regions. Accordingly, Christy conducted dye experiments to determine whether or not a correlation exists between the weight of clot formed in the wake region of each test-body and the fluid residence time in, and size of, this region. For each test-body shape, the wake region vortex residence time (figure 4.10) and size (figure 4.11) were estimated, by studying the behaviour of the dye and time taken for it to dissipate from this region, in a water flow\(^{[24]}\) injected with dye\(^{12}\).

In steady flow milk experiments, regions of stasis were associated with curd deposition but the extent of curd did not appear to be directly related to the fluid residence time in, or size of, these regions. Christy observed that in steady flows past test-bodies (where \( \text{Re}_{\text{test-body}} \approx 330 \)) a trapped vortex formed downstream of each test-body (figure 4.11) - the site where all four test-objects developed deposits. In steady flow, the smallest vortex was observed for the teardrop yet this object developed the second largest downstream clot. This might be partly because the vortex formed behind the teardrop was the most long-lived compared to the other test-bodies, but this would not

\(^{12}\)Since Christy's dye experiments were conducted using unfouled test-bodies and water, and water has a kinematic viscosity of about 3/4 that of milk, the predicted residence times and relative sizes of regions of stasis are approximations of the initial conditions only.
explain why the sphere, which had a similar vortex residence time and larger vortex, accumulated about half the amount of clot formed in the wake of the teardrop. In addition, although the vortices behind the sphere, disc and cone test-bodies were of similar size in steady flow, the relative length of their residence times did not seem to determine their ordering with respect to clot extent. The second most long-lived vortex was found to occur behind the sphere, but this test-body developed the least downstream clot.

In half-wave-rectified sinusoidal (pulsatile) flow, the duration of stasis appeared to be the dominant factor determining the extent of deposition. The dye-study revealed long-lived trapped vortices oscillating within the wake region of the teardrop (lasting for 14 s) and sphere (lasting for 8 s), while no vortex remained trapped downstream of either the disc or cone for more than one cycle of the flow. While the wake regions behind the teardrop and sphere were smaller than those behind the disc and cone, the wake region clot extent was greatest for the teardrop, followed by the sphere, while the wake of the cone and disc remained virtually free of clot. It is, however, apparent that factors other than residence time are also relevant to clot extent; the extent of clot was much greater in the disc's wake region relative to that behind the cone, although the

Figure 4.11: Christy's postulated flow patterns for steady and pulsatile flows past unfouled test-bodies[24].
residence times of the vortices trapped behind the cone and disc-shaped test-bodies were both about 1 s, and the wake regions were of similar size. The explanation might be that a change in the level of mixing, due to a change in test-body shape, may alter the aggregate collision rate and thus curd adhesion.

During both steady and pulsatile flow dye experiments, Christy observed dye residing adjacent to the test-chamber wall for 20-25 s, indicating that curd deposition at this wall is likely. Wall clot did indeed form in all milk experiments, and its final thickness was said to be greater in steady than in pulsatile flow experiments. The greatly increased level of agitation in pulsatile flow might be expected to produce a well adhered clot, given Christy's findings with regard to curd adhesion and agitation (section 4.1.3); however, the high peak shear stresses and transient boundary layer occurring in the pulsatile regime may limit clot growth. Another possibility is that wall clot loss occurred, making comparison of the final wall clot thicknesses meaningless.

Christy's results indicate that there is no simple relationship between clot extent and the size of a region of stasis or its residence time; other factors, such as the level of mixing, may also influence the extent of clot formed. Merely making an object more streamlined does not guarantee it will accumulate less deposit when flow separation still occurs. Christy's findings suggest that deposition tends to occur where regions of stasis occur and that, while no one flow phenomenon always controls deposition, variation in local flow conditions can affect deposit extent. For instance, sometimes the influence of local fluid residence time may predominate over that of the level of agitation while in other circumstances the order of these influences is reversed. In Christy's experiments, the vortex trapped behind the sphere had a much smaller residence time in pulsatile flow than in steady flow, yet the clot weight was much greater in the former experiment. This was also the case for the teardrop. The disc and cone also had much smaller vortex residence times in pulsed flow, but accumulated less clot in pulsatile flow than in steady flow experiments. Apparently the increased agitation when changing from steady to pulsatile flow did not compensate for the reduction in the residence times of the vortices behind the disc and cone. The different wall clot extents in steady and pulsatile flows
may have reflected differences in agitation and shear stresses, since the fluid residence
times at the wall appeared to be similar. Stasis and agitation may combine to enhance
coagulation when both occur at sufficient levels, while very short fluid residence times
may limit the extent of clot formation, particularly when accompanied by elevated
shear stresses.

Discussion
The analogy between deposition from milk and blood flows appears to be satisfactory.
The phenomenon of platelet thrombi exuding a particular species, which in turn diffuses
and promotes more aggregation, is very different from the milk clotting system, where
the source of the activating species (rennet and CaCl₂) is well upstream and these
species are mixed throughout the fluid. However, a critical concentration of activated
platelets or destabilized micelles appears to be needed in both cases, in order for
collisions to result in fusion and a deposit to develop. Fulfilment of both 'concentration
requirements' and agglomeration/adhesion appears to need stasis and collisions near a
foreign surface. This similarity in deposition mechanisms is exemplified by comparing
the results of net experiments with blood and milk. Both fluids had activating species
present in their bulk yet deposited clot/thrombus only at the same particular sites,
those associated with both stasis and mixing.

While the previously reported milk clotting results are promising, with regard to the use
of renneted milk to investigate the relationship between haemodynamics and thrombus
deposition, the limitations of this work with milk should be realized.

There are a number of differences between thrombosis in vivo and the milk clotting
occurring in this system, in particular with regard to the effect of fluid phenomena;
the sensitivity of blood, milk, thrombi and milk clots to shear stress, with regard to
degradation and embolization, may differ such that deposit extent in one system does
not indicate the extent that would occur in the other system; diffusion is enhanced
by red cell tumbling in blood flow, but not in milk flow; while the release of clotting
agents into the blood is likely to be highly localised, the active enzyme in the milk
experiments is added to the bulk milk mixture, so that all of the milk clots after a known time and the possibility exists that milk coagulation will occur in regions of stasis not associated with the test-object. In addition, processes exist in vivo for deactivating the proteolytic enzyme, but not in the milk experiments, and the extent of curd and thrombus deposition may differ on the same material. It is thus important to realize that the milk flow model is not intended to provide an estimate of the extent of thrombus that would form, but to indicate sites prone to thrombus deposition.

The large differences between the results from Christy’s steady and pulsatile milk flow experiments add fuel to the long-term debate over the usefulness of steady flow experiments. The effects of vortex shedding and elevated shear/agitation can be absent in steady flow experiments, but these experiments can still be used to study the effect of those flow phenomena that occur in both steady and pulsatile flows. In flow situations where the flow is damped, being only weakly pulsatile, correlation with steady flow experiments becomes more appropriate.

4.3 The challenges

The goal of my research was to extend and improve upon Christy’s milk experiments, making them more fruitful. The usefulness of Christy’s pioneering studies was perceived to have been limited by experimental uncertainties and the absence of a way to continuously observe the events occurring during these experiments.

4.3.1 Wall clot

Christy, and Lewis before him, showed concern about the presence of bubbles and wall clot in their experiments. The effect of these on the test-body clot was uncertain. Reduction of the quantity of vapour bubbles present in the milk flow is an issue addressed in section 6.1.3, while the potentially more significant problem of wall clot formation, and ways to prevent or minimize it, has been considered here. The formation of wall clot can cause flow irregularities and, when the volumetric flow rate is kept constant, can result in an increase in the milk flow velocity by reducing the flow cross-
section, leading to increased shear stresses and a shorter mean fluid residence time in the test-section. Wall clot can also make it difficult to remove the test-object, for examination at the end of an experiment, without loss of its deposit by contacting this clot. The biggest concern, however, lies with wall clot loss. There is the possibility that all, or portions of, the wall deposit may become detached and impinge on the test-body, either adhering to or dislodging clot already formed there, rendering uncertain the origin of any clot deposit then found on it.

Methods that have been adopted to tackle adhesion of coagulating milk to solid surfaces include calcium removal via a membrane[239]; the coating of surfaces[227][240]; periodic back-surfing[241] and use of pulsed flow[242]; and provision of a layer of non-coagulating fluid at the solid boundary[243]. Each of these approaches to inhibiting wall clot formation are considered below, with the conclusion that a method which inhibits clot growth altogether is most suitable, but that if this is not possible, the wall clot extent should be minimized and its loss prevented.

The endothelium stays patent by being permeable to certain chemicals and by it synthesizing prostacyclin[244], a potent inhibitor of platelet aggregation. Using a 'calcium sink', as opposed to injecting a clot inhibiting chemical, may be a way to achieve a patent test-chamber in the milk experiments. Berridge[227][239] found that a cellulose dialysis membrane containing EDTA acts as a Ca\(^{2+}\) sink, providing a curd-free surface.

If such a Ca\(^{2+}\) sink could not be established here, one could search for clot-inhibiting materials by firstly looking at the materials used in the dairy industry. In the dairy business, stainless steel surfaces tend to be used[245] for ease of cleaning and for rust prevention but, according to Berridge's results[227], use of stainless steel will promote adherence of coagulum; Berridge found that curd of about 5 to 9 g adhered to his test pieces of stainless steel, after 90 min of the milk clotting experiment described in section 4.2.1. The low clot extent and curd-releasing tendencies of lecithin-covered surfaces have led many to adopt this composite in order to minimize curd adhesion.
However, wall clot loss is not acceptable here; a strongly adhesive curd bond is needed. Unfortunately, Berridge found that when the bond of curd to surface was stronger than the cohesive curd bond (as for copper and stainless steel), the extent of curd was substantial. A compromise may be feasible; materials assessed in Berridge’s experiments that did not experience large amounts of curd adhesion or curd loss, within the first 40 min (i.e. within the duration of a milk experiment), might be adequate. The candidate materials tested by Berridge which fall into this category and which had less than 1 g of clot adhered to them during this time, include a film of lecithin, a film of an equimolar mixture of cholesterol and lecithin, and regenerated cellulose (viscose). Achieving a uniform layer of these films, and maintaining them during experiments and post-experiment cleansing, may be difficult. Alternatively, hydrophilic regenerated cellulose looks worthy of a trial, as Berridge found it to have the lowest rate of deposition (0.2 mg min\(^{-1}\) cm\(^{-2}\)) and only about 0.5 g of curd adhered after 60 min, up to which point no clot loss had occurred.

Whilst pulsation and momentary reversal of flow have been reported as methods for preventing blockage, they appear to be ways of minimizing, as opposed to preventing, wall clot buildup by intermittent clot dislodgement, which is undesirable here. Lipatov and Fridenberg passed acidified milk through flat and round cross-section channels, at a pulsation frequency of 0.6–3.0 Hz, because they found this ‘prevented’ curd from adhering to the coagulator channel walls. Whether or not clot actually formed and was dislodged, or did not form at all, was not made clear in this document. Although a correlation between Lipatov’s and Christy’s experiments might be informative, with regard to the potential of wall clot loss occurring in Christy’s pulsatile flow experiments (in which the pulsation frequency was about 1.2 Hz), this was not attempted. The reason was that the channel dimensions in the former’s work, and thus the Reynolds number and shear stresses occurring, were not available. Also, acid curds are more easily displaced from surfaces and are known to be softer than enzyme-induced curds.

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\(^{13}\) The flow conditions in Berridge’s experiments are very different from those in Christy’s tests, but this is a starting point.
Hain and Jacob\textsuperscript{[243]} used an approximately similar configuration to Christy's test-section for the continuous production of milk curd from upward flowing renneted milk, and they too observed curd deposition at the wall. In order to overcome this wall clot formation, they used a thin fluid film of milk or whey to act as a lubricant between the walls of the vessel and the curd. Four slits, each extending round the whole circumference of the cylinder near its base, allowed milk or whey to be pumped into the cylinder. The walls of the cylinder were heated, possibly because this would reduce the viscosity and density of the lubricating fluid and thus promote its steady rise up the wall\textsuperscript{[248]}.

Preliminary research into wall clot prevention has been conducted in this laboratory. An undergraduate Honours research project was conducted by Stuart Williamson and myself, in search of a way to eliminate or limit the occurrence of wall clot whilst causing minimal interference to the milk flow. Several new designs or modifications of Christy's test-section were considered. Of the ideas mentioned above, coating of the test-chamber wall and use of an annular film of fluid were further considered. Coating of the wall was, however, not attempted, due to the difficulties involved with coating (stated above) and because coating the wall would not remove the clot-promoting stagnant layer of fluid adjacent to the wall and so may permit the formation of a weakly adhered wall clot. During the course of the undergraduate project, a concurrent liquid/liquid core-annular flow column was designed and constructed and preliminary tests with this column were performed. The purpose of this column was to provide a stable flow of inert, immiscible fluid at the test-chamber wall, in order to inhibit contact of the coagulable core fluid with the wall whilst causing negligible interference with the core-flow of renneted milk. The preliminary results, obtained using steady flow, were encouraging: with water as the core-fluid, and $C_2Cl_3F_3$ as the film fluid, a continuous annular film was established and no entrainment into the core fluid occurred.

Further core-annular flow work was conducted by myself during my postgraduate research. A computer model, intended to act as a guide for evaluating the flow parameters most likely to permit continuous core-annular flow, was developed and
based on several assumptions: the flow in the column is fully developed; there is smooth annular flow with no waves forming at the liquid/liquid interface; there are only axial pressure gradients; and laminar flow occurs. This model enabled evaluation of velocity profiles, interfacial shear stresses, interfacial velocity gradients, axial pressure gradients, fluid film thicknesses, and film and core fluid Reynolds numbers, indicating what values of such parameters as density ($\rho$), viscosity ($\mu$) and flow rate ($Q$) of the two fluids would be most likely to provide a 'stable' core-annular flow regime for steady flow experiments. The velocity profile of a stable column of this type would probably be characterized by a maximum velocity at the centre-line of the tube, with a gradually decreasing velocity with distance from the centre. Therefore, if the velocity profile was found to contain negative velocities or an inflexion, for a given set of conditions, then instability was predicted. The ratios indicated as being the most feasible and practical for the core (c) and film (f) fluids, in steady flow, were $\rho_f/\rho_c = 1.0$, $Q_f/Q_c = 0.01$, and $\mu_f/\mu_c = 0.5$, which were in agreement with expectations based on the experimental findings obtained during the undergraduate research. Most of this work is detailed in a paper[249] reproduced in Appendix A. This core-annular flow regime has yet to be tried in milk experiments; it may not be feasible for pulsatile flow studies, as there is acceleration and deceleration in this flow and an inflexional profile is created in the decelerating phase, making instability and entrainment more likely[27].

4.3.2 Clot growth analysis

Christy's milk experiments were limited to providing a single measurement of clot deposited after a fixed time period and were unable to provide information on the deposition process, such as test-body clot growth rates and concomitant wake region changes. If by some real-time imaging technique one could study what is occurring during the milk experiments, not only could more deposition information be obtained but a thorough investigation of reproducibility could be performed. Clot deposition rates and the extent of the effects of bubbles and wall clot could be quantified, and methods for inhibiting wall clot formation could be assessed. Furthermore, if the imaging technique allowed observation or measurement of the deposition at earlier stages of the experiment, the cost of milk experiments could be reduced by shortening their duration. Shortening the experiment might also reduce the probability of wall clot
loss, if this dislodgement occurs only above a critical curd weight or thickness\textsuperscript{[227]}. An assessment of a visualization technique, intended for continuous imaging of deposition in Christy's milk experiment, is given in chapter 5.

Attempts at recommissioning Christy's equipment were met with many problems, including extensive clotting. Duplicating his pre-run consistency tests, such as modified Lee-White tests, did not provide all the answers to these problems. In order to achieve reproducibility, equipment tests (reported in chapter 6) and a study of the dependence of enzyme-induced milk coagulation on system conditions were carried out, with the findings of the latter work reported in the following section.

### 4.4 Variation of milk, rennet and coagulation

In order to obtain truly reproducible results and allow comprehensive comparisons of results obtained in different laboratories, details of the dependence of milk and rennet properties on their source, composition and pretreatment were sought, as well as information regarding the influence of system conditions on rennet-coagulation of milk.

#### 4.4.1 Milk source

The type of herd from which milk comes can make a difference to the curd it forms. For instance, milk from a Jersey herd has been found to give firmer curd than that from a Holstein-Friesian herd, when formed under similar conditions\textsuperscript{[250]}. "Hamdy et al.\textsuperscript{[256]} studied the effect of varying some milk constituent concentrations on chymosin-induced coagulation of cow's milk. They found that increasing the fat"
content of cow milk, by adding cream or butter-fat oil, extended the coagulation time, whereas Bastian et al.\[150]\ found that the milk clotting time decreases with increasing protein and fat content. Hamdy and his colleagues also found that the clotting time was lengthened when either the %SnF (solids-not-fat) was elevated or the milk was diluted by water. Addition of CaCl₂, so that the soluble calcium in the milk increased from 0.118% to 0.175% (wt.), had the most significant effect – a reduction in the coagulation time by more than four-fold.

κ-casein is the only casein that contains sugars; it has been found that these sugars play a role in milk clotting, possibly by retarding the rate of chymosin action on κ-casein\[257]\. Wheelock and Knight\[258]\ have observed that peptides which lack sugar are released much more quickly by the action of chymosin on κ-casein\[259]\, compared to sugar-rich κ-casein\[257]\.

The molar ratio of αs/κ and β/κ is also relevant to precipitation of para-κ-casein. The αs- and β-caseins accelerate precipitation at low concentrations but are inhibitors at high concentrations\[194]\.

‘Heat stability’ is the length of time required for the onset of flocculation of a milk sample at a given temperature, and can be altered by artificially adjusting the concentration of various salt and protein factors\[260]\. Most of the colloidal and soluble constituents individually modify the heat stability of milk\[165]\. For instance, removal of colloidal calcium phosphate (CCP) from the casein micelle results in drastic changes in micellar properties, including an increase in heat stability\[261]\. The heat coagulation time (HCT) varies with pH, and inter-species differences (including composition differences) result in considerable differences in the shape of the HCT/pH curve\[1886]\. Whilst heat stability characteristics of cow’s milk have been found to be relatively consistent throughout a lactation (excluding very late lactation\[14]\) and over consecutive lactations\[263]\, these stability characteristics are influenced by seasonal\[264]\ and dietary\[265]\ changes. The results of an extensive survey\[266]\ of the heat stability of

\[14]\ The protein concentration of milk increases significantly with advancing lactation\[1888]\.
bulk Scottish milk indicate that milk produced during July–November is most stable, while milk that has very poor heat stability is produced during May and June; the increased stability of milk from cows on pasture was associated with a compositional difference: an increase in the concentration of urea. Webb and Holm[267] also found a relation between milk composition and stability; a solids-not-fat (SnF) content of about 13% was found by them to be the critical value determining whether pre-heating has a stabilizing (greater than 13% SnF) or destabilizing (less than 13% SnF) effect.

4.4.3 Milk pretreatment

The properties of milk and milk products are determined to a high degree by the functional properties of the casein micelles, and are thus influenced by the micellar changes induced by processing conditions. Milk pretreatment is necessary because raw milk is not very stable15 at its milking temperature of 37°C, being susceptible to rapid spoilage by naturally occurring enzymes and contaminating micro-organisms. The best or least alterative way to preserve milk is debatable.

To prevent property changes and inhibit the growth of contaminating micro-organisms, many workers choose to store raw milk at a reduced temperature of about 2–6°C16; however, the resultant milk is considered by some to be ‘processed milk’[268]. The microflora of raw milk can be altered by bulk storage at this temperature[269], as can the rennet coagulation time (RCT) and curd firmness17[270][271]. Berridge[227] has found that stale milk, or milk kept at 4°C for 3 to 5 days before adding rennet, tends to produce more adhesive clot when renneted than when fresh milk is renneted. On the other hand, Davies and White[260] and others[272] have found that cow’s milk can be stored at low temperature, such as 4°C, for up to one week in the absence of pretreatment or preservatives, without significant change in RCT or any other aspect of clotting.

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15 Davies and White[260] have found coagulability of some individual-cow milks to be altered even by exposure to light.
16 Milk is destabilized by freezing and many chemical preservatives[216].
17 Changes in RCT and curd firmness can result from cold storage, due to it causing reversible changes in the physical and chemical status of the raw milk (involving protein and salt[210]).
Modification of many factors, including pH and calcium phosphate/citrate and casein solubility, can occur during cold storage and affect the protein equilibrium in milk and micelle stability. The salts equilibrium and heat stability are much affected by changes in milk pH (table 4.2), which is about 0.3–0.4 units higher at 2–6°C compared with 37°C. Davies et al. and others have found that the calcium and phosphate content of serum increase on cold storage. Soluble casein concentration increases substantially during cold storage, with up to 42% of the total casein being dissolved after storage for 48 hours at 4°C. The equilibrium of αs-casein between serum and micelles is almost independent of temperature, unlike β-casein which dissociates from the micelles into milk serum at low temperature. This is illustrated in figure 4.12, where values were recorded after 4 hours of storage. During longer storage times the αs-κ-casein equilibrium is also affected and both caseins are dissolved to some extent into milk serum, but at lower concentrations than β-casein.

<table>
<thead>
<tr>
<th>Component</th>
<th>pH 5.0</th>
<th>pH 6.6</th>
<th>pH 7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>casein (%)</td>
<td>15.5</td>
<td>19.6</td>
<td>15.8</td>
</tr>
<tr>
<td>calcium (%)</td>
<td>0.16</td>
<td>0.67</td>
<td>0.66</td>
</tr>
<tr>
<td>phosphorous, inorganic (%)</td>
<td>0.05</td>
<td>0.25</td>
<td>0.22</td>
</tr>
<tr>
<td>phosphorous, total (%)</td>
<td>0.09</td>
<td>0.42</td>
<td>0.41</td>
</tr>
<tr>
<td>water (%)</td>
<td>79</td>
<td>74</td>
<td>78</td>
</tr>
</tbody>
</table>

(Temperature: 0–5°C)

Table 4.2: Casein micelle composition variation with pH

<table>
<thead>
<tr>
<th>Component</th>
<th>0 - 5 °C</th>
<th>35 - 40 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>casein (%)</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td>calcium (%)</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>phosphorous, inorganic (%)</td>
<td>0.24</td>
<td>0.29</td>
</tr>
<tr>
<td>phosphorous, total (%)</td>
<td>0.45</td>
<td>0.63</td>
</tr>
<tr>
<td>water (%)</td>
<td>76</td>
<td>62</td>
</tr>
</tbody>
</table>

Table 4.3: Casein micelle composition variation with temperature

Attempts have been made to re-establish the properties of the original raw milk after cold storage. Heat treatment can cause reabsorption of solubilized casein by the micelles and has a considerable effect on the distribution of calcium in milk, which inevitably affects the RCT. Morr found that cooling freshly drawn milk to 0–5°C causes transfer of calcium and soluble casein (table 4.3), mainly β-casein, from
Figure 4.12: The concentration of $\alpha_s$-casein (unshaded) and $\beta$-casein (shaded) in milk serum after storage for 4 hours at various temperatures. The micellar protein was sedimented by ultracentrifugation (190 kg, 2 hours) at the storage temperatures$^{[171]}$.

...the micelles$^{[189b]}$, and that upon warming this process is reversed; at 0–5°C, micellar casein/total casein is 75–80%, whereas at 25°C this percentage increases to 95–98%.

The duration over which Morr cooled and heated the milk to achieve these changes is unknown, but Reimerdes$^{[282]}$ found that maintaining milk at 60°C for 30 min, that has undergone changes in serum and micelle composition due to cold storage, can re-establish a protein content in the serum equal to that in the original fresh raw milk.

Another way of limiting quality changes caused by contaminating microorganisms is heat treatment, but this can cause changes in milk properties$^{[283]}$.$^{[188]}$. Heating milk at 60°C and below, even for prolonged periods, has been found to have little effect on the rennet coagulation time$^{[18]}$.$^{[272]}$, but this is not the case at much higher temperatures. While whey proteins are sensitive to temperatures above 60°C$^{[189a]}$, and undergo complete denaturation in 5 min at 90°C$^{[284]}$, isolated $\kappa$-casein experiences thermal denaturation at 90°C$^{[285]}$.$^{[286]}$. Heat treatment of milk can increase the rennet coagulation time (RCT) to varying extents, depending upon the severity of the heat treatment$^{[188]}$. In the UHT (ultraheat treatment) sterilization of milk, milk

$^{18}$While changes do occur in milk at these temperatures, such as loss of lipoprotein lipase activity when stored at 37°C for 4–24 hours$^{[289]}$, the properties related to RCT appear unaltered.
is sometimes heated at 132°C for 1 s resulting in a longer RCT than for raw milk. Rollema and Brinkhuism have found, using NMR spectroscopy, that elevating the temperature, especially in the range 60–98°C, causes the rigid structure of the casein micelle to disintegrate, giving increased solubility to most of the micelle proteins. Milk is normally pasteurized at or above 72.5°C, for 15 s, altering milk reactivity but not causing denaturation.

One way to avoid the need for milk pretreatment would be to have a few cows in the laboratory, but this option itself poses a few problems and is not practical.

### 4.4.4 Rennet

The origin, preparation and storage of chymosin, as well as the physical and chemical conditions of the medium, influence chymosin activity.

Rennet can come from a whole range of natural sources, such as plants and mammals, with its reactivity and response to changes in the physical and chemical conditions of the medium depending on its origin. According to their origin, their optimum temperature and their heat stability vary, they are more or less sensitive to alkalis, and are more or less activated by acids or by calcium salts. Vegetable rennets of different properties exist, yet none are identical to those obtained from ruminants, each possessing a distinctive behaviour towards exterior agents; vegetable rennets resist the action of heat better, have an optimum temperature that is higher and, in general, act on a scale of temperature more extended than animal rennets.

There are several proteolytic enzymes present in rennet obtained from the stomach of a calf, so special procedures have to be followed to isolate the specific protease – chymosin. Unless calf rennet is prepared in a very precise fashion (section B.2), it will contain other proteolytic enzymes and foreign substances which influence the effect.

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19 Most commercial rennets contain pepsin, the proportion of pepsin depending on the age of the calf when sacrificed. Chymosin appears to be more specific in its attack, with pepsin showing a more general proteolytic activity.
of temperature on its activity and thus the RCT.

The storage conditions of rennet in solution can alter its activity. Rennet in solution maintains its activity at 0°C for a very long time. However, at warmer temperatures (i.e. 20–30°C), a perceptible diminution of enzymic activity is perceived, more so at higher temperatures[232]. Aguhlon[291] found rennet to be considerably influenced in an unfavourable way by ultra-violet rays in the presence of oxygen or in a vacuum.

The activity of rennet in solution is strongly influenced by the solution temperature and pH. Most enzymes work best within narrow ranges of pH and temperature, with both the pH-activity and temperature-activity profiles tending to be bell-shaped[292]. For the interaction with κ-casein, chymosin has a pH optimum between 5 and 5.5[293] and a temperature optimum between 40 and 41°C. The activity of chymosin decreases as the pH or temperature are increased above their optimum value, ultimately being irreversibly denatured, although sufficient activity remains for the enzyme to be effective at the normal pH of milk (~ 6.6).

4.4.5 The effect of certain system conditions on milk coagulation

There are several physical and chemical conditions, such as pH, temperature and Ca\(^{2+}\) ion concentration, which can influence the primary and secondary phases of the overall milk clotting process[294] and, in turn, affect the properties of the final curd[194][232].

The coagulation of milk by calf rennet occurs more rapidly in an acid milk than in a normal milk. Small amounts of acid produce very obvious results; a decrease of pH from 6.8 to 6.3 accelerates the aggregation of renneted micelles by a factor of 2[295], acting on casein in such a way as to make it more apt to coagulate[232] and increasing the activity of the rennet. The general importance of pH is related to a reduction of micelle repulsion and increased enzyme activity, except at low pH (i.e. less than 6) where the acidic nature has similar cleavage action as chymosin (i.e. acid coagulation)[248]. One result of the reduced micellar charge, in acid coagulation, is a reduction in both the micellar calcium and the contribution of calcium to gel-strength, hence the finding that
acid coagulation gives softer cheese\textsuperscript{[247]}. 

Temperature variation not only modifies the micelle composition (table 4.3), but also alters the rapidity of both phases of the milk clotting reaction and the clot rheology\textsuperscript{[232]}. Sharma \textit{et al.}\textsuperscript{[206a]}, using a vibrating sphere viscometer and skimmed milk, found that the initial rate of $\kappa$-casein hydrolysis (determined by measuring the amount of free amino acids per gram of protein per minute), the rate of aggregation and the curd firmness increased with increasing temperature. 

For most single enzymic reactions, the reaction rate is approximately doubled by a 10°C rise in temperature\textsuperscript{[292]}; however, the temperature coefficient varies somewhat from one enzyme to another, depending on the activation energy of the catalyzed reaction and the influence of temperature on the enzyme activity. The milk clotting reaction has at least two stages occurring at the same time, which have different activation energies and different levels of dependence on temperature, so there is a combined temperature effect on the whole system. For instance, Effront\textsuperscript{[232]} found that coagulation occurs quickest at 41°C, whereas at 15°C and 66°C, no coagulation occurs. At 66°C, the rennet deteriorates, losing its ability to coagulate casein. At temperatures below 15°C\textsuperscript{[297],[232]}, enzymic degradation of $\kappa$-casein can occur, but without concomitant coagulation of milk\textsuperscript{[206a],[183]}; subsequent heating of the treated milk, after the rennet action is completed, causes clotting to occur rapidly (at 30°C) or instantaneously (at 45°C). Chymosin can complete its action of cleaving about 90% of $\kappa$-casein on each micelle within 18 to 24 hours\textsuperscript{[227]} at low temperatures, and the second phase is an even longer process at low temperatures. The heat-induced acceleration of the lag-phase and the second phase of milk clotting is possibly respectively due to chymosin activity increasing with temperature and increasing thermal motion and hydrophobic interaction\textsuperscript{[247]}. 

Addition of Ca$^{2+}$ ions to renneted milk improves curd firmness and reduces the clotting time. The effect calcium ions have on the rheology of the clot is possibly linked to the rate of reaction; a high concentration of Ca$^{2+}$ gives a fast rate of reaction, and this may
make the chains link in a much more favourable way to form strong crosslinks. The effect of Ca\(^{2+}\) addition on each of the phases in milk coagulation is not fully understood.

Ca\(^{2+}\) may or may not act as a catalyst or coenzyme in the first phase of milk coagulation, affecting chymosin action on the Phe-Met bond. Whether or not it enhances chymosin activity and the mechanism by which it would do this is uncertain; there are results that indicate that Ca\(^{2+}\) addition does affect the primary phase of milk clotting and other results that indicate the opposite.

Wheelock and Penney\(^{291}\) observed that the initial rate of chymosin action on milk is directly proportional to the amount of peptide released. Based on this observation, Wilson and Wheelock\(^{272}\) investigated the possibility that addition of Ca\(^{2+}\) to milk could alter the primary phase of milk clotting; the RCT was found to decrease, on addition of Ca\(^{2+}\), but the number of peptides released by chymosin did not change, indicating that addition of Ca\(^{2+}\) does not alter the initial rate of the enzymic phase. Contrary to these findings, it has been reported that the addition of Ca\(^{2+}\) does influence the rate of the enzymic reaction; addition of Ca\(^{2+}\) appears to increase the affinity of rennet for the micelle. Although the number of peptides released was similar with and without Ca\(^{2+}\) addition, Green and Marshall\(^{300}\) found marked increases of enzymic as well as clotting activity when up to 5 mM Ca\(^{2+}\) was added to milk. The RCT, and plots of macropeptide released against incubation time with rennet, when different amounts (0 to 5.0 mM) of Ca\(^{2+}\) were added to simulated milk ultrafiltrate, indicate that addition of Ca\(^{2+}\) slightly accelerates the initial rate of the enzymic action of rennet and has a more marked effect on the latter part of this phase.

While there is doubt regarding the effect that Ca\(^{2+}\) addition has on the enzymic phase, much more research has been done on the aggregation (2nd) phase than on the proteolytic stage of milk clotting. Chymosin can cleave \(\kappa\)-casein but cannot coagulate milk at temperatures below 15°C, but if the milk is sensitized by increasing the amount of alkaline-earth salts present renneted milk can coagulate at such temperatures\(^{232}\); this clearly indicates that Ca\(^{2+}\) addition does affect the aggregation phase. This effect of
Ca$^{2+}$ on the aggregation phase appears to be due to a reduction in the activation energy of the second phase, so that an increase in temperature is not essential for coagulation to occur; this may be achieved by the Ca$^{2+}$ ions increasing the hydrophobicity of the micelles and reducing micellar repulsion.
Chapter 5

Ultrasound and its Feasibility for Imaging Curd Growth

In order to extract more information from Christy’s milk experiment, with regard to the course and mechanism of clot formation, a method which allows continuous quantitative measurement of curd deposition on the test-objects was sought. From the various techniques that could be used for studying this growth, ultrasonic imaging was chosen. This chapter attempts to provide a basic understanding of ultrasound, including its nature, how it can be generated and used for imaging, the factors leading to improvement or degradation of images, and the use of ultrasonic imaging in relevant applications. Results are provided of analyses of ultrasound-induced phenomena and of preliminary tests, aimed at determining the feasibility of this ultrasound application and how it can best be implemented.
5.1 Deposition measurement techniques

The nature of the system to be interrogated places limitations on the type of imaging technique that will be suitable. Since the milk mixture is opaque and its clotting is sensitive to both temperature and flow disturbances, optical, thermal and invasive imaging techniques are inappropriate. However, one can consider non-invasive techniques currently used in the diagnosis of thrombosis, such as radioactive labelling\textsuperscript{302}, computed tomography (C.T.)\textsuperscript{303}, magnetic resonance imaging (M.R.I.)\textsuperscript{304} and ultrasonography\textsuperscript{305}. Platelets\textsuperscript{306}, or enzymes that bind to fibrin\textsuperscript{307}, can be marked with radioactive tracer and studied using a scintillation counter; regions of high radionuclide concentration correspond to thrombus. Similarly, in the study of milk clotting, a constituent of milk that is found throughout curd could likewise be labelled. How accurately the deposit dimensions could be measured using this method is uncertain, however and, since the milk is not recirculating but is used in large quantities, much tracer would be needed. C.T. scans have been used for diagnosis of deep venous thrombosis (DVT), where the X-rays produce shadow images which broadly represent the electron density of thrombotic structures. A less hazardous and more accurate technique for measurement of deposit is M.R.I., where the body under investigation is subjected to a magnetic field of a non-hazardous intensity and the energy transferred to the protons of the medium is returned in the form of radio waves; however, the high equipment cost (about $ 2 Million) and unavailability of magnetic resonance machines is discouraging. Ultrasonic scanning was chosen for further study, since this imaging technique offers a rapid, reliable\textsuperscript{309}, relatively inexpensive and easily conducted way of constantly monitoring the progression of thrombus position and size\textsuperscript{310}, while producing no known side effects\textsuperscript{312}. 
5.2 Ultrasound production and its use for imaging

5.2.1 Ultrasound

Ultrasound is energy propagated through a medium by cyclic pressure variation, with high and low pressure regions occurring at frequencies above the range of human hearing (i.e. > \(\sim 20\)kHz). This sound can be thought of as high frequency longitudinal waves, with the motion of the particles of the transmitting medium being parallel to the direction of wave travel, and can be transmitted continuously or in short, regular pulses through any substance, solid, liquid or gas, which possesses elastic properties. The push and pull action of a vibrating sound source, in the pulsed form, results in a short burst of vibration travelling through the medium and influencing a limited number of particles at any one time. These vibrating molecules, in turn, collide with their neighbours and pass on some of that energy, so that a sinusoidal variation in the pressure along the medium occurs. The wave of acoustic pressure flows away from the source, with the part of the wave where the particles are crowded together (the compression region) having a pressure higher than normal, and the region where the particles are furthest apart (the rarefaction region) having a pressure lower than normal (figure 5.1(a)). For simplicity, a sound wave is generally depicted as an oscillatory trace (figure 5.1(b)), where its wavelength (\(\lambda\)) is the distance between two adjacent bands of compression or rarefaction, and its amplitude (\(P_0\)) is the magnitude of the maximum change in pressure caused by the wave.

5.2.2 Ultrasound Transceivers

Ultrasound transducers that both generate and detect ultrasound waves are called transceivers, and can have one or many crystals. A basic ultrasonic single-crystal transceiver takes the form of a small cylindrical tube covered at one end by a disc-shaped crystal, which has thin metallic-film electrodes evaporated on its front and back faces. Multi-element array transducers are commonly 4 to 10 cm long and contain 64 to 256 such crystals that measure 1 to 2 mm wide by 5 to 10 mm long[313]. The crystals employed in ultrasonic transceivers exhibit the property of piezoelectricity; they produce a voltage when deformed by an applied pressure, and conversely change
Particle distribution along a line

(a) compression rarefaction

Sound Source

(b) P direction of wave

\[ \text{Figure 5.1: Sound waves can be depicted as (a) a series of vertical lines, whose spacing represents intensity, or as (b) a sinusoidal continuous wave, where the amplitude (P_0) denotes intensity and the wavelength (\lambda) is the distance between adjacent regions of rarefaction or compression\[313].} \]

shape when a voltage is applied. On applying a high frequency oscillating electric signal to a piezoelectric element, a sequence of crystal contractions and expansions will occur at the same frequency, such that the electric signal is converted into high frequency pressure waves (ultrasound). A piezoelectric crystal can also be used to detect pressure fluctuations, as these cause the crystal to expand and contract which generates an oscillating electric signal\[305]\[313\]. The operation of piezoelectric transceivers is optimum at the natural resonance frequency of the crystal(s), which depends upon the crystal thickness\[314\].

5.2.3 Ultrasonic imaging

Reflection is the primary mode of interaction with matter which enables ultrasound images to be achieved. Pulsed ultrasound transceivers\[1\] can be used to obtain images by use of the echo information received from interfaces which reflect sound. The percentage of the ultrasound beam reflected back to the transceiver from an interface depends on the angle of incidence of the beam and on the difference between the acoustic impedances (Z_1 and Z_2) of the media on either side of the interface (figure 5.2). With

\[1\]Pulsed, rather than continuous wave, ultrasound is used for imaging with a single probe, as a transceiver cannot produce and detect sound waves simultaneously.
Figure 5.2: A schematic representation of the redistribution of energy in an ultrasonic wave incident on an interface (B), where $Z_2 \neq Z_1$. Generally, when sound waves strike a surface, the energy may be divided into three portions: the incident (i), the reflected (r) and the transmitted (t) energy. "s" represents the small shear wave and surface wave components that sometimes occur\[313].

For normal incidence at a flat interface, the percentage of the incident beam energy reflected (R) is expressed by the equation\[305][315]:

$$R = \frac{I_r}{I_i} \times 100\% = \left( \frac{Z_2 - Z_1}{Z_2 + Z_1} \right)^2 \times 100\% \ldots (5.1)$$

where $I_r$ is the intensity of the reflected wave, $I_i$ is the intensity of the incident wave, and $Z_1$ and $Z_2$ are the acoustic impedances of the media on either side of the interface, respectively. The calculation of R becomes more complex when the angle of incidence...
Using information from the backscattered ultrasound signal, diagnostic ultrasound instruments can permit essentially instantaneous, real-time images of both static and moving structures. Similar to the echo-return technique used by bats and dolphins, the ultrasound imager measures three things of importance: the time between transmission and reception of a wave, the direction from which the echo came, and the intensity of the echo. The lapse of time between sending and receiving echoes allows the depth of reflective tissues to be evaluated, using a simple range equation and the average velocity of sound in the medium\(^2\). The direction in which the echo is travelling provides information on the interface alignment, relative to the ultrasound beam, and the amplitude of the echo signal represents the acoustic impedance change across the reflective interface. The two-dimensional cross-sectional representation of structures, deducible from such information from an array of ultrasound beams, is usually displayed on a cathode ray tube synthesized line by line, with each line of the image corresponding to the echo information from an individual pulsed beam.

The part of the ultrasound beam that is of high enough intensity to be effective in producing detectable echoes corresponds to the 'effective beam'. The intensity of the beam corresponds to the rate at which energy is transported by the wave per unit area, and falls off with distance along the axis of the transducer, more rapidly so in the direction at right angles to the central axis. Considering a multi-element transducer to be composed of a large number of small ultrasound sources, each emitting a spherical wave, the intensity at each point in the field, at a given time, is the algebraic sum of the hundreds of individual positive and negative pressure waves arriving at that point. Proceeding along the ultrasound beam, away from the transducer, a great variation in ultrasound intensity from wave front to wave front occurs, with a series of pressure maxima and nulls occurring along the beam axis (figure 5.3). The difference in the path length from different parts of the transducer becomes smaller and smaller, until at the last axial maximum (depicted by X in figure 5.4) nearly all of the acoustic waves

\(^2\)Most ultrasonic imaging monitors that are intended for medical use are programmed to assume the velocity of sound to be that in soft tissue (i.e. 1.54 km/s).
Figure 5.3: A diagrammatic representation of parts of the heterogeneous acoustic beam emitted by the transducer, T. The transverse beam plot at position X shows the last axial maximum and marks the transition between the highly non-uniform near field and the diverging beam of the far field[515].

arrive in phase, and the acoustic intensity is highest (if none of the energy has been absorbed en route). This point is termed the beam focus or focal point, at which the the beam width is a minimum and the best imaging is achieved. The effective beam has a gradually narrowing conical region, which leads up to the focal point and is termed 'the near field', and a diverging region, which occurs beyond the focal point and is called 'the far field' (figure 5.4).

The resolution of an ultrasonic real-time scanning instrument corresponds to the smallest detail that it can depict. More specifically, the contrast resolution defines the smallest detectable difference in tissue reflectivity, while the axial and lateral resolution
5 Ultrasound and its Feasibility for Imaging Curd Growth

determine the minimum size of structure detectable.

Contrast resolution is the minimum change in a physical quantity depicted in an image, characterizing the effectiveness of the instrument to display small changes in grey shade/reflectivity of neighbouring tissues, and is not as affected by frequency variation as are the axial and lateral resolution.

The axial resolution is the minimum separation of two targets that lie on the beam axis, for which separate echo signals can be detected, and in theory is equal to half the pulse length\(^{313}\). This resolution is improved when the spatial pulse length is reduced, the ultrasound source is 'damped' (which makes the emitted and received pulses sharper), and when the ultrasound frequency is increased. Most ultrasound pulse-echo systems emit pulses of 3 to 5 cycles in duration and cannot be altered by the operator; however, for a fixed number of cycles in a pulse, increasing the frequency reduces the wavelength and thus the pulse length (equation 5.2). As a result better axial resolution is achieved, but a reduction in the depth of penetration also occurs, as ultrasound is more easily absorbed at higher frequencies (section 5.3).

The minimum separation of two structures that are side by side, lying in a plane perpendicular to the ultrasound beam axis, for which two separate echo signals can be obtained, is the lateral resolution. The lateral resolution is usually the parameter that limits image quality, as it is always worse than the axial resolution\(^{312,314}\), and is approximately equal to the effective beam width, which varies with distance from the transducer and is best at the focus. Consequently it is dependent on the operating frequency of the transducer, and on the element size or focusing ability. Beam width and focusing are strongly related to frequency since they are both determined by diffraction. The only way you can improve the focusing independently of the frequency is to increase the diameter (i.e. size) of the transducer (section 5.3.3). This is likely to give a bulky transducer which is difficult to work with. Another option is to use a linear-array transducer whose focusing can be controlled electronically (section 5.2.4). In general, increasing the ultrasound frequency results in a longer near field and less divergent
far field (equations 5.3 and 5.4), producing a narrower beam deeper into the tissue and consequently better lateral and axial resolution. These ideas apply equally to multicrystal and single crystal transducers. A group of small crystals is equivalent to a single crystal of the same size\[314\].

The velocity of sound, $c$, depends on the medium through which it is transmitted, is a constant for a given uniform medium, and has a relationship with the frequency, $f$, and wavelength, $\lambda$, of sound which is denoted by the equation\[316\]:

$$c = f\lambda \ldots (5.2)$$

For versatile, high resolution imaging it is desirable to generate a beam with a long, narrow near field and focal zone. The focal zone is the region extending along the beam axis near the focal point, over which the beam width is less than twice that at the focal point. At a given ultrasound frequency, the length of the near field ($L_{nf}$) varies as the square of the crystal radius, $r$, and the angle of divergence in the far field, $\theta$, varies inversely with that radius, denoted by\[305][313]:

$$L_{nf} \simeq \frac{r^2 f}{c} = \frac{r^2}{\lambda} \ldots (5.3)$$

$$\sin \theta \simeq 0.61\frac{\lambda}{r} \ldots (5.4)$$

5.2.4 Electronic and mechanical focusing

Focusing is very useful for enhancing ultrasonic imaging, and can be achieved electronically and/or mechanically. An increased maximum intensity and a smaller width of the ultrasound beam can be achieved by focusing, giving stronger echoes and better lateral resolution on the beam axis. The more extreme the focusing, the better the lateral resolution at the focal point but the smaller the focal zone, as the field strongly converges/diverges on either side of the focal point.
A directional, focused beam can be achieved by activating the strip elements in a multi-crystal transducer with slight time delays between each activation, called 'electronic focusing'. By controlling the delay between excitation of the different elements, the degree of focusing and the focal length can be controlled. The pressure waves coming from the elements can be made to arrive in phase at one particular point, at which constructive interference provides a high intensity focus, with the position of the focal region being determined by the sequence of delays chosen. Large transducers, which contain many strip elements, provide a very sharp focus, as the greater the ratio of transducer width to the wavelength of ultrasound the better the focusing ability of the probe.

Ultrasound can be focused by means of lenses. While electronic focusing in the plane of the array of a rectangular probe does not appreciably affect the width of the beam in the direction perpendicular to this plane, focusing in both directions can be achieved by using annular elements and a circular as opposed to rectangular probe face, or by using concave crystals or an acoustic lens (figure 5.5).

![Figure 5.5: Effects on ultrasound of different lenses.](image)

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3 Control of crystal activation can also be utilized to change the beam direction without physically moving the device.
5.3 Ultrasound attenuation

Reduction of the intensity of an ultrasonic wave as it propagates is described as attenuation. It may arise as a result of the wave energy being absorbed, refracted, diffracted, scattered, or reflected. The size of the energy loss is dependent on the strengths of these attenuating processes, and determines the depth and quality of an ultrasound scan. The attenuation coefficient, \( \mu \), represents the fraction of energy lost due to the various attenuative processes, and is expressed as the rate of decrease of intensity in units of decibel per cm depth of tissue (dB/cm) and given by the equation\(^{313}\):

\[
\mu = \frac{10}{x} \log_{10}(\frac{I_x}{I_o}) \quad (5.5)
\]

where \( I_x \) is the intensity level at a point in the beam a distance \( x \) from the source, and \( I_o \) is a reference value.

5.3.1 Absorption

Absorption of ultrasound is due to internal frictional forces that oppose the vibration of molecules and cause the conversion of some of the wave energy into heat, reducing the intensity of the particle vibrations and the size of the 'effective beam'. The absorption coefficient, \( \alpha \), is the fraction of wave energy absorbed in unit path length and is defined by an equation of the same form as equation 5.5, in which \( \mu \) is replaced by \( \alpha \) when attenuation is by absorption alone\(^{318}\). The amount of ultrasound absorption increases with increasing viscosity of the medium, 'relaxation time' of the molecules and frequency of the ultrasound. Elevating viscosity increases the internal friction of moving molecules so that energy absorption and heat production are increased. The time for a molecule to return to its equilibrium position after it has been moved by the ultrasound wave is termed the 'relaxation time'. If this time is long, such that the molecule does not return to its original position before the next compression wave arrives, extra energy is required to stop and reverse the direction of such a molecule, resulting in increased absorption. The higher the frequency of ultrasound transmitted through a viscous medium, the more rapidly the molecules vibrate and the quicker the compression waves arrive, giving molecules less time to relax between cycles, resulting in more absorption. The upper limit of frequency, for ultrasound propagation, is approximately 5 MHz in gases and 500 MHz in liquids and solids.
5.3.2 Refraction

Refraction is the deviation of an ultrasonic beam from its original direction as it strikes an interface between two media, in which the velocity of ultrasound differs. When the ultrasound wave strikes this interface perpendicularly, no change in beam direction occurs. However, when the incident wave strikes this boundary at another angle (figure 5.6), the transmitted wave does not continue along the same line of propagation as it originally occupied in the first medium, but is refracted. Refraction can cause objects to appear to be in a different location than they actually are, or may cause the shape of an object to be incorrectly depicted by ultrasound, giving rise to refraction artefacts.

The relationship between the incident angle, $\theta_1$, and the refraction/transmission angle, $\theta_2$, when ultrasound strikes an interface comprising two media where $c_1$ and $c_2$ are the velocity of sound in the first and second medium, respectively, is given by Snell's law:

$$\frac{\sin \theta_1}{\sin \theta_2} = \frac{c_1}{c_2} \ldots (5.6)$$

The degree to which the beam is bent or refracted at an interface depends on the difference in the velocity of sound in each medium comprising the interface. As this difference in velocity increases, so does the degree of refraction. When an ultrasonic wave crosses an interface, into a medium in which sound travels more slowly (i.e. $c_1 > c_2$), the beam bends towards the normal, and conversely, the beam bends away from the normal when $c_1 < c_2$. When ultrasound strikes a soft tissue/bone interface at an angle of incidence greater than 22°, the ratio of $c_1/c_2$ for these media is such that the angle of refraction is larger than 90° (i.e. the beam does not enter the bone but is reflected back into the soft tissue).

5.3.3 Diffraction

Diffraction is the spreading of an ultrasonic wave, after it interacts with an obstacle, and strongly affects the lateral resolution. It can occur when small openings or apertures, which act as small ‘secondary sources’ of sound, are present in the medium scanned.

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4 An artefact is any information on an image that is due to a spurious signal, and does not truly represent the physical conditions existing within the scan field.
The extent of diffraction of an ultrasound beam, as it moves away from a 'secondary source', is dependent on the size of this source relative to the wavelength of the ultrasound wave transmitted. For any given frequency, the larger the sound source, with respect to the wavelength of the sound, the more directional the sound beam becomes. If the wavelength of the sound wave is larger than the source that produced it, the sound will proceed in the form of a spherical wave away from that source.

5.3.4 Scattering

The size and shape of reflectors affects the amplitude of the echoes reflected back to the transceiver. Scattering causes ultrasound energy to be dispersed in many directions, increasing beam attenuation, and occurs when the ultrasound waves interact with obstacles which have surface irregularities, are obliquely aligned to the beam axis or are of a size similar to (or less than) the wavelength (figure 5.7). Small objects which give very weak echoes, such as the filaments of fishing nets, are difficult to detect sonically and that is why dolphins tend to get caught in these nets. The overlap of scattered echoes generates wave interference patterns and results in a speckle-pattern on the ultrasound image.
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5.4 Ultrasound uses and associated phenomena

To facilitate the adaptation of ultrasonic imaging to Christy's milk clotting system, by determining which ultrasound beam and transceiver characteristics are most appropriate for this application, reviews of ultrasound-induced phenomena and ultrasound applications, and preliminary tests (reported in section 5.5), were carried out.

5.4.1 Ultrasound-induced phenomena

Certain forms and applications of ultrasound can induce phenomena, such as acoustic streaming, standing waves, cavitation and temperature rise, capable of disrupting the milk clotting experiment.

Acoustic streaming is the circulation of fluid induced by radiation forces\(^{[319]}^{[320]}\). When induced near a bubble, the bubble dynamics lead to eddying movements that are of microscopic proportions yet can produce high viscous shear stresses capable of damaging or changing fluid constituents\(^{[321]}\). Acoustic streaming is less likely to occur when the intensity and frequency of the ultrasound are reduced. If this conversion of sound energy to kinetic energy occurs in the milk experiments, where bubbles are present in the milk flow, the improved mass transport might enhance the aggregation phase of the milk clotting process, and the shear stresses might cause deterioration of clot and the constituents of the milk mixture.

Standing waves occur when two waves travelling in opposite directions overlap. For example, a reflected wave can be superimposed on an incident wave, resulting in a standing wave which consists of high and low intensity regions fixed in space relative to
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The reflective interface. This can result in a higher local intensity than in the absence of reflection. The creation of a standing wave can impede or even arrest the movement of small particulates or cells in a liquid, trapping them at pressure nodes and promoting particle separation and agglomeration\[^{305}\] \[^{322}\] \[^{323}\]. Standing waves are less disruptive when the ultrasound intensity is low, and are less likely to be generated when the ultrasound beam axis is not perpendicular to the reflective interfaces, the applicator or reflective interfaces are not stationary and when pulsed, as opposed to continuous wave, ultrasound is used (since in the pulsed regime the superimposition of waves can only occur when the pulses overlap). Standing wave formation might interfere with the milk clotting process, when ultrasound is applied to the milk experiment, as bubbles and aggregates might be trapped and made to resonate and agglomerate, respectively.

Cavitation\[^{324}\] is a phenomenon (which may be hydrodynamic, thermal, or acoustic in origin) that results in the formation, and in some cases enlargement and implosion, of gaseous and vaporous cavities in a liquid. Cavitational activity occurs in two forms under the influence of the pressure fluctuations of a sound wave; the stable type of cavitation involves the enhanced oscillation of bubbles, whereas the transient form involves the growth and violent collapse of bubbles, which causes localized high shear stresses, temperatures and pressures. The 'cavitation threshold' corresponds to the intensity of ultrasound above which cavitation occurs in a fluid. High intensity ultrasound imposes large pressure fluctuations on the medium in which it is travelling; the compression cycles exert a positive pressure on the liquid, pushing the molecules together, while the expansion cycles exert a negative pressure, pulling the molecules away from one another. When a sufficiently large negative pressure is applied to a liquid, such that the average distance between the molecules exceeds the critical molecular distance necessary to hold the liquid intact, the liquid will break down and cavitation bubbles will be formed and in some cases collapse. The acoustic streaming and extreme conditions arising from cavitational activities would no doubt interfere with the milk experiment. In order to reduce the likelihood of cavitation occurring one should choose values of certain system and ultrasound features which increase the cavitation threshold.
The cavitation threshold is a function of a number of physical parameters, including solution properties (viscosity, surface tension, vapour pressure, type and concentration of dissolved gas and solid particles), temperature, the external pressure, and the intensity, frequency and attenuation of the ultrasound.\[325]\]

Cavitation is more difficult to generate when the vapour pressure of a liquid is reduced and its viscosity or surface tension is increased. The pressure in the rarefaction cycle must be lower than the vapour pressure and overcome the natural cohesive forces acting in a liquid, in order to induce cavitation. When the fluid viscosity is increased, not only do these cohesive forces increase but the amount of ultrasound absorption also increases (section 5.3.1), reducing the intensity of the ultrasound beam and the likelihood of cavitation. For example, the threshold intensity varies between 0.5 to 2.0 W/cm\(^2\) for tap water, but can be tens of W/cm\(^2\) for higher viscosity liquids.\[326]\[327]\]

Degassing and filtration have been found to increase the cavitation threshold. Estimates of the acoustic pressure necessary to cause cavitation in water have been approximately 1500 atm; however, in practice cavitation usually occurs at considerably lower values, because most liquids are sufficiently contaminated by gas molecules or particulate matter (with gas-filled crevices), both of which lower the cavitation threshold.\[328]\] For example, in tap water an acoustic pressure of only a few atmospheres will form bubbles. Roy \textit{et al.}\[329]\] found that the smaller the filter size used for ultrafiltrating a medium prior to its irradiation, the greater the cavitation threshold. Figure 5.8 illustrates the effect of degassing on the threshold intensity, at different ultrasound frequencies.

Increasing the temperature of a liquid allows cavitation to be achieved at lower acoustic intensity, because it reduces the liquid's viscosity and surface tension and increases its vapour pressure. Pressurizing the system, above atmospheric pressure, makes the formation of bubbles more difficult to achieve and thus increases the cavitation threshold.\[330]\]
The intensity is the main factor determining whether or not cavitation occurs. Cavity growth also is largely dependent upon the intensity of sound. High intensity ultrasound can expand the cavity so rapidly during the negative-pressure cycle that the cavity never has a chance to shrink during the positive-pressure cycle, resulting in cavities growing rapidly in the course of a single cycle of sound (transient cavitation). For low intensity ultrasound the size of the cavity oscillates in phase with the expansion and compression cycles, and over many cycles the cavity may grow gradually. At intensities above the cavitation threshold, an increase in intensity will cause more violent bubble collapse and thus greater mechanical and chemical effects.

As is clear from figure 5.8, the frequency of ultrasound strongly affects the cavitation threshold. At lower ultrasound frequencies (kHz) cavitation is more readily induced, as the rarefaction phase has sufficient time to form cavitation bubbles and greater sound energy can be transmitted further into the medium. At higher ultrasound frequencies, the absorption of ultrasound is greater and the duration of the rarefaction phase shorter, resulting in more power being required to maintain the same cavitational effects. Even if a bubble were produced during rarefaction, the time required to collapse that bubble might be longer than is available in the compression half-cycle of a high frequency sound.
wave. For example, using 7.5 MHz ultrasound, the rarefaction cycle lasts 0.067\(\mu s\) (\(1/7\)), less than the necessary collapse time of a bubble of radius 10\(^{-3}\)cm, which is approximately 1 \(\mu s\)\(^{-3}\).

Vibrational energy can be transformed into heat\(^{312}\). The amount of local heating induced by ultrasound depends on the ultrasonic intensity\(^5\), the type of tissue irradiated, the rate at which heat is dissipated, and the duration of exposure and frequency of the ultrasound\(^{319}\). The temperature rise (\(\Delta T\)) induced by transmission of ultrasound of intensity \(I\), for a time \(t\), in the absence of any cooling mechanism, can be estimated by using the equation\(^{316}\):

\[
\Delta T = \frac{2\alpha It}{\rho C_m} \ldots (5.7)
\]

where \(\alpha\) = absorption coefficient, which increases approximately linearly with frequency\(^{313}\)\(^{316}\), \(\rho\) = tissue density, and \(C_m\) = specific heat per unit mass of tissue. The higher the intensity of the beam and the longer the exposure time, the more vigorous the vibration of the tissue and the greater the tissue temperature rise. Figure 5.9 provides a guide to the approximate ultrasound intensity necessary to produce a 1\(^\circ\)C temperature rise in tissue, at different ultrasound frequencies and for a fixed duration. A temperature rise in the milk experiment would have a noticeable affect on the clotting (section 4.4.5).

5.4.2 Ultrasound characteristics suitable for the milk application

The test-chamber to be studied comprises a solid tube, of 32 mm i.d. and 40 mm o.d., which contains an axially located curved test-object that is 10 mm in diameter at its thickest part. The thickness of clot over the whole test-body is of primary interest and may be between 0 and 4 mm. A suitable transceiver would be one which emits an ultrasound beam having a focal length and focal zone both of about 20 mm, and an axial resolution less than, say, 1 mm. The lateral resolution need not be so small, but

\(^5\)There are six intensities that can be defined: \(I_{SP}\), \(I_{SP}\), \(I_{SP}\), \(I_{SP}\), \(I_{SP}\), \(I_{SP}\), \(I_{SP}\), \(I_{SP}\), \(I_{SP}\), \(I_{SP}\), \(I_{SP}\), and \(I_{SP}\); where \(SP\) = spatial peak, \(SA\) = spatial average, \(TP\) = temporal peak, \(TA\) = temporal average, and \(PA\) = pulse average.
Figure 5.9: The approximate ultrasound intensity necessary to produce a $1^\circ C$ rise in local tissue temperature, as a function of frequency.$^{[312]}$

as close to 1 mm as possible.

The main characteristics influencing the selection of an ultrasonic scanner for the curd deposition application were: the cost, the depth of penetration, the effective beam shape and resolution, the accuracy of the measurement system and its effect(s) on milk clotting. These aspects depend on the frequency and intensity of the ultrasound, as well as the transducer head size and shape, focusing and duration of irradiation.

Frequency determines, to a large extent, pulse length and beam width and hence axial and lateral resolution. The greater the frequency the finer the resolution and the smaller the likelihood of cavitation for a given intensity, but the greater the cost of the transducer and the shallower the depth of penetration. Table 5.1 provides guidelines for frequencies, regarding their corresponding depth of penetration and resolution. A 10 MHz ultrasound probe may provide adequate penetration and an agreeable axial resolution for clot thickness measurements, while the lateral resolution, which is diffraction limited, is better the smaller the wavelength, and can be enhanced by beam focusing. While the great difficulty involved in constructing transducer arrays with very small crystals that have a resonant frequency greater than 10 MHz could be side-stepped by using a reciprocating single-crystal probe, the depth of penetration would be too shallow for the application in mind. The curve in figure 5.10 illustrates the
Table 5.1: Frequency, penetration and resolution[^313]

<table>
<thead>
<tr>
<th>Freq. (MHz)</th>
<th>Penetration (cm)</th>
<th>Axial R. (mm)</th>
<th>Lateral R. (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>4.6</td>
<td>/</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>2.3</td>
<td>~5.0</td>
</tr>
<tr>
<td>3</td>
<td>/</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>0.9</td>
<td>2.0</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>~0.65</td>
<td>~1.3</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>0.5</td>
<td>~1.0</td>
</tr>
<tr>
<td>15</td>
<td>/</td>
<td>0.3</td>
<td>/</td>
</tr>
<tr>
<td>25</td>
<td>/</td>
<td>0.2</td>
<td>/</td>
</tr>
</tbody>
</table>

[^313]: Improvement in axial resolution attainable with increasing frequency; the concomitant increase in transducer cost becomes less attractive above 10 MHz, as the corresponding improvement in axial resolution is small. In addition, the depiction of the test-body and clot may be impaired by scatter, and thus might not be improved markedly by employing a more expensive, higher frequency probe.

Figure 5.10: The axial resolution as a function of the operating frequency, for optimally damped transducers[^305].

Higher intensity corresponds to greater vibrational effects and likelihood of cavitation, but an improved amplitude of echoes returned from weakly reflecting interfaces.

To image the region of interest and to avoid diffraction, which occurs when the transducer size is comparable to that of the sound wavelength, a probe length of about 5 cm is necessary. The curved elements of the probe illustrated in figure 5.5(d) would probably be appropriate, allowing good coupling and focusing, where the beam is made...
narrower in the direction at right angles to the plane of the scan.

Focusing the transmitted pulse will result in tissue being irradiated at higher intensity than if unfocused, but larger echo amplitudes are achievable and better image detail due to an improved lateral resolution. Sequential crystal activation that allows the beam to be steered is also desired, as this allows echoes from interfaces aligned obliquely to the unsteered beam to be received at greater amplitude.

The duration of irradiation should be kept low, to avoid the formation of standing waves and reduce the time-averaged intensity.

In order to estimate the characteristics of ultrasound for which non-disruptive imaging of this milk system is possible, different ultrasound applications were reviewed. A few ultrasound applications only were looked at, because the effects and imaging ability of applying ultrasound to one system will not necessarily be the same for another. For instance, since threshold values above which ultrasound-induced phenomena occur depend on several of the ultrasound beam and system conditions (section 5.4.1), the exact effect of an ultrasound beam on each system is unique. The results obtained from in vivo ultrasonic studies may not provide an accurate indication of that occurring when the same ultrasound field is applied to an in vitro system, as the geometry, the occurrence and type of nuclei that provide the potential for cavitation, the mechanism of action and the type and response of molecules may differ considerably between the two systems\textsuperscript{[305][312][313][333][334]}. Reviewing in vitro results, as opposed to in vivo ones, may be more appropriate but can still only provide a very approximate guide to which ultrasound beam characteristics do not disrupt the milk clotting system.

Ultrasonic thickness measurements of very thin films (of the order of 10 µm)\textsuperscript{[335][336]} have been achieved with great precision (down to 1% accuracy), but due to the high frequencies used (90–510 MHz) the depth of penetration has been far too shallow to be of use in the milk experiment. The range of frequencies used in diagnostic ultrasound\textsuperscript{6}

\textsuperscript{6} Diagnosis of valvular heart disease\textsuperscript{[341]}, peripheral vascular disease and a range of tissue

Diagnosis of valvular heart disease\textsuperscript{[341]}, peripheral vascular disease and a range of tissue
are more likely to provide beam characteristics appropriate for the milk application.

Present-day pulsed wave diagnostic ultrasound typically has a frequency of 1–20 MHz and SATA intensities of 5–20 mW/cm², with SPTP intensities up to 10 W/cm². In diagnostic ultrasound, the typical ultrasound frequency range is 2–5 MHz for abdominal[309][337] and heart scans, and 5–20 MHz for examinations of superficial regions, such as the eyes[313]. Pulse wavelengths and the corresponding resolution features of ultrasound images are of the order of 1 mm.

Clark et al.[338] used real-time ultrasonic imaging to measure the thickness of soft tissues over bone, an application not much different from the intended milk clot investigation, where clot is deposited on a solid surface. The range of soft tissue thicknesses measured was 4.0–37.6 mm. A linear sector 7.5 MHz ultrasound transceiver was employed, with an axial resolution less than 1 mm, a lateral resolution of 4 mm and a focused penetration of 4–5 cm deep. A soft silicone 'stand-off' block was placed between the transceiver and the skin to allow the area of interest to lie within the focal region of the ultrasound beam. While certain beam characteristics of this probe are not acceptable for the milk clot application, the success of this tissue/bone imaging application is very encouraging. A higher frequency and/or more focused ultrasound beam would provide better resolution and a focal length more appropriate for the milk application. For instance, a frequency of 20 MHz would improve axial and lateral resolution by a factor of 3, relative to 7.5 MHz[314].

The ultrasound intensities used in clinical diagnostics appear to cause no noticeable bioeffects[312]. However, unlike mammalian tissues and blood flow, the milk system contains bubbles, making the occurrence of ultrasound-induced phenomena more likely (section 5.4.1). The effect of irradiating the aerated milk system depends on several factors, including the size and quantity of the bubbles and the ultrasound beam characteristics.

characterizations, such as cross-sectional area, diameter, wall and plaque thickness[311], as well as thrombus and cyst depiction[309][310], are possible using ultrasonic scanning equipment.
Flynn[339] and Carstensen[340] have calculated transient cavitation thresholds for microsecond-length pulses of ultrasound in a gaseous solution that indicate thresholds are dependent on the ultrasound frequency and the size of the gas nuclei in the medium. They found that the cavitation threshold may be as low as 1–2 W/cm², at 1 MHz, and 20–40 W/cm², at 10 MHz. These intensities are above those typically used in clinical diagnostics.

If bubbles in the milk flow were to reside in the ultrasound field, they might oscillate with undesirable consequences. Bubbles might become trapped in the recirculating flow in the wake of the test-bodies or, as has been observed on post-experimental examination, adhere to the test-object and its clot. If this were the case, the bubbles might be made to oscillate and the work of Miller and his colleagues[342] might be indicative of what to expect. Miller et al. exposed human platelet-rich plasma to 2.1 MHz ultrasound in the presence of 4 μm diameter gas bubbles stabilized in the hydrophobic pores of a membrane. Two different sources (one generating spatial peak intensities of 8–250 mW/cm², and the other, a commercial Doppler device, producing a spatial peak intensity of 80 mW/cm²) were used to expose the plasma and immersed membrane for 10 minutes. The platelets gathered around the pores, forming reversible aggregates at 8 mW/cm² (spatial peak intensity) and irreversible aggregates above 16–32 mW/cm². The size of the aggregates formed increased with increasing intensity. They also found that applying diagnostic ultrasound of a spatial peak intensity greater than 32 mW/cm² induced platelet aggregation under similar conditions. Application of diagnostic ultrasound to the milk experiments might thus promote aggregation of destabilized micelles, unless the milk is efficiently degassed.

Ultrasonic treatment of enzymes, notably the active enzymes in rennet (pepsin and chymosin), has been found capable of altering their properties. The response of an enzyme to ultrasound is determined by several factors, including the enzyme's molecular weight, structure and concentration in solution, the solution temperature and pH[343] and the ultrasound beam characteristics. The harsh conditions resulting from cavitation and acoustic streaming, induced by high intensity, low frequency
ultrasound, have been found to deactivate or denature pepsin and chymosin\textsuperscript{[344][345]}. The use of diagnostic ultrasound is unlikely to affect chymosin in this way, as not only is cavitation activity unlikely but the temperature rise resulting from the use of diagnostic ultrasound is usually negligible and the milk flow would rapidly dissipate heat.

Based upon the above findings, diagnostic ultrasound appears worthy of further investigation. In section 5.5, preliminary tests and their results are reported and discussed in the endeavour to find a suitable transducer for imaging milk clot deposition and to determine how best to adapt this probe to the test-chamber.

**5.5 Application of ultrasonic imaging to the milk system**

The preceding review of ultrasound has provided guidelines for the ultrasound beam characteristics suitable for the milk application, but certain questions remain; for instance:

- Can ultrasonic imaging be used to measure the thickness of milk clot immersed in flowing milk?
- Is enzymic milk clotting affected by the presence of diagnostic ultrasound?
- How can an ultrasonic scanner be physically adapted to the test-chamber of the milk experiment, in order to give acceptable image quality while causing minimal interference?

In order to answer these questions, preliminary tests were carried out with diagnostic ultrasound equipment. Many of the technical difficulties that were encountered with the milk apparatus, and detailed in chapter 6, were unresolved at this stage of the research, so models of the milk system were used.

**5.5.1 Imaging of the milk system**

The aim of the first test was to determine whether or not it is possible to image ultrasonically the interfaces between milk clot, aqueous milk and P.V.C. A bell-shaped solid P.V.C. test-body, covered in freshly prepared milk coagulum and suspended on a
steel rod, was immersed in a basin of milk and imaged using a real-time, multi-element, 3 MHz ultrasound transceiver. The ultrasound probe was submerged slightly below the milk free surface and aligned parallel to the axis of the test-object, and a rubber strip was placed on the base of the basin to avoid undesirable reflections from the back wall. The interfaces formed by the clot and milk and the rod and milk were roughly delineated, but the test-body/clot interface was not depicted. A subsequent test was performed using the same arrangement and a 7.5 MHz probe, which provided a better, less hazy image of the milk/clot interface and what appeared to be the test-body/clot interface, meriting further research.

A simple static model was devised (figure 5.11) for assessing the feasibility of using ultrasound to image milk clot. This model consisted of the test-chamber from the milk apparatus, with clot on some of its inner wall, plugged at both ends, and containing milk or water and a partially clotted teardrop-shaped P.V.C. test-body on a stainless steel rod. The preclothing of the test-body was achieved by fitting the test-body onto a steel rod and using this to stir a mixture of warm milk, rennet and CaCl₂, and then leaving it to clot. Wall clot was prepared by trying different large-scale clotting procedures, not much different from that of the Lee-White test, until a combination of reagents was discovered that allowed a well-adhered clot to be formed on the test-chamber wall.

Since the intended application is a novel one, the type of scanner which would be most suitable was not readily apparent. Selection of the transducer by testing a wide range of probes of different design (i.e. flat or curved crystals (figure 5.5(d)), electronic focusing, and different frequencies (5-15MHz)) would be most appropriate, but the required selection of probes for such comprehensive tests was not available. However, three transducers were available for these tests: two 7.5 MHz transceivers (one linear (6 cm x 1 cm), the other convex), and a point probe with an estimated frequency of 10 MHz. The suppliers⁷ estimated the resolution and focal length of all three probes to be 1 mm and 20 mm, respectively. The 7.5 MHz linear-array transceiver proved to be the most appropriate of the three probes, as it was able to both couple well to the

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⁷Dynamic Imaging Ltd., Young square, Brucefield industrial park, Livingston, Scotland.
wall of the static model and scan the full length of the test-body. However, it did not provide imaging of a quality adequate for test-body clot thickness measurement.

There are various ways to improve image quality, but for further tests to be conducted an ultrasound scanner had to be purchased. The most suitable scanner available was a "Concept 2000", which came equipped with a linear-array piezoelectric transceiver operating at 7.5 MHz, SATA intensity of 9.6 mW/cm$^2$ and a beam power of 462 $\mu$W. This transceiver has a face that measures 6 cm x 1 cm, has 79 crystals, which are sequentially activated to focus the ultrasound beam to one of two optional focal lengths (35 mm or 60 mm), and provides pulses of ultrasound of 0.45 $\mu$s width in water, with a pulse repetition frequency between 2 to 8 kHz, and a fixed frame rate of $\sim$25 f/s.

Assessments of this scanner's effect on milk clotting (section 5.5.2) and of ways to improve its imaging of the milk system (section 5.5.3), as well as its resolution, were conducted.
5.5.2 Investigation of ultrasonically-induced effects

While most bubbles in the milk flow will spend very little time in the ultrasound beam, bubbles that are trapped in the wake and clot of the test-body, or in standing ultrasound waves, may be irradiated for the full duration of the test-chamber scan. These trapped bubbles might resonate in the ultrasound field, resulting in acoustic streaming and enhanced mass transfer.

In order to investigate whether or not the purchased ultrasound system induces any undesirable effects, a few simple static8 tests were conducted. Any thermal, chemical or mechanical effects induced by ultrasound during these tests were negligible, as no noticeable changes in clotting, clot appearance or bubble behaviour resulted from the use of ultrasound. No temperature rise occurred during 80 minutes of irradiation of a stagnant, water-filled perspex test-chamber. Application of ultrasound through the wall of the test-tube, during a Lee-White test with milk, resulted in no change in clotting time or clot adhesion. Bubbles studied under magnification, both in a warm water flow and adhered to the P.V.C. test-body, were seen to behave no differently when irradiated with ultrasound. Irradiation of a preclotted test-body submerged in stagnant water caused no dissolution or disturbance of the clot, at both macroscopic and microscopic levels.

5.5.3 Image enhancement

Ultrasound transmission through the perspex test-chamber wall is associated with unacceptable attenuation and consequently poor imaging of the clotted test-body. This image degradation is a result of ultrasound reflection, absorption and refraction. While both the reflection and absorption of ultrasound will result simply in a reduction of the amplitude of echoes from the test-chamber contents, the consequence of refraction can be an image which incorrectly depicts the depth, thickness and shape of structures. In order to improve imaging, (i) redesign of the test-chamber was attempted; (ii) different materials of construction of the test-chamber and test-object were tested; (iii) a 'stand-off' was tried; (iv) and the optimal settings of the machine controls were established.

8 Reproducible milk clotting in the milk rig had not been achieved at this stage of the research.
(i) **Test-chamber redesign**

Different test-chamber designs were considered. One could seal the transducer into the test-chamber, with its active face flush with the surface of the inner-wall, but this would not allow the operator to scan different regions of the test-chamber, as is desired. Alternative designs included a test-chamber with a ‘shaved flat’ (figure 5.12(a)), and ones with a flat, thin window (figure 5.12(b) and (c)).

If the site of probe application was a flat section machined on the outside of the test-chamber (figure 5.12(a)), the probe would not disrupt the flow within the test-chamber, would be more easily applied and ultrasound would be transmitted through less attenuative material; however, refraction would still take place.

Comparisons were made between images obtained by transmission and reception of ultrasound through the original perspex test-chamber wall, the wall of a P.V.C. test-chamber, and the flat window of a test-chamber of the design illustrated in figure 5.12(b). Transmission through the silicone-rubber window provided a better test-body image than that obtained with the P.V.C. pipe wall which, in turn, was better than that with perspex. It was, however, difficult to obtain a water-tight seal between the rubber window and the test-chamber, and this window was bumpy; capable of disturbing the flow within the test-chamber. Another problem associated with the ‘windowed’ test-chamber, that would be likely to affect the milk clotting experiment, is that it renders the test-chamber unsymmetrical. Compositional, as opposed to structural, modifications might be an easier and more versatile way of improving imaging.

![Figure 5.12: Three test-chamber design ideas.](image-url)
(ii) Composition

The attenuation caused by the different media within the field of scan (i.e. the milk clot, aqueous milk, the test-body and its support rod, and the test-chamber wall) was assessed, and improved where necessary.

The images achieved when the static models illustrated in figures 5.11 and 5.12(b) were used, with and without wall clot present, indicated that the wall clot does not attenuate the ultrasound beam markedly. Minimal attenuation also is caused by aqueous milk\[^{546}\], over the shallow depth to be scanned here.

When the ultrasound beam interacts with the test-objects, refraction, absorption, reflection and scattering lead to incomplete imaging of the posterior side of the test-body and of structures lying directly behind it, and extensive reverberation\(^9\) arises due to the presence of the highly reflective \((Z=45.7\times10^6 \text{ kg/m}^2\text{s})\), low loss \((0.43 \text{ dB/cm at } 10 \text{ MHz})\), 1.6 mm diameter stainless steel rod, which is aligned perpendicular to the ultrasound beam axis. Unambiguous imaging of the near-side of the test-bodies was thus the best that could be hoped for. The criteria used for choosing the material for construction of the test-body and its support rod were that there should be a large difference in acoustic impedance between it and the milk and curd, so that strong reflection can be achieved at both the test-object/milk and test-object/curd interfaces; that the material should be acoustically absorbent to avoid reverberation artefacts; and that it should be inexpensive and rigid even when thin. The acoustic impedance of aqueous milk, \(Z_{\text{milk}} = \rho_{\text{milk}} \times c_{\text{milk}} \approx 1030[210] \times 1540[349] = 1.59 \times 10^6 \text{ kg/m}^2\text{s}\). The speed of sound in curd \((c_{\text{curd}})\) is believed to be 1540 m/s, similar to that in aqueous milk and soft tissue[^313]; it was found that the scanner, which assumes the speed of sound to be 1540 m/s in its computations, displayed a given interface to be at the same depth of immersion in milk, whether coated with milk clot or not. \(Z_{\text{curd}}\) was assumed to be about \(1.63 \times 10^6 \text{ kg/m}^2\text{s}\) (the average value for soft tissue[^313]).

\[^9\]Reverberation occurs only when the ultrasound beam is perpendicular to strongly reflective interfaces and the medium between them is not highly attenuative. It is manifested in the image by equidistant lines parallel to the strong reflector, which are caused by internal reflections between the proximal and distal interfaces prior to the echo returning to the transceiver.
The ideal material for construction of the test-chamber is one that is inexpensive, rigid and acoustically matched to its surroundings. Such a material would have a similar acoustic impedance and similar speed of propagation of sound to that of milk and the elastomer cover on the transceiver crystals, and cause no loss (absorption or reflection) of ultrasound. The acoustic impedance of the probe face is \(1.63 \times 10^6\) kg/m\(^2\)s\(^{-1}\).

A preliminary series of non-quantitative tests was carried out with readily available cylindrical tubes of different materials, in order to determine whether or not one of these materials might be suitable for construction of the test-chamber. The tubular vessels were filled with water and a preclotted P.V.C. teardrop test-body immersed within them. Glass (pyrex) containers gave the worst imaging, resulting in extensive refraction and reflection and an image riddled with artefacts. Perspex receptacles of different sizes were also found to cause unacceptable levels of beam distortion. A P.V.C. pipe allowed a better, but still poor, test-body image to be obtained. Transmission through either high density polyethylene or polycarbonate gave better imaging of the test-body, and transmission through polypropylene gave the best test-body imaging. Variation of the thickness of polycarbonate (0.75–3.0 mm) and polypropylene (2.25–4.0 mm) did not markedly alter the quality of image achieved.

A quantitative, more extensive and systematic approach was subsequently adopted in the search for suitable materials of construction for the test-body and test-chamber; data from the literature (given in table 5.2) and values obtained from experiments were used.

In table 5.2, "loss" corresponds to the reduction of ultrasound intensity as it propagates through a material, as given by equation 5.5. For example, if ultrasound of 1 MHz frequency has an intensity \(I_0\) of 9.6 mW/cm\(^2\) on entering soft tissue (a medium which incurs a loss of 0.7 dB/cm), then \(I_x\), the intensity of the wave, say, 1 cm into the soft tissue, would be about 8.2 mW/cm\(^2\).
### Table 5.2: Approximate material properties

<table>
<thead>
<tr>
<th>Material</th>
<th>$c_l$ (m/s)</th>
<th>$\rho$ (kg/m³)</th>
<th>$Z_L$</th>
<th>Loss (dB/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>adiprene</td>
<td>/</td>
<td>1160</td>
<td>1.94</td>
<td>/</td>
</tr>
<tr>
<td>air (dry) (0°C)</td>
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<td>0.00043</td>
<td>12 at 1 MHz</td>
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<td>17.33</td>
<td>0.018 at 1 MHz</td>
</tr>
<tr>
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<td>1110</td>
<td>2.0</td>
<td>/</td>
</tr>
<tr>
<td>carbon (pyrolytic)</td>
<td>/</td>
<td>2210</td>
<td>7.31</td>
<td>/</td>
</tr>
<tr>
<td>carbon (vitreous)</td>
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<td>1470</td>
<td>6.26</td>
<td>/</td>
</tr>
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<td>/</td>
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<td>3.9 at 5 MHz</td>
</tr>
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<td>1300-1450</td>
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<td>11.2 at 5 MHz</td>
</tr>
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<td>900</td>
<td>1.4</td>
<td>/</td>
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<td>/</td>
<td>1.63</td>
<td>0.7 at 1 MHz</td>
</tr>
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<td>stainless steel</td>
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<td>water (25°C)</td>
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<td>997.0</td>
<td>1.49</td>
<td>1.91 $\times 10^{-3}$</td>
</tr>
<tr>
<td>wood (pine)</td>
<td>/</td>
<td>450</td>
<td>1.57</td>
<td>/</td>
</tr>
</tbody>
</table>

$Z_L$ = acoustic impedance $= \rho \times c_l \times (\times 10^6)$ kg/sm²; $c_l$ = speed of longitudinal bulk waves.
In the search for a material suitable for the construction of the test-object, it was found that a high percentage of ultrasound is reflected at the interfaces between water and aluminium (70%), carbon (43%), glass (65%), porcelain (64%) and stainless steel (87%), but that these materials are not very absorbent. Less reflective polymers (listed in table 5.3) were chosen for preliminary tests, due to the ease with which they can be shaped and their greater absorption of ultrasound.

Of the materials listed in table 5.2, those which roughly satisfy the above mentioned test-chamber material criteria and/or were promising in early imaging tests were wood, polyurethane and a few of the materials listed in table 5.3. Berridge found\[227] in his milk deposition studies (described in section 4.2.1) that the rate of curd deposition on wood is very small (0.06 mg/mincm$^2$), so its use here might limit wall clot growth, but unfortunately wood is too attenuative to allow transmission of ultrasound. Michael Finsterwald\[350] recommended a certain type of soft polyurethane, which has very good acoustic properties (e.g. $c = 1540$ m/s; $Z = 1.6$ kg/sm$^2$; and is 'low loss'), but a pipe of this type of polyurethane might be insufficiently rigid to be practicable and was in any case unavailable. The different polyurethane samples available were rigid but highly attenuative. The remaining materials worthy of further consideration were included in the test described below.

**Speed of sound tests**

The values in the literature of $Z$, $\rho$ and $c$, of the polymers chosen for further consideration, either varied from source to source or were not found. An existing ultrasonic measurement system, at the Royal Infirmary of Edinburgh, was thus used to obtain a more accurate and complete quantitative guide to which of the most promising materials would be best suited for construction of the test-chamber and test-object, with regard to the criteria listed above.

The test system (figure 5.13(b)) consisted of a water tank, a clamp stand for mounting the sample perpendicular to the ultrasound beam, a 3.5 MHz ultrasound transducer and receiver both applied against special windows on opposite sides of the water
Figure 5.13: (a) An A-scan trace depicting reflecting interfaces, and (b) a schematic of the water tank apparatus used for determining the speed of propagation of sound in different test-materials.

Tank, and a demodulator through which the recorded signal passed on its way to the oscilloscope. Flat, square samples, composed of the first six materials listed in table 5.3, were prepared and individually mounted in the measurement tank. A pulsed-echo one-dimensional scan was performed, called an A-scan, in which echoes are displayed as vertical deflections of the trace on a CRT screen at locations corresponding to the interfaces which produced the echoes (figure 5.13(a)). The size of the vertical deflection indicated the magnitude of the echo signal, and the difference between the position of the deflections along the horizontal axis was a measure of the time taken for sound to travel through the test-material.

The difference in sound transmission time, when ultrasound was passed through the water tank with and without a sample present, was used with the value of the sample thickness to calculate the longitudinal plane wave velocity in that specimen. The acoustic impedance \((Z)\) of each material was then determined, by substituting density values from the literature and the empirically obtained sound speeds into the equation: \(Z = \rho \times c\); the values obtained are listed in table 5.3. Using these values, and a value for \(Z_{\text{milk}}\) of \(1.59 \times 10^6 \text{ kg/m}^2\text{s}\) and \(c_{\text{milk}}\) of 1540 m/s\(^3\), the reflection and refraction of ultrasound caused by interfaces consisting of milk and these materials were evaluated as explained below.
Material | c (m/s) | ρ (kg/m³) | Z × 10⁶ (kg/m²s) |
--- | --- | --- | --- |
P.V.C. | 2282 ; 2300 | ~1375 | 3.138; 3.162 |
P.T.F.E. | 1425 ; 1350 | ~2170 | 3.092; 2.930 |
perspex | 2598 ; ~2705 | ~1185 | 3.079; 3.205 |
nylon | 2517 ; ~2440 | ~1145 | 2.882 ; 2.794 |
polycarbonate | 2082 ; / | ~1190 | 2.478; 2.69 |
polypropylene | 2303 ; / | ~898 | 2.068 ; 2.36 |
polyethylene (HDPE) | / ; 1950 | ~935† | / ; 1.823 |

The ratio of the speed of sound in the different media to that in milk ($\frac{c}{c_\infty}$) were calculated and listed in table 5.4, to allow one to compare the different solid/milk interfaces, with respect to the extent of ultrasound refraction (equation 5.6) they cause. In addition, the empirical values of Z in table 5.3 and the value of $Z_{milk}$ were substituted into equation 5.1, in order to estimate the relative extent of ultrasound reflection at the different solid/milk interfaces, and the values obtained are given in table 5.4.

Table 5.4: Refractivity, reflectivity and absorption of ultrasound with different materials interfaced with milk
Choice of test-chamber material

From examination of table 5.4, none of the materials considered are ideal for use in the construction of the test-chamber, since all reflect, refract and absorb ultrasound. In order to determine which of these materials is most suitable for construction of the test-chamber, assessment of the combined importance of $(\frac{A}{O})$, percentage reflection and absorption was attempted.

Since ordering of materials with regard to transparency to ultrasound and based solely on material properties is not possible without quantitative knowledge of the relative importance of the various properties involved, and no such information was available, the results of imaging tests were used here. Comparison of the results of the preliminary series of image quality tests reported earlier with the material properties in table 5.4 indicates that no single property of a given material uniquely determines the quality of ultrasound imaging of an object enclosed by walls of that material. It was found that ultrasonic imaging was better when ultrasound was transmitted through the P.V.C. pipe wall than through the wall of the perspex pipe. The amount of ultrasound absorbed in, and reflected at, the P.V.C. wall is slightly greater than with the perspex wall; however, perspex causes more refraction $(\frac{A}{O})$ and scattering than P.V.C., and it is the refraction and back-scattering artefacts, which riddle the images obtained by transmitting ultrasound through perspex, that make imaging through perspex worse than that through P.V.C. On the other hand, tests conducted with P.V.C. and polypropylene showed that imaging was best when ultrasound was transmitted through polypropylene. This appears to be because the greater percentage reflection of ultrasound at the milk/P.V.C. interface, relative to that at the milk/polypropylene interface, has a greater influence on imaging than the refraction $(\frac{A}{O})$ and absorption occurring, which are greater in polypropylene than in P.V.C. The quality of imaging when ultrasound was transmitted through polyethylene and polycarbonate was found in preliminary experiments to be similar, although a greater percentage reflection and refraction $(\frac{A}{O})$ are likely to occur with the polycarbonate. These images were better than those obtained with P.V.C., although P.V.C. causes less absorption; presumably, the lower absorption is offset by the greater percentage reflection and refraction $(\frac{A}{O})$ occurring with P.V.C. Yet, it is anomalous that polypropylene allowed better...
imaging than polyethylene; while causing less sound absorption than polyethylene, polypropylene is more reflective and refractive.

In order to confirm that of the materials considered polypropylene is the best material of construction for the test-chamber, test-chambers were constructed of the two materials most likely to be of use – polypropylene and polyethylene. Comparisons between the quality of images obtained using these test-chambers indicated that the polypropylene test-chamber with a 4 mm thick wall does indeed provide the best imaging, followed closely by the polyethylene test-chamber with a 3 mm thick wall.

A polypropylene test-chamber, with a bore of 32 mm and a wall thickness of 4 mm, was used in further tests unless otherwise stated. The choice of this pipe wall thickness was guided by its rigidity, as imaging was not noticeably affected when the thickness of this wall was varied between 2.25–4.0 mm.

Choice of test-object materials

Of the materials listed in table 5.4, P.V.C., P.T.F.E. and perspex are most likely to provide a good image on normal irradiation and observation, based on the percentage reflection estimated to occur at their solid/milk interface when perpendicular to the ultrasound beam axis. However, the surfaces of the test-bodies intended for use in the milk experiments are not, in most places, perpendicular to the ultrasound beam axis. Since this axis is also the line of observation, such surfaces are mainly viewed by scattered (diffusely reflected) radiation. The amount of ultrasound returned by diffuse reflection to the transceiver is not only dependent upon the change in acoustic impedance across the milk-test-body interface but also on the angle of incidence and the roughness of the surface\textsuperscript{10}. It was decided, therefore, that imaging tests should be conducted with test-bodies constructed from all of these materials, except polyethylene.

\textsuperscript{10} The size of an echo from a smooth object varies rapidly with the angle of incidence of the ultrasound beam; the amplitude of echoes received by specular reflection from a smooth surface perpendicular to the beam axis can be about 100 times greater than that of echoes received by diffuse reflection from the same surface obliquely aligned to the ultrasound beam axis\textsuperscript{[13]}.
Tests were conducted using a milk-filled polypropylene test-chamber and a preclotted or clean teardrop-shaped test-body, and some of the results are given in table 5.5. The region of the test-body with the smallest angle of incidence (i.e. the widest part) produced the strongest echoes, while the more oblique areas were delineated poorly, if at all. Although P.V.C., P.T.F.E. and perspex all give approximately the same percentage reflection at interfaces with milk perpendicular to the ultrasound beam axis, P.V.C. and P.T.F.E. provided better images than perspex for interfaces perpendicular and oblique to the beam. The perspex test-body produced a glare of intense local specular reflection on its near-side, which made clot detection difficult, and its oblique parts were less well depicted; presumably the incident radiation was mostly specularly reflected away from the observation direction, probably due to the much smoother surface of the perspex test-body. On the other hand, the nylon test-body was imaged with a quality similar to that of P.V.C. and P.T.F.E.; though it is less specularly reflective, the nylon specimen used may have had a rougher, more diffusely reflective surface than P.V.C. and P.T.F.E. The polycarbonate and polypropylene have the smallest differences of acoustic impedance from that of milk, of the group of materials tested, so it was no surprise that test-bodies constructed of these materials were least well depicted, particularly on their oblique sides. Of the test-body materials providing the best image, P.V.C. is the most absorbent (thus the least likely to cause reverberation artefacts) so was chosen as the test-body material. The teardrop, cone and disc-shaped test-bodies used by Christy, and illustrated in figure 4.8, are constructed of P.V.C. and so were used in the tests reported later.
The depiction of the P.V.C. test-bodies within the polypropylene test-chamber was not ideal, particularly on the oblique sections and when clot was present. Although the near face of the P.V.C. teardrop could be completely delineated, the oblique parts were more fuzzily depicted and any clot on these parts obscured these regions of the test-body surface. This problem might be overcome by making the surface of the test-bodies sufficiently rough, as is sometimes done with biopsy needles in invasive ultrasonic procedures\cite{313}, allowing more sound to be scattered back to the transceiver. However, such surface modification might alter the test-body clotting propensity and would be difficult to do uniformly. The use of a stand-off is a recommended way to improve the percentage of ultrasound returned to the transceiver; tests carried out with a makeshift stand-off proved disappointing, however, and are reported later.

The reverberation caused by the steel rod supporting the test-body was reduced by finding a replacement for steel that is more absorbent, less reflective and rigid even when thin. In order to determine which of the available materials was most rigid (i.e. has the largest Young's modulus \(E\)), the values of \(c\) and \(\rho\) (from table 5.3) were substituted into the equation\cite{355}:

\[
c = \sqrt[3]{\frac{E}{\rho}} \quad (5.8)
\]

and the Young's moduli calculated are given in table 5.6.

<table>
<thead>
<tr>
<th>Material</th>
<th>(E \times 10^9) kg/ms(^2)</th>
<th>dB/cm at 5MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>perspex</td>
<td>7.97</td>
<td>10</td>
</tr>
<tr>
<td>Nylon</td>
<td>7.36</td>
<td>2.9</td>
</tr>
<tr>
<td>P.V.C.</td>
<td>7.03</td>
<td>11.2</td>
</tr>
<tr>
<td>polycarbonate</td>
<td>5.207</td>
<td>24.9</td>
</tr>
<tr>
<td>polypropylene</td>
<td>4.80</td>
<td>18.2</td>
</tr>
<tr>
<td>P.T.F.E.</td>
<td>4.41</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Table 5.6: Young's moduli of, and loss in, different materials

Perspex was the material chosen for construction of the new support rod. The new rod is illustrated in chapter 6 (figure 6.4) and has a greater diameter than the steel rod (for rigidity), is of greater length, and is tapered so that it can be inserted into the test-bodies. This rod was found to cause less reverberation than the steel rod;
especially at its tapered part, since this section of the rod is not perpendicular to the ultrasound beam axis. Thus the region of interest, that is the test-body and its immediate-wake region, is now free from reverberation and even less likely to cause standing wave formation.

(iii) Use of a stand-off

A stand-off is usually used to either reduce image degradation due to reverberation, by increasing the distance between the reflective structure and the sound source, or to enable the operator to position the probe so that the object under view lies at the beam's focal point. Neither reverberation nor the fact that the test-bodies are not at the beam's focal point\(^1\) was a major problem here. A simple test was conducted that indicated that the ultrasound beam is weakly focused and that the image quality is insensitive to variation in object depth; a bell-shaped solid P.V.C. test-body was depicted with similar quality, when 19 mm and 35 mm from the probe face in a water bath. A flexible stand-off could still be useful, however, as it would allow the operator to achieve angles of incidence closer to 90° on the otherwise oblique and poorly delineated test-body surfaces.

A deaerated water balloon, coated with gel couplant on both sides and known to act as a good stand-off clinically, was inserted between the probe and the test-chamber wall. This stand-off was awkward to use and gave poorer images than those obtained without it. A similar result was achieved using a gel-filled sanitary probe cover. A more useful stand-off for this application might be a customized one; however, the cost of a customized stand-off is extremely high. Thus a stand-off was not used in the work reported hereafter.

(iv) Instrument settings

The image quality is highly dependent on the operator, who determines the effective beam shape by using sensitivity controls. Once the optimal settings of these controls

\(^1\)The focal length of the 7.5 MHz probe is about 35 mm, and the distance between the test-chamber outer wall and the centre-line of the pipe is only 20 mm.
were established (section 6.1.11), the axial and lateral resolution were estimated. The best axial resolution (AR) for soft tissue is denoted by\[\text{(5.9)}\]:

$$AR = \frac{1}{2} SPL$$

where the spatial pulse length (SPL) is the product of the number of cycles in the pulse and \(\lambda\). The 7.5 MHz transceiver emits pulsed ultrasound at a speed of 1540 m/s (in 30°C milk)\[349\] with 3 cycles in each pulse; using equation 5.9, the best axial resolution to be expected is about 0.31 mm. The axial resolution of diagnostic ultrasound is always better than lateral resolution\[312\]; the lateral resolution is usually about three times poorer than axial resolution\[314\] and was estimated by the instrument manufacturers to be 1 mm. The resolution or detail, achievable when imaging a non-flat clot, may not be as good as these predictions, so resolution tests were conducted.

Since extensive, well adhered coagulum on the test-body was not easy or cheap to achieve, a small object was taped onto the P.V.C. teardrop, and this model of the clotted test-body was immersed in a water-filled polypropylene test-chamber. Paper was rolled into a ball and taped onto the teardrop in two different places. Thickness measurements of the ‘clot’ taken from the display screen were accurate to within about 0.4 mm, and lateral resolution was about 1 mm.

### 5.5.4 Interpretation of ultrasound images

Interpretation of ultrasonic images can be difficult, with image detail being coarse and some structures causing beam attenuation and misleading artefacts. An ultrasonic image is dependent on the equipment, the media under study and the operator. The operator bias can be minimized by familiarity with the limitations and capabilities of the ultrasound scanner, and by learning what patterns or display characteristics correspond to specific structures and which are artefacts in the ultrasonic images.
All of the systems illustrated in figures 5.14(a), 5.15(a) and 5.16(a) contain a test-chamber, in which a flow (from left to right in the image) of renneted milk passes a centrally positioned test-body, and are displayed on the ultrasound monitor as shown in figures 5.14(b), 5.15(b) and 5.16(b), respectively. The scale graduations on the perimeter of the ultrasound image are separated by 10 mm, while the grey-scale wedge on the left side of the image indicates the range of grey in which echoes are depicted. Aspects of the ultrasound images worth consideration are denoted by the letters A—G, and are discussed below.

The milk clot provided diffuse reflections, arising due to scattering of the ultrasound beam and resulting in a speckle-pattern depiction, and was imaged better when in some locations than in others. The curved test-body and clot adhered to it were depicted, but not well enough to allow clot thickness measurements to be made. The clot on the wall nearest the probe was partially depicted, but was too near the probe to be imaged unambiguously. There exists a region next to the transducer face of about 4–5 mm deep, termed the ‘transducer dead space’, which cannot be imaged due to the fact that transceivers cannot detect echoes while they are transmitting. A cloudy region (A) occurred in this superficial region, due to echoes from structures near the probe face being returned to the transceiver during transmission. A consolation was that the milk/clot and wall/clot interfaces at the far-side of the pipe (F) were depicted well enough for thickness measurements to be made, except when lying directly behind the test-body. This wall clot was usually depicted as consisting of a bright layer adjacent to the wall, probably trapped bubbles, covered by a dark or speckled clot whose interface with the milk flow was a grey line highlighted by bubbles flowing over it.

Nearly all of the ultrasound beam intensity was reflected or absorbed, where air bubbles and the test-body were present in the beam. A glare was sometimes present, due to strong reflection at the surface of the test-body and bubbles, which made image interpretation of the adjacent structures difficult. Bubbles in the flow (B) and on the test-body (C) were depicted brightly and gave rise to scatter and shadowing. The frame rate was sufficient for the bubbles in the flow not to appear as if they were jumping. Shadowing is an artefact that occurs when ultrasound is greatly attenuated by strongly reflective
Figure 5.14: (a) The actual system, and (b) how it was ultrasonically depicted.
Figure 5.15: (a) The actual system, and (b) how it was ultrasonically depicted.
Figure 5.16: (a) The actual system, and (b) how it was ultrasonically depicted.
highly attenuative test-body caused a shadow region (D), which made interpretation of images on its far-side impossible. Bubbles, and even a highly reflective brass rod ($Z = 40.6 \times 10^6 \text{kg/m}^2\text{s}^2$) placed perpendicular to the ultrasound beam, were undetectable when in the shadow region of the test-body.

Reverberation artefacts occurred due to multiple reflections of the ultrasound waves. 'Ghost' interfaces were present in the ultrasound images, on the posterior side of the support rod (E), due to internal reflections between the front and back faces of the support rod.

The depth of a structure is displayed, following a calculation made by the signal processor (using the speed of sound in soft tissue, 1540 m/s). Since the speed of sound in curd is similar to that in soft tissue, the curd thickness depicted on the display was the actual thickness. However, the ultrasonically depicted thickness of other media within the plane of the ultrasound scan, in which sound travels at speeds substantially different to that in soft tissue, was not similar to the actual thickness of these media. For instance, the 10 mm diameter teardrop-shaped test-body was depicted as being less than 10 mm wide (G in figure 5.14(b)).

Given the fixed parameters of the ultrasound transducer, the fixed shape of the test-bodies, the small difference in acoustic impedance between the curd and milk, limited time and no effective stand-off available, it was not felt that much more progress in image enhancement could be made. At this point, therefore, it was decided to proceed to the next stage: conducting ultrasonically imaged milk experiments. The results obtained from ultrasonic scans in numerous milk clotting experiments are reported and discussed in chapter 7. Chapter 6 details the apparatus, how it was used and the way in which technical difficulties were approached.

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interfaces and/or highly absorbent layers. Structures on the posterior side of these highly attenuative media can produce only weak echoes, which must cross the highly attenuative structures before reaching the transceiver. This can result in a dark shadow region on the far-side of highly attenuative structures.
Chapter 6

Apparatus and Recommissioning

Numerous difficulties in securing controlled and reproducible operation were experienced when attempting to recommission a previously constructed milk clotting rig. A particular problem was the formation of extensive, poorly adhesive milk clots. This chapter provides descriptions of the various pieces of apparatus composing the milk rig and their inherent problems, if any, and the measures taken to overcome these problems. In addition, details of consistency checks, including consistency assessments of reagents, wall clot growth and test-body clot shape and weight, and an investigation of the effect of using ultrasound in the milk experiments are given.

The experimental procedure and apparatus (figure 6.1) employed here are similar to that used by Christy and described in section 4.2.3, with a few exceptions. 15 gallons of fresh, unpasteurized milk was used in each experiment. The accumulation of clot on the test-object and test-section wall was visualized by applying an ultrasound transceiver, which was linked to a scanner, video-tape recorder and printer, to the test-section wall. Other equipment additions or changes are detailed below, and the experimental procedure is described, in full, in appendix B.
6.1 Apparatus

6.1.1 Dispensation of milk
The milk was used on the same day that it was obtained, and dispensed from three 5 gallon P.V.C. containers with built-in taps. A manifold was employed to allow convenient changeover between water and milk, at the start and end of milk experiments, and from one milk container to the next during experiments.

6.1.2 Heat exchanger and temperature controller
A single-pass plate heat exchanger was used, with 6 parallel hot water channels alternating with 5 parallel milk channels, to elevate the temperature of the milk to 37°C. The heat exchanger was constructed of 316 stainless steel, with Paracril gaskets to minimize fouling by milk proteins. Three heating elements (one single-phase 2 kW, one 3-phase 4.5 kW and one 3-phase 6 kW element) submerged in a water tank, which supplies hot water to the heat exchanger, were linked to a temperature controller. This

Figure 6.1: Schematic diagram of the milk clotting apparatus.
controller was equipped with a negative feedback loop to control the water\(^1\) and thus milk temperature.

Inaccurate temperature readings and a major lack of temperature control were discovered, so tests were subsequently conducted to identify the sources of these faults.

The testing of thermocouples was facilitated by implementing a pocket arrangement; this made removal of the thermocouples for testing easier than the original arrangement, as the thermocouples were previously fixed in place (in direct contact with the flowing fluid). Tests on all five of the thermocouples, using flasks of water at different temperatures, indicated these detectors were unreliable, giving inconsistent and incorrect values. The problematic temperature sensors were found to be made of a T-type alloy, which is incompatible with this specific temperature controller. The thermocouple wires were thus replaced with wires composed of a compatible (K-type) alloy. These new thermocouples were tested, in the same way as before (using water samples at different temperatures), and were found to provide correct temperature readings.

The temperature controller was unable to provide a steady-state temperature of 37\(^\circ\)C, so attempts were made to achieve temperature control. Insulation was applied to vessels and tubing but with little effect, indicating that the temperature fluctuations due to heat losses occurring through the system were not the main cause of the problem. The 2 kW heating element was found to be faulty, but this was not of concern as this heating element and the 4.5 kW element, present to enable heating of flows of milk up to 6 l/min, were found to be unnecessary for heating the 2 l/min milk flow used here. The third heating element, that is the 6 kW one, proved capable of supplying all of the heat duty required. The temperature control system itself was studied, and it was found that only the 'thermal cut-off' mechanism, which limits temperature runaway, was operating correctly. Following extensive checking and repairs to the existing control

\(^1\)To prevent the risk of heat-induced changes in the milk (section 4.4.3), the water temperature was kept below 60\(^\circ\)C.
system further testing showed little improvement in temperature control, with the temperature fluctuating continuously by 3–8 °C. The controller was deemed to be of poor design and performance and was thus replaced.

A “91e Eurotherm” temperature controller was chosen as the new controller. This self-tuning PID controller, which automatically calculates the PID control parameters when a setpoint is chosen, provided precision control (± 0.2°C) and enabled the set-point temperature to be achieved with circulating water within about 15 minutes.

6.1.3 Degassing unit

The degassing unit illustrated in figure 6.2(a) consisted of a 5 litre stainless steel vessel containing 1/2” ceramic spheres (to improve the surface area / volume ratio and promote bubble coalescence), and was accompanied by two 7 litre perspex capacitance chambers, with vacuum supplied by use of a water-jet ejector nozzle.

The purpose of the degassing unit was to remove excess gases from the milk, by bursting and discharge of bubbles at this liquid’s free surface. The milk contains gas in solution and in the form of bubbles. When it is heated from 4 to 37°C, the dissolved gas content is reduced but the number of bubbles in the milk increases. If the bubbles of gas in the milk are not removed, or at least significantly reduced in number, their presence may cause significant unwanted effects: they might disturb the flow and clot deposition; scatter the ultrasound beam and thus reduce the quality of imaging (section 5.3.4); and provide nucleation sites for cavitation or acoustic streaming phenomena induced by ultrasound (section 5.4.1).

The inability of the existing equipment to deliver deaerated milk to the test-chamber became apparent by means of ultrasonic imaging; it was discovered that the milk entering the test-chamber was riddled with bubbles. Unfortunately, it was found that this was due not only to an ineffective degassing unit but also to the diaphragm and peristaltic pumps causing bubble formation. Therefore, additional degassing, downstream of these pumps, was considered to be necessary. This was tried in steady
Figure 6.2: Degassing section ideas.
flow, using an available deaerator, but with disappointing results; the bubble content of the milk entering the test-chamber appeared to be slightly, if at all, reduced, but nowhere near as much as desired. The use of a degassing vessel downstream of the diaphragm pump would not be ideal in any case, as it would cause damping of the pulse.

Further preliminary attempts to improve degassing resulted in improving the vacuum achievable, in some cases, but still did not enable the milk to be satisfactorily degassed. All the points in the system with the potential of allowing gas intrusion were checked and sealed where necessary, and the number of joints in the flow system was reduced. The two perspex vacuum chambers were both cracked, having experienced contraction and expansion due to pressure fluctuations, and the glue used on their end plates was no longer providing an air-tight seal. These two chambers were therefore replaced by two similarly sized glass vessels, and their inlet and outlet stoppers sealed tightly with vacuum grease. The water-jet ejector was found to be faulty and was replaced. It was discovered that the gauze in the deaerator had somehow moved out of its intended position, resulting in some ceramic balls actually blocking the flow outlet; the gauze was subsequently rearranged.

The creation of bubbles by the pumps was unavoidable, but modification or redesign of the degassing unit might be able to reduce the gas content of the milk flow. Modification of the original device was first considered, since a simple alteration would save the time and expense of a new deaerator. During the milk experiments the level in the first vacuum chamber was maintained at about one inch. This means the deaerator was completely full of heated, aerated liquid. With these conditions in mind, the following modifications were considered:

- The original design allowed the heated milk to enter the deaerator and flow straight to the outlet, limiting the duration of exposure of the milk to the vacuum. An inlet baffle could be inserted to reduce the range of fluid residence times and, as a result, increase the degree of gas disengagement (figure 6.2(b)).
- The effect of varying the number of ceramic balls could be assessed.

- Gauze can act not only as a packing support but also as a trap for bubbles, so another gauze could be placed on top of the packing (figure 6.2(c)).

- To promote the rise of bubbles that have passed through the packing and gauze, a column, such as that shown in figure 6.2(d), could be incorporated into the deaerator design.

- To reduce the number of 'dead zones', which lead to large residence time variation, the deaerator base could be made conical. This more gradual flow constriction would also reduce acceleration at the deaerator outlet, hopefully reducing the number of bubbles being carried downstream.

- A high vacuum is desired. The vacuum could be increased, and a gas membrane used so that the milk is not drawn into the vacuum line.

Some of the above modifications for degassing enhancement were attempted, and it was found that none of the inlet-baffle, variation of the number of ceramic balls, or additional gauze (40mesh (0.236 mm diameter)) were effective when tried. The other proposals (i.e. conical-base; extra column; and increased vacuum) were not implemented. A new degassing unit was deemed necessary.

An 8 litre, baffled P.V.C. vessel was designed (figure 6.2(e)), with a large nitrile rubber O-ring fitted in a grooved lid for air-tight performance. A 40mesh gauze was employed at the outlet side of the system (to trap bubbles, promoting their coalescence and rise, and increase the mean residence time of the bubbles) and a baffle was used to increase the mean fluid residence time and to force all milk to pass the inlet of the vacuum chamber. It was calculated that the rise velocity of bubbles in this new unit is such that 8 ml of milk flows out of the deaerator in the time it takes a bubble to reach the top of the deaerator.

The new degassing unit appeared to reduce the size and number of bubbles passing through the test-section, but a large number of bubbles were still present. Radically
different deaerating techniques were subsequently considered. Some of the numerous methods assessed for degassing the milk are listed below, accompanied by their inherent advantages and/or shortcomings.

(i) Ultrasonic degassing: Swallowe et al.[359] used a 36 kHz source in direct contact with a solidifying melt, to induce gas disengagement from the melt. Whilst ultrasound can indeed be used for degassing, this action is facilitated by induced transient cavitation—a phenomenon that would be likely to denature milk proteins.

(ii) The inability of the original and new degassing units to thoroughly degas the milk, perhaps is due to an insufficient residence time of the milk in the degassing unit. If the residence time of the milk in the present degassing vessel was markedly increased, the flow from this vessel would be much less than that desired (i.e. \( < 2 \text{l/min} \)). An alternative way to degas the milk for a longer period of time is by degassing the milk prior to each experiment.

Consideration was given to the idea of degassing the milk prior to each experiment, by application of a vacuum, but the increased bubble content when the milk is then heated during the experiment would still be unresolved. If the milk was not clotted at 37°C but at, say, 20°C (recall that rennet will not clot milk below 15°C), the bubble population might be lower; however, the clotting time would be much greater and the clot rheology different (section 4.4.5).

(iii) An alternative way of degassing the milk before experiments, which would not be made ineffective by the preference that the milk be at 37°C in experiments, is by preheating the milk at, or above, 37°C.

Many degassing methods involve heating at temperatures of about 100°C. For instance, boiling water for 20 min, or heating it above 80°C for one hour or more, is very effective[360]. However, milk is sensitive to such high temperatures. Heating milk at 100°C for 15 to 20 min causes extensive denaturation and aggregation of whey
different deaerating techniques were subsequently considered. Some of the numerous methods assessed for degassing the milk are listed below, accompanied by their inherent advantages and/or shortcomings.

(i) Ultrasonic degassing: Swallowe et al.\textsuperscript{[359]} used a 36 kHz source in direct contact with a solidifying melt, to induce gas disengagement from the melt. Whilst ultrasound can indeed be used for degassing, this action is facilitated by induced transient cavitation—a phenomenon that would be likely to denature milk proteins.

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proteins and flocculation of casein micelles\textsuperscript{189a}. Heating milk at lower temperatures, on the other hand, might be feasible. It has been found that heating milk at 60°C and below, even for prolonged periods (up to 5 hours), has little effect on the rennet coagulation time (RCT)\textsuperscript{272} (section 4.4.3). This may not always be the case, however, as such heating could have an adverse effect on milk, according to Webb and Holm\textsuperscript{267}. They found that there exists a critical value of about 13% SnF (solids-not-fat) below which thermal destabilization occurs (section 4.4.2). The cow milk used in the present experiments contained about 8.5% SnF, so heating this milk prior to milk experiments might destabilize it.

In order to determine whether or not the RCT is unaffected and satisfactory degassing is achieved when heating the milk used here, small-scale tests were conducted. Heating a 200 ml milk sample at 34°C, for about 20 min, degassed this milk well enough to improve ultrasound imaging, while not causing any noticeable changes in the milk's properties. This heat treatment, and slight variations of it, were found to be inconsequential to the RCT. Three 5 ml samples of unpasteurized milk were maintained at 34°C for 20, 25, and 30 min, respectively, before being used in modified Lee-White tests (where inversions were every 15 s). All three tests gave both similar clotting times and solid plugs of curd.

Time constraints and delays due to extensive equipment revamping led to some of the ideas described above not being tried. The most hopeful degassing idea appears to be the preheating of milk prior to an experiment. If the milk were preheated in a large diameter container and this container was elevated, so that it acts as a header vessel and makes the peristaltic pump no longer necessary, a steady flow of bubble-free milk through the test-chamber may be achieved. On the other hand, heating milk prior to pulsatile flow experiments would not be expected to allow one to achieve a flow of bubble-free milk through the test-chamber, as the diaphragm pumps' pressure fluctuations cause bubble formation. The results reported in chapter 7 were obtained with the aid of the degassing unit shown in figure 6.2(e), and without preheating the milk since equipment for such preheating was not available at the time.
6.1.4 Peristaltic pump

A Watson Marlow 603U/R peristaltic pump, with 12.7 mm i.d. tubing, was used to draw the milk from the milk containers through the heat exchanger and degassing unit and into the header vessel. This pump was operated with feedback control from a level controller in the header vessel, and was capable of providing flow rates up to 8 l/min.

6.1.5 Header vessel

An insulated stainless steel container of 5 litre capacity was used as a header tank, providing a supply of heated milk at a constant head to the test-section downstream. The level control was facilitated by the use of a ‘Telstor Series 80’ level sensor and controller, with feedback to the power supply of the peristaltic pump. A baffle-ring surrounding the exit of the header vessel was used to inhibit bubbles being carried straight through to the test-section.

The steady flow experiments were conducted using the gravity driving force provided by the height difference between the liquid level of the header vessel and the outlet of the test-section exit tube. Pulsatile flow experiments, on the other hand, were carried out with the outlet of the test-section exit tube at a height of about 8 cm above the liquid level in the header vessel, in order to eliminate the steady flow component. The changing of drain or header vessel height, when trying to establish the correct flow rate, was made easier by installing a variable-height header vessel support, two drain-pipe inlet nozzles of different height, and height graduations for both of these pieces of apparatus.

The need to maintain a flow rate through the test-section of 2 l/min, even when clot development impedes flow, requires monitoring of this flow rate. The header vessel height could then be altered or, in pulsatile flow studies, the stroke volume of the diaphragm pump changed. A rotameter was incorporated but removed, as the resistance it caused to the flow made 2 l/min unachievable. A turbine or magnetic flow meter was not used either, due to lack of availability or expense, so flow checks at the
sink were conducted with a flask and stopwatch.

6.1.6 Diaphragm pump

Pulsatile flow experiments were conducted using a hydraulic diaphragm pump capable of producing a range of pulse frequencies (20–150 pulses/min), stroke volumes (0–100 ml), and systolic/diastolic duration ratios (0.4–2.0). The pulse frequency was set at 70 pulses/min, the stroke volume at about 28.6 ml, and the systolic/diastolic duration ratio at unity, for all of the pulsatile flow experiments.

A few minor alterations of the pump were required, after which this pump was able to provide consistently a flow rate of 2 l/min. Air in the pump’s lines led to inefficiency and long tubing led to pulse damping, so the lines between the bellows and diaphragm were shortened and well sealed. Diaphragm and bellow repairs were also necessary.

6.1.7 Dosing pumps

Since the clotting time (RCT) of the milk mixture was about 35 s in Lewis’s net experiments (section 4.2.3), here the CaCl₂ and rennet were added to the milk stream immediately prior to the test-section to ensure that the mean residence time of the mixture in the test-section was significantly less than 35 s. This injection of rennet and aqueous CaCl₂ was performed by two identical Liquid Metronics Series A1 metering pumps, which can produce flows with dose frequencies in the range 15–100 doses/min and stroke volumes between 0–0.63 ml.

Difficulties arose with calibration, as these pumps needed extensive servicing and none of the readings on the dials directly corresponded to the actual flow and pulse rates produced. The pumps were repaired, cleaned and leaks were eliminated, allowing accurate calibration. Twenty ml/min of each reagent was supplied continuously, at a frequency of 70 beats/min and a stroke volume of about 0.29 ml, for all of the experiments.
6.1.8 Test-section

The test-section used by Christy is illustrated in figure 6.3(a). It consisted of three 32 mm i.d., equal-length, flanged perspex pipes, a flow converger and flow diverger, and a test-body on a support rod fixed to a circular seat. Silicone rubber tubing of 12.7 mm i.d. was used at the entrance and exit of the test-section, and a diffuser and converger were employed in the cylindrical test-section to allow the flow area to change gradually. The diffuser promoted plug flow in pulsatile flow, and laminar flow in the steady flow regime, limiting the spread of residence times of the activated milk mixture.

Changes were made here, to the original test-section, mostly for ultrasonic imaging or procedural purposes (figure 6.3(b)). The middle pipe, housing the test-body and referred to as the test-chamber, was replaced by a polypropylene pipe. Leakage at the flange/pipe join, previously experienced with the perspex test-chamber, was prevented by having the polypropylene test-chamber flanges friction-welded rather than glued. The numbers I to VIII were marked on one of the test-chamber’s flanges, to allow ultrasound probe position to be recorded when conducting clot symmetry checks. The stainless steel rod on which the test-body was suspended was changed to a perspex rod (figure 6.4), and a new test-object seat was made for dismantling reasons (section 6.1.10).

6.1.9 Waste disposal

The spent milk flows from the test-section to a vertical drain-pipe, which has a high flow rate of cold water introduced at its top-end via a conical diffuser. The previous drainage system consisted of a flushed drain-pipe, positioned over a sink, with about 8 feet of tubing attached between this pipe and the exit of the test-section. This arrangement allowed the residence time of activated milk to exceed the RCT while in the system, leading to extensive clotting in this tubing and consequently flow resistance. Accordingly, dilution of the milk stream was performed closer to the test-section exit, to reduce the residence time of activated, undiluted milk in the system and thus the resistance to flow. A pipe at the base of the water-flushed drain-pipe directed the effluent to the sink.
Figure 6.3: The (a) original and (b) modified test-sections.
6 Apparatus and Recommissioning

6.1.10 Test-objects and their removal

The shapes of the test-bodies employed were teardrop, cone and disc, and are illustrated in figure 4.8. The conical and disc-shaped test-bodies were constructed of P.V.C., while five teardrop-shaped test-bodies were used, each being composed of a different material (P.V.C., P.T.F.E., nylon, perspex or polycarbonate). Each body in turn was mounted on the perspex support rod (figure 6.4) axially aligned with the flow and positioned at the downstream end of the test-chamber.

Dismantling the test-section is a delicate stage of the milk experiment that has been troublesome in the past. The opening of a valve or flange, upstream or downstream of the test-object, had to be carried out carefully and in such a way as to prevent a sudden flow of fluid or influx of bubbles which could interfere with the final clot on the test-object. At the end of a milk experiment, when the test-section contained stagnant unrenneted milk, drainage was originally carried out by either slightly opening a drainage valve upstream of the test-section or by raising the test-section until the milk free surface had passed by the test-body. Both of the above drainage methods remove the fluid pressure helping to keep wall clot in place, allowing wall clot to slip from the downstream perspex pipe into the test-chamber. Also, the P.V.C. test-object seat was previously inserted in a cavity in the downstream perspex flange (figure 6.3(a)), and this caused problems with dismantling - the seat was prone to slipping as the two
flanges were separated, causing disruption of the clot on the test-object.

A new test-object seat arrangement was devised and a new dismantling technique developed, allowing the above mentioned problems with the test-object seat and wall clot to be solved. The cavity in the perspex flange, previously occupied by the test-object seat, was filled and a new test-object seat was made that fitted between the perspex and polypropylene flanges. This new arrangement allowed a new dismantling procedure to be carried out, after flow was stopped at the end of a milk experiment. Wing nuts were used to hold the test-body seat and the polypropylene and perspex flanges together, to enable quick dismantling of the test-chamber with minimal disruption. After slight loosening of all six of these screws and removal of one of them, a thin, 45 mm wide, lightly greased stainless steel plate was inserted between the perspex and test-body seat (figure 6.3 (b)), completely occluding the bore of the test-section. Once this steel plate separated the contents of the downstream perspex pipe and that of the polypropylene pipe, the remaining five screws and perspex pipe were removed with no consequence on the test-chamber contents. The dismantling procedure is described in more detail in appendix B.

6.1.11 Ultrasound transceiver and monitor, and recording equipment

The 7.5 MHz piezoelectric transceiver, used in the milk experiments to obtain information about the instantaneous state of the clot and its growth pattern, was described in section 5.5.1 and is illustrated in figure 6.5.

The ultrasound monitor is shown in figure 6.6. It was equipped with nine buttons on the left, and five knobs on the right, of its display screen, as well as a keyboard and a trackerball.

The operational controls, on the left side of the display screen, were used as follows. The invert button allowed the image to be displayed upside down. Transpose enabled the user to alter the image orientation from left to right or vice versa. If focus was selected, the focal length was reduced from 60 mm to 35 mm. This option was not
Figure 6.5: The ultrasound probe, its support and the test-chamber.

Figure 6.6: The ultrasound monitor.
used in the milk experiments, because the region of interest was within 20 mm from the probe. The *scale up/scale down* buttons allowed any one of three magnifications to be chosen: 1.5, 2.0 and 3.0, enabling the operator to make a closer study of any area on the display screen. The *far* and *near* buttons enabled the ultrasound image to be moved up or down, so that the lower or upper parts of the image could be viewed more closely\(^2\). The *screen* button allowed storage of two full size images in memory, while another screen was provided for real-time imaging. The *freeze* button, and a foot-switch, allowed the user to still-frame the image on the screen.

The way in which echoes were processed and displayed on the screen was governed by the use of the controls to the right of the display screen. Image enhancement was performed by varying the *brightness* and *contrast* controls, while observing the ultrasound image and guided by the grey-scale on the left side of the display screen (which should gradually go from black to white). Too high a setting led to a lack of image detail, but too low a value led to weak echoes not being displayed. The amplification of received echoes can be modified by varying the *overall* gain, using a special screwdriver, or by using time-gain compensation (TGC). While increasing the *overall* gain influences all echo signals, TGC increases amplification of weak echoes only. The controls available for establishing the appropriate TGC included: *initial* gain, which enables amplification of echoes received from superficial reflectors and was kept low to keep the transducer 'dead space' artefacts (i.e. the bright, cloudy area near probe) from saturating the display; *near* gain, which provides a small level of amplification for the echoes received from shallow structures (in the first 5 to 7 cm of the beam, approximately) and was kept at about 50% of the range permitted; and *far* gain, which was not of use here, because this control enables amplification of echoes received only from distant reflectors in the region 7 to 13 cm away from the transceiver, which is outside the region of interest here. While TGC was of limited use, a more versatile form of selective amplification compensation, called automatic gain control (AGC), was available. AGC automatically compensated for the general decrease in echo magnitude with depth, providing the best achievable images by not increasing the

\(^2\)The arrow cursor keys, on the keyboard, also allowed the operator to move the image to the near and far fields and to the left and right, when the image was scaled-up.
overall echo size but only the size of weak echoes, and reduced the demands on the operator and the variation in image quality due to the subjectiveness of imaging. Image quality was reproduced by establishing good baseline settings. Once it was determined how best to enhance imaging, only one or two controls needed to be adjusted during scanning.

The caliper and mark switches and trackerball were used to measure the dimensions of structures in the ultrasonic images, to the nearest whole number of millimetres. The trackerball was used to position the calipers once the appropriate programme was selected from the menu (i.e. programme A was chosen for area and circumference measurements, and D for distance measurements). Pressing the caliper button made the first caliper appear on the screen, and the trackerball was rolled to position this caliper, and the mark button pressed to fix it in place. The second caliper subsequently appeared, which was positioned and fixed as before, enabling a measurement to be made. Three distance measurements were able to be held on the screen at any one time.

The information obtained during the milk experiments was recorded using a video-tape recorder and an ultrasound UP-850 thermal (black and white image) printer, enabling detailed analysis of clot deposition and wall clot loss. Replaying the video-tape of magnified ultrasound images, on a television screen much larger than the display screen on the ultrasound monitor, allowed precise measurements to be made (to the nearest 0.01 mm) with hand-held digital calipers applied to the television screen. The distance between two adjacent scale graduations around the perimeter of the ultrasound image corresponds to 10 mm, providing a scale from which to relate measurements made in this way.

6.1.12 Ultrasound probe support

The probe can be well-coupled acoustically to the curved test-chamber wall, by using plenty of gel and manually holding the probe against the test-chamber. However, continuous imaging of the milk experiment for its full duration cannot be achieved in
this way by one experimenter, as there are numerous experimental tasks which must also be performed as the run proceeds. This problem could be resolved by fixing the probe permanently to the wall of the test-chamber; however, versatility is essential when attempting to establish a new technique, and imaging of the far side of the test-body and other regions of interest would not be possible with such an arrangement nor would probe maintenance. To resolve this problem, a P.V.C. probe support was designed and constructed (figure 6.5), in order to allow a versatile way of clamping the ultrasound probe to the test-chamber without interfering with the milk experiment or greatly increasing the demands of the experimental procedure. Using this transducer support, the probe can be firmly applied to the test-chamber (without the need for hand support) and be easily moved to other areas.

6.2 Reagents and reproducibility

6.2.1 Consistency of CaCl₂ concentration and milk & rennet activity

With the primary aim of achieving reproducible milk experiments, tests were conducted to confirm the consistency of composition and properties of the fluids used in the milk experiments (CaCl₂, cow milk and calf rennet). The procedures used for conducting these pre-experiment tests are described in appendix B, and their results are reported here.

Calcium Chloride

Variation of CaCl₂ concentration can alter milk clotting time \[301\] and rheology of the clot formed \[189a\] (section 4.4.5), so consistency, or accurate knowledge, of this concentration is necessary for the type of milk experiments conducted here. Contrary to expectation, the available CaCl₂ solid, previously used in similar researches, did not allow one to achieve consistent, saturated solutions by prolonged contact of excess CaCl₂ solid with water. It is possible that this batch of solid CaCl₂ was contaminated in some way; but no clear explanation of this anomalous behaviour has so far been found. Unfortunately, this impure CaCl₂ was used in a number of milk experiments. It was therefore necessary to determine the concentration of the CaCl₂ solutions concerned,
and this was achieved by titration (section B.1). Tests showed the chosen method of titration to be reliable with solutions made not only with distilled water but also with Edinburgh tap water, so the latter was then accordingly used (for convenience) in these analyses. A new batch of CaCl\(_2\) was later obtained, which enabled solutions of consistent CaCl\(_2\) concentration to be achieved.

**Milk and Rennet**

The milk used here was unskimmed, unpasteurized whole milk containing no preservatives and of a relatively uniform composition. The effects of seasonal, source and dietary variations on milk composition (section 4.4) were not considered to be significant here. It so happens that the experiments were conducted using milk produced during the most heat stable period of the year\[^{[266]}\], and by cows of the same type and on a uniform diet; the milk contained 4.38 ± 0.07% butter-fat, 3.48 ± 0.05% protein, and ~8.5% SnF (solids-not-fat), and the herd of cows from which it came\[^3\] consisted solely of pedigree Holstein Friesians at the University farm\[^{[361]}\].

The only milk treatment prior to experiments was cold storage and this was considered unlikely to alter the rennet coagulation time (RCT). The milk was stored at 4\(^\circ\)C for an average duration of about 7 hours, prior to each experiment. Given the findings of Davies and White\[^{[260]}\] and others\[^{[270][272]}\] (section 4.4.3), regarding cold storage and RCT, any alterations to the raw milk properties during this cold storage would not have been sufficient to alter the RCT.

Commercial calf rennet was used in the milk experiments conducted here. The reproducibility of this rennet was achieved by similar preparations of each batch (section B.2) and proper storage. The rennet age was of concern with regard to variation and maintenance of rennet activity. It was desired to know whether or not this rennet maintains its activity for a long time; if it does, one large batch of rennet could be purchased, as opposed to the 2 litre batches originally purchased, in order to avoid the problems which can be caused by differences between rennet batches. The duration

\[^3\]The herd was 'closed'; in other words, it did not take in any other cows.
for which the rennet maintains its initial activity was assessed by conducting modified Lee-White tests, using samples from the same rennet batch and of different ages. It was found that the rennet did not experience significant loss in activity over a period of 8 months, when stored in a cool (2-5°C), clean and dark place, in closed containers (the appropriate storage conditions based on section 4.4.4).

In order to ensure the milk and rennet activity were consistent, prior to each milk experiment pH measurements4 and modified Lee-White tests were conducted on both fluids (section B.3).

The pH of the different milk and rennet batches was found to be very consistent. At about 14 to 15°C, the average value of the rennet pH was 5.385 (SD = 0.6%), and when the rennet temperature was elevated to about 37–38°C, and maintained for 20 min, the rennet pH rose slightly to an average value of 5.480 (SD = 0.6%). The average pH of the milk was found to be 6.707 (SD = 0.2%), at about 11 to 12°C, and 6.646 (SD = 0.6%), after being maintained at 37°C for 20 min.

Three modified Lee-White tests were conducted, at a fixed temperature and CaCl₂ concentration, with the consistency of the rennet and milk activities deduced from the time taken to form a non-flowing plug of curd. The activity of both the calf rennet and milk was expected to be consistent, based on the suppliers’ consistency measures and the above findings; however, the Lee-White clotting times were not always reproducible. These findings appear to be associated with the variation of the CaCl₂ concentrations. The impurities present in the original batch of CaCl₂ were at first thought to be the cause of the inconsistent clotting times, but it now appears that the use of solutions of low CaCl₂ concentration could be the cause. 15 of 21 tests had similar results, when using CaCl₂ solutions of similar concentration (3.00-3.05 M) prepared with the new batch of CaCl₂(s), and 14 of 15 Lee-White clotting times were similar, when more concentrated CaCl₂ solutions (3.30–3.38 M) prepared with the impure CaCl₂(s) were

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4The activity of calf rennet and coagulability of unpasteurized milk are both sensitive to pH (section 4.4.4 and 4.4.5).
used. However, when solutions of low CaCl₂ concentration (1.72–2.51 M) were used, a wide range of clotting times, from 45 s to 105 s, were obtained.

Reproducible results were obtained in the milk flow experiments (section 6.2.2), but not always in the Lee-White tests, perhaps due to certain differences between the two systems. The explanation for these differences in ability to achieve reproducible results might be that at low CaCl₂ concentrations, the RCT and rheology of clot may be very sensitive to the level of agitation and the temperature (section 4.4.5). Both of these variables were greater in the milk experiments than in Lee-White tests.

6.2.2 Reproducibility of clot deposition

Christy found the shape and extent of clot deposited on test-objects to be reproducible in his milk experiments. Reproducible clot deposition, with the revised milk clotting apparatus of Christy, was also indicated here by test-body clot extent and shape, and by wall clot growth patterns.

The final weight of clot on the test-object was recorded in two categories: 'test-body clot' weight, which corresponds to the weight of clot associated with the test-body (that is clot on the test-body itself and in its immediate downstream region/wake), and the 'test-object clot' weight, which is the total weight of clot on the test-body and its support rod. When wall clot loss occurred, during experiments or drainage of the apparatus afterwards, it did not in all cases interfere with the 'test-body clot' but always impinged on the radial portion of the support rod, so the weight of 'test-body clot', as opposed to that of the 'test-object clot', was used in most analyses. The boundary between the downstream part of the 'test-body clot' and that due to the rod was taken, somewhat subjectively, as the location (on the rod) beyond which clot shape appeared unaffected by the presence of the test-body.

Comparison of results from comparable experiments, conducted with pulsatile milk flow past a teardrop-shaped test-body, indicates the reproducibility of the test-body clot extent (table 6.1).
6 Apparatus and Recommissioning

<table>
<thead>
<tr>
<th>CaCl$_2$ conc$^a$ (M)</th>
<th>Test-body clot weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.05±0.01</td>
<td>0.334±0.001</td>
</tr>
<tr>
<td>3.30</td>
<td>0.314</td>
</tr>
<tr>
<td>3.38</td>
<td>0.361</td>
</tr>
<tr>
<td>/</td>
<td>Average (g) = 0.336 (SD 6%)</td>
</tr>
</tbody>
</table>

Table 6.1: Weights of clot deposited on a teardrop-shaped test-body

For a given test-body shape and flow condition, the final test-body clot shape at the end of an experiment was reproducible. For example, the clot on the teardrop-shaped test-body was typically bubbly upstream and smooth elsewhere, in steady flow (figure 6.7(a)); and, in pulsatile flow, the clot on this body was bubbly upstream, somewhat barrel-shaped along its middle and tapered at its downstream end (figure 6.7(b)).

The plots in figure 7.6, of clot growth in steady and pulsatile milk flow studies using similar CaCl$_2$ concentrations, illustrate that wall clot growth rates were reproducible.

6.2.3 The effect of ultrasound on clotting

An assessment of the effect of ultrasound on milk clotting, further to that reported in section 5.5.2, was conducted by comparing results from experiments where ultrasound was applied for different durations. The application of diagnostic ultrasound to the milk clotting experiments gave results that indicate no effect on clot shape or extent. It was found that even when the probe was applied to one part of the clotting chamber for the full duration of an experiment (i.e. about 35 min), no clot irregularities were apparent. Acoustic streaming, as defined in chapter 5, might have occurred, as some bubbles resided within the scan plane, but no significant effect on the ultimate clot extent and shape were observed.

With the reproducibility problems resolved, milk experiments were conducted and their results are reported in chapter 7.
Figure 6.7: Photographs of the clot on the teardrop-shaped test-body, after (a) steady and (b) pulsatile milk flow experiments.
Chapter 7

Results and Discussion

The aims of the present research were to investigate further the potential and limitations of a previously performed milk clotting experiment and assess the application of ultrasonic imaging to it, in order to elucidate the relation between milk clotting and flow pattern of renneted milk. The results presented in this chapter provide, in particular: insights into the clot growth pattern at the wall of a pipe and on differently shaped test-objects axially located in the pipe flow; the frequency of, and interference caused by, wall clot loss in such experiments; the effect of varying CaCl$_2$ concentration and flow regime on the extent and rate of clot deposition and on wall clot loss; the relationship between clot extent and the test-body material; and the ability and limitations of the ultrasonic imaging equipment used to aid and extend these enquiries.

Table 7.1 provides details of the milk experiments conducted here, including values of clot extent on the test-object and test-body (in grams) and the thickness of clot at the test-chamber wall just upstream of the test-body (in mm) at the end of each milk experiment. $X$ denotes a value that is suspect, due to wall clot interference during the experiment or in the dismantling stage, and $*$ denotes an experiment where only 14
gallons of milk were used, not the usual 15 gallons. If wall clot loss occurred during a milk experiment, denoted by a † in table 7.1, the wall clot thickness given is the thickest achieved. Additional information is provided by the ultrasound images recorded during these experiments and the photographs of the clotted test-objects removed at the end of experiments (figures 6.7, 7.1 and 7.2). The disc-shaped test-body had very little clot on it after a pulsatile flow experiment, and a somewhat conical wake-region clot in steady flow. This clot pattern was also observed for the conical test-body. An approximately uniform layer of bubbles and clot were found on the upstream face of each test-body.

<table>
<thead>
<tr>
<th>CaCl$_2$ conc$^a$ (M)</th>
<th>Flow type</th>
<th>Test-object</th>
<th>T-object clot (g)</th>
<th>T-body clot (g)</th>
<th>Wall clot (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.12±0.01</td>
<td>Steady</td>
<td>P.V.C. teardrop</td>
<td>0.267</td>
<td>0.125±0.001</td>
<td>2.0†</td>
</tr>
<tr>
<td>1.72</td>
<td>Steady</td>
<td>P.V.C. teardrop</td>
<td>0.619X</td>
<td>0.194X</td>
<td>3.5</td>
</tr>
<tr>
<td>1.72</td>
<td>Pulsatile</td>
<td>P.V.C. teardrop</td>
<td>0.200</td>
<td>0.07</td>
<td>0.8</td>
</tr>
<tr>
<td>2.12</td>
<td>Steady</td>
<td>P.V.C. teardrop</td>
<td>1.800X</td>
<td>0.944X</td>
<td>3.5†</td>
</tr>
<tr>
<td>2.12</td>
<td>Steady</td>
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<td>1.7†</td>
</tr>
</tbody>
</table>

Table 7.1: Details of the milk flow experiments
Figure 7.1: Photographs of the clot on the disc-shaped test-body, after (a) steady and (b) pulsatile milk flow experiments. Figure (a) includes a photograph of the upstream view of the disc-shaped test-body, after a steady flow experiment.
Figure 7.2: Photographs of the clot on the conical test-body, after (a) steady and (b) pulsatile milk flow experiments. Symmetrical clot deposition developed on the test-object in steady flow; however, on removal of the test-object from the test-chamber, the test-body came away from the support rod and was thus replaced before the photograph in (a) was taken. The two clotted test-objects pictured in (b) both have wall clot deposited on them; wall clot loss occurred in the final stages of both the experiments from which these objects came.
7.1 Ability and limitations of the imaging of clot

The amount of information that could be drawn from the ultrasound images was limited by their quality. The ultrasound transceiver and monitor, described in sections 5.5.1 and 6.1.11, enabled images of clot both on the test-bodies and test-chamber to be obtained, but of very different quality. Ultrasonic images of test-bodies and their clot were inadequate for the intended aim of continuous measurement of clot growth on the test-body; however, the images of clot adhered to the far inner-wall of the test-chamber were of a quality sufficient to allow the growth of this clot to be continuously measured.

Geometrical and instrumental limitations were the reasons behind the variation in quality of images of curd deposit. In particular, the focus of the ultrasound beam was closer to the far wall of the test-chamber than to the test-body, and this wall and the clot formed on it were perpendicular to the ultrasound beam axis, while the surfaces of the cone, disc and teardrop-shaped test-bodies were curved and mainly at an oblique angle thus providing diffuse reflections of the ultrasound. The clot on the near inner-wall of the test-chamber was not depicted clearly, presumably due to the 'transducer dead space' (defined in section 5.5.4), and this region gave rise to spurious images. Accordingly, the imaging of clot on the far inner-wall of the pipe was further analysed.

The accuracy of the continuous ultrasonic measurements of clot deposition was estimated. There were several factors determining this accuracy, such as: (a) uncertainty about the velocity of ultrasound within the clot, assumed in the calculation converting echo signals into distances; (b) the ultrasound beam intensity and resolution, and how these were affected by structures within the beam; and (c) the placement and accuracy of the calliper markers. Each of these factors is considered, in turn, below.

(a) In order to be able to convert ultrasonically depicted curd thicknesses into actual thicknesses, one must know the speed of sound propagation in milk curd ($c_{\text{curd}}$) and that used in the depth calculations made by the ultrasound monitor. 1540 m/s, which is the speed of sound propagation in soft tissue, was the value used by the ultrasound monitor when evaluating the depth of reflective structures. The value of $c_{\text{curd}}$ was not
found in the literature but, as explained in section 5.5.3, test results indicate $c_{\text{curd}} \approx 1540$ m/s. It is accordingly assumed that the relative error in $c_{\text{curd}}$ is less than 10%, which means that the maximum relative error in thickness measurement arising from this source of error will be 10%.

(b) Due to the limiting axial resolution of the ultrasound beam of about 0.31 mm (calculated in section 5.5.3), interfaces lying along the beam axis cannot be distinguished when less than 0.31 mm apart. However, when the interfaces composed of clot/milk and clot/wall are separated by distances greater than 0.31 mm, the error involved in clot thickness measurements is less than 0.31 mm.

The accuracy of measurements of wall clot thickness was expected to be adversely affected by the marked attenuation which arose mainly from the bubbles in the milk; however, the attenuation caused by the bubbles was compensated by the highly reflective bubbles flowing over the surface of the wall clot, highlighting the clot/milk interface. Bubbles in the milk flow led to poor imaging of clot due to attenuation of the ultrasound beam and to the fact that when clot and highly reflective bubbles resided side-by-side on the test-object they were sometimes not distinguishable. By comparing the ultrasound images in figures 7.3(a) and (b), which were obtained with and without bubble-riddled milk flow, it is apparent that bubbles are degradative to ultrasonic imaging. Arresting the milk flow at the end of each milk experiment resulted in a large reduction in the number of bubbles in the field of scan and significantly better imaging of clot on the test-body and far inner-wall. Placing the clotted test-object in an unfouled, duplicate polypropylene test-chamber filled with non-flowing, partially degassed milk, allowed even better depiction of the test-body clot, indicating that while the near-wall clot has properties which suggest it will cause little attenuation, the bubbles in this clot can still degrade image quality. The flow of bubbles over the smooth clot surface did, however, highlight the clot/milk interface, helping one to establish the exact location of the clot/milk interface and thus to measure wall clot thickness.
Figure 7.3: Ultrasound images of a clotted teardrop-shaped test-body (a) in the presence of a bubble-riddled milk flow, and (b) in stagnant, partially deaerated milk. The clotted test-object featured in these ultrasound images is shown in (c).
(c) The on-screen callipers provided measurements to the nearest millimetre, which is not a satisfactory accuracy for the present purpose. A consistent measurement technique was developed (described in section 6.1.11), where hand-held callipers (with 0.01 mm accuracy) and close observation of enlarged ultrasound images and the bubble flow over the clot/milk interface allowed one to measure clot thicknesses more accurately.

The growth rate of wall clot was of primary importance here, as opposed to the absolute value of clot thickness. The error in calculating the change in clot thickness, in order to determine clot growth rate, is believed to have been much smaller than the error in the absolute thickness of clot, and is estimated to be ±0.1 mm.

7.2 Clot development and flow conditions

The deposition rate of clot on a surface in a milk flow will be dependent on the balance between flow forces and the clot’s adhesive and cohesive bonds, which may vary with time as cross-linking and other physical changes within the curd take place, and also on the rate at which activated micelles or clot-promoting constituents are conveyed to that surface relative to the rate of convective removal. The intention of this section is to provide the results of clot deposition both at the test-chamber wall and test-body and discuss how flow conditions affected this clot deposition.

7.2.1 Wall clot growth in steady and pulsatile flow experiments

Detailed growth rates of wall clot were measured ultrasonically. The four ultrasound images shown in figure 7.4 were recorded at different times during the course of a milk experiment and illustrate the growth of wall clot with time. The plots of wall clot thickness against time, shown in figure 7.6, were constructed using information from ultrasonic images recorded during four steady and four pulsatile flow experiments. From these plots it can be seen that an apparent lag phase occurs during which no clot is observed; that an initially constant rate followed by a diminishing rate of growth occurred in the steady flow studies; and that clot grew at an approximately constant.
rate in the pulsatile flow experiments. In order to determine why such profiles of clot growth occurred, the changes in flow conditions over the duration of the steady and pulsatile flow experiments were considered.

The apparent lag phase observed for all flow experiments, during which no clot was observed, was expected. The limiting axial resolution of the ultrasonic imaging delays curd depiction in the early stages of the experiment, and curd deposition itself is delayed by the enzymic lag phase of milk coagulation.

In steady flow experiments, the milk flowed steadily under the influence of gravity through the test-chamber. The flow rate decreased as these experiments proceeded, perhaps due to progressive blockage of the tubing downstream of the test-chamber. Consideration of the relative resistances in the test-section and other parts of the flow system leads to the conclusion that the mean fluid velocity ($\bar{u}$) would have increased as the cross-sectional flow area and volumetric flow rate decreased, as wall clot developed. This change in velocity, coupled with the reduced flow area, would mean that the shear stress at the wall would increase markedly as clot deposited at the pipe wall. The mean fluid residence time within the test-chamber ($t_{res}$) would decrease as a result of this change in velocity. These changes in shear stress and $t_{res}$ would be expected to cause a diminishing rate of clot growth similar to that observed (figure 7.6).

In pulsatile flow experiments, the milk flow was produced by a hydraulically driven diaphragm pump, which is believed to have maintained a constant volumetric flow rate throughout these experiments despite the progressively increasing flow resistance as wall clot built up. Both $\bar{u}$ and the shear stress at the wall will have increased, while $t_{res}$ will have decreased, as wall clot developed. The reduction in $t_{res}$ would result in the milk being less likely to reside in the test-chamber for a time greater than the clotting time; this, and the increase in shear stress, would be expected to reduce the growth rate as the experiment proceeded. However, the clot growth rate was approximately constant. A possible explanation for this is that a balance may have occurred between
Figure 7.4: Ultrasound images of a test-chamber containing a disc-shaped test-body, recorded 5.25, 6, 7 and 9.5 minutes from the start of a milk experiment.

Figure 7.5: An ultrasound image illustrating wall clot thinning in the region adjacent to the test-body. The flow was from left to right.
Figure 7.6: The growth profiles of clot at the test-chamber wall immediately upstream of the test-body, in steady (S) and pulsatile (P) flow experiments, using 3.0 M CaCl₂ solutions.

The adverse effects of increasing shear stress and decreasing $t_{res}$ and the favourable effects of agitation on mass transfer and curd adhesion (recall the positive effect of agitation on clot adhesion observed by Christy and reported in section 4.1.3).

It can be seen from the plots in figure 7.6 that both the initial rate of deposition and final extent of clot were greater in steady than in pulsatile flow experiments. If the above suggested changes in flow conditions did occur, during the steady and pulsatile flow studies, the differences in the mass transfer rate, shear stress and velocity profile may have caused this difference in clot deposition. Mass transfer to the wall of the test-chamber would be expected to be greater in pulsatile flow than in steady flow, therefore, the greater extent of clotting in steady flow is unlikely to be due to enhanced mass transfer. On the other hand, the peak shear stresses will have been greater in the pulsatile than in the steady flow experiments; this, the transient nature of the boundary layer and perhaps a smaller stagnant reactive region at the wall, in pulsatile flow, may be the main causes of the smaller extent of clotting in pulsatile flow experiments.

On scanning the different pipes composing the test-section, it was found that the smooth wall clot was thicker with distance downstream in both steady and pulsatile flow
experiments. This might have been due to $\Delta t$ (the average time taken by the fluid to travel from the reagents' injection point to a given distance downstream in the system) and the thickness of the boundary layer both increasing with distance downstream and, downstream of the test-body, to the mixing induced by the test-body. However, since the flow conditions and composition of the pipe wall were not uniform throughout the test-section, this cannot be certain. This is further discussed in section 7.4.

7.2.2 Wall clot growth adjacent to the test-body in steady and pulsatile flows

The effect that a test-body has on the local flow conditions depends on its shape, size and the flow Reynolds number. The mean pipe and test-body Reynolds numbers, based on the initial experimental conditions, were 1051 and 328, respectively. Each 10 mm diameter test-body caused a significant decrease in the effective flow cross-section and thus local flow acceleration, elevated shear stresses and reduced fluid residence time. These changes in flow conditions would explain why thinning of clot at the wall occurred opposite the test-body, as they would be expected to inhibit or reduce local clot growth.

Adjacent to each test-body, the local flow conditions were found to have a marked effect on wall clot (figure 7.5). The results of a study of the thickness of wall clot adjacent to the test-body, at the end of each of the experiments, indicate that wall clot adjacent to the test-body was thinner than if no test-object had been present, and the level of thinning that occurred was dependent on the shape of the test-body and the flow regime. The mean values of percentage reduction of wall clot thickness adjacent to the test-bodies, in steady (S) and pulsatile (P) flow were: 11% (S) and 75% (P) for the disc; 17% (S) when the cone was in place; and 4% (S) and 16% (P) for the teardrop-shaped test-body.

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1 Wall clot loss occurred during the pulsatile flow experiments that were conducted with the conical test-body, so measurements of the extent of clot thinning at the end of these experiments were of little use.
The extent of thinning of the wall clot may be dependent upon the flow acceleration (which determines the changes in shear stress and fluid residence time at the wall) and the extent of redirection of flow towards the wall, which are a result of the test-body being present in the flow. The disc causes a greater flow acceleration and a more abrupt change in flow direction than the cone and, in turn, the teardrop-shaped test-body. Given this, it was expected that the disc would cause more clot thinning than the cone and that the cone would be associated with a greater extent of clot thinning than the teardrop-shaped test-body. The extent of wall clot thinning adjacent to the test-body was indeed smaller with the teardrop than with the other test-bodies, in both flow regimes. In steady flow, the conical test-body was associated with the greatest reduction in wall clot thickness. The reason for this finding is unknown.

7.2.3 Variation of test-body clot extent with flow conditions

More clot deposition results were obtained which indicate that flow characteristics are important with regard to clotting. It was found that the shape and extent of test-body clot differed with varying flow conditions. The flow conditions were modified by varying the test-body shape (cone, disc or teardrop) and the flow regime (steady or pulsatile).

The approximate sizes and residence times of vortices formed in the wake region of the unfouled test-bodies were determined by Christy[24] and reported in section 4.2.3. For convenience, the ordering of test-bodies, based on their wake vortex residence times, is reiterated here alongside the weights of test-body clot recorded at the end of experiments in which CaCl₂ solutions of about 3.0 M were used (figure 7.7). The size of the wake vortices, which are sketched in figure 4.11, are such that the teardrop has the smallest vortex and the cone and disc have vortices of similar size. It should be realized that this information on the wake vortices was obtained from dye experiments which had conditions that were approximately similar to the initial conditions of the milk experiments.

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2The weight of clot found on the conical test-body at the end of pulsatile flow experiments was deemed void due to wall clot interference.

3Water, which has a kinematic viscosity of about 3/4 that of milk, and an unfouled test-body and test-chamber were used in the dye experiments.
Steady flow:  
- Disc (8sec) < Cone (12sec) < Teardrop (>18sec)  

Pulsatile flow:  
- Cone (1sec) = Disc (1sec) < Teardrop (14sec)  

Figure 7.7: Ordering of test-bodies, based on the residence times of the vortices formed in their wake region.

A rough indication of how the test-body wake region changed during experiments was obtained from echo-information received from bubbles passing over the clotted test-object. Bubbles in the flow gave rough indications of certain features of the flow pattern in one plane, including the sudden stop and reversal of flow during the transition from systole to diastole, the point of flow separation and reattachment, and recirculation in the wake region of the test-body. Bubbles recirculated in the wake region and flowed along the poorly depicted clot/milk interface and not into dark 'no go areas' which were believed to correspond to clot on the ultrasound image. On studying the flow of bubbles around the test-object, it appeared that the recirculation zone in the test-body wake region decreased in size as clot developed there. Ultrasonic imaging of the wake region behind the teardrop-shaped test-body appeared to show this region both elongating and becoming thinner, as clot developed in this region. The flow separation point appeared to move further upstream, along the test-body, and the flow reattachment point on the support rod extended further downstream, as the size of the recirculation zone decreased and the wake region filled with clot, in steady flow milk experiments. In the pulsatile flow milk experiments the clot extent in the test-body wake region was too small for such deductions to be made.

Correlations between the shape and extent of test-body clot and the size and residence times of the test-body wake vortices were attempted, under the assumption that the relative values of test-body vortex sizes and residence times were maintained during

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4In microbubble echocardiography (ME)\textsuperscript{362}, where microbubbles of air are injected into the bloodstream, the highly reflective blood/air interface provides a strong echo signal, allowing remarkably enhanced imaging of the heart chambers, permitting computation of ejection fractions and noninvasive diagnosis of coronary artery disease\textsuperscript{363}\textsuperscript{364}.
Residence time and clot extent. Due to the fact that the wake vortex is a smaller and less turbulent than the disc's, the residence time is shorter. The wake vortex is also much more intense than the disc, resulting in a shorter residence time.

Residence time and clot extent. The wake vortex is smaller and less intense than the disc, resulting in a shorter residence time. The wake vortex is also much more intense than the disc, resulting in a shorter residence time.

Residence time and clot extent. The wake vortex is smaller and less intense than the disc, resulting in a shorter residence time. The wake vortex is also much more intense than the disc, resulting in a shorter residence time.

Residence time and clot extent. The wake vortex is smaller and less intense than the disc, resulting in a shorter residence time. The wake vortex is also much more intense than the disc, resulting in a shorter residence time.

Residence time and clot extent. The wake vortex is smaller and less intense than the disc, resulting in a shorter residence time. The wake vortex is also much more intense than the disc, resulting in a shorter residence time.

Residence time and clot extent. The wake vortex is smaller and less intense than the disc, resulting in a shorter residence time. The wake vortex is also much more intense than the disc, resulting in a shorter residence time.

Results and Discussion
These results are by no means exhaustive, but may indicate that when wake vortex size is similar the difference in vortex residence time may determine the relative extents of clot and *vice versa*. On the other hand, when wake vortex residence time and size are both different it cannot be certain which of these two vortex characteristics will predominate.

The extent of clot on a given test-body was smaller in pulsatile than in steady flow experiments. This reduction in clot extent might be explained by the reasoning given in section 7.2.1 and/or by the change in wake vortex residence time. The residence times of wake vortices in pulsatile flow were less than those in steady flow, for each test-body; a reduction in wake vortex residence time may act to inhibit clot growth, by reducing the amount of milk present in this region that has resided for a time in excess of the clotting time.

The differences between the present experiments and those of Christy are numerous<sup>5</sup>, so the comparisons that can be made between the results from these two pieces of work are limited. The ordering of the test-bodies, based on the weight of test-body clot at the end of experiments of fixed duration, was similar for both sets of experiments. The final clot found on each of the test-bodies were quite similar in shape for both sets of experiments, but the extent of this clot was in all cases much greater in the work done here. Both sets of results failed to indicate a simple relationship between the residence time or size of the wake vortex and clot extent. However, the importance of stasis was indicated by the finding that all sites where clot formed were regions of stasis.

<sup>5</sup>The differences were in CaCl<sub>2</sub> concentration; the shape, diameter and material of the test-body support rod; the way the flow conditions changed during the experiment; and the criteria used for choosing the section of clot on the test-object for weighing.
7.3 Clot development and CaCl₂ concentration

With the aim of controlling or enhancing curd adhesion, it was considered a worthwhile endeavour to investigate the effect on clot growth of varying the concentration of CaCl₂ and of changing the material of the surfaces on which curd develops in the milk experiments. The effect of the former change is considered here, while the latter change is addressed in the section 7.4.

In the milk flow experiments, increasing the CaCl₂ solution concentration from 1.12 M to 3.38 M, of which 1% (vol.) was injected, tended to increase the extent of clotting in both steady and pulsatile flow experiments, as indicated in tables 7.2 and 7.3. In pulsatile milk flow experiments, use of 1.72 M CaCl₂ produced a wall clot of about 0.6 mm thickness, while use of 2.51 M or higher CaCl₂ concentration produced wall clot of 1.7 mm or thicker, 21 minutes into the experiment. In steady flow milk experiments, a small increase in CaCl₂ concentration from 1.12 M to 1.72 M caused no significant change in wall clot thickness, giving about 2.0 mm thick wall clot 21 minutes into the experiment. A larger concentration increase, to 2.12 M and above, produced a marked increase in wall clot extent; wall clot thickness increased up to 4.8 mm, 21 minutes into the experiment.

Figure 7.8 illustrates the general dependence of wall clot growth on CaCl₂ concentration; both the clot growth rate and final extent tend to increase with increasing CaCl₂ concentration.

Increasing the concentration of CaCl₂ increased the extent of test-body clot also (table 7.1). For example, on the P.V.C. teardrop-shaped test-body, in pulsatile milk flow experiments, use of 1.72 M CaCl₂ gave a final test-body clot of 0.07 g, while use of 3.38 M gave a much larger clot weight of 0.316 g. In steady flow milk studies, the CaCl₂ concentrations employed and the corresponding clot extents on the P.V.C. teardrop-

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6Wall clot loss occurred during a significant number of the milk experiments, generally later than 21 minutes after the rennet and CaCl₂ dosing pumps were turned on, so comparisons of wall clot thicknesses recorded 21 minutes into each experiment were accordingly carried out.
Calcium ions are known to accelerate the rate of milk clotting and affect curd rheology (section 4.4.5). The apparent relationship between clot extent and CaCl₂ concentration, found under the milk experiment conditions used here (i.e. 37°C, initial flow rate of 2 l/min, and duration of about 35 min), may arise from the increase in CaCl₂ concentration altering curd rheology and reducing the rennet coagulation time (RCT) and thus the fluid residence time necessary for milk to clot. These changes are the likely causes of the observed increases in growth rate of wall clot and extent of clot on the pipe wall and test-body.

The effect on the rennet coagulation time of varying the concentration of the CaCl₂ solution in milk is made apparent by Lewis's curve of clotting time vs. percent added saturated aqueous CaCl₂ (figure 4.6). The range of CaCl₂ concentrations used here...
lie on the part of this curve which indicates that an increase in this concentration would be expected to reduce the RCT. On the other hand, when using much greater concentrations of CaCl$_2$ solution than those used here, the opposite effect would be expected according to Lewis’s curve of clotting time vs. CaCl$_2$ concentration.

### 7.4 Clot development and surface material

The choice of test-body materials was made mainly on the basis of reflectivity, while those materials chosen for the test-chamber were ones sufficiently transparent to ultrasound. Two test-chambers composed of different materials were employed, namely polyethylene and polypropylene, and the results used to relate wall clot extent to test-section material. The teardrop was the best delineated test-body shape, and thus was the shape chosen for the test-body material assessments. The teardrop-shaped test-bodies constructed of the hydrophobic materials P.T.F.E., P.V.C., polycarbonate, perspex and nylon for use in the ultrasound imaging assessments described in chapter 5 were used in these tests.
Wall clot

Comparison of the wall clot growth with time in steady flow milk experiments, using the polyethylene and polypropylene test-chambers and the same CaCl$_2$ concentration of 2.12 M, was made impractical by the loss of wall clot after about 10 minutes into the latter experiment. However, comparing the plot of clot growth against time from the experiment with the polyethylene test-chamber with the plot from a steady flow milk experiment conducted with the polypropylene test-chamber and a 2.51 M CaCl$_2$ solution, it was found that a slightly greater extent of wall clot occurred in the latter experiment. This could be accounted for by the higher CaCl$_2$ concentration (figure 7.8).

The test-section was constructed from three flanged pipes in series, two made of perspex and the middle one, the test-chamber, made of polypropylene (section 6.1.8). This difference in pipe composition was chosen for the ultrasound imaging analyses detailed in chapter 5, not for the purpose of correlating clot extent with differences in test-section wall material. Direct comparison between the clot adhesiveness and extent on these pipes and their material differences was not possible, because the flow conditions and $\Delta t$ (the average time taken by the fluid to travel from the reagents' injection point to a given distance downstream in the system) were different in each of the pipe sections composing the test-section. Thus, although the test-section wall clot was tapered, gradually increasing in thickness with distance downstream, this does not inform one of the effect of material type on clot thickness, as the conditions in each pipe were different. In the perspex pipe, upstream of the test-chamber, the flow was not fully developed and $\Delta t$ was relatively small; $\Delta t$ increased as the milk mixture flowed through the test-chamber, and this milk flow was considered to be fully developed and then disturbed in the latter half of this pipe by the presence of the test-body; the milk entering the perspex pipe downstream of the test-chamber was better mixed, due to the mixing induced by the test-body, and, being furthest downstream, had milk flowing through it which had the greatest values of $\Delta t$. 

7 Results and Discussion

Test-body clot

Test-body clot weights measured at the end of experiments, known to be unaffected by lost wall clot, were used to compare the clot-forming propensities of different test-body materials. Test-body clot was not depicted ultrasonically with a quality sufficient to allow its growth rate to be estimated. The ordering of materials, based on the weight of clot found on the differently composed teardrop-shaped test-bodies at the end of steady and pulsatile milk flow experiments, together with the concentrations of CaCl₂ used, are given in tables 7.4 and 7.5; clot weights were measured to the nearest milligram.

<table>
<thead>
<tr>
<th>Quantity\ T-body Material</th>
<th>Nylon</th>
<th>P.T.F.E.</th>
<th>P.V.C.</th>
<th>P.V.C.</th>
<th>Perspex</th>
</tr>
</thead>
<tbody>
<tr>
<td>[CaCl₂] (M)</td>
<td>2.51±0.01</td>
<td>3.05</td>
<td>3.38</td>
<td>2.12</td>
<td>3.30</td>
</tr>
<tr>
<td>Test-body clot (g)</td>
<td>0.858</td>
<td>0.614</td>
<td>0.591</td>
<td>0.500</td>
<td>0.430</td>
</tr>
</tbody>
</table>

Table 7.4: Clot weights in steady flows past teardrop-shaped test-bodies

<table>
<thead>
<tr>
<th>Quantity\ T-body Material</th>
<th>P.V.C.</th>
<th>Polycarb.</th>
<th>Perspex</th>
<th>Nylon</th>
<th>P.V.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>[CaCl₂] (M)</td>
<td>3.38±0.01</td>
<td>3.05</td>
<td>3.30</td>
<td>2.51</td>
<td>1.72</td>
</tr>
<tr>
<td>Test-body clot (g)</td>
<td>0.361</td>
<td>0.334</td>
<td>0.314</td>
<td>0.168</td>
<td>0.070</td>
</tr>
</tbody>
</table>

Table 7.5: Clot weights in pulsatile flows past teardrop-shaped test-bodies

The differences in material and CaCl₂ concentration were expected to determine the extent of clot on the test-bodies, and indeed this was apparent in the results, but to different degrees depending on the flow regime. In steady flow, the influence of test-body material on clot extent was obvious and appeared to predominate over the influence of variations in CaCl₂ concentration. With test-bodies composed of different materials, use of similar CaCl₂ concentration did not result in similar test-body clot weights, and increasing the CaCl₂ concentration did not affect the order of the test-bodies, based on their clot extents. In pulsatile flow trials, it was found that the CaCl₂ concentration predominated, with increases in this concentration generally leading to greater clot extent.

The extent of test-body clot and ordering of materials in steady and pulsatile flows, based on clot extent, have been compared. The final clot extent is greater in steady flows, and it appears that in pulsatile flows the dependence of clot extent on CaCl₂ concentration is greater than that on materials whereas in steady flows the converse
appears to be true. This may arise because the change in flow regime from steady to pulsatile results in a shorter mean fluid residence time in the wake of the test-body, as well as greater flow velocities and more agitation. The consequence of these changes may be more forceful collisions between aggregates and the test-body surface but for a shorter duration; the greater the CaCl$_2$ concentration the more aggregates will be formed in a given time and the more prominent the effect of these changes in flow conditions on clot extent. In addition, it may be that the effect of changes in curd rheology, on increasing the CaCl$_2$ concentration, becomes more obvious when the more extreme conditions of pulsatile flow are present.

7.5 Wall clot loss

7.5.1 The consequences of wall clot loss

Wall clot interference with test-object clot has been suspected but unverified in previous milk studies (section 4.3.1). Bubbles in the milk flow have been observed (ultrasonically) impinging on and adhering to both the test-object and its adhered clot, resulting in crater-shaped clots. Irregular clot formations on the upstream-face of the test-body, other than those known to be due to bubbles, that were observed by Christy (section 4.2.3) are now believed to have been due to wall clot interference. Such deposits were observed at the end of the present experiments only when loss of wall clot was known to have occurred.

It should be realized that, under the conditions of the present experiments, even when clot lost from the pipe wall during an experiment does not directly interfere with the test-object clot, it does tend to restore the flow rate towards the initial 2 l/min and thus makes the conditions, such as shear stress and mean fluid residence time, vary in a way different from that in milk experiments where no wall clot loss occurred. On one occasion only, dislodged wall clot temporarily arrested the flow, presumably due to momentary blockage of the converger at the exit of the test-section.

Eight of the 20 milk experiments discussed in this chapter experienced wall clot loss
during their course. Table 7.6 provides details of these wall clot losses, including the thickness of wall clot immediately prior to its loss, denoted by $x_{ clot }$. On studying the video cassette-tape records of ultrasonic images depicting the loss of wall clot, it was found that:

- Following the loss of wall clot, a new layer of clot develops at the wall.

- When this loss occurred, all of the wall clot was lost and in some cases impinged on the test-body. The results for test-body clot shape and extent were considered to be of use (i.e. not noticeably affected by the loss of wall clot) at the end of 3 of the 8 experiments in which wall clot loss occurred.

- No pattern of wall clot loss became apparent for either flow regime. That is, there was no specific time, thickness or CaCl$_2$ concentration at which wall clot was consistently lost.

- There were warning signs prior to wall clot loss in pulsatile, but not in steady, flow experiments. Prior to its loss, the wall clot was in all cases observed shaking. This was followed by the clot pulsing; it began to move slightly up- and downstream synchronistically with the systolic and diastolic phases of each pulse, before moving downstream.

<table>
<thead>
<tr>
<th>Regime</th>
<th>CaCl$_2$ conc$^a$ (M)</th>
<th>$x_{ clot }$ (mm)</th>
<th>Time of loss (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>steady</td>
<td>2.12±0.01</td>
<td>2.0</td>
<td>10</td>
</tr>
<tr>
<td>steady</td>
<td>3.05</td>
<td>5.5</td>
<td>37</td>
</tr>
<tr>
<td>steady</td>
<td>3.38</td>
<td>2.1</td>
<td>8</td>
</tr>
<tr>
<td>pulsed</td>
<td>3.00</td>
<td>3.0</td>
<td>32</td>
</tr>
<tr>
<td>pulsed</td>
<td>3.05</td>
<td>2.4</td>
<td>33</td>
</tr>
<tr>
<td>pulsed</td>
<td>3.30</td>
<td>1.7</td>
<td>26</td>
</tr>
<tr>
<td>pulsed</td>
<td>3.38</td>
<td>1.7</td>
<td>23</td>
</tr>
<tr>
<td>pulsed</td>
<td>3.38</td>
<td>3.25</td>
<td>34</td>
</tr>
</tbody>
</table>

Table 7.6: Wall clot loss details

The prevention of wall clot formation has been addressed (section 4.3.1 and appendix A), but there appears to be no simple way to achieve this in both steady and pulsatile flows without disrupting the milk coagulation studies. Until the formation of clot at the test-chamber wall can be prevented, the avoidance of wall clot loss will remain of primary importance. The effect of certain system conditions on wall clot integrity have been assessed and the findings reported below.
7.5.2 Wall clot loss and flow regime

The choice of flow regime, either steady or pulsatile, appears strongly to affect wall clot integrity. 27% (3 of 11) of the steady flow milk experiments and 56% (5 of 9) of the pulsatile flow milk studies experienced wall clot loss. Pulsatile flow is accompanied not only by greater agitation and peak shear stresses than is steady flow, but also by an increase in the number of bubbles in the milk flow (section 6.1.3). Some or all of these factors may promote wall clot loss\(^8\), thus explaining why twice as many experiments of the pulsatile regime lost wall clot. But it is possible that not all of the changes, when going from steady to pulsatile flow, have an adverse effect on clot adhesion. On studying table 7.6, it can be seen that two of the three steady flow experiments, which experienced wall clot loss, lost wall clot at a much earlier stage than any of the pulsatile flow experiments. The better adhesion of clot in the early stages of the pulsatile flow experiments may arise due to the greater agitation (section 4.1.3) which occurs in these flows. The greater shear stresses present in these flows (and perhaps the increase in the number of bubbles) do, however, appear to take their toll; in principle, the shear stress at the wall increases as the pulsatile flow experiment proceeds and the increasing wall clot reduces the flow area, and this may be why in an extended experiment clot was lost more frequently in pulsatile, as opposed to steady, flows.

These findings appear to indicate that enhanced mixing and thus better clot adhesion is obtained in pulsatile, rather than steady, flow and that this improved adhesion may just postpone wall clot loss; the adhesive bonds of the wall clot may be overcome by the injurious effects of elevated shear stresses.

7.5.3 Wall clot loss and \(\text{CaCl}_2\) concentration

There appears to be a link between the occurrence of wall clot loss and the concentration of \(\text{CaCl}_2\) used. Increasing the \(\text{CaCl}_2\) concentration was associated with an increasing occurrence of wall clot loss (table 7.1). When the concentration of the \(\text{CaCl}_2\) solution

\(^{7}\)The pulsatile flow experiments involved the use of two pumps, whereas only one pump was used in the steady flow studies. Both pumps were found to generate bubbles.

\(^{8}\)Berridge spoke of the "fragile nature of the curd at the moment of formation", and pulsation has been used in cheesemaking to shift wall clot or inhibit clot adhesion\textsuperscript{246}\textsuperscript{248}.\n
was 2.51 M or less, 1 of 7 experiments experienced wall clot loss; where a CaCl₂ concentration of 3.00 M or greater was used, 7 of 13 experiments experienced wall clot loss; and when a CaCl₂ concentration of 3.30 M or greater was used 4 of 5 experiments suffered wall clot loss. Increasing the CaCl₂ concentration may make wall clot loss more likely by the consequent changes in clot rheology and clot extent. A more rigid clot is expected to occur when increasing the CaCl₂ concentration, and such a clot may be more likely to dislodge from the pipe wall than a less rigid clot. An increase in clot extent may cause greater shear stresses at the wall, as it results in a smaller flow area, which will be more likely to overcome the adhesive bond between curd and the pipe wall.

While the implications of the above findings on wall clot loss are interesting, these results are too few to be conclusive. Vibrations of the test-chamber or slight jolts to it, for instance when the ultrasound probe is contacted with the test-chamber, could also affect the integrity of the adhesive bonds between the pipe wall and clot. Extensive testing would be needed to determine the factors relevant to wall clot loss. Further analysis of the reasons behind wall clot loss was not attempted here.
Chapter 8

Conclusions and
Recommendations for Future
Work

8.1 Conclusions

1. Curd deposition rate can be measured non-invasively using ultrasonic imaging
equipment similar to that used in clinical practice. No influence of ultrasound on
the final extent or shape of clot was apparent, even when the test-chamber was
sonicated for the full 35 minute experimental duration.

2. Deposition of curd on the test-bodies used here was difficult to measure
ultrasonically due to scattering of the ultrasound beam reflected from the test-
bodies’ surfaces. Deposition on the wall of the cylindrical test-chamber, on the
other hand, was clearly depicted and its growth and shedding ultrasonically
observed.
3. The extent and deposition rate of curd are affected by the flow conditions. For both steady and pulsatile flows, following a short 'lag phase', the wall clot growth rate was at first constant. In steady flow, the deposition rate fell rapidly after about 20 minutes, before tapering-off to zero to give what appears to be a limiting deposit thickness. For pulsatile flows, the wall clot continued to grow at an approximately constant rate. This growth rate was lower than that in the initial stages of steady flow and, over the time interval used in both kinds of experiments, the extent of clot deposited in pulsatile flows was markedly less. The influence of flow conditions on clot extent was also apparent adjacent to the test-body, where wall clot thinning was found to occur.

4. The extent and rate of curd deposition are influenced by the concentration of CaCl$_2$. Increasing this concentration over the range studied here tended to increase the deposition rate and the ultimate extent of curd.

5. Ultrasonic imaging allows one to determine whether or not wall clot loss occurs. By this means it was sometimes found that all clot at the pipe wall is dislodged and washed downstream, usually towards the end of the experiment, after which a new layer of clot begins to form at this wall.

6. Wall clot loss was found to occur more often with pulsatile flow, as opposed to steady flow, and at higher CaCl$_2$ concentrations. Uncontrolled factors may also affect wall clot integrity; there was no specific time, curd thickness or CaCl$_2$ concentration at which clot was consistently lost from the pipe wall.

7. The cause of some of the irregular clot formations on the upstream-face of test-bodies observed by Christy and myself appears to be wall clot impingement and, in some cases, adherence.

8. Ultrasonic imaging of the deposition of clot, from renneted milk which is flowing past an implant, appears to have potential as a method for non-invasively determining the location, extent and deposition rate of clot in studies aimed at predicting the flow-related thrombogenicity of artificial implants. The full potential of this ultrasound application has yet to be seen. In the present arrangement, the growth rate of deposition on the wall but not on the test-bodies
was successfully observed. It is conceivable, however, that ultrasonic imaging could be of use for studying curd deposition on test-bodies, such as artificial heart valves, if certain modifications to the initial set-up are made (section 8.2).

8.2 Recommendations for future work

1. Milk experiments with the flow apparatus and ultrasound equipment used here could be performed with test-objects whose surfaces are perpendicular to the ultrasound beam axis (i.e. parallel to the test-chamber pipe wall), notably with the flow shunts described by Stein and Sabbah and with ridged Gott rings (reported in section 3.3.3). Results from such experiments with milk would allow one to compare further milk and blood deposition, and would enable one to assess further the effect of varying flow conditions on curd deposition.

2. The ultrasonic imaging equipment used here requires to be refined, or perhaps replaced by a more appropriate ultrasound system, to allow measurement of the thickness of curd adhered to surfaces not perpendicular to the ultrasound beam axis, such as those surfaces of the test-bodies used here. A phased-array transceiver with a beam-steering facility could be used to make the angle of incidence more favourable. This probe could also overcome the problem associated with ultrasonic imaging of artificial heart valves, namely attenuation by their housing, by positioning this probe either just upstream or downstream of the valve. Alternatively, a flexible stand-off would enable the operator to position the probe at better angles to heart valves and the otherwise oblique surfaces of the test-bodies used here, while maintaining good coupling; this may also allow the ultrasound beam’s focal point to be positioned at the centre of the region of interest. Perhaps a fluid, or gel-filled, stand-off, in the form of a band encircling the test-chamber, would be useful.

3. If the above recommendation was implemented, one would be able to image curd on test-bodies; however, information on changes in the local flow conditions, as clot developed on these bodies, would still be needed to correlate flow conditions to the deposition rates and extent of curd.
A combination of particle image velocimetry (PIV) with ultrasonic measurements could be very fruitful. One way to achieve this combination, would be to utilize the bubbles in the milk flow by using them as tracer particles in ultrasonic studies. Adaptation of ultrasonic Doppler and imaging technology to the milk clotting system used here may be a useful way of obtaining information both on flow velocities and deposition rates. Alternatively, if the population of bubbles in the milk flow is inadequate for this purpose, one could use PIV with tracer particles and an optically clear fluid in experiments with test-bodies of progressively different shapes. If the ultrasonically recorded changes in effective test-object shape were mimicked, using moulds that represent the progression from the initial to the final shape of the test-object, one could study the change in size, level of mixing and fluid residence time of vortices in the wake region of test-bodies, over the duration of the milk experiment. This might throw light on the hydrodynamic causes of the ultrasonically observed rate and extent of clot deposition on the test-bodies.

4. Efforts should be made to identify, and perhaps remove, the cause(s) of the progressive decline in flow rate during the steady flow experiments. Calculations indicate that the mean fluid velocity in the test-chamber increased, as wall clot developed in steady flow experiments, but this was not ascertained directly. An experimental programme of controlled and measured variation of mean fluid velocity, and thus knowledge of the changes in mean fluid residence time and shear stress in the test-chamber, would throw light on the effect on clot deposition of such changes.

5. Difficulties with degassing warm, flowing milk, and bubble creation by the pumps, limited the success of achieving bubble-free milk flow in the test-chamber, which had an impact on imaging and possibly clot adhesion. Preheating stagnant milk prior to its use in flow experiments, and removal of the peristaltic pump previously used in these experiments, may be an effective way to ensure bubble-free milk flows through the test-chamber in steady flow studies. In addition to these measures, for pulsatile flow experiments, use of a degassing unit downstream of the diaphragm pump may be fruitful, but at a price - the pulsations may be
markedly damped.

6. Wall clot loss alters flow conditions and has the potential to interfere with test-object clot, so the prevention of wall clot formation is desired. To this end, the feasibility of such means as a test-section whose walls act as a Ca$^{2+}$ sink, or of the core-annular flow column designed in this laboratory, could be assessed using ultrasonic imaging.

Tests could also be carried out to determine the effects on clot adherence and rheology of Ca$^{2+}$ concentration and temperature.

7. The colloidal and soluble calcium in raw milk will vary with the diet of the source animal. Since the Ca$^{2+}$ concentration strongly influences coagulation, this should be checked prior to each experiment. Such testing was not performed here, given the fixed diet of the herd and lack of variation in milk composition data, but is a recommended addition to pre-experiment tests.
Appendix A

Publications

The co-authors of the following papers have given their permission for these papers to be included here.
Paper presented at the
1st IChemE Research Event

Cambridge, England
January, 1991
Appendix A: Publications

THE 1991 ICHEME RESEARCH EVENT

Analysis of Proteinaceous Deposition Rates from Ultrasonic measurements
Christy J.R.E., Macleod N., Marosek K. - University of Edinburgh

Abstract
Fluid flow in the vicinity of artificial cardiovascular implants is known strongly to influence the deposition of thrombus around such devices. However, the relationship between flow characteristics and deposition tendency is not clear. Previous work in this laboratory, using a blood analogue fluid, has shown that stasis is not the only factor controlling clot deposition; high shear stresses may limit deposit growth and high mass transfer rates may promote it. In order to appraise these factors, it is necessary to observe the growth of deposit as it occurs. Since the fluid is opaque, ultrasound is to be used for such observation.

Introduction
The purpose of this research is to throw light on mechanisms of flow-related thrombus formation in artificial heart valves, thus facilitating the identification and design of valves of minimal clotting tendency.

As we have indicated in reference 1, in the continuing search for an athrombogenic mechanical prosthetic heart valve the influence of fluid pattern on blood clotting has received relatively little systematic attention compared with that given to chemical and surface material properties. Accordingly, artificial materials are now available for mechanical valve construction which are intrinsically less thrombogenic than those made from them. In these circumstances, the logical way forward toward development of a mechanical valve usable without anticoagulants is to identify those features of the flow through existing valves that lead to thrombus formation, with a view to eliminating or reducing such undesired fluid effects in new designs.

Many physical flow characteristics have been proposed as determinants of thrombus formation, including:

a) high local values of shear stress
b) high local values of mass transfer coefficient
c) stagnation points
d) regions of stasis.

Experimental evidence, such as that of Tiederman2 and Taylor2, show no correlation between a) and b) and the formation of particulate deposits from flowing blood. Vorhauer's experiments4 with bodies of revolution supported in the canine descending aorta point, on the other hand, to wake regions behind bluff bodies, at which c) and d) occur together, as favoured site for thrombus growth. Figliola5 and Yoganathan6 in their studies of prosthetic valves have likewise linked observed sites of coagulation in vivo with regions where separated flow and stasis are found in vitro under steady flow conditions at the same mean Reynolds number. Since such regions are associated with high local mass transfer, stagnation points and stasis together, identifying the effects of any particular flow property remains a problem.

An Analogue of Thrombus Formation
With the aim of studying these similarities in simpler and more determinate flow situations, we have more recently examined the extent of the resemblance between the clotting of blood flowing past certain solids of revolution placed in the canine aorta, observed in vivo by Vorhauer, and that of comparable flows of milk about similar test bodies in our own experiments. We find that milk clotting in steady and pulsatile flow around four simple bodies of revolution (Fig 1) shows many similarities of location and extent with in vivo canine thrombosis formation for similar bodies as reported by Vorhauer and Tarnay7. With the making of in vitro observations. It appears that such a coagulable 'analogue fluid need not mimic the cascade of reactions leading to the formation of thrombin, but only the action of thrombin on fibrinogen and the subsequent formation of a fibrin thrombus. Heated milk, to which are added rennet (containing the enzyme Chymosin) and Calcium Chloride (which accelerates clotting and controls the rheology of the formed clot), provides an analogue for this. Chemically the final blood and milk clotting reactions are similar, both systems involving the enzymic cleavage of a soluble protein to yield an insoluble protein and soluble glycopolypeptides, the insoluble protein undergoing a second order polymerisation reaction to form a fibrous mesh. On a macroscopic scale both reactions exhibit an induction period, during which no solid material is visible. Lewis8 has shown that the behaviour of this analogue liquid in impinging jet experiments11 and Hasovec's net-clotting experiments12 is remarkably similar to that of blood; and that the milk curd formed around Bjork-Shiley and Starr-Edwards prostheses in an experimental heart chamber occur at the same sites as are prone to thrombosis in vivo.

Findings and Further Questions
A dye injection investigation of the fluid residence time distribution under the hydrodynamic conditions of our milk flow experiments reveals that a permanent or trapped vortex (Fig 2) persists at each downstream site where clot is found, implying that stasis, or stagnation, is of predominant importance in causing milk clotting. While clot deposition in pulsatile flow occurred always within regions of stasis, it did not, however, occur everywhere in such regions. Thus, whilst it is apparent that stasis is a prerequisite for thrombus formation, the extent and subsequent shape of the clot would appear to be governed by other factors.

These might be mass transfer or sheering effects. Indeed it is likely that the growth of the clot deposit will be limited either by the rate of transfer of the clotting species to the deposit surface or by the action of sheering forces preventing adhesion of further material

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Evidently, it is important to distinguish between these two possibilities in the case of any particular device that shows promise of remaining clot-free in the fluid stream. To be able to make this distinction experimentally it is necessary to observe the growth of deposit continuously during the experiment rather than solely at the end as is done at present. We propose to use an ultrasound technique for such continuous monitoring of the deposit growth in the opaque liquid.

A New Experimental Technique

We have found in experiments using pre-formed milk clot suspended in either water or fresh milk, that it is possible to observe the location and shape of the fluid/solid boundaries ultrasonically in such systems. A conventional 7.5MHz a-scan ultrasonic probe was used in these tests, loaned to us for these trials by the manufacturer, Dynamic Imaging Ltd of Livingston. With this equipment we have further identified means of incorporating the probe into the wall of our milk flow-system in such a way that it can continuously and non-invasively monitor the deposition of clot on the test body within.

Applications of the New Technique

Following installation of the ultrasonic equipment thus proven in preliminary trials we intend:

a) To extend further our fundamental enquiries into the course and mechanism of clot formation in disturbed flows of milk and, by inference, of blood;

b) To set up a test procedure or facility for rapidly evaluating and ranking artificial heart valves with respect to their liability to thrombose.

Figures

Fig 1. Photographs: (a-d) steady flow. (e-h) pulsatile flow.

Fig 2. Dye movements during pulsatile flow cycle. Disc resembled the cone and sphere resembled the tear-drop. Permanent wake vortex observed for all objects in steady flow.

References


Paper published in
Chemical Engineering Science,
DEVELOPMENT OF A CONCURRENT LIQUID–LIQUID CORE-ANNULAR FLOW COLUMN TO PREVENT WALL DEPOSITION IN COAGULATION STUDIES

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(First received 29 August 1991; accepted in revised form 26 May 1992)

Abstract—This paper describes a method of avoiding the formation of deposits at the wall of a vertical cylindrical tube carrying a steady flow of coagulable fluid (rennetised milk) by providing a cocurrent annular film of inert, immiscible fluid adjacent to the tube wall. A liquid–liquid core–annular flow column, 24 cm long and 3.2 cm diameter, intended for use as a cardiac valve test chamber, was designed and tested to determine the flow conditions under which a continuous annular film of fluid down the wall could be maintained in the presence of a downward flow of relatively low-viscosity aqueous core fluid. Ultimately, for film fluid Reynolds numbers of 880 and above, it was found that a continuous film can be maintained throughout this vertical cylindrical test chamber for all core fluid flow rates attainable in our apparatus. The results indicate that a downward coaxial-flow chamber for milk experiments is indeed feasible. Such coaxial flow systems have been used in reducing pressure drops in the pumping of viscous fluids. Our results suggest also that a core–annular flow might be used to provide protection of pipe walls of short length from fouling or corrosion by a core fluid.

1. INTRODUCTION

Lewis and Macleod (1983) have shown that the clotting behaviour of a flowing milk mixture is remarkably similar to that of blood in similar flow situations. In a further study of fluid-mechanical influences on the clotting of milk and blood, Christy and Macleod (1989) examined the clotting of this milk mixture in steady and pulsatile flows past certain axisymmetrical objects located axially in a cylindrical test chamber, and compared their results with those reported for clot deposition from blood flow around similar test bodies suspended in the canine descending aorta (Vorhauer and Tarnay, 1967) in an attempt to relate both to known flow patterns. However, milk clotting along the wall of the cylindrical test chamber, presumably due to a slow-moving boundary layer in this region, leads to problems in analysing clot deposition on the test body; this layer of deposit on the wall not only makes it difficult to remove the test object for examination undisturbed, but also renders uncertain the origin of any clot deposit then found on the test object. For instance, wall clot dislodged upstream of the test body may impinge on the test body during an experiment, either adhering to or dislodging the clot already formed on it.

The purpose of this research was to find means either of preventing formation of wall clot or of reducing its influence by modification of the wall conditions in the cylindrical test chamber. The following possibilities were considered:

(i) Liquid–liquid core–annular flow. Removal of the reactive fluid from the wall region by providing an annular film flowing coaxially and cocurrently with the core fluid.

(ii) Enlargement of the test-chamber diameter so as to reduce the possibility of dislodged wall clot coming into contact with the axially mounted test body.

(iii) Coating of the test chamber wall to inhibit clot deposition.

(iv) Arranging for a thin sheath of flexible solid material, acting as an inner lining to the test chamber, to be pulled through the column, thus removing the wall clot as it forms.

The potential of each of these ideas was considered systematically. Solution (i) would be suitable if it were indeed possible to maintain a continuous annular film at the wall. Facilitating option (ii) would make the removal of the test body easier but would not solve the whole problem; the possibility of dislodged wall clot impinging on the test body would remain, and a larger volumetric flow rate of milk would be necessary to maintain the desired Reynolds number. Option (iii) would probably fail, as the stagnant layer adjacent to the wall would still promote clotting and the poorly adhered clots would probably, and most undesirably, be carried downstream, impinging on the test object. Alternative (iv) would prevent the formation of stationary clots on the wall of the test chamber without disturbing the flow significantly. However, apart from the obvious mechanical difficulties of such an arrangement, the possibility of clot formed on this material, upstream of the test object, becoming dislodged and impinging on the test body would still be present.
Option (i), two-phase core-annular flow was, therefore, chosen for investigation as offering the best prospect of success.

The main aim of the present research was accordingly to ascertain whether this liquid–liquid co-current annular flow mode of operation is possible with a core fluid of relatively low viscosity, determining the flow conditions, if any, under which a liquid–liquid core-annular flow column can be operated to give a continuous annular film throughout the length of the test chamber.

Several investigators have analysed core-annular flow in liquid–liquid systems. Hickox (1971) carried out a theoretical analysis of stability using a standard method of small perturbations on a laminar, core-annular flow. His analysis indicates that core-annular flow is unstable for any case where the viscosity of the outer phase is greater than the viscosity of the inner phase. This result agrees well with the experimental findings and has been predicted by thermodynamic considerations. [From the minimum-energy approach (Everage, 1973; Maclean, 1973), the two liquids minimize their viscous dissipation in core-annular flow when the more viscous phase is surrounded by a less viscous phase.] Hickox (1971) also examined the influence of the velocities of the phases on the stability of the core-annular flow. However, Hickox's linear analysis is valid only for small perturbations whose wavelength is long, not for large amplitude or high-frequency disturbances.

Charles et al. (1961) worked with horizontal pipeline flow of equal-density oil–water mixtures. He investigated the reduction of the pressure gradient for oil flowing along a pipeline; on introducing a concentric flow of water, less viscous than oil, a uniform annulus in the region next to the pipe wall was obtained. From experimental observations of the flow patterns obtained with different oil flow rates and constant water flow rates, Charles (1961) found that there was, in his equipment, a low critical oil (core) velocity, below which core–annular flow cannot be maintained. An upper critical oil velocity was also found to exist, above which the shear between the water annulus and the oil core renders the core-annular flow regime unstable.

Shertok (1976) substituted the fluid properties used by Charles (1961) into the linear stability theory equations developed by Hickox (1971). The agreement between the theory and the experimental findings is good for low superficial velocities of the oil and water. However, the results diverge at higher superficial velocities due to a breakdown in the constraints of Hickox's formulation.

The experimental work conducted by Shertok (1976) involved co-current liquid–liquid upward vertical core-annular flow. He found, using a density ratio substantially different from unity, unlike Charles, that there are upper limiting core and film fluid superficial velocities, beyond which core-annular flow is unattainable. Shertok's employment of laser Doppler velocimetry led him to conclude that although vertical core-annular flow is characterized by ripples at the fluid–fluid interface, these have little measurable effect on the bulk flow.

Oliemans and Ooms (1986), like Charles et al. (1961), sought to facilitate the flow of a highly viscous heavy crude oil through a horizontal pipe by attainment of a core-annular flow, with the annulus of water. However, their application was for heavy oils with \( \mu > 500 \text{ cP} \) and \( \rho > 900 \text{ kg/m}^3 \); the difference in viscosity from the annular fluid was so great that the core approximated to a rippled solid rod; their results are of limited use to us.

Thus, it appears that no complete theoretical treatment is available for the stability of downward vertical core-annular flows, adequate for the design of our proposed non-fouling milk column, and that there is no published experimental work directly applicable to our system. Because our aim was unique, our experiments having a much smaller viscosity ratio (i.e. 1.5) than other studies (all in excess of 6.3), the literature was able to provide us with approximate guidelines only. We, therefore, undertook the preliminary analysis described in Section 2 as a guide to the experimental work described in Section 3.

2. DESIGN AND STABILITY CRITERIA

The following parameters were considered relevant to the stability of a liquid–liquid core-annular flow column with a continuous liquid film (Fig. 1): interfacial tension, \( \sigma \), component densities, \( \rho_1, \rho_2 \), component viscosities, \( \mu_1, \mu_2 \), component flow rates, \( W_1, W_2 \) and the geometry of the entrance section. The following preliminary analysis aided the choice of film fluid and materials of construction. Throughout this discussion, it is assumed for simplicity that the fluids are Newtonian.

(i) Surface tension (\( \sigma \)). This tension may govern the waviness and stability of the fluid–fluid interface (Wallis, 1969) and, hence, affect the ease of emulsification. Energy considerations would suggest that for stability the wetting propensity of the film fluid on the wall should be greater than that of the core fluid. Oliemans and Ooms (1986) used physicochemical agents both to facilitate the wetting of the pipe wall by the film fluid and to improve the stability of the fluid–fluid interface.

Regarding the liquid–liquid interface, Hutchinson and Whalley (1973) suggest that the entrainment rate for core-annular flow columns is an increasing function of \( \gamma/x/\sigma \), where the interfacial shear stress, \( \gamma \), is competing against the containment effect of the surface tension, \( \sigma \), and \( x \) is the film thickness. This would imply that we desire a high surface tension at the liquid–liquid interface to prevent droplet formation.

(ii) Fluid densities (\( \rho_1, \rho_2 \)). A vertical configuration was chosen to minimize gravitational stratification problems. For stability at the liquid–liquid interface, it would seem preferable to employ liquids of equal density in the film and core. However, in order to aid
Prevention of wall deposition in coagulation studies

(iii) Fluid viscosities ($\mu_c$, $\mu_f$). A source of instability, according to Ooms (1971, 1972), is an imbalance of the interfacial velocities of the fluids. If the core and film fluid viscosities differ, then the velocity gradients in the two fluids at the interface will be different: the abrupt change in the velocity profile will prove to be the primary cause of instability. Hickox (1971) showed that having the film fluid viscosity larger than the core fluid viscosity always leads to instability.

(iv) Core and film fluid flow rates ($W_c$, $W_f$). The core-annular flow configuration is quite dependent on the velocities of the two phases. For stability it is preferable to have a low interfacial shear stress to minimize the variation in velocity gradients across the interface. From the work by Hutchinson and Whalley (1973), mentioned above, it would appear that the interfacial shear stress should be kept low to prevent entrainment. For constant liquid viscosities, keeping the fluid-fluid interfacial velocity gradients small will keep $\tau$ low, if waves are not present. However, to ensure low velocity gradients either the overall flow rate, $W_c + W_f$, must be kept low or the ratio $W_f/W_c$ must not be too small. Since the Reynolds number for the overall flow is to be fixed, the ratio $W_f/W_c$ must be chosen large enough to keep $\tau$ low. This may lead to a large film thickness. Unfortunately, if the film flow rate and thickness are large then, as is commonly found experimentally, the tendency for wave formation increases. For instance, Gill et al. (1964) found that in an upward core-annular air-water flow the entrainment flow rates increase continuously with increasing $W_f$, at all air flow rates. Some compromise between $W_f$ and $x$ may be necessary.

(v) History of the flow. Charles et al. (1961), Shertok (1976) and Oliemans and Ooms (1986), all utilized special inlet nozzles to prevent disorderly mixing of the two fluids prior to their introduction to the test chamber. There are a number of ways to initiate core-annular flow (Kubie and Gardner, 1977) with minimum interfacial disturbance (Fig. 2). For example, a porous circumferential zone of the wall through which unreactive fluid diffuses into the region adjacent to the wall, or a conical diffuser with a uniform circumferential gap, may be provided to introduce the two fluids cocurrently at the top of the test chamber. The latter configuration was chosen here since it appeared to be the easiest one to install.

From the above analysis it was decided that for downward core-annular flow the film fluid should possess the following properties. It should wet the test-chamber wall preferentially, relative to the core fluid (i.e. have a lower surface tension than the core fluid) and be immiscible with, less viscous than, and have a density equal to or greater than the core fluid. Since water was chosen to be the core fluid, a non-ionic (or covalent) compound was sought for use as the film fluid so as to ensure a high interfacial tension.
between them and easy separation of the two fluids downstream of the test-chamber. Of the suitable organic fluids available, 1,1,2-trichloro-1,2,2-trifluoroethane \((\text{C}_2\text{Cl}_3\text{F}_3)\) was chosen. At 25°C, water and \(\text{C}_2\text{Cl}_3\text{F}_3\) have surface tensions of about \(72 \times 10^{-3}\) and \(19 \times 10^{-3}\) N/m, viscosities of about 1.002 and 0.680 cP, and densities of 998 and 1565 kg/m³, respectively. An interfacial tension between the two liquids of about \(40 \times 10^{-3}\) N/m is expected.

Simple wetting propensity tests were conducted to aid the choice of a suitable material from which to construct the test chamber, the criterion being that the material should be wet more easily by the film fluid than by water. Several different materials (Perspex, PTFE, PVC, aluminium, brass and stainless steel) were tested. It was found that trichlorotrifluoroethane will wet most of the materials tested preferentially to water, with PVC and Perspex performing best in these tests.

### 3. EQUIPMENT AND PROCEDURE

The apparatus for providing a steady flow of core and film fluid to the test chamber containing the test body is illustrated in Fig. 3. Water flows by gravity through the test chamber from a constant head tank, and the film fluid \((\text{C}_2\text{Cl}_3\text{F}_3)\), which is stored at the base of the settling tank, is pumped to the top of the test chamber. Within the test chamber the test body is fixed at its downstream end to a vertical 50 mm long metal "sting" coaxial with the vertical test chamber, as shown in Fig. 4.

Cocurrent core—annular flow is produced by the water flowing within the diffuser and the \(\text{C}_2\text{Cl}_3\text{F}_3\) flowing between the test-chamber wall and the outside of this diffuser. The PVC diffuser was designed to minimize flow separation. A "reservoir" for the \(\text{C}_2\text{Cl}_3\text{F}_3\) was incorporated into the diffuser to assist in distributing the flow. The width of the gap between the diffuser and test-chamber wall, from which the film fluid is introduced, was not designed to be variable since the film thickness will not depend on the gap width once the film is established on the wall.

The water—\(\text{C}_2\text{Cl}_3\text{F}_3\) stream leaving the base of the test chamber enters a settling tank. A baffle is positioned in this tank to dissipate the kinetic energy of the inlet fluid and to provide a way of keeping the denser recyclable film fluid from leaving through the overflow to the drain. A large-diameter settling tank (about 50 cm) was used so that no large circulation and relatively small velocities are present.

By varying both the film and core fluid flow rates, it was possible to evaluate the flow ranges and Reynolds numbers for which a continuous film is obtainable within the test chamber. To enable observation of the
Prevention of wall deposition in coagulation studies

Fig. 3. Schematic diagram of the two-phase annular flow column apparatus.

Fig. 4. The two-phase annular flow test chamber.

film's performance, a red dye (Sudan IV), miscible with the film fluid but immiscible with water, was used. The use of this dye allowed a definite interface to be seen when the film became discontinuous along the test-chamber wall. In certain cases, where a continuous film was obtained, photographs were taken through the transparent wall of the test chamber for use in further analysis.

The fact that the dye is immiscible with water may appear to imply that the dye will not affect the film
and core fluid interaction, as it will be contained within the film fluid. However, Davies and Rideal (1961) have observed that wetting by liquids is poorer when the liquids are contaminated rather than pure. Consequently, all preliminary tests were performed with the dye present, as the significance of the dye for the film's performance was unknown.

When the dyed film fluid was continuous throughout the test chamber, the appearance to the naked eye, was a uniform pale red. Still photographs showed, however, that this colour was not uniform, but was crossed by circumferential, horizontal lines of a deeper red, the spacing between such lines increasing from the top to the bottom of the test chamber. These lines may have indicated the presence of waves or ripples. No entrainment of film fluid was apparent when the film was continuous.

When film discontinuity occurred, a clear and definite ragged interface between the two fluids was observed at the wall; the film fluid in contact with the wall had a pale red appearance whilst the water was transparent, containing some red droplets whose colour was deeper than that of the film, suggesting that they were thicker than the film. Isolated drops of film fluid can be seen even through a continuous film, because the overlap of dyed film fluid is depicted by a deeper shade of red. It is, therefore, probable that entrainment from a continuous dyed film would have been observed had it occurred.

4. RESULTS

The desired result, a vertical liquid-liquid core-annular flow column with a continuous film down the test-chamber wall, was achieved over a range of flows. It was found that, for film fluid Reynolds numbers of 880 or above, any non-zero core fluid flow rate achievable with our equipment could be employed to obtain a continuous film both upstream and downstream of the test body. Values of the flow rates, the corresponding Reynolds numbers (as defined below), and the observed flow characteristics for the film and core fluids (C2Cl3F3 and water, respectively) are tabulated in Table 1. The film and core Reynolds numbers were defined using the following two equations:

\[ Re_f = \frac{2p_f W_f}{\mu_f \pi D} \quad (1) \]

<table>
<thead>
<tr>
<th>( W_f ) (l/min)</th>
<th>( Re_f )</th>
<th>( W_c ) (l/min)</th>
<th>( Re_c )</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.12</td>
<td>90</td>
<td>0–5.78</td>
<td>0–3565</td>
<td>Deficiency of C2Cl3F3 results in only droplets of the film fluid, at all core fluid flow rates</td>
</tr>
<tr>
<td>0.22</td>
<td>170</td>
<td>0–5.78</td>
<td>0–3565</td>
<td>This increase in film fluid flow rate does not improve the result</td>
</tr>
<tr>
<td>0.34</td>
<td>260</td>
<td>0–5.78</td>
<td>0–3565</td>
<td>Incipient film at the diffuser exit</td>
</tr>
<tr>
<td>0.47</td>
<td>360</td>
<td>0–5.78</td>
<td>0–3565</td>
<td>Similar results as those found at ( Re_f ) of 260 ± 20</td>
</tr>
<tr>
<td>0.61</td>
<td>465</td>
<td>0–5.78</td>
<td>0–3565</td>
<td>Very short film at diffuser outlet</td>
</tr>
<tr>
<td>0.74</td>
<td>565</td>
<td>0–5.78</td>
<td>0–3565</td>
<td>Increased water flow rate extends the film slightly</td>
</tr>
<tr>
<td>0.88</td>
<td>670</td>
<td>0–5.78</td>
<td>0–3565</td>
<td>Longest film observed is about a third of the length of the test chamber, at ( Re_c ) of 700. Higher water flow rates resulted in film break-up</td>
</tr>
<tr>
<td>1.02</td>
<td>780</td>
<td>0.38</td>
<td>250</td>
<td>Similar results as those obtained at ( Re_f ) of 565; however, the film is slightly longer here</td>
</tr>
<tr>
<td>1.02</td>
<td>780</td>
<td>1.18</td>
<td>780</td>
<td>A continuous film is obtained down to the test body, after which discontinuity in the film occurs</td>
</tr>
<tr>
<td>1.02</td>
<td>780</td>
<td>2.68</td>
<td>1780</td>
<td>Continuous film obtained throughout test chamber</td>
</tr>
<tr>
<td>1.02</td>
<td>780</td>
<td>5.78</td>
<td>3565</td>
<td>Continuous film obtained throughout test chamber</td>
</tr>
<tr>
<td>1.15</td>
<td>880</td>
<td>0–5.78</td>
<td>0–3565</td>
<td>Continuous film throughout the test chamber over the full range of attainable core flows</td>
</tr>
<tr>
<td>1.28</td>
<td>975</td>
<td>0–5.78</td>
<td>0–3565</td>
<td>Continuous film throughout the test chamber over the full range of attainable core flows</td>
</tr>
<tr>
<td>1.54</td>
<td>1175</td>
<td>0–5.78</td>
<td>0–3565</td>
<td>Continuous film throughout the test chamber over the full range of attainable core flows</td>
</tr>
</tbody>
</table>

Table 1. The results obtained using the vertical two-phase liquid-liquid core-annular flow column, where \( W_f, Re_f, W_c, \) and \( Re_c \) are the film and core fluid flow rates and Reynolds numbers, respectively.
The equipment available limited the range of Reynolds numbers we could explore. So, here, no upper limit was found for the Reynolds number range for which a continuous film is obtainable. The maximum Reynolds numbers attainable with our pumps were 1175 (± 20) for the film fluid and 3565 (± 135) for the core fluid.

At the different values of flow rate in Table 1, the dependence of the column’s performance on the film and core fluid Reynolds numbers was determined by visual observations of the dyed film.

At low film fluid flow rates (i.e. $Re_f \leq 670$), any continuous film formed was fragmentary and did not extend from the diffuser down as far as the test body. Increasing $Re_f$ was found either to increase the extent of the film slightly or, under certain circumstances, to cause its disruption.

With $Re_f \geq 780$, a continuous film from the diffuser down to the test body was achieved irrespective of the value of $Re_c$. This continuous film extended past the test body when $Re_f \approx 880$, for all values of $Re_c$. At $Re_f = 780$, the continuous film extended downstream of the test body only when $Re_c \geq 780$ (corresponding to a Reynolds number based on the diameter of the test body, $Re_{test\ body} \geq 250$).

5. DISCUSSION

A range of film Reynolds numbers was sought for which a continuous annular film is attained in a relatively short length of vertical pipe (24 cm). For our milk coagulation studies, a core Reynolds number of about 1000 is employed. A stable regime is, therefore, in which the core flow is laminar and not disturbed by the presence of the film fluid. These requirements appear to have been satisfied.

When the film is not continuous down to the test body, an increase in the core fluid flow rate may either extend or disrupt it (see Table 1).

At the critical film fluid Reynolds number, increasing $Re_f$ stabilizes the film; beyond this $Re_f$, the value of $Re_c$ appears to have little significance with regard to the stability of the film within the range of $Re_f$ attainable here.

The film fluid Reynolds number was, thus, found to be of primary importance for film stability. For $Re_f \geq 780$, a continuous film in the region up to the test body is obtained. It is remarkable that for film fluid Reynolds numbers above 880 the film remains continuous throughout the test chamber, even beyond the test body, in spite of the expected tendency of disturbances around this body to destabilize the core-annular flow system beyond it.

At the transition film Reynolds number of 780, it was found that the extension of the continuous film beyond the test body occurred only if the core fluid Reynolds number was 780 or more. At this $Re_f$, and core fluid Reynolds number values less than 780, the film breaks down in the region adjacent to the test body; for core fluid Reynolds numbers greater than 780, the film remains continuous. This stabilizing effect of the core fluid at the transition film Reynolds number of 780 is at first surprising, since at other film fluid Reynolds numbers the core fluid does not appear to have any appreciable influence on the behaviour of the film. It may be significant that for a core fluid Reynolds number of 780 the Reynolds number based on the diameter of the test body is 240, whereas at the next lowest core Reynolds number measured (250) this test body Reynolds number is only 80. At a body Reynolds number of 80, it is unlikely that a turbulent wake will be present downstream of the test body, whereas at a body Reynolds number of 250 or above, a wake will almost certainly form. Perhaps this turbulent wake permits the film to remain continuous, possibly by reducing the degree of local deceleration of the core fluid in the region near its interface with the film fluid.

Viscosity and density were considered very important for flow stability by several authors; for instance, when the two phases in horizontal flow have unequal density, it was found that for establishment of a core–annular regime the core fluid viscosity needs to be very large (i.e. greater than 500 cP; corresponding to a viscosity ratio between the two fluids that exceeds 500), and the density difference less than 100 kg/m$^3$.

Charles et al. (1961) adopted a similar orientation, finding that the use of equal density fluids allows lower viscosity core fluids to be used (viscosity ratios from 6.3 to 65) whilst still attaining core-annular flow. The experimental work in the literature most closely resembling ours is that carried out by Shertok (1976). The differences between our configuration and his, such as flow direction and viscosity ratio, render a direct comparison meaningless. Shertok’s results do, however, indicate the dependence of core-annular flow on the two fluid flow rates, with critical velocities defining the velocity window in which such a flow is obtainable. Here we observed the lower critical Reynolds number but not the upper, due, no doubt, to the limitations of our pumps’ maximum capacities.

6. CONCLUSIONS

A cocurrent downward-flow vertical core–annular flow column was designed and tested using trichlorotrifluoroethane ($C_3F_7Cl$) as the film fluid, immiscible with the core fluid, water, to determine the feasibility of a proposed non-fouling chamber for milk coagulation experiments.

It was discovered that this mode of cocurrent contacting can be established, even with a viscosity ratio as small as 1.5, for film and core fluid flows of Reynolds numbers in excess of 780. Relating this result to our blood analogue experiments, the use of treated milk in this core–annular flow column may be able to show whether the clotting on the walls of the conventional test chamber significantly affects observations of test-body clotting. This information should lead to a more reliable comparison between the
characteristics of the clotting of milk and blood. For our application, that of providing a clot-free boundary layer in thrombogenicity experiments, these results are, therefore, promising, a continuous thin fluid annulus being achieved with apparently minimal disturbance of the core fluid.

**NOTATION**

- \( D \): internal diameter of the test chamber, m
- \( R_c \): core fluid Reynolds number
- \( R_f \): film fluid Reynolds number
- \( R_{test \ body} \): Reynolds number based on the diameter of the test body
- \( U_c \): core fluid velocity
- \( W_c \): core fluid volumetric flow rate, \( m^3/s \), unless otherwise stated
- \( W_f \): film fluid volumetric flow rate, \( m^3/s \), unless otherwise stated
- \( x \): mean thickness of fluid film, m
- \( z \): axial distance through test chamber, m

**Greek letters**

- \( \rho_c \): core fluid density, \( kg/m^3 \)
- \( \rho_f \): film fluid density, \( kg/m^3 \)
- \( \mu_c \): core fluid viscosity, \( kg/m \cdot s \)
- \( \mu_f \): film fluid viscosity, \( kg/m \cdot s \)
- \( \sigma \): interfacial tension, \( N/m \)
- \( \tau_i \): interfacial shear stress, \( N/m^2 \)

**REFERENCES**


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Edinburgh, Scotland
January, 1995
DETERMINATION OF MILK CLOT DEPOSITION RATES BY ULTRASONIC IMAGING

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Previous studies [1,2], using milk to which cheesemakers' rennet is added as a blood analogue for thrombosis, have revealed a dependence of the extent of milk clot deposition on flow characteristics such as stasis and shear stresses. Such studies, however, were limited in relation to determining deposition rates as the deposit was only measured on dismantling the apparatus. The present study involves the use of ultrasonic imaging equipment to determine the thickness of clot adhering to the wall of a vertical, cylindrical test chamber as the experiment proceeds. The maximum deposition rate in pulsatile flow is found to be significantly less than in steady flow at the same mean flowrate (Re = 1040).

INTRODUCTION

Previously enzymic coagulation of milk has been studied [1] in a vertical pipe around axially mounted test-bodies, in order to assess the relationship between clot deposition and flow characteristics. That study, however, was limited in relation to determining deposition rates in that the extent of deposition was only ascertained at the end of an experiment of fixed duration. In some experiments it was suspected that clot formed on the test-chamber wall upstream of the test-body may have become dislodged, impinging on the test-body, but this could not be confirmed. Ways to eliminate wall clot formation have been considered [3], but there appears to be no simple way of maintaining clot-free walls in both steady and pulsatile flows without disrupting the milk coagulation studies. Therefore, imaging of clot deposition was considered necessary to obtain information on clot growth rates and wall clot behaviour. Ultrasonic imaging was the technique chosen, as it has been successfully used non-invasively for in vivo deposition studies [4] and the results of preliminary imaging tests indicated its feasibility.

Ultrasonic imaging equipment, such as is used clinically for foetal measurements in the womb, is used here for continuous measurement of deposit thickness during the experiment. Ultrasonic measurement of the clot thickness is made possible by the interfaces between both the clot & fluid and clot & surface to which it is adhered reflecting some of the incident ultrasound beam, with the monitor transforming the time delay between ultrasound transmission and echo reception into interface depths and displaying the interfaces one-dimensionally. The depiction of clot forming on each test-body using ultrasound was poor, as the test-body shapes cause extensive ultrasound scatter. Ultrasonic imaging of clot deposition at the far inner-wall of the test-chamber, on the other hand, was sufficient to allow clot growth rates to be determined and these are reported here.

EXPERIMENTAL APPARATUS AND PROCEDURE

The experimental arrangement used in earlier milk studies has been described elsewhere [1]. Equipment additions/modifications made since these studies include the use of a polypropylene, as opposed to perspex, test-chamber, a perspex rather than stainless steel test-body support, a Concept 2000 ultrasound scanner from Dynamic Imaging equipped with a linear-array piezoelectric transceiver operating at 7.5MHz (intensity 9.6mW/cm², beam power 462uW) and supported in a customised holder, a video recorder and printer.

The milk apparatus under ultrasonic study comprises a cylindrical test-chamber, on the axis of which is mounted an axially symmetrical test-body and through which is passed a steady or pulsatile flow of a heated, unpasteurized milk mixture containing 1% (vol) cheesemakers' rennet and CaCl₂ (aq.). The concentration of the CaCl₂ solution ranged from 1.12 to 3.38M. The milk flow rate adopted at the onset of each experiment was 2 l/min and the duration of each study was about 35 minutes. The ultrasound beam scanned a diametrical

1 Dynamic Imaging Ltd., Young Square, Brucefield industrial park, Livingston, Scotland.
section of the chamber around the test-body. Deposition rates were determined by analysing the video-tape records of the continuous ultrasound imaging of each experiment, using a large television screen and hand-held digital calipers.

RESULTS

Four ultrasound images taken at different stages of a milk experiment using 3M CaCl₂ are given in figure 1. Here the flow is from left to right and the wall clot thickness is indicated by markers ('+', 'x') at the bottom left of each image. The wall clot was depicted as a smooth deposit, symmetrical about the pipe axis, and its growth was found to be dependent on flow regime. From the graph of deposit thickness against time (figure 2), it is clear that the initial rate of deposition and maximum thickness of deposit are both significantly greater in steady flow (~1.0mm/min and ~5mm) than in pulsatile flow (~0.1mm/min and ~3mm) at the same mean flow rate (pipe Reynolds number, based on mean velocity, of 1040).

DISCUSSION

There are at least two possible explanations for the different initial rates of clot growth in steady and pulsatile flows. It is known that the two stage reaction sequence leading to coagulation exhibits a 'lag' phase during which no particulate matter is visible, followed by rapid coagulation to form a solid clot\cite{2,3,4}. The extent of deposition in previous studies\cite{2} has been explained in terms of stasis being required for \(\kappa\)-casein in the milk to undergo enzymic transformation before coagulation will occur. This is borne out by clot deposition only occurring in regions where the local residence time approaches the duration of the 'lag' phase. Indeed, in Figure 2, extrapolation of the growth curves back to zero deposit suggests the existence of a lag phase of between 1 and 1.5 minutes duration from commencing injection of enzyme into the fresh milk upstream of the test chamber. One explanation is therefore that in pulsatile flow, due to the transient nature of the boundary layer, the region having a residence time in excess of the 'lag' phase duration is smaller than that for steady flow, resulting in less fluid being in an active coagulating state and hence a lower rate of coagulation. The mass transfer coefficient predicted (1 \(\mu\)m/s) for proteins in steady flow is low suggesting limited diffusion into the bulk fluid (of maximum clot growth rate 20 \(\mu\)m/s). Another explanation would be that in pulsatile flow the maximum fluid shear forces near the wall of the test chamber are greater than those in steady flow and that these fluid shear forces act against the cohesive forces of the coagulating species, reducing the net rate of deposition.

The reduction in rate of deposition in steady flow as the experiment proceeds may be due to thinning of the effective region of the boundary layer as the flow channel becomes more restricted or to a balance between the forces of clot cohesion and the fluid shear forces, or indeed to depletion of coagulating species from the boundary layer as material deposits. In pulsatile flow it is possible that the clot did not grow sufficiently to significantly alter the nature of the transient boundary layer nor the fluid shear forces acting.

In both steady and pulsatile flow experiments, shedding of the wall clot was observed in some experiments after 20-34 minutes. This may be due to hydrolysis of the clot (a natural process in milk following coagulation). Renewed growth of clot commences immediately following such shedding of deposit without a further lag phase, presumably because activated species are already present. This growth occurs at a rate comparable to that in the early stages of the initial clot formation.

It has been found that reduction of the CaCl₂ concentration leads to a reduction in the maximum rate of deposition and in the maximum thickness of clot and appears to lead to an increase in length of the lag phase. These would support previous observations\cite{5} that calcium ions enhance the coagulation process, both increasing the rate of coagulation and stabilising the resultant clot.

CONCLUSIONS

Ultrasound has been shown to provide a convenient measurement of milk clot deposition rates on a cylindrical test chamber wall. Deposition rates are higher in steady rather than pulsatile flow, presumably due to differences in boundary layer characteristics between these flow regimes.

REFERENCES

Figure 1: Ultrasound images of a test-chamber containing a disc-shaped test-body, taken 3.25, 6.7, and 9.3 minutes from the start of a milk experiment.

Figure 2: The growth profiles of clot at the test-chamber wall immediately upstream of the test-body, in steady (S) and pulsatile milk (P) flow experiments, using 3.0M CaCl₂ solutions.
Appendix B

Experimental Details

This appendix provides descriptions of the tests carried out before each milk experiment, the rennet used, and the procedures adopted when performing experiments, shutdown, dismantling and cleansing.

B.1 Titrations

Titrations were conducted to ensure the concentration of the CaCl₂ solutions was consistent, as milk coagulation is sensitive to fluctuations of this concentration (figure 4.6). The aqueous CaCl₂ solution was prepared by adding crushed CaCl₂ flakes to about 2 litres of soft tap water, which was then shaken, allowed to dissolve and then filtered. The method adopted for titrating the filtrate is described below, preceded by the theory on which it is based.

Ethylenediaminetetra-acetic acid (EDTA) is very effective in forming complexes with metal cations, and reacts with most metals in a 1:1 ratio. Since EDTA is a complexing agent for hydrogen ions, pH has a marked effect on its complex formation; the complexes formed with divalent metals are stable in ammoniacal solution but tend to decompose
in acid, and most EDTA-metal complexes are very soluble in water.

In solution, an EDTA-metal chelate exists in equilibrium with its component ions; for example, an EDTA-calcium (Y-Ca) complex:

\[
\text{Ca}^{2-}Y^2 = \text{Ca}^{2+} + Y^4^-
\]

In alkaline solution, the equilibrium lies far to the left and very few free Ca\(^{2+}\) ions exist. The stability constant for the EDTA-calcium complex is defined as:

\[
K = \frac{[\text{Ca}^{2-}Y^2]}{[\text{Ca}^{2+}][Y^4^-]}
\]

The metal-specific indicator method of titration requires an indicator to be added to the reaction mixture to form a distinctively coloured product with the metal cation being titrated. In this case, Ca\(^{2+}\) was the cation. The indicator must have a colour that is different from the Ca-indicator complex, and this complex must form under the same conditions as, and be significantly less stable than, the EDTA-metal complex. Solochrome Black T (SBT), which is blue when free, was chosen as the most suitable indicator for this task. Ca-indicator complexes are unstable and thus give poor end-point indication, so a small amount of magnesium was used to give a (pink) Mg-SBT complex more stable than the Ca-SBT complex but less stable than the corresponding EDTA complexes of the metals concerned (table B.1).

<table>
<thead>
<tr>
<th>Metal</th>
<th>logK of SBT complex</th>
<th>logK of EDTA complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>7.0</td>
<td>8.7</td>
</tr>
<tr>
<td>Ca</td>
<td>5.4</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Table B.1: The stability constants for the complexes formed by SBT\(^{[356]}\) and EDTA\(^{[357]}\), with Mg and Ca at 20°C.

The CaCl\(_2\) concentration was evaluated by conducting a titration where 1 M EDTA (pH 8.5) was gradually added from a burette to a conical flask containing a solution at pH 11 and consisting of 6.75 g NH\(_4\)Cl, 57 ml NH\(_3\) (conc.), 40 ml distilled water, 2 ml Solochrome Black T (SBT) indicator, 0.0616 g of MgSO\(_4\).7H\(_2\)O and a chosen volume of the CaCl\(_2\) solution - a modified version of the procedure detailed in West and Sykes' publication\(^{[356]}\). The solution was initially pink, due to the formation of the Mg-SBT complex but, by addition of a sufficient volume of EDTA, the solution became blue. Addition of EDTA leads to EDTA-Ca complexes being formed. EDTA-Mg complexes
form, once the Ca\(^{2+}\) is consumed in this way, resulting in the breakdown of SBT-Mg complexes. When there are no more SBT-Mg complexes (i.e. when all the Mg has complexed with EDTA), the SBT free-state colour of blue is once more resumed. A small amount of Mg\(^{2+}\) was used so that the colour change, from pink to blue, occurred close to the equivalence point and thus an accurate value for the CaCl\(_2\) concentration was obtained. The volume of EDTA sufficient to achieve a colour change \(V_{\text{EDTA}}\) was used to determine the concentration of the CaCl\(_2\) solution \(M_{\text{CaCl}_2}\), using equation B.1.

\[
\text{EDTA}^{4-} + \text{CaCl}_2 \rightleftharpoons [\text{Ca(EDTA)}]^{2-}
\]

\[
\text{No. of moles of EDTA} = \text{No. of moles of CaCl}_2
\]

\[
\Rightarrow M_{\text{EDTA}} \times V_{\text{EDTA}} = M_{\text{CaCl}_2} \times V_{\text{CaCl}_2}
\]

\[
\Rightarrow M_{\text{CaCl}_2} = M_{\text{EDTA}} \times V_{\text{EDTA}} / V_{\text{CaCl}_2} \quad \ldots \quad \text{(B.1)}
\]

### B.2 Rennet used in this laboratory

The details of the calf rennet\(^1\) used here are as follows. The commercial calf rennet (also called essence of rennet) was an aqueous solution of rennet enzymes with added salt (min. 16%) and sodium benzoate (max. 0.3%). The active milk-clotting enzymes present in the rennet were chymosin and bovine pepsin, extracted from the fourth stomach of bovine animals using methods developed by “Chr. Hansen’s Laboratory” (CHL). 85–95% of the rennet’s milk clotting activity was related to chymosin, and 5–15% to bovine pepsin\(^2\). The strength of the rennet\(^3\), controlled by comparison against a known standard, was 10.0 CHU/ml ±2. The specific gravity and pH of the rennet

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\(^1\)Rennet was supplied by Bannerman (J.M.) & Co., 7 Grange Road, Edinburgh, Scotland.

\(^2\)Consistent pepsin content in the calf rennet is important, because this concentration determines the dependence of clotting activity on pH; bovine pepsin is much more sensitive to pH than chymosin, and pepsin has a more general proteolytic activity than chymosin.

\(^3\)1 CHU (Chr. Hansen’s Unit) is defined as the milk clotting activity present when 1 ml of enzyme solution is able to clot 50 g of substrate (low heat-treated non-fat dry milk, Peake, grade A, Galloway West) at pH 6.5 in 420 seconds at 32°C.
were $1.165 \pm 0.015$ and $5.50 \pm 0.2$, respectively.

**B.3 Milk and rennet tests**

A "Philips PW9421" pH meter, which covers the range pH 0.000 to 14.000 with a resolution of 0.001 pH, was employed for milk and rennet pH readings. Two buffer solutions were made: a pH 7.0 tablet was added to a bottle containing 100 ml of distilled water, sealed and shaken; this was repeated with a pH 4.0 tablet. The two containers were sealed to avoid the effect of air interference while the tablets dissolved overnight. On the following day, the day of the experiment, the pH meter was prepared by: first immersing the Pt100 resistance thermometer and glass probe in the pH 7 buffer solution; then pressing the ATC and pH buttons to select automatic temperature compensation and pH measurement; followed by setting the slope to 100% and adjusting the 'asymp pot' until the pH value of the buffer (at the buffer temperature) was displayed. The electrode and probe were then rinsed in distilled water and wiped dry with a clean cloth. This was repeated using the pH 4.0 buffer solution, since the pH range involved here lies within the range pH 4.0 - 7.0. Two samples of both rennet and milk were prepared in flasks, and one of each immersed in a water bath at $37^\circ C$ for 20 minutes. The probe and electrode were immersed in each sample and the pH of the rennet and the milk recorded, at both $37^\circ C$ and ambient temperature.

To avoid unproductive experiments, due to discrepancy in the reagents or a variation in the quality of the day's milk, Christy and myself conducted three modified Lee-White tests prior to each milk experiment. These tests were carried out at 33.5°C, with inversions every 15 seconds, using samples from the rennet, CaCl$_2$ and milk to be used in the subsequent milk experiment. Each test required a dry, 0.8 cm diameter test-tube, a rubber cork, one 5 ml syringe, two microsyringes and a water bath with temperature control. Three test-tubes, each containing 5 ml of milk, were immersed in the water bath for about 15 minutes. Each test-tube was then removed from the bath, in turn, and the tests performed. 1% (vol.) of aqueous calcium chloride and rennet were added, the test-tube was then stoppered and briefly shaken and the time set at
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$t=0$. This mixture was then inverted every 15 seconds, starting at $t=0$, until no flow was present. The point at which the non-flowing milk-plug was achieved was taken as the clotting time. The reason for using a temperature and inversion time less than those used in the standard Lee-White procedure (viz. 37°C and 30 seconds) was that a more sensitive test was achieved; a lower temperature was used to prolong the clotting time, so that any variations in this time would be more noticeable, and more frequent inversions were made to allow a more accurate detection of changes in clotting time.

B.4 Experimental procedure

The experimental procedure adopted for the main milk experiments is described below (refer to figure 6.1).

The test-body was thoroughly cleaned and mild adhesive was applied to the tip of its support rod, to ensure the test-body did not fall off. The test-section was then reassembled with the test-body in place. The water supply to the water tank was sent to the sink, to expel sediment in the water pipes, before being redirected to the top of the water tank, at which point a slight water tank overflow was established. By activating the water-jet ejector, a vacuum was achieved and water (from the water tank) was allowed to flow through the milk rig. When water began to appear in the first vacuum chamber, the peristaltic pump was turned on. The liquid level setpoint for the header vessel was set at 40%, and the level controller set to regulate the speed of the peristaltic pump based on this value. For pulsatile flow experiments, the outlet of the test-section exit tube was positioned about 8 cm above the level in the header vessel. Once the level in the header vessel had risen to over 50% the diaphragm pump was started. The frequency and stroke volume of the diaphragm pump were then adjusted to provide the desired 70 pulses/min and 2 l/min. In preparation for steady flow experiments, the diaphragm pump chamber was removed from the flow system and the header vessel height was raised until the desired flow rate was achieved (by gravity).
A preliminary water wash of the milk rig was performed to prevent the formation of an air-milk interface and the trapping of air bubbles and to heat up the equipment, whilst adjusting the water-jet ejector to obtain a water level of approximately one inch in the first vacuum chamber. A flow rate of 2 l/min was established and residual air, not removed by the vacuum chamber, was bled-off at the high points within the system or helped out by manipulation of the tubing. The heating-fluid pump and 6 kW heater were turned on and the temperature controller tuned, to give a setpoint temperature of 37°C.

The outlets from the three 5 gallon milk containers were connected to the main flow line. The lids were removed from these containers and from the rennet and CaCl₂ vessels, to ensure no vacuum was created. The flow rate at the test-section outlet was checked and the ultrasound probe, with gel on its face, was clamped firmly against the test-chamber wall, using the probe support. The best image of the test-object was then achieved by manipulation of the various monitor settings. Experimental details were typed on the monitor screen and the video recorder started. The ultrasound probe was repositioned, when desired, by slightly loosening the wing-nuts of the probe support or by removing the support backing which allows removal of the probe from the support.

Once the desired temperature and flow rate (37°C, 2 l/min) were achieved, the apparatus was ready for a milk experiment. The water supply to the water tank was stopped and a changeover, from water to milk flow, was implemented by opening the outlet valve of the milk container closest to the heat exchanger. This changeover corresponded to 'time = 0'. Water was supplied to the top of the drain-pipe downstream of the test-section, to prevent extensive clotting there, and milk was allowed to flow through the system. As many bubbles as possible were dislodged from the test-section and test-object, percussively and by tube manipulation, prior to the stage where milk entered the test-section. The milk was allowed to run through the test-section for about one minute before starting the rennet and CaCl₂ dosing pumps.
Throughout the experiment ultrasound images (of the test-section contents) and the milk temperature were recorded, and the calipers on the display screen were used for clot measurements. When time allowed, the flow rate was measured using a measuring cylinder at the sink. Once the level of milk in the first milk container dropped significantly, a wooden wedge was used to tilt this container to allow most of its contents to be used. To prevent air intrusion and discontinuity in milk flow on changeover between containers, the valve on the next milk container was opened before closing the valve of the emptying milk container and before the level in this container was as low as the outlet valve. This changeover procedure was repeated for the changeover from the second to the third milk container, with the remnants of the previous container being poured into the top of the next milk container.

**B.5 Shutdown and dismantling**

When the last milk container was almost empty, flow from the water tank was reinstated (to replace the milk flow) and this milk container's outlet valve closed. The water supply to the top of the water tank was also reintroduced. Once water appeared in the line to the header vessel, the peristaltic, heating-fluid and dosing pumps were turned off, the water supply to the water tank and the water-jet ejector was terminated, and the header vessel was allowed to empty; the milk remaining in the header vessel was allowed to flow through the test-chamber, flushing out the enzyme-activated milk. After a minute, or when the liquid level in the header vessel had dropped to about 10%, the pulsatile pump was turned off (if it was on-line). At this point, the fluid-content of the test-section was unrenneted milk, and not water or activated milk. A final ultrasound image was recorded and the transducer and its support were removed from the test-chamber.

The dismantling of the test-section was performed very carefully, so as not to dislodge any clot on the walls or test-object. A Hoffman clip was put on the tube between the header vessel and test-section, to prevent milk surges occurring. The exit-tubing of the test-section was removed and the supply of water to the top of the drain-pipe terminated. A steel plate was used to section-off the upper perspex pipe from the polypropylene test-chamber, by sliding it between their flanges (as described in section
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6.1.10). A basin was placed directly below the test-section to collect spillage from the perspex pipe, when this pipe was removed from the top of the test-section. The clotted test-object was then delicately raised, passing through the free surface of the milk, and removed from the test-chamber. It was sketched and photographed, before hand-held digital calipers were used to measure the diameter of the clotted test-object in several different places, and the test-object was weighed.

B.6 Cleansing

The transducer was cleaned with great care, as the piezoelectric crystals are brittle and surface scratches on the plastic protective layer affect the focusing and the "matching" properties of this layer.

The partly-disassembled test-section was cleaned with a large brush and then reassembled, in preparation for a thorough cleansing method. The pulsatile pump (if not on-line) was inserted into the milk flow line, and the system flushed with water, as if preparing for another experiment but with the water at 55°C. The flow to the water tank was stopped so that its liquid level would drop to about 15 cm. At this stage the pumps, water-jet ejector and heaters were turned off. The system was then rearranged into two cleaning loops. The outlet of the test-section exit tube was detached from the drain-pipe and inserted into the top of the header vessel, which now contained hot water. 35 g of Tergazyme, in solution, was added to the header vessel and the diaphragm pump started. To clean the heating and degassing section of the rig (i.e. the side of the rig upstream of the header vessel), the flow from the peristaltic pump was diverted from the top of the header vessel to the top of the water tank, which now contained a dilute caustic soda solution. The peristaltic pump was turned on to circulate this mixture, and the flow rate achieved by modifying the level controller setpoint and by use of the vacuum line. Some of this mixture was later used to clean the milk containers. The single-pass milk flow system was reinstated, after cleaning was complete and this system was flushed with water for about one hour.
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