A BLOOD ANALOGUE

FOR

THROMBOGENICITY ASSESSMENT

by

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The work described in this thesis is the original work of the author except where specific reference is made to other sources. It has not been submitted, in whole or in part, for any degree at any other University.

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PREFACE

The chief problem at present with artificial heart valves is their thrombogenicity, which requires the recipients to undergo dangerous anticoagulant therapy, which is not always successful. The present accepted method of testing valve thrombogenicity is implantation in experimental animals. This technique is unsatisfactory because of its high cost, the lack of control, the length of the experiments and because differences between the blood of various species render the results of dubious value. A more fundamental approach to the problem based on fluid mechanics fails because of the complexity of the situation and because of the opposed effects of a given fluid mechanical phenomenon. The in vitro use of blood is not possible due to the need for a single pass system to avoid recirculating clots, necessitating some thirty gallons of blood for a run of only one hour.

An analogue method using a fluid which does not have the disadvantages of the alternatives described above, therefore, seems worth exploring. Rennetized milk is a possible fluid for such a purpose and experiments were performed to ascertain whether it behaves in a manner analogous to blood in respect of its essential clotting properties. The first indications from the Lee-White inverted test tube test were very promising with milk behaving in a manner apparently identical to blood. Further experiments using the Stagnation Point Flow Chamber showed that there was a striking similarity in the microscopic appearance of the deposits forming at and around the stagnation point when a jet of milk/blood impinges normally onto a glass slide. A subsequent experiment, in which the fluids are pumped through a mesh, revealed that the sequence of clot growth as measured by the pressure variation upstream of the mesh is the same for both fluids. Finally, full scale tests of the clotting propensity of various heart valves were performed using an artificial heart system, which showed that the location and appearance of the

clots forming on the valves with rennetized milk were similar to those found in humans and that the results were reproducible. It is, therefore, apparent that rennetized milk shows great potential as an analogue for the flow related clotting of blood and can be used for testing artificial heart valves provided care is taken in the choice of materials for construction.

CHAPTER 1

INTRODUCTION

With the dramatic increase in the number and variety of artificial devices implanted over the past twenty years, it has become increasingly important to find a method for predicting the thrombogenicity, not only of the materials used, but of the whole device.

1.1 Animal Implantation

At present the accepted method of estimating the probable clotting effects in human patients is to implant the device in animals of various species; if the results are satisfactory, clinical trials are supposed to be justified. However, the differences in the blood characteristics of these species are very great, and the usefulness of animals as models for the clotting response of the human system is questionable. This uncertainty is highlighted by Grabowski et al, who studied platelet adhesion to foreign surfaces, under controlled conditions of whole blood flow, using an in vitro system in which the platelets adhere to the wall of the flow channel.

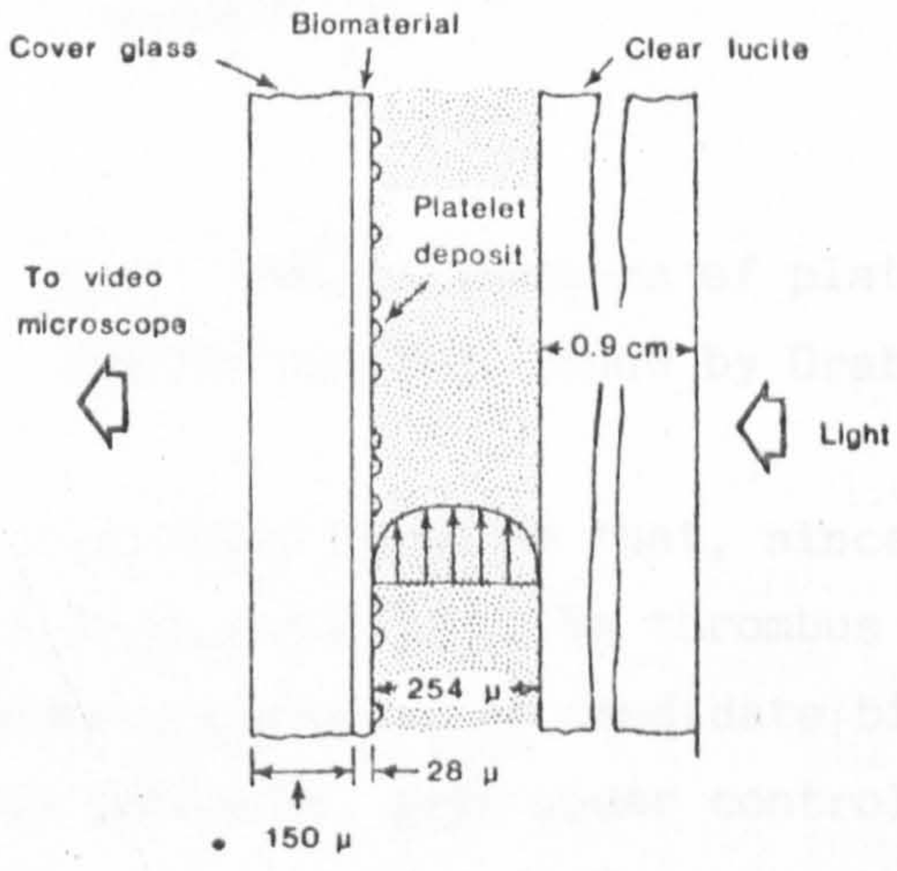

Fig 1. Flow chamber used by Grabowski et al.

Human, rabbit, dog, calf, sheep, pig, macaque and baboon blood were used and they reached the following conclusions:

1) At a shear rate characteristic of those found in mammalian arteries, human platelet adhesion (and that of calf, baboon, macaque, hog or sheep) is negligible in comparison to dog or rabbit platelet adhesion after 10 minutes of blood flow through their chamber.

2) The species differences are biomaterial dependent; the human-dog difference being present with Cuprophan or Avcothane, but absent with compressed Goretex or fluorinated ethyl-cellulose.

PLATELETS/mm^2

(Mean ± SE,N)* After 10 mins of Blood Flow at a Surface Shear Rate of 986 sec^{-1}

Species	Cuprophan PT-150	Avcothane 51	Compressed Gore-Tex	Fluorinated Ethylcellulose
Human	< 100 (4)	< 100	5,600 ± 1,500(4)	5,600 ± 5,000(4)
Dog	27,400 ± 4,600(5)	19,400 ± 9,300(4)	3,600 ± 1,600(4)	5,500 ± 3,500(4)
Rabbit	78,400 ± 6,400(4)	-	-	-
Calf	< 100 (4)	-	13,500 ± 9,800(5)	-
Baboon, Macaque, Hog, or Sheep	< 100 (4)+	-	-	-

*N is the number of different subjects or animals.
+N = 4 for each of the 4 species listed.

Fig. 2 Table showing numbers of platelets adhering to chamber surface found by Grabowski et al.

Their final conclusion is that, since platelet adhesion plays a fundamental role in thrombus formation, tests for thromboresistance of candidate biomaterials and for platelet adhesion, even under controlled flow conditions, must take into account differences between human blood and that of other species.

A further implication is that the ranking order of such materials may vary with the animal species used in the experiments. Mills (2), using a turbidimetric technique to measure the degree of platelet aggregation by ADP on blood derived from animals of difference species, also found marked differences between the species. He concluded that investigations of the participation of platelets in thrombosis, in experimental models, could lead to some very misleading results, if species differences were not somehow taken into account.

There are other disadvantages in relying on animal trials as the primary means of testing thrombogenicity:

1) As well as the differences in the blood characteristics of the various species, there are other marked physiological differences, which may affect the results. For instance, the pulse rate of the dog is very much faster than that of man; and the calf, much used in definitive tests of artificial hearts and heart valves, is an infant which will grow throughout an experiment and it is not clear how that fact will affect its blood clotting. An example of animal trials yielding misleading results if given by Starr (3), who found excessive tissue growth on his ball valve, when it was tested in dogs, which was not found in clinical trials. To overcome this he designed a silicone shield for the valves when they were being tested in dogs.

2) The cost of the experiments is high, as a full surgical team is required, operating under sterile conditions, and the animal must be maintained and housed throughout the trial. The high cost means that in many cases it is only possible to test a "finished" device and it is not possible to test the thrombogenicity of alternative versions or modifications throughout the development period.

3) Animal tests are very lengthy.

4) There is a lack of control of the experiments by the designer of the device as he is dependent on the skill of the surgical team, the suppliers of the animals producing healthy specimens, the animal keeper maintaining the animals properly, and the vet or surgeon performing the removal of the device without disturbing any formed clot.

5) Ethically, if there is a possible substitute for animal experimentation, it should be developed and used (4).

1.2 The Hydrodynamic Correlates of Thrombosis

The crudely empirical method of using animal trials for testing the complete device having proved unsatisfactory, it is natural to enquire whether a more fundamental or mechanistic approach to blood clotting effects might not prove more advantageous. Virchow in 1856 (5) announced that a triad of factors was important in coagulation: (i) surface chemistry, (ii) hydrodynamics, (iii) blood chemistry. Accordingly, a plausible technique for evaluating thrombogenicity, associated with the physical design of a prosthesis, depends on the assumption that one can identify the hydrodynamic factors predominant in causing clotting. By measuring the extent to which a prothesis is associated with these factors, it should in principle be possible to infer its likely propensity to cause clotting.

Much work has been done on the hydrodynamic aspects of clotting and among the phenomena which have been considered to be important are:

(1) Shear Stress,
(2) Stagnation,
(3) Mass Transfer.

These will now be considered in turn.

1.2.1 Shear Stress

The effect of shear on thrombogenesis is confused; by damaging the cellular components of blood, shear stress can cause the release of chemicals, such as A.D.P. and A.T.P., into the plasma, which enhance coagulation and platelet aggregation. On the other hand shear can also damage the species involved in the clotting mechanism, such as the platelets, factor X and prothombin, and hence, inhibit coagulation. Shear can also prevent adhesion to the surface on which it acts and affect the structure of any clot forming on the surface. It may also cause damage to to the lining of blood vessels, which can in turn lead to thrombus formation.

ADP and ATP Effects

The red blood cells or erythrocytes contain A.D.P., which is a powerful platelet aggregating agent, and a clot promoting factor known as erythrocytin. If the cell is damaged or ruptured, (haemolysed) these substances are released into the blood plasma (6,7,), which may leaḋ to a hypercoagulable state (8,9) and platelet aggregation-adhesion resulting in thrombus formation (10 - 17). Thus all haemolytic agencies are potentially thrombogenic and since thrombosis leads to further haemolysis of the cells involved in the thrombus the effects are cumulative.

Tillmann et al (18,19) suggest that a local platelet activation seems possible during diastolic regurgitation by the ATP released from sublethally damaged erythrocytes. During the following ejection phase, platelet aggregation can take place in the next deadwater region, which is located downstream of the small orifice area at the disc of the Bjork-Shiley heart valve, or the

Lillehei-Kaster valve and it is here that thrombus formation is often observed in clinical practice (20 - 23).

Shear stress appears, in some circumstances, to enhance and in others to reduce, deposition and aggregation effects. It is not clear how these opposite effects are related to different shear situations.

Anderson et al (24), using a rotational viscometer to study the effects of shear stress on human platelets in platelet rich plasma, found that the platelet count in the shear field was reduced by aggregation, from an initial count of 300,000, to as low as 60,000, for a shear stress of 200 - 400 dynes / cm^2 applied for 30 seconds. They found no measurable lysis under these conditions, but, the function of the remaining unaggregated platelets was impaired, as indicated by the reduced aggregation by ADP. This result is supported by work by Dewitz et al (17), from the same laboratory, using whole blood instead of platelet rich plasma, and by Hung et al (25).

Inhibition by Damage to Clot Promoters

As well as its ability to inhibit the ADP aggregation of platelets, shear stress has been shown to decrease the activation rate of factor X in the intrinsic pathway (26), and Spaeth et al (27), using a laminar flow tubular reactor to generate a well defined shear stress environment, have shown a dramatic reducing effect of fluid shear on the conversion of prothombin to thrombin. Furthermore, Charm and Wong (28, 29) have shown that some enzymes, when subjected to shear, lose activity as a function of time and shear rate and that plasma fibrinogen, in vitro, suffers a loss of clottability due to shearing.

Effect of Shear on Adhesion and Clot Structure

Glover et al, (30, 31) and Roberts et al (32) have shown that graded shear stress can have marked effects on clot structure formation. Under shear the mechanical strength of the clot formed can be a factor of five weaker, the initiation time shortened and the initial polymerization rate doubled. They also found that shear stress can cause the formation of platelet aggregates, which may be important in the thromboembolic phenomena or thrombosis associated with the extended use of blood contacting devices.

Petschek et al (11, 12, 33,) using the stagnation point flow chamber described later, found that increasing the shear stress decreased the amount of material deposited onto a surface, leading to discrete white cell circles of radius limited by the applied shear stress.

Grabowski et al (15, 16) found that platelet aggregate growth rates appear to be limited at low shear rates by platelet convection, but at higher shear rates by dilution of A.D.P., finite platelet - A.D.P. reaction times and surface shear stress. This may resolve the foregoing conflicts.

Another means by which shear can lead to thrombosis is described by Yoganathan et al (34, 35) who found that at a downstream distance of about 12 cm from a prosthetic heart valve, the wall shear stresses were of a magnitude that could damage the endothelial lining of the ascending aorta and the coronary arteries, and lead to thrombus formation.

Physical Aggregation

The basic theory of the purely physical aggregation of particles by simple contact in a colloidal suspension was worked out by Smoluchowski (36). When coagulation is controlled by Brownian diffusion, the kinetics of the decay

of the total particle concentration, N*, is approximately 2nd order, i.e.

$\frac{dN^*}{dt} = -\frac{4\ a\ G\ b\ N^*}{\pi}$, where a is the collision frequency in shear flow, G is the shear rate and b is the volume fraction of suspended particles.

This supports the theory that by increasing the shear rate, the rate of aggregation is increased.

Conclusion

In conclusion, shear will not normally cause clotting to occur in situ and the presence of a high level of shear on a surface will tend to prevent the build up of any thrombus. However, by causing aggregating agents to be released into the plasma, it may well promote thrombosis elsewhere by causing the blood to become hypercoagulable.

1.2.2 Stagnation

Stagnation is considered by many authors to be one of the prime causes of thrombosis (11, 12, 20, 33, 37 - 42). There are two distinct types of stagnation which are considered important, the first of which is caused by flow impinging at right angles to a surface (stagnation in the strict fluid dynamicist's sense), while the second, more accurately termed stasis, is associated with flow regions in which the residence time is greater than $\left(\frac{\text{local volume}}{\text{mean fluid velocity}}\right)$. These are sometimes quiet pockets of fluid and sometimes regions of recirculating flow. Petschek's group (11, 12, 33) have looked at the first of these two types of stagnation and its effect on blood deposition, using their stagnation point flow chamber in which blood from the carotid artery of an anaesthetized dog impinges perpendicularly onto a slide on which the

thrombus forms. This work is described later. Most other investigations have been concerned with stasis.

Leonard (42) and Morton (43) have shown that the presence of a captured vortex greatly enhances platelet deposition, and Yoganathan (20) has shown a direct correlation between the areas of stasis on the Bjork-Shiley heart valve and the areas of thrombus formation and apparent tissue overgrowth.

As the presence of a foreign surface is known to promote thrombus formation (10, 12, 41, 44-50) it is not suprising that increased blood residence time in the vicinity of the surface should lead to enhanced thrombus deposition.

1.2.3 Mass Transfer

The rate of deposition of blood particles onto a surface will be affected by the rate at which those particles are brought to the surface. Hence, it is reasonable to suppose that the mass transfer coefficient, which is a measure of this transport phenomenon, might be related to the blood clotting propensity of a given flow environment. The mass transfer coefficient at a point on a surface is generally closely related to the shear stress at that point and this is certainly true of flow parallel to a surface. However, if one considers the case of a jet of fluid impinging perpendicularly onto a surface, at the central stagnation point the shear stress is zero and rises radially outwards within the impingement zone, while the mass transfer coefficient remains constant throughout the same region.

Since impingement is one of the causes of deposition of blood onto surfaces (11, 12, 33, 41) and the relationship between mass transfer and shear stress clearly breaks down under that circumstance, it is worthwhile to check whether the mass transfer coefficient is more closely related to blood clotting propensity than is the shear stress.

There is considerable evidence to support a correlation between blood clotting and mass transfer rates, Knight et al (51) found clot on the unfaired edge of the ring and on the upstream side of the disc at the location of which the blood leaving the sharp edged orifice would impinge. The mass transfer rates are very high at these points and when a faired ring was substituted, these patterns of deposition disappeared (52). Gott (53) found that when short segments of rigid tube were implanted in dogs, those with bluff or abrupt end faces or edges were much worse than those tubes with streamlined trailing and leading edges, at causing thrombus deposition. This one would expect from the mass transfer characteristics of these flow situations.

Several other authors (12, 13, 40, 54) have done work which supports the assertion that mass transfer and blood clotting propensity are related, but Taylor (55, 56) and Vorhauer (57) have shown that there are exceptions to the correlation. Vorhauer implanted a variety of bodies into the canine aorta and weighed any shed thrombus downstream. The order of thrombogenicity was found to be: disc, ball, upstream apex-cone, tear-drop. However, in terms of mass transfer characteristics the order would be: cone, disc, ball, tear-drop, so there are limits to the relationship. Furthermore, Taylor found that, when investigating the deposition phenomena found in Petschek's stagnation point flow chambers, there was no correlation between the mass transfer coefficients and the radius of the white cell circles formed.

Although there is clearly likely to be a relationship between mass transfer and blood deposition, it evidently does not hold universally in the expected form.

1.2.4 Other Fluid Mechanical Phenomena

There are other fluid mechanical pehnomena, which may be important in coagulation. Heller (58, 59) has shown that colloidal coagulation in a turbulent regime is greatly enhanced by the presence of a single bubble.

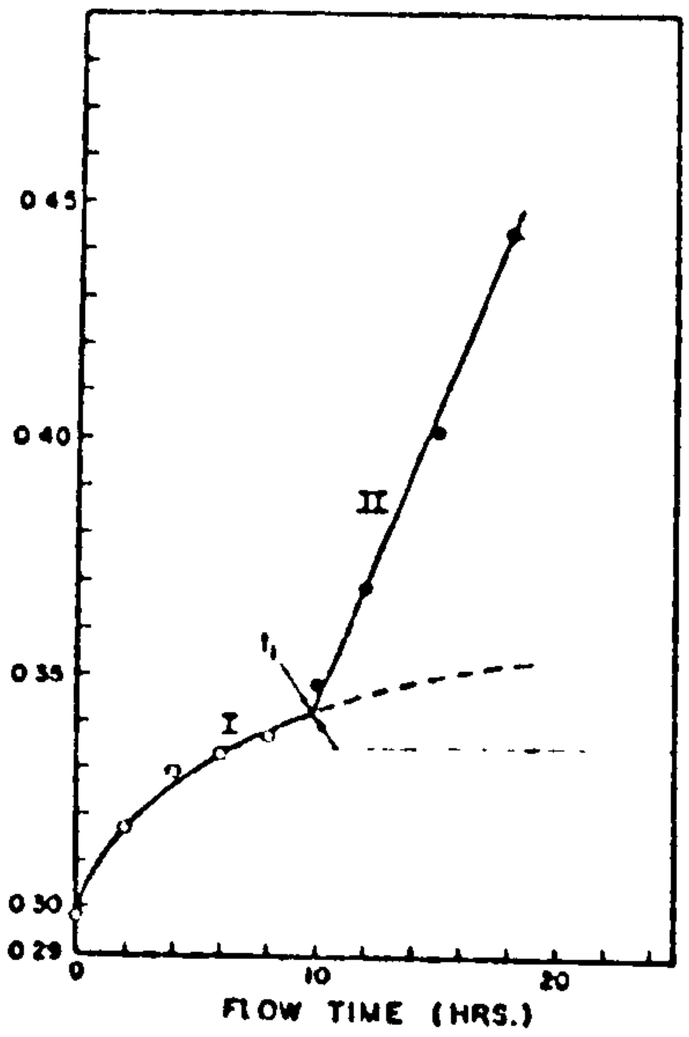

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Figure 10. Coagulation of α-FeOOH sol during locally turbulent flow in loop of Tygon tubing (28). I. In absence of any liquid–gas interface. II. In presence of single air bubble in turbulent section of flow. t_1: time at which bubble is introduced. Flowrate: 150 ml/min.

Fig. 3. The Effect of the Presence of a Single Air Bubble on the Rate of Coagulation of a FeOOH sol (Heller).

Ward (60) and Balderman (61) have shown that the removal of gases from blood decreases its propensity to form clots, both in vitro and during cardiac surgery, if a single bubble were introduced into, or generated by cavitation in, the fluid in the region of a heart valve, for instance, platelet aggregation might well be greatly enhanced. This is borne out by observations of deep sea divers during decompression (101-103), where microbubbles are released into the blood stream and enhanced platelet aggregation occurs. On the Bjork-Shiley valve, one of the heart valves associated with thrombosis problems (21, 23, 62-64), the presence of an obstruction to the flow - in this case a disc not opening fully - can create vortices in the valve field. If a series of small bubbles were introduced into these vortices, greatly enhanced coagulation and deposition onto the valves might be expected. Since blood contains large volumes of dissolved gases, a small negative pressure, which has been shown to exist in the region of the valve (65), may be sufficient to dissociate

bubbles, and hence cause clotting and aggregation. However, Bernstein et al (66) found that, using a jet stream injection device designed by Forstrom (67) to provide controlled shear stress fields, no significant changes in the oxy-haemoglobin dissocation curve were observed.

Thus the incidence of clotting on, for instance, the Bjork-Shiley valve might be due to any or all of the following fluid mechanical factors:- negative pressure (causing cavitation), high shear stress during the opening and closing cycles, trapped turbulent vortices and stagnation over a large area. There is, at present, no obvious way of isolating these possible causes, or assessing the effect of design changes on individual factors for the Bjork-Shiley valve.

The hydrodynamic correlates of clotting phenomena are therefore extremely complex, and it is accordingly unlikely that a hydrodynamic study by itself using continuum fluid mechanics could ever satisfactorily be used to predict the propensity of a device to clot. Effler (68) goes so far as to suggest that the deposits found on heart valves are not clots in the accepted sense of the word, but, a rejection mechanism due to the treatment of the sewing rings in the bleaching process.

An explanation for some of the confusion as to the cause of clots by different authors may be as follows. It seems probable that there are two distinct types of deposition which may occur. One is caused by the presence of a site, which due to some local fluid mechanical, or surface effect, acts as an inception point from which a thrombus may grow. A clot caused by this will be of a discrete nature. The other type of deposition is caused by a change in the blood chemistry, possibly due to haemolysis, which creates a state of hypercoagulability. The deposit found then will be very much more general in nature, much as described by Effler, which seems

much more plausible than a rejection mechanism.

1.3 The Use of Blood In Vitro

In view of these difficulties, in the way of a more fundamental approach to the prediction of thrombogenicity, one is forced to consider more directly empirical methods. One such technique, alternative to the use of in vivo methods of assessing thrombogenicity, is the use of blood in an in vitro system. Working in vitro removes the problem of lack of control over the experiment, but, due to the differences between human and animal blood, the use of human blood would be desirable in such experiments.

A single pass system is vital to avoid recirculating clots, and the flow rates and conditions must approximate to those found in vivo. For this reason, an apparatus such as that used by Wright (69) for testing the haemolysis caused by prosthetic heart valves could not be used to give realistic indications as to the likely propensity of valves to cause clots. It is also very difficult to use blood in vitro without treating it with anti-coagulants at some stage, and, although the anti-coagulant properties can be reversed, one cannot know exactly how this will affect the coagulative properties of the blood. The risk of infection to the experimenters, many of whom may be engineers, unused to working with blood, cannot be overlooked, and it is difficult to obtain sufficient blood, even animal, for experiments on the most moderate scale.

1.4 Blood Analogue

A possible solution to these difficulties would be to use a blood analogue, that is to say, a fluid whose behaviour in the relevant circumstances has been shown to be similar to the behaviour of blood. This fluid must be:- readily available, of reproducible properties and composition, cheap, safe to use, and as similar as possible to blood in respect of its

essential clotting properties and in the adhesiveness and rheology of the formed clot. The last of these is important, since thrombus formation is a cumulative process and once a deposit has begun to form it is important to be able to observe its development.

Several fluids have been used for coagulation studies, FeOOH sol by Heller (58), FeOOH and CuO sols by Freundlich (70, 71) Dow polychlorostyrene latex by Heller and Delauder (72), and magnetite or Fe_3O_4 was used by Newson (73).

If one is to mimic the coagulation and deposition of blood which is a biological fluid, and whose clotting is due to the action of a specific enzyme, thrombin, it would be appropriate for the analogue to have similar characteristics. None of the fluids listed above has these properties, whereas rennetized milk does. Other less well known and more obscure enzymatic clotting processes are;- the conversion of ovalbumin into plakalbumin by subtilisin (74), and the formation of collagen fibres from procollagen by procollagen peptides (75).

Since it is not proposed that the analogue should be able to mimic those effects which are attributable to blood chemistry or surface effects, but merely those relating to hydrodynamic effects, it is not essential that there should be any more than a gross overall similarity between the fluid used and blood itself. However, it is worthwhile to consider milk, blood and their coagulation reactions to see if there is any great similarity between them, as several authors have remarked upon this.

The idea that milk should behave analogously to blood is not new, for as Thomas Coghan said in 1584 (76), "Milke is made of bloude twice concocted.....for until it comes to the paps it is plain bloude, but afterwards by the proper nature of the paps it is turned into milke," and in the 9th century

Isaac Israeli said, "Lac non est aliud nisi sanguis coctis in uberibus" (77).

Many people have noted the similarity between the coagulation of blood and milk, notably, Berridge (78, 79), in whose work on the adhesion of coagulating casein to various surfaces, a curious phenomenon was observed: when a section of beef aorta had coagulating casein passed over it, no deposition was found on the surface which would normally be in contact with the blood, but a large quantity of deposit was observed on the cut ends. Scott-Blair (80, 81, 82) has done a great deal of work on the coagulation of both milk and blood, and has noted the similarities between the two. Jolles (83, 84) 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. He concludes as follows:- "That the enzymatic cleavage of both fibrinogen and k-casein may proceed via similar mechanisms is suggested from the evidence that (a) a certain degree of homology exists between the primary structures of cow k-casein and the B_b and chains of human fibrinogen and (b) the sequences surrounding the thrombin/chymosin-sensitive linkages of these two proteins determine in their part their susceptibility to thrombin/chymosin hydrolysis. The chemical modification studies on fibrinogen together with the recent work carried out on k-casein, support this hypothesis and indicate that the histidine residues of both k-casein and fibrinogen, especially those of the B_b chain, play an important part in the interactions between these two proteins and their respective proteases".

1.5 The Milk Clotting Process

The clotting of milk by chymosin, the predominant protease in the fourth stomach of the young calf, includes three main phases. The first of the phases is the action of the chymosin on k-casein, the protein in milk involved in the clotting process, and involves the release of caseino(glyco)peptide (85). The second is the formation of the coagulum, which is caused by the casein micelles aggregating in the presence of calcium ions, and in the third phase (86) the casein components are slowly hydrolized.

The approximate composition of cow's milk is as follows (87, 88, 89):-

water	87%
fat	4%
sugar	5%
casein	3%
other protein	0.4%
ash	0.6%

Approximately 80% of the protein in milk is present as a stable suspension of relatively large spherical calcium proteinate-phosphate particles, called micelles, which vary in diameter between 40 and 300 nm. (90). The micellar protein, or casein, consists of two groups of components, one soluble and the other insoluble in the presence of Ca^{2+}; the insoluble components are a_s-casein and b-casein, whereas k-casein is soluble. The k-casein stabilizes colloidal dispersions of calcium caseinate and is identified by Waugh and von Hippel (91) as the protective colloid. A number of different models have been proposed to describe the structure of the casein micelle. There are two distinct fields of thought: on

the one hand that all the k-casein is located on the coat (92); alternatively, that it is distributed throughout the micelle (93). However, recent evidence suggests that both are true and k-casein is located within the micelle as well as on the surface (94).

The micelle-stabilizing properties of the k-casein are destroyed by the action of the chymosin; a phenylalanyl-methionine linkage is split (95), yielding a soluble fraction containing the caseinoglyco-peptide and an insoluble para-k-casein, which complexes with a_s-casein and forms the strands of the clot (96). This can be summarized as follows:

$$\text{k-casein} \xrightarrow[\text{ph } 6.8]{\text{chymosin}} \underset{\text{(insoluble)}}{\text{para-k-casein}} + \underset{\text{(soluble)}}{\text{caseinoglyco-peptide}}$$

Green (97) and Doolittle (98) have shown the importance of Ca^{2+} concentration on the rate of aggregation in the secondary phase of the milk clotting process. The secondary phase is not yet fully understood, but it does not appear to happen until the substate has been completely modified (99), although there is some doubt about this (100).

The clotting time, being the time till completion of the secondary stage, is usually inversely proportional to the concentration of chymosin. Temperature is very important; increasing temperature decreases the clotting time, although temperatures above 43^{o}c will render the enzyme less active. Ph is also important; the clotting time falling with falling Ph (104).

There are other mechanisms by which milk can be coagulated namely; heat coagulation (105, 106) and through souring,

where development of lactic acid hastens coagulation (104).

The process of milk coagulation is not yet fully understood, but a great deal of recent work has been done (78, 83, 84, 99, 100, 107-113).

1.5.1 Fluid Mechanical Effects on Milk Coagulation

In contrast to the great volume of literature on the effects of fluid mechanical phenomena on blood coagulation, little has been written on the effects of the same phenomena on milk coagulation.

Agitation, particularly in the presence of air, and homogenization in which milk is forced through small passages under pressure (e.g. 2000 psi) at velocities of approximately 600 ft/sec, both result in the breakdown of the fat globules. This results in the formation of smaller globules whose membrane is of different composition to the original. As well as causing smaller globules to be formed, agitation can cause the release of free fat into the plasma, and aggregates of fat globules to be formed (114).

The destruction of the original fat globule membrane allows enzymes, such as lipase, to attack the fat causing lipolysis (115-117), which is an enzymatic digestion of the fat to give free fatty acids, which will inhibit the action of rennin. However, homogenization or agitation will hasten coagulation, in spite of the decreased rennin activity, although the firmness of the milk curd formed will be reduced (224).

It seems probable that fluid mechanical phenomena will have the same effect on the deposition of milk as they do on blood. However, to establish that this is the case,

a series of experiments have been performed. These are described later and the results obtained suggest that the analogy does, in fact, hold.

1.6 Blood Coagulation

There are two distinct types of blood coagulation:- haemostasis and thrombosis. Haemostasis occurs in stationary blood and is the "normal" coagulation (118). Thrombosis is the deposition of blood constituents from flowing blood, and is not equivalent to haemostasis, but is a distortion of the haemostatic process.

1.6.1 Haemostasis

Several biochemically orientated reviews of the clotting factors invlved in blood coagulation have appeared in recent years (119-121). A 13 factor cascade mechanism has been postulated comprising two separate pathways, the intrinsic and extrinsic systems.

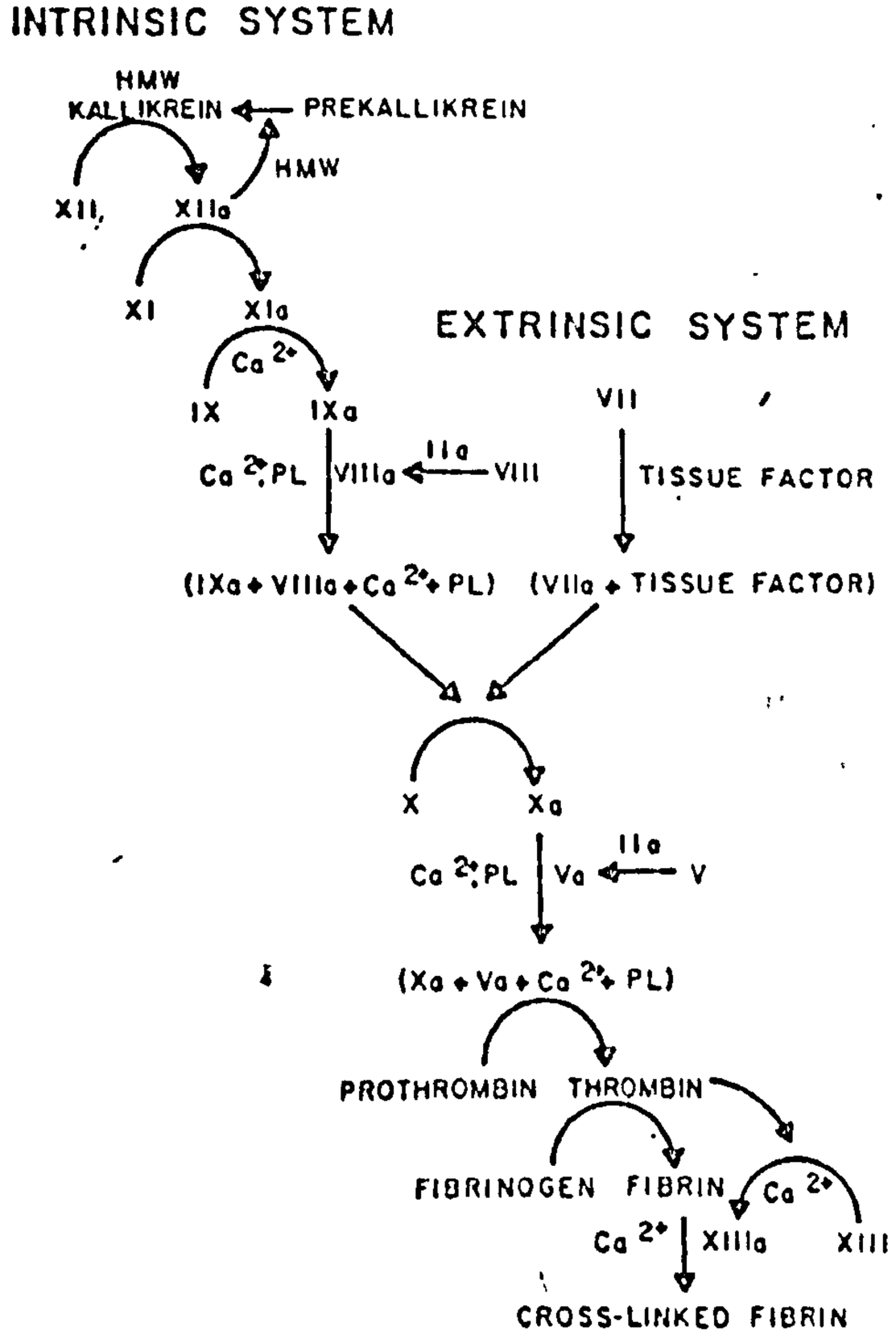


Figure 4. Cascade Mechanism of Blood Coagulation

The intrinsic pathway involves only blood factors, whereas the extrinsic pathway requires the participation of a tissue lipoprotein (tissue factor). The pathways converge at the level of activation of factor X. The operation of both seems necessary for effective haemostasis; deficiencies of factors in either result in a haemorrhagic state.

	Common name	Molecular weight[a]	≃Concentration in 1 ml plasma
Factor I	Fibrinogen	340 000 (330 000)	3 mg
Factor II	Prothrombin	72 000 (38 000)	200 µg
Factor III	Tissue factor	220 000–320 000	0
Factor IV	Calcium ions	–	–
Factor V	Proaccelerin	290 000–400 000	?
Factor VI	Activated V	–	
Factor VII	Proconvertin	63 000 (63 000)	2 µg
Factor VIII	Antihaemophilic factor	?	?
Factor IX	Christmas factor	55 400 (46 500)	3–4 µg
Factor X	Stuart factor	55 000 (40 000)	6–8 µg
Factor XI	Plasma thromboplastin antecedent	160 000 (160 000)	7 µg
Factor XII	Hageman factor	90 000 (90 000)	40 µg
Factor XIII	Fibrin stabilizing factor	320 000 (140 000)	
	Prekallikrein	88 000 (88 000)	25–40 µg
	High molecular weight kininogen	160 000	80 µg
	Protein C	62 000 (60 000)	5 µg
	Protein S	69 000	?
	von Willebrand factor	>1.5 million	7 µg

[a] Molecular weight on activated form given in parentheses.

Figure 5. Blood Clotting Factors.

Many of the reactions share a requirement for phospholipids and Ca^{2+} for rapid reaction rates. Tissue factor probably provides the lipid for the extrinsic pathway, and platelets supply the phospholipid for the intrinsic pathway (10, 39). The phospholipid is thought to play an important part in organising and localizing the participants in the reactions (122), and also by preventing inactivation of the factors by naturally occurring inhibitors in plasma (123). The rate of conversion of prothrombin to thrombin is increased five hundred times by the presence of phospholipid.

1.6.2 Fibrinolysis

The reverse process to blood coagulation is known as fibrinolysis, which causes the dissolution of a clot by the degradation of fibrin to water-soluble fibrin degradation products. This hydrolysis is accomplished by a serine protease, plasmin, which is generated from its zymogen, plasminogen, by substances termed plasminogen activators, which are present in many body fluids and tissues. Factor XXII plays a role in initiating fibrinolysis and recent studies show that this activity may be activated by kallikrein (124).

The existence of fibrinolysis is important with prosthetic implant devices for it means that if a clot forms, for example on a heart valve, it will not remain in situ indefinitely, but will eventually dislodge and enter the circulation.

1.6.3 Thrombosis

Much has been written about thrombosis over the past few years (12, 14-16, 33, 39, 45, 125-128). Petschek's group (33) have done much work on the sequence of thrombus formation, using their stagnation point flow chambers (see later). The sequence of thrombus formation in this particular flow system, in which a jet of fresh arterial blood was made to impinge normally on a plane surface, was found to be as follows:-

1) Within ½-1 minute a protein layer is deposited evenly over the surface (129).
2) This is followed by the formation of a monolayer of platelets (126).
3) If a high flow rate exists over the surface, no further development takes place for periods of up to 3 hours, or, thrombus may form and grow on local

imperfections, leading to wedge thrombi.

4) Under conditions of low flow, a white cell circle of shear limited radius, centred on the stagnation point, is formed.

5) Symmetric thrombi may form in the centre of the stagnation area.

Thus the sequence of deposition onto a prosthetic device would be:-

a) Protein layer deposition, which occurs in all cases independent of material.

b) Platelet adhesion, which occurs in all cases independent of material.

c) White cell deposition in areas of low flow, or on thrombogenic materials.

d) Thrombus growth in areas of low flow and on thrombogenic materials.

The thrombus growth will be similar to the blood coagulation mechanism described above.

1.7 Comparison Between Milk and Blood Clotting

Proteolysis seems to be the triggering mechanism for both clotting reactions, and is generally casued by the action of thrombin on the fibrinogen in blood and of chymosin on the casein in milk. The presence of Ca^{2+} enhances the final stage polymerisation in both cases. Both reactions have similar kinetics (100), are favoured by the same temperature effects, and mechanical effects may cause aggregation in both cases (80, 114). Although the membranes of the fat globule and the milk micelle are much thinner than that of the blood cells, it may be possible to obtain a meaningful analogue for haemolysis in blood (115, 116, 80).

Perhaps the greatest difference between the clotting of milk and blood is that all the reactants required for the coagulation of blood are present in the fluid, whereas the clotting agent for milk (chymosin) is in the stomach of the natural recipient (119, 130).

Several experiments have already been performed comparing the coagulation of milk and blood, and there are others in which the technique used for the milk tests is sufficiently similar to one used for blood, that the results are comparable.

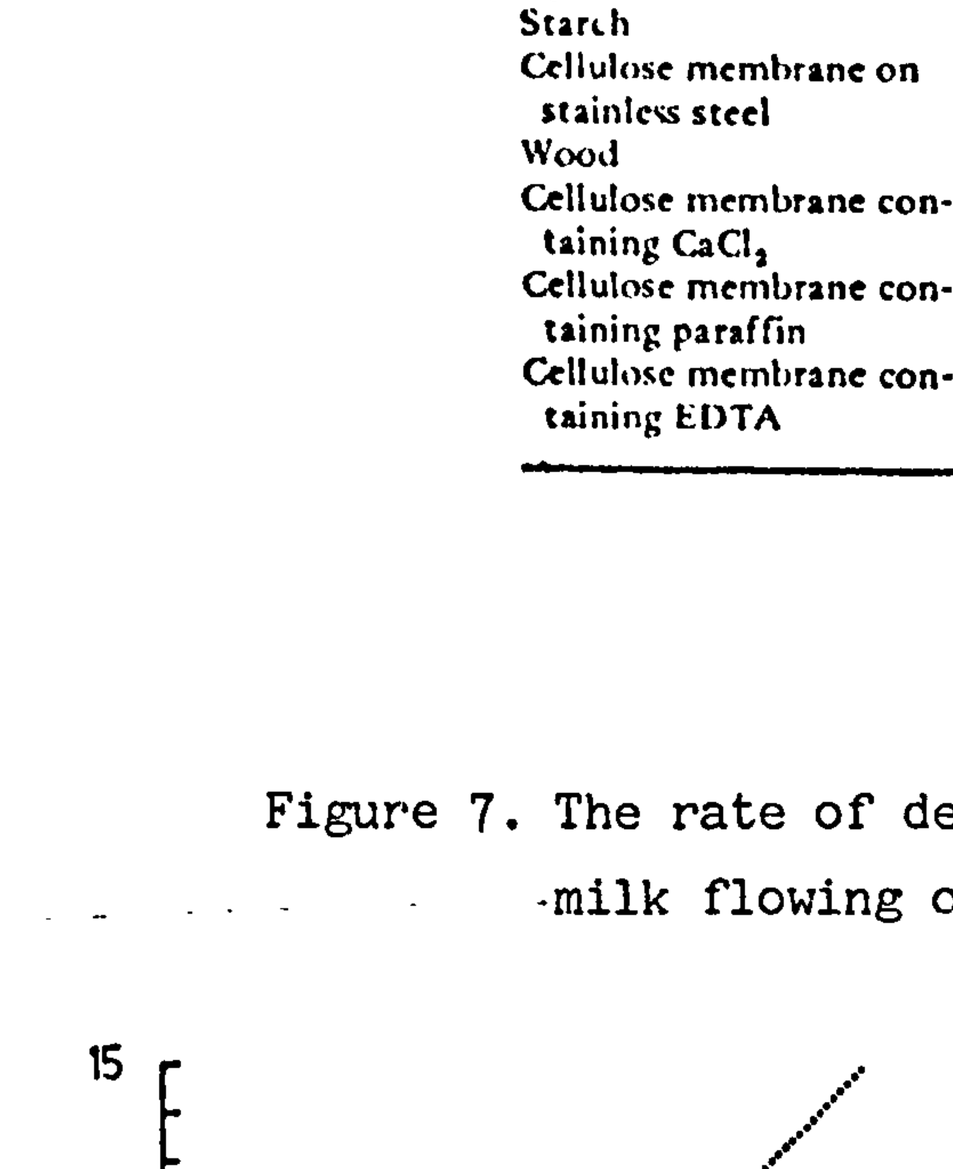
The experiments of Gott (45) and Berridge (79), in which, rings of different materials are implanted in the venous circulation, and clotting milk flows over the strips of different materials respectively, demonstrate the importance of surface chemistry (Virchow's triad) in coagulation. The effect of coating the surface of a milk test piece with lecithin is very similar to the effect of coating a Gott ring, for use in blood, with heparin, and the suggestion that the protection they afford surfaces is due to a delay in the absorption of proteins, is tenable in both cases.

Prosthetic rings	Time of implant	Amount of thrombus in lumen				
Polycarbonate	2 h	0	0	0	0	0
Polypropylene	2 h	0	0	0	0	0
Teflon	2 h	0	0	0	0	0
Silicone rubber	2 h	0	0	0	0	0
No. 304 stainless steel	2 h	0	0	0	0	0
G. B. H. coated polycarbonate	2 weeks	C	C	C	C	C
Polypropylene —H*	2 weeks	C	C	C	C	C

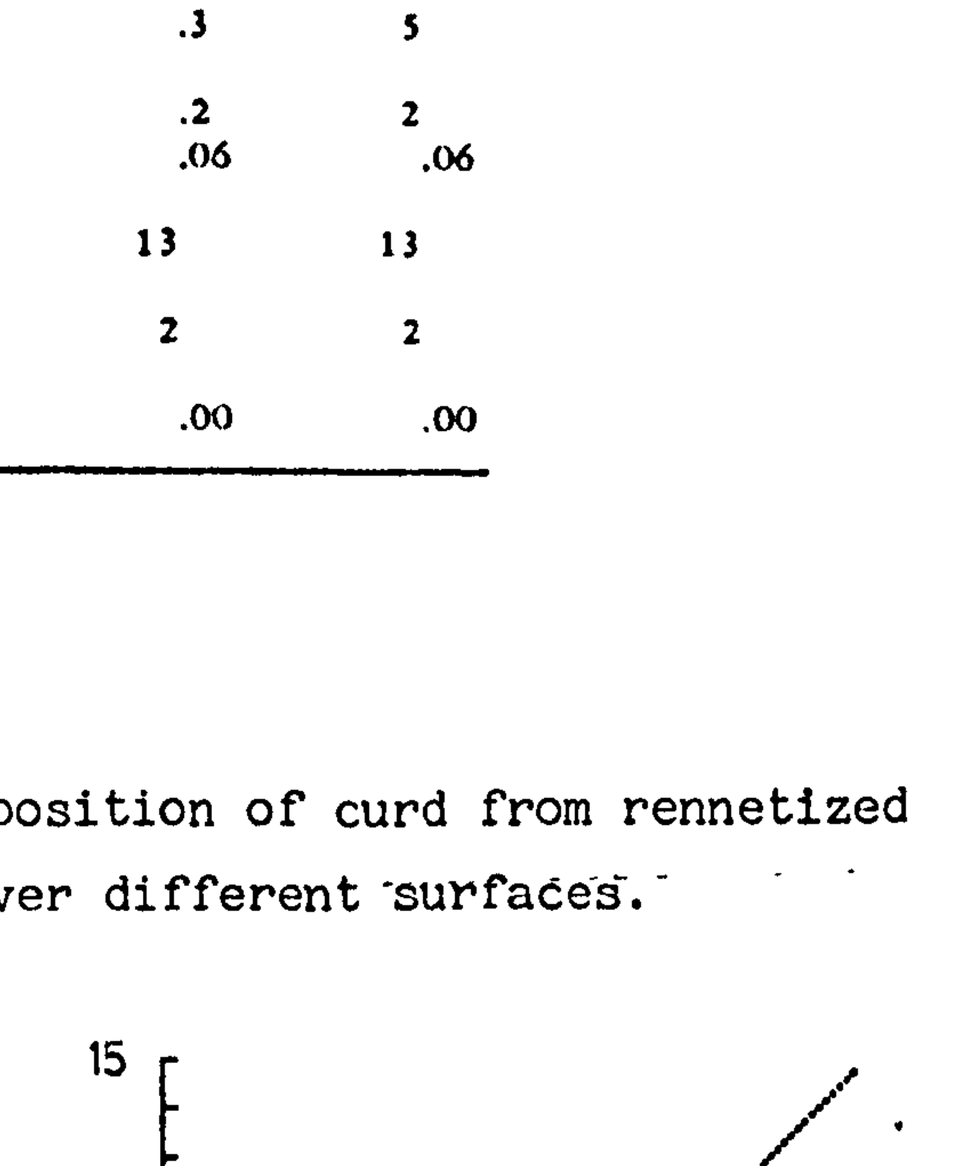
Figure 6. The Degree of Thrombosis on Gott Rings of Various Materials.

Surface	Rate of deposition	
	Mini-mum	Maxi-mum
Stainless steel	2	10
Other metals	3	20
Soft paraffin	4	4
Hard paraffin	2	5
PTFE	2	6
Cholesterol	3	3
Aorta	3	16
Polyacrylamide	4	8
Collodion	4	5
Lecithin	1	2
Lecithin and cholesterol	.6	3
Agar	.5	3
Starch	.3	5
Cellulose membrane on stainless steel	.2	2
Wood	.06	.06
Cellulose membrane containing $CaCl_2$	13	13
Cellulose membrane containing paraffin	2	2
Cellulose membrane containing EDTA	.00	.00

Figure 7. The rate of deposition of curd from rennetized milk flowing over different surfaces.



Weight of curd adhering to a stainless steel strip; • covered with lecithin, o with an equimolar mixture of lecithin and cholesterol. Dotted lines from Fig. 3.



The effect of various hydrophobic materials on stainless steel. o cholesterol, • polytetrafluorethylene, □ hard paraffin mixture, ■ soft paraffin ("Vaseline").

Figure 8. The process of deposition of curd to various surfaces.

The growth of milk curd, on the surface, found by Berridge is not steady in all cases. The solid can be dislodged in a manner similar to that of an embolus being "thrown off" in a blood system. The result for milk curd on wood is very interesting and an investigation as to whether wood is also athrombogenic would be justified.

Payens (100, 112, 113) has analysed the kinetics of the clotting of milk and blood. He has shown that the lag phase in the clotting is due to the difference in reaction order of enzymatic production and flocculation, instead of being due to the need for the substrate to have been completely modified before flocculation can begin (99). He finds that the condition for the clotting time is $t/\overline{k_s V}/2 = C$ where t is the clotting time, k_s the flocculation rate constant, V the maximum rate of enzymatic product formation and C a constant. Double logarithmic plots of t versus enzyme dilution are always observed to be linear over a wide range of enzyme concentrations, for both milk and blood.

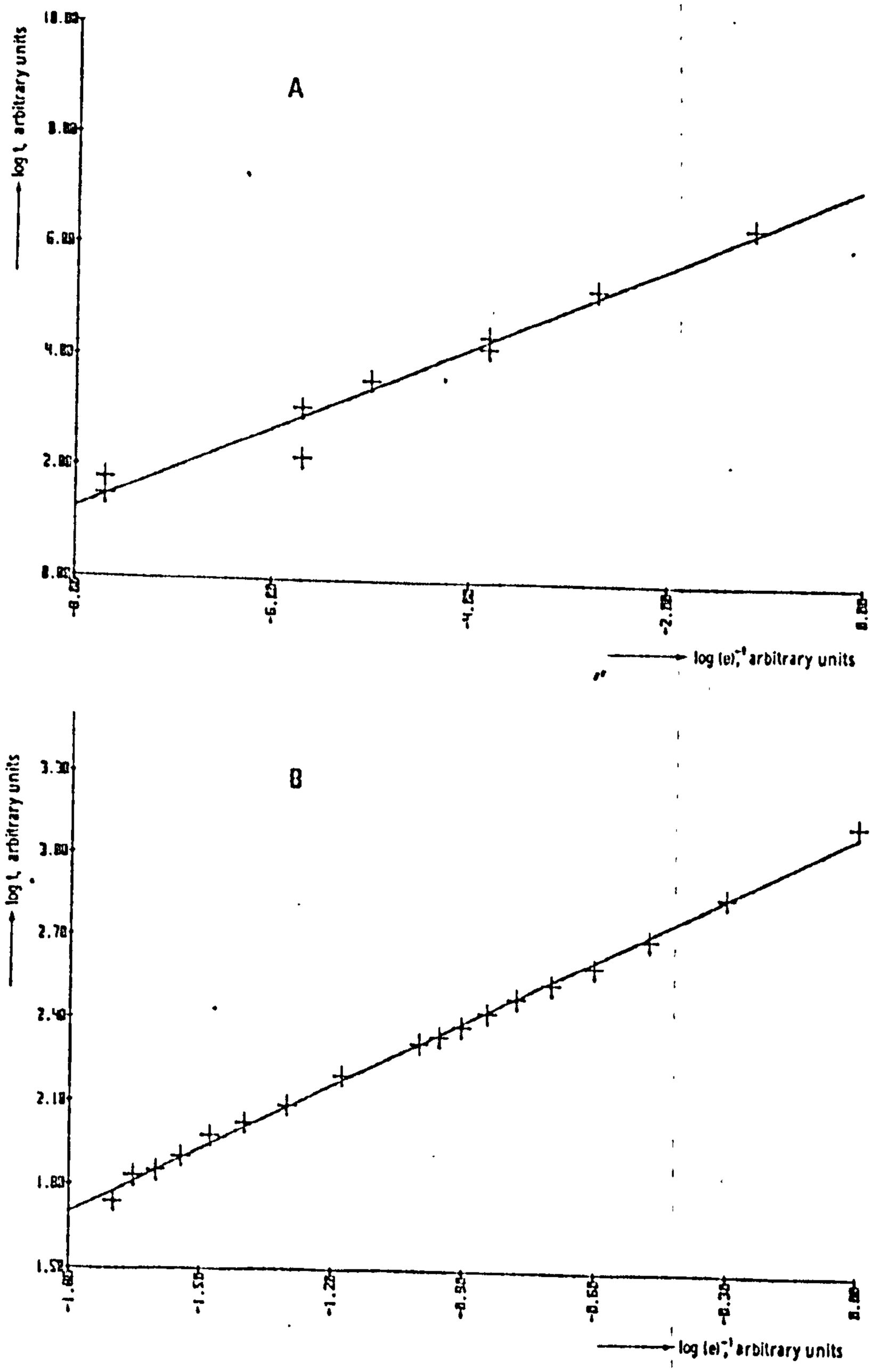


Figure 9. Typical examples of the regression of log clotting time on log enzyme dilution. (A) The clotting of citrated horse plasma by thrombin. (B) The clotting of milk by rennet.

Substrate	Enzyme prep.	Slope	$R^{2a)}$	Number of-exps.
blood plasma	lung tissue extract	0.29	0.995	18
blood plasma	chicken embryon. extract	0.41	–	–
blood plasma	chicken embryon. extract	0.50	–	–
blood plasma	chicken embryon. extract	0.68	–	–
blood plasma	thromboplastin	1.08	0.999	4
citrated horse plasma	thrombin	0.73	0.967	9
human fibrinogen	human thrombin	1.0	–	–
milk	calf rennet	0.77	0.998	18
milk	calf rennet	0.95	0.999	16
micellar casein	calf rennet	0.99	0.976	5
κ-casein	calf chymosin	0.87	0.987	4
κ-casein	M. Pusillus protease	0.83	0.992	4

Figure 10. Double logarithmic regression of clotting time versus enzyme dilution observed with the clotting of blood and milk.

Sinitsina and Palmin (131) studied the effect of salt solution soluble and water soluble proteases, isolated from leucocytes, upon milk. They found that the water soluble fraction was capable of clotting milk and raised the rennin activity, whereas the salt solution soluble fraction did not clot milk and tended to inhibit the action of rennin on the milk. They concluded with the supposition that the salt solution soluble fraction of leucocyte proteases are in their action closely related to trypsin, which does not clot milk, whereas the water soluble fraction, which does clot milk, resembles chymosin. They do not mention whether these fractions are capable of clotting blood, nor indeed do they identify which proteases they have dissolved. Nevertheless, that it should be possible to clot milk with an extract of blood so simply obtained, suggests a possible parallel between the clotting processes in milk and blood.

That the two fluids are genetically related is supported by work done by Fiat et al (132), who found that there is an immunological cross reactivity between bovine fibrinogen and bovine k-casein.

In the U-tube gelometer experiment described by Scott-Blair (80), pressure is applied alternately to one side and the other in a U-tube containing clotted fluid and the strain in the clot for a given stress is measured.

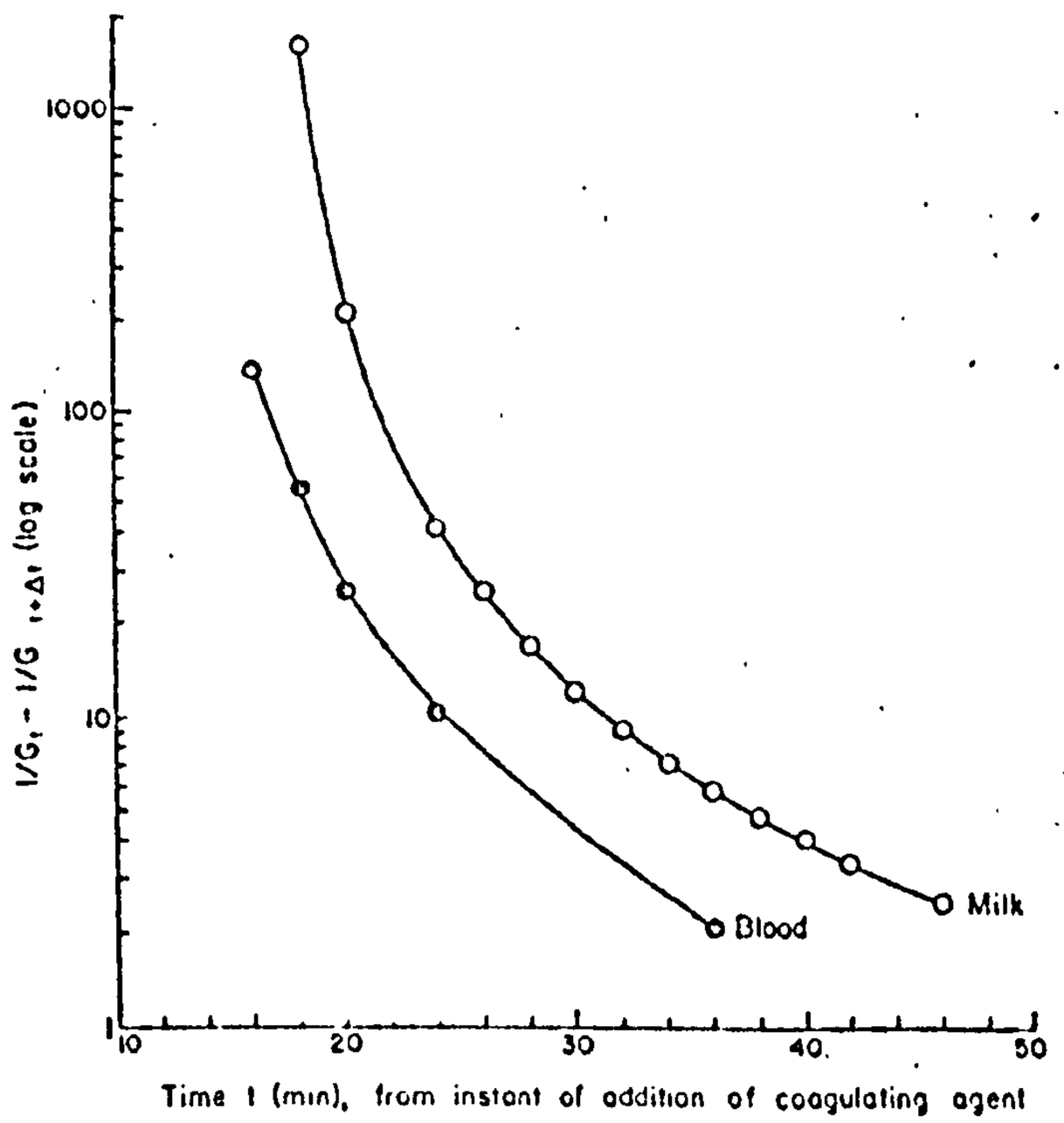


Figure 11. Plots of Logistic Equation for coagulation of milk by rennet and blood by plasma geloplastin.

This experiment was originally used in a comparison between milk and blood, and shows the "general similarity of behaviour in the rheological properties of bovine

blood coagulated by plasma geloplastin and of milk coagulated by rennin". This result is important because, as has been pointed out above, one of the desirable properties of a blood analogue is that, as well as clotting in a way similar to that of blood, it should behave analogously once it has clotted.

Conclusions

Milk and blood are in many respects very different fluids. Blood, with its platelets, erythrocytes and leucocytes, and containing all the reactants required to activate the clotting mechanism within these cells and the plasma, is a much more complex fluid than milk. Milk has micelles and fat globules, which like the blood cells both have membranes. The fat globules can aggregate under agitation as can the platelets in blood. The size range of the fat globules in whole milk is from 0.1 - 6 microns, whereas the platelets are roughly 1 micron.

The protein involved in milk coagulation is k-casein and the final stage of the rennin action is:-

$$\text{k-casein} \xrightarrow{\text{chymosin}} \underset{\text{(insolbule)}}{\text{para-k-casein}} + \underset{\text{(soluble)}}{\text{caseino(glyco)peptide}}$$

The main protein involved in blood coagulation is fibrinogen and the final stage of thrombin action is:-

$$\text{Fibrinogen} \xrightarrow{\text{thrombin}} \underset{\text{(insoluble)}}{\text{Fibrin gel}} + \underset{\text{(soluble)}}{\text{fibrinopeptides}}$$

Both reactions occur significantly faster in the presence of calcium ions. In a situation in which the blood clotting reaction has already been activated, i.e. if the blood has become hypercoagulable due to the clotting agents being

released into the blood stream, the clotting reactions are very similar. Likewise, as will be shown later, if thrombosis similar to that studied by Petschek et al (12) is occurring the analogy holds. However, milk cannot be used to predict how the blood chemistry will be modified by a hydrodynamic disturbance, e.g. whether or not the presence of a heart valve will cause a state of hypercoagulability, and it is unlikely that it will be possible to use the milk analogue for testing the effects of different surface chemistry. If these limitations are borne in mind, there appears to be sufficient similarity between milk and blood, for milk to be used for predicting the flow related thrombogenicity of artificial implant devices.

1.8 Choice of Experimental Procedure

There is a wealth of published techniques for the investigation of blood coagulation (12, 13, 17, 24, 27 31, 40, 42, 45 - 48, 80, 133 - 138), and the procedure adopted for trying to establish the milk anaolgue is the repetition of a sample of techniques, using rennetized milk instead of blood and comparing the results obtained.

Some of these require specialised pieces of equipment, such as a cone and plate viscometer (17, 24, 138), or a thrombelastograph (47, 48), which were not readily available and could, therefore, be discounted as possible techniques. With others, such as the Gott ring experiments (40, 45, 46) similar experiments have already been performed using rennetized milk and there is little to be gained by repeating them (79). Others would be very difficult to repeat with milk or would give results which would be difficult to interpret (12, 13, 27, 47, 135).

The Lee White test tube study (139) is a very simple experiment, which consists of taking one ml. of blood from a vein and placing it in a small glass test tube (8 mm diameter). The tube is rotated endwise every 30 seconds, and the endpoint is marked when the blood no longer flows. This experiment is easily duplicated using a milk-rennet-$CaCl_2$ mixture and was used to find the desired concentrations of rennet and calcium chloride and the optimum temperature for uses in the early stagnation point flow experiments described later, using a clotting time of 5 mins. In this experiment the milk with 1% conc. $CaCl_2$ solution and 1% rennet essence at room temperature, behaves in a manner analogous to that of blood, the milk ceasing to flow after it has clotted, with a reasonably sharp endpoint.

The two main experiments chosen to test the extent of the analogy between the coagulation characteristics of flowing blood and those of a milk mixture having the same Lee-White clotting behaviour, were Petschek's Stagnation Point Flow Experiment (11) and Hladovec's Net Experiment (140).

In the stagnation point flow experiment blood or rennetized milk impinges normally onto a glass slide, and the patterns of deposition occurring at and around the stagnation point are observed. In the net experiment the pressure variation upstream of a net attached to the end of a tube through which coagulating blood/milk flows is continuously monitored.

CHAPTER 2

THE STAGNATION POINT FLOW EXPERIMENT

2.1 Introduction

This technique isolates the hydrodynamic and chemical factors controlling clotting on foreign surfaces exposed to flowing blood.

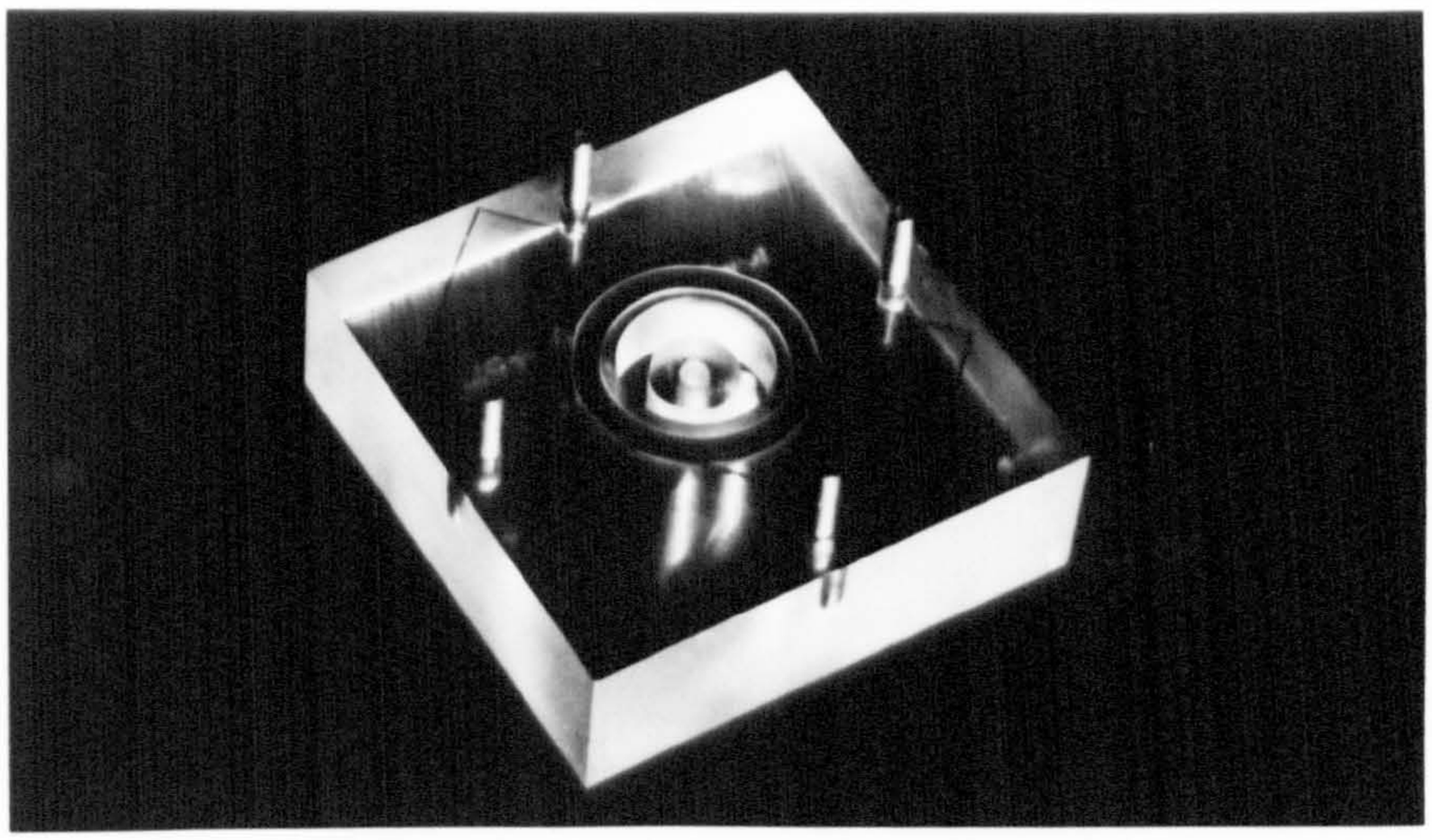


Fig 12. Photograph of perspex flow chamber used for milk deposition studies.

A jet of fresh arterial canine blood impinges normally on the flat surface under test, which forms an end wall of a closed flat cylindircal chamber coaxial with the jet. Surface deposits are examined microscopically in situ when the specimen is transparent. The system operates completely full of blood, the spent fluid being withdrawn at a controlled rate from the perimeter of the chamber at a point sufficiently far from the axis so as not to disturb the symmetry of the flow near the stagnation point. In studies of the effects of changes in nature of the test surface and the flow conditions, many patterns of deposition

phenomena occur.

The sequence of these can be summarized as follws:-

1) Initial deposition of a layer of protein over the entire surface. This occurs very quickly and Leininger (141) suggests that, for plastics, the normally negative charge is then lost.
2) A monolayer of platelets is deposited in an apparently random manner over the surface.
3) The platelets may aggregate into "clumps" randomly over the surface and/or white cells may be deposited in a circular configuration.
4) Thrombi may grow on the surface either symmetrically around the stagnation point or in wedge shaped deposits downstream of surface imperfections.

2.2 Fluid Mechanics

The formation of a white cell circle of limited radius diminishing with increasing flow is explained by Petschek et al in terms of a supposed radial distribution of shear stress.

$$\tau = \rho\beta\nu r$$

where τ is the shear stress
ρ is the density of blood
β is an empirically determined flow parameter characterizing each of the flow chambers used
and ν is the kinematic viscosity of blood
r is the radial distance measured from the central streamline.

Thus, for a given flow parameter, β, the shear rate should be zero at the stagnation point, and would appear to increase linearly as the fluid moves radially outward. The boundary

of the white cell circle is from this viewpoint, at the radius where the shear stress has a value sufficient to overcome the adhesion bond between the white cells and the surface.

An investigation of the fluid mechanics of the chamber flow, using the laser holographic technique developed in this laboratory (142) to measure mass transfer coefficients, has shown that the shear stress rises radially outwards within the inner impingement zone according to the above equation but that outside the jet orifice area shear stress falls off with increasing radius (55, 56). Thus the analysis by Petschek et al holds only within the jet region and fails to explain those white cell circles which are of diameter greater than that of the jet. It is possible that the deposition of white cells is indeed limited by shear stress, but, blood is not a continuum and one explanation of those circles may be that the fluid shear acts on the particles or cells attached to the surface and although the force may be insufficient to pull them completely away from the surface, it is sufficient to drag them radially outwards until the fluid shear force is equal to the tangential adhesive force between the cells and the surface. Furthermore, since the particles being dragged along the surface will have acquired momentum, if their density is greater than that of the bulk fluid, they may not come to rest until some distance beyond the point at which the fluid force balances the tangential adhesion force. Thus it is possible to obtain shear limited circles whose radius is such that the perimeter is outwith the range of rising shear stress but into the region of diminishing shear stress.

2.3 Milk Experiments

Three chambers similar to those used by Petschek et al were constructed in perspex. Petschek had used seven flow chambers with five different β values. Each value for β was determined empirically, and gives a measure of the shear in the chamber. β is a function of the chamber depth and the diameter of the inlet hole.

Chamber	β, (mm-sec)$^{-1}$	Support Height, h, mm	Entrance Hole Diameter, d, mm
A	1.9	1.9	2.9
B	1.9	1.9	2.9
C	8.6	0.69	2.9
D	17	0.31	2.5
E	157	0.20	1.6
F	157	0.20	1.6
G	200	0 10	1 2

Fig 13. Flow Parameter β and dimensions for Petschek's flow chambers.

In order to give a range of shear the dimensions of chambers A, D and E were used giving β values of 2, 17 and 157 respectively.

The milk was pumped into the chambers using Meltec D.N. Infusion pumps. These are variable speed screw drive syringe pumps capable of pumping between 0.13ml/hr and 133.3ml/hr per 50ml syringe. In order to give the 2ml/min which Petschek et al had used, two syringes were filled with milk and connected to one of the two pumps used, which was usually set to 66.65ml/hr giving a milk flow rate of 133.3ml/hr and a run time of up to 45 mins. The two syringes on the other pump were filled with rennet essence and a saturated solution of $CaCl_2$ respectively. The rennet essence used was a standard cheese-making preparation containing the enzyme chymosin, sodium chloride and sodium benzoate.

The liquids were pumped down separate tubes and were introduced through hypodermic needles to a piece of flexible tubing connected to the inlet to the chamber. Small bore needles were used to improve mixing, which was a problem with the very small flows used.

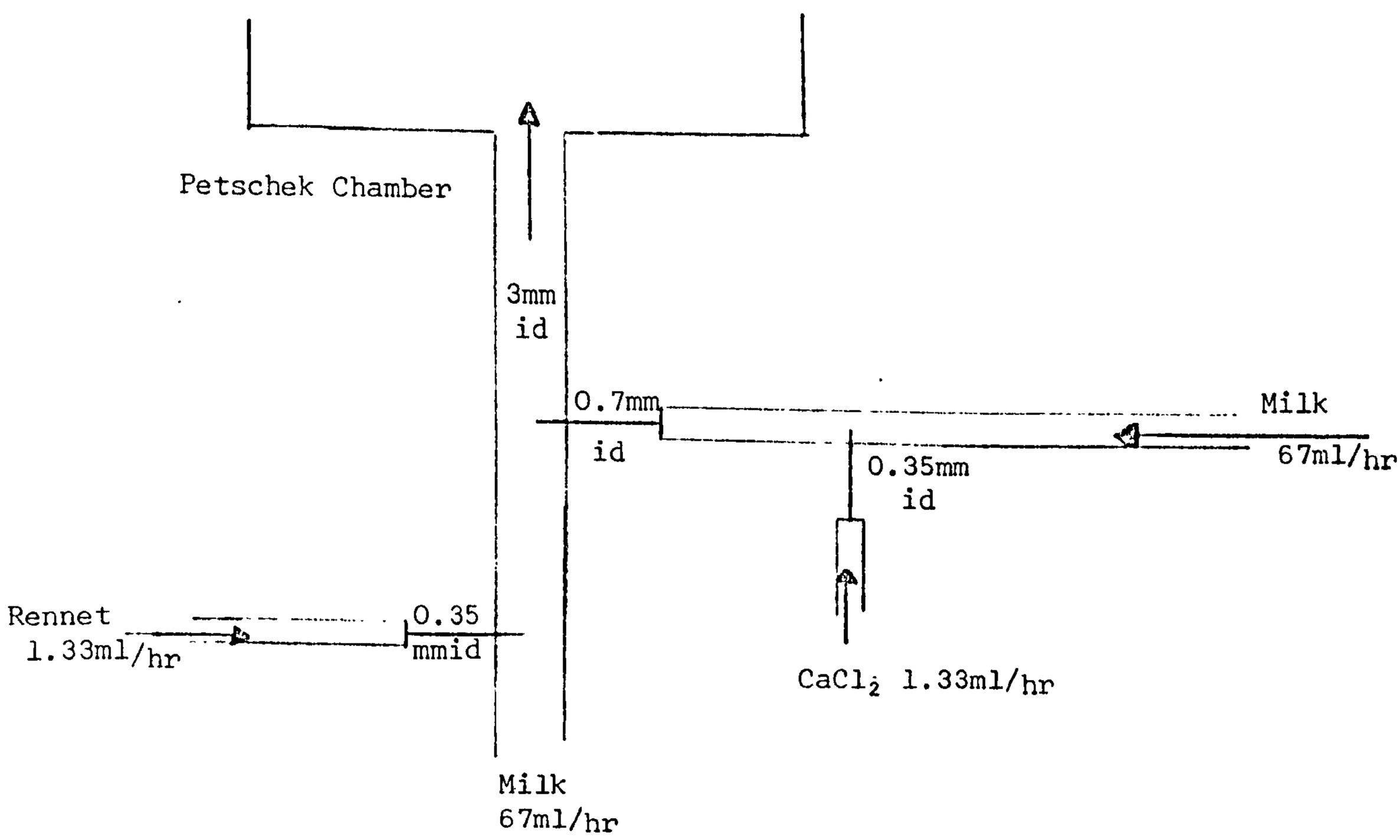


Fig 14. Arrangement used to obtain good mixing.

The pumps and syringes were placed in a thermostatically controlled oven set to the temperature required for each run. The chambers were placed on the stage of a Zeiss photomicroscope so that continual observation of the underside of the coverslips on which the milk was impinging was possible. Thin slides were required to allow continuous observation and Chance Propper No 1 24 x 50mm microscope glass cover slips were found to be sufficiently thin. Unfortunately the thin glass slides were very easy to break and great care had to be taken whilst the microscope was in use.

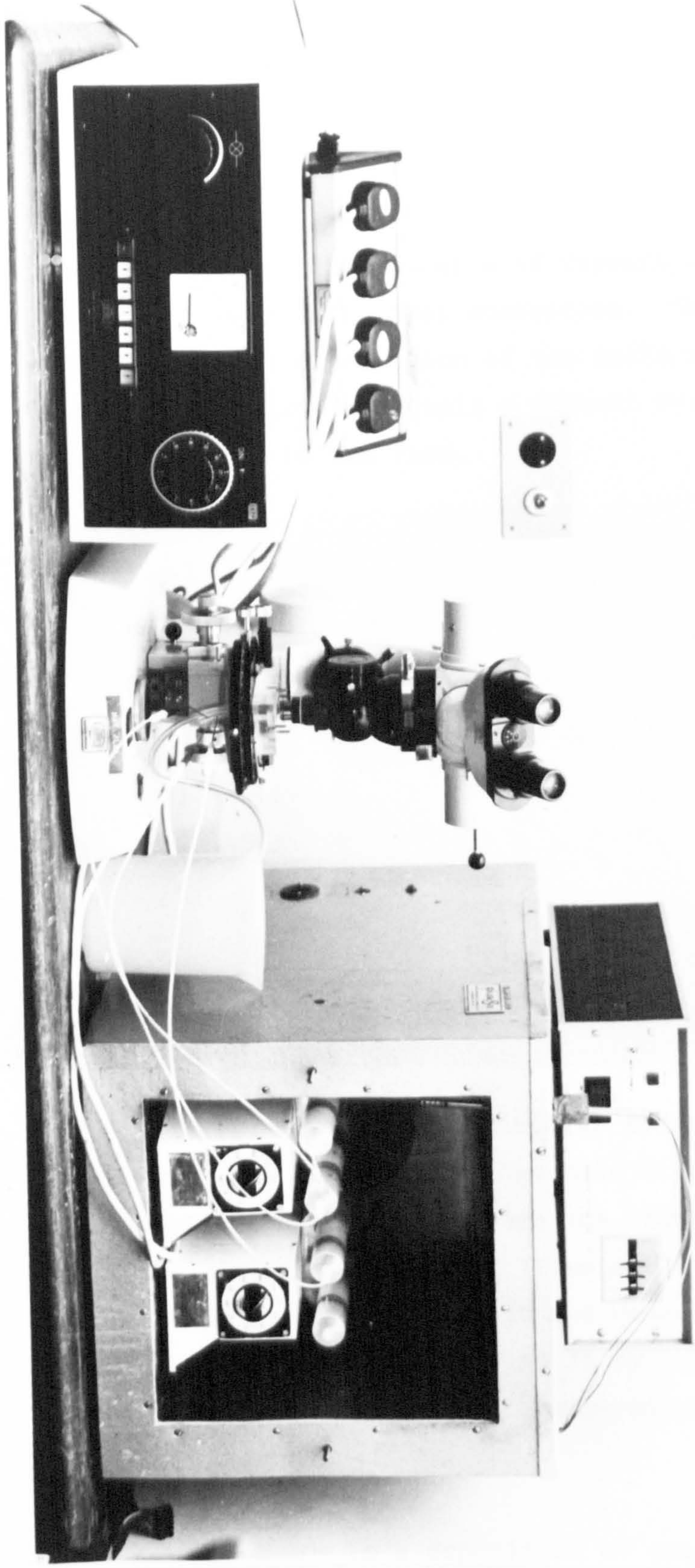


Fig 15. The apparatus used in experiments.

2.4 Results

The first step in the sequence of deposition with milk appears to be protein layer adsorption. This is largely conjectural, but observation of the surface under 800-900 magnification reveals a deposit over the entire surface exposed to the flow.

Fig 16. Milk. Slide surface outside stagnation point after run. Standard chamber has hole diameter of 2,5mm and a distance of 0.31mm between the chamber column and the slide giving a β value of 17$(mm\ sec)^{-1}$. The milk flow rate is 2ml/min throughout and rennet and cone $CaCl_2$ solution are both added to 1% by volume. The run duration was 5 minutes and the temperature 38^{o}C. Magnification 840x.

Subsequently there is a deposition of small particles onto the surface

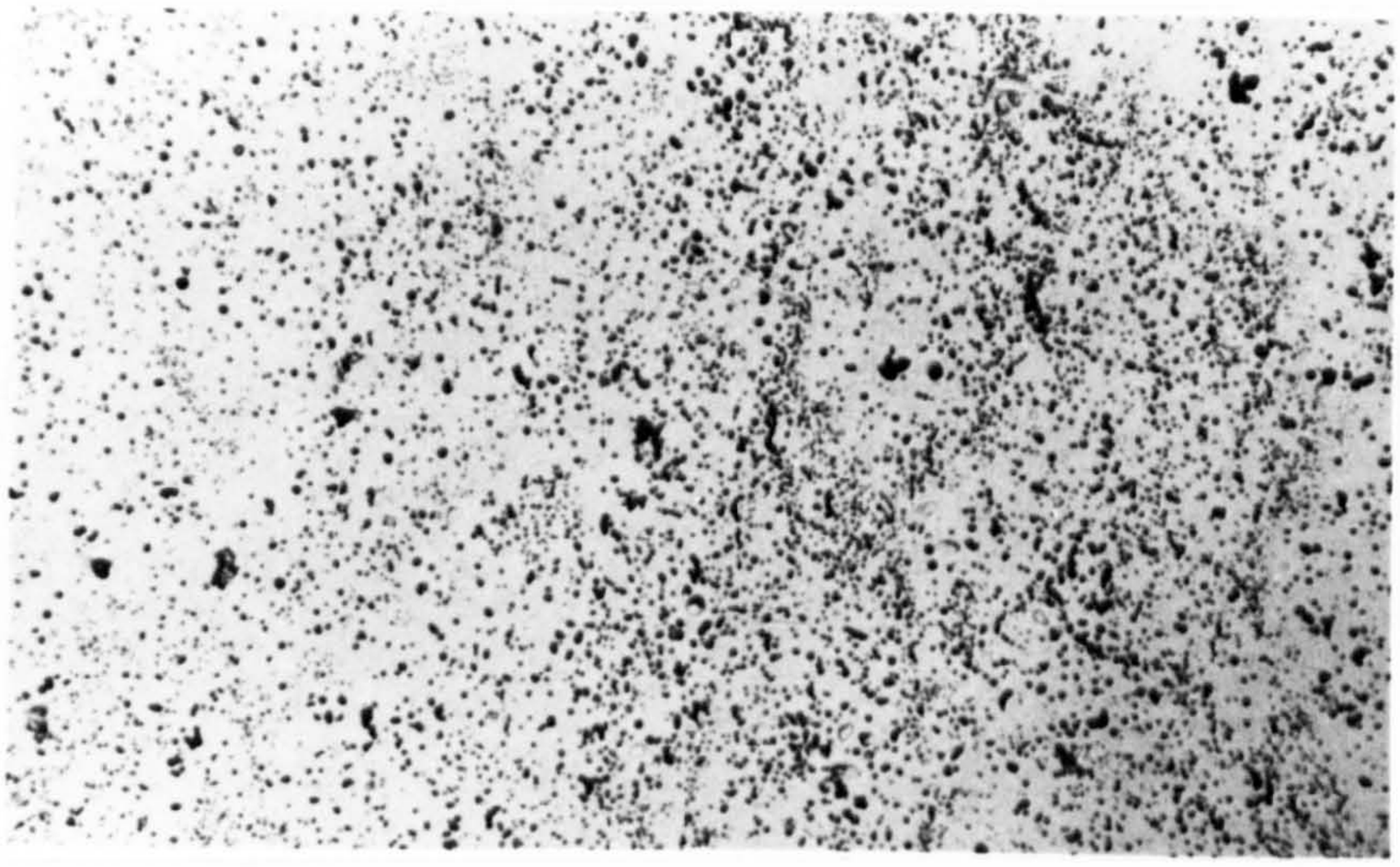


Fig 17. Milk. Slide surface at stagnation point. Low shear chamber with hole diameter 2.9mm and depth 1.9mm giving a β value of 1.9 $(mm\ sec)^{-1}$. A low temperature of 15°C and a low $CaCl_2$ concentration were used. Run Duration 30 minutes. Magnification 135x.

This parrallels Petschek's platelet monlayer deposition which was found with blood.

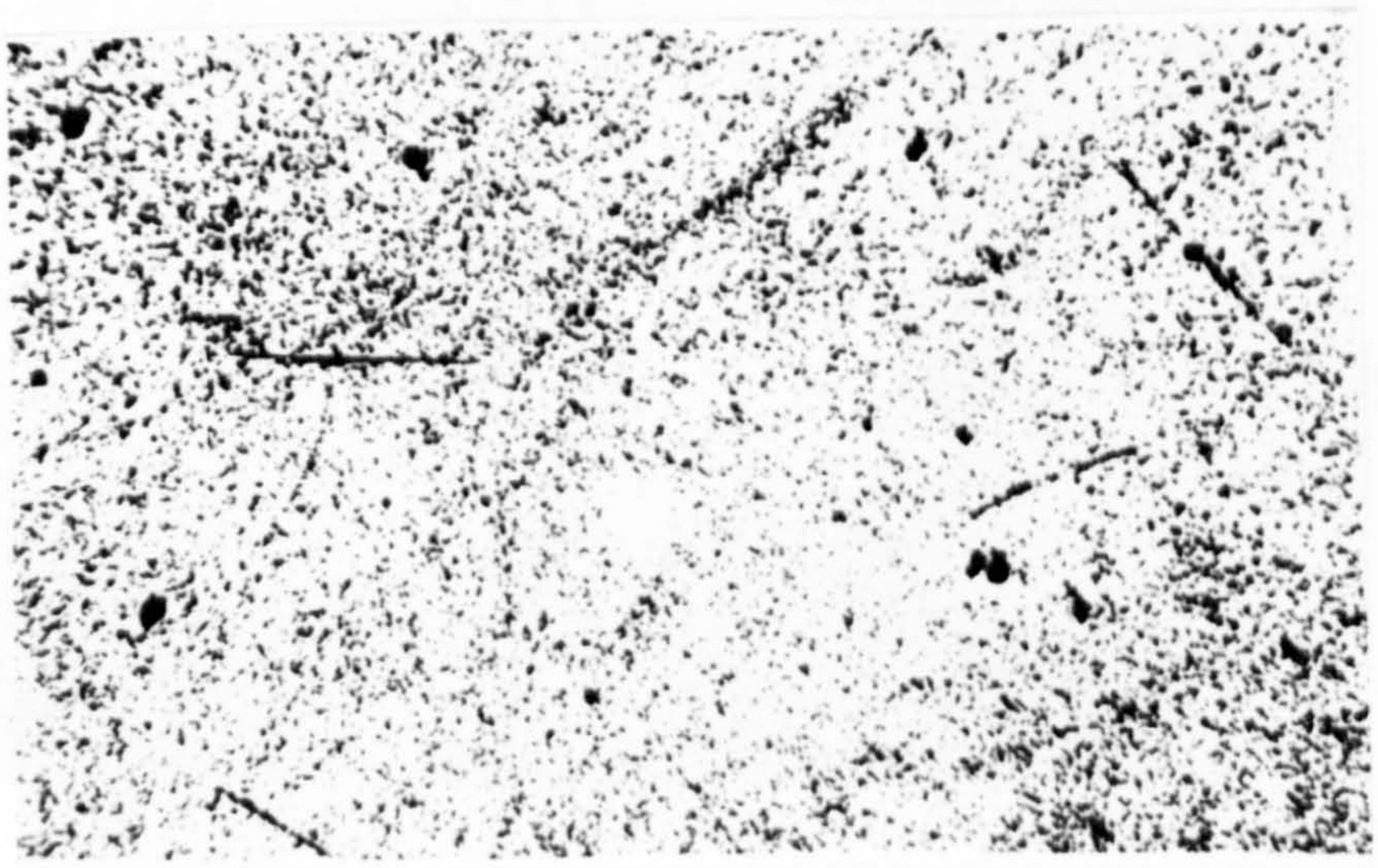


Fig 18. Blood. (Avco Everett Research Laboratory). A similar deposition pattern to that shown for milk in Fig 17 onto a LT1 carbon disc in a chamber of β-value 31$(mm\ sec)^{-1}$ after 30 minutes. Magnification 115x.

This may be followed by a deposition of larger particles similar to Petschek's white cell circles.

Fig 19. Milk. High shear chamber with hole diameter 1.6mm, and depth 0.2 mm giving a β-value of 157$(mm\ sec)^{-1}$. Deposition at stagnation point after 10 minutes. Temperature 25-30°C. Magnification 270x.

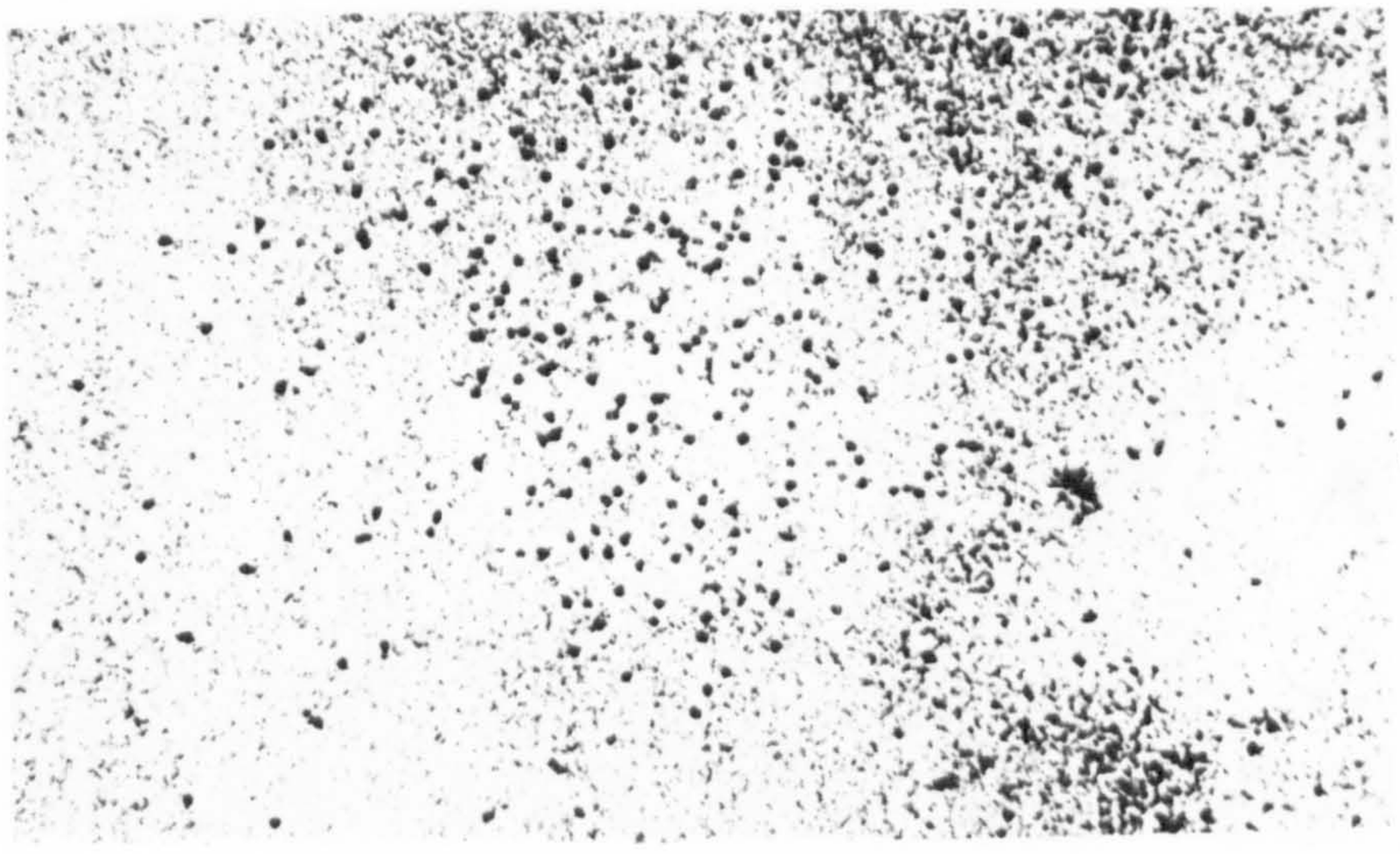


Fig 20. Blood. (Avco Everett Research Laboratory). A similar deposition pattern to that shown for milk in Fig 19 onto an ion plated carbon slide in a chamber of β-value 31$(mm\ sec)^{-1}$ after 30 minutes. This is referred to as a diffuse white cell circle. Magnification 120x.

This may be followed by a further deposition of larger particles to give a well established circle.

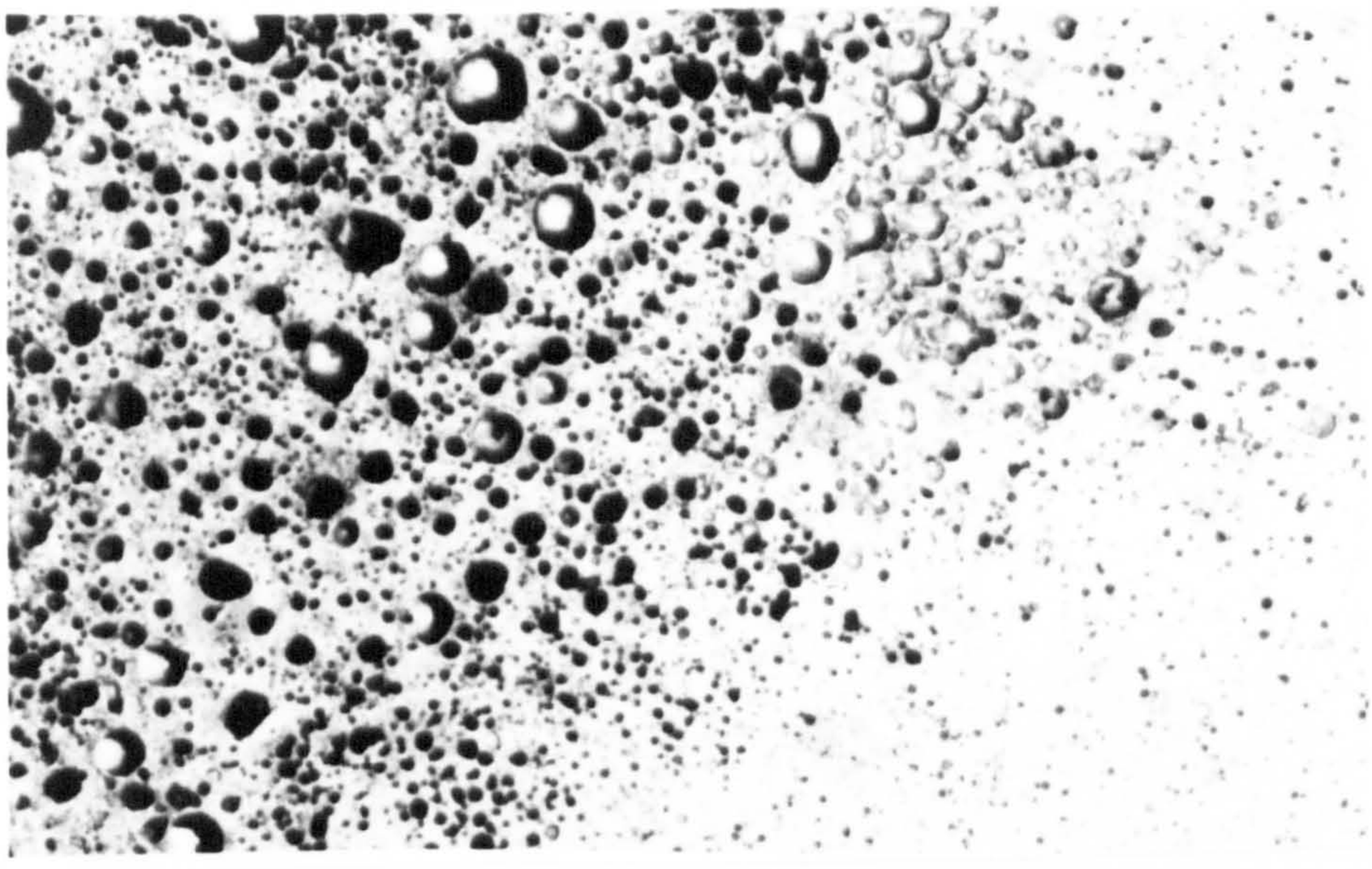


Fig 21. Milk. Stagnation point deposition in standard chamber at $25^{o}C$ after 30 minutes. Magnification 270x.

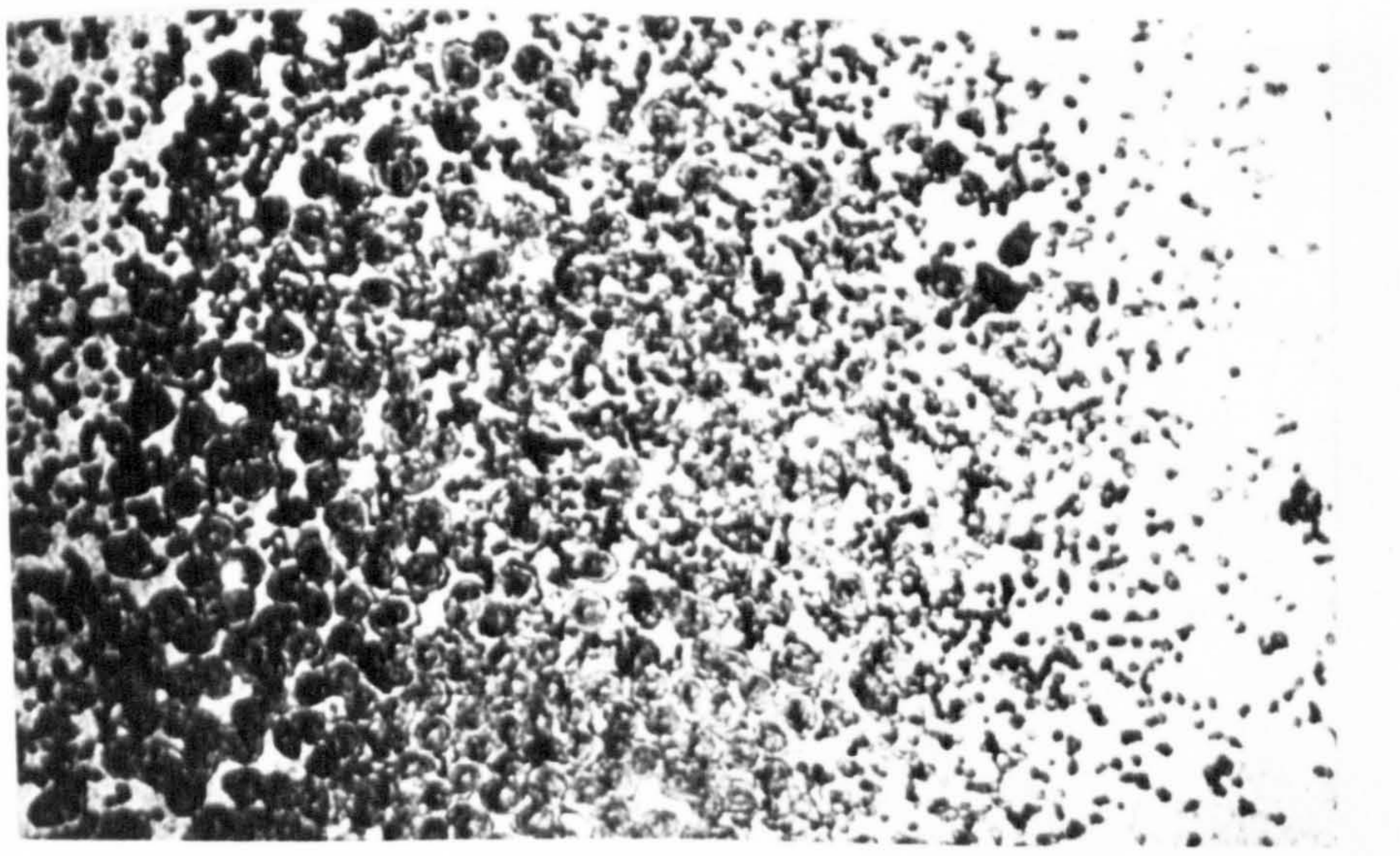


Fig 22. Blood(Avco Everett Research Laboratory). A circular deposition pattern at stagnation point on an LT1 carbon disc in a chamber with β value 2.8 $(mm\ sec)^{-1}$ after 30 minutes. Magnification 270x.

Alternatively, it is possible to observe a phenomenon similar to the start of a wedge thrombus.

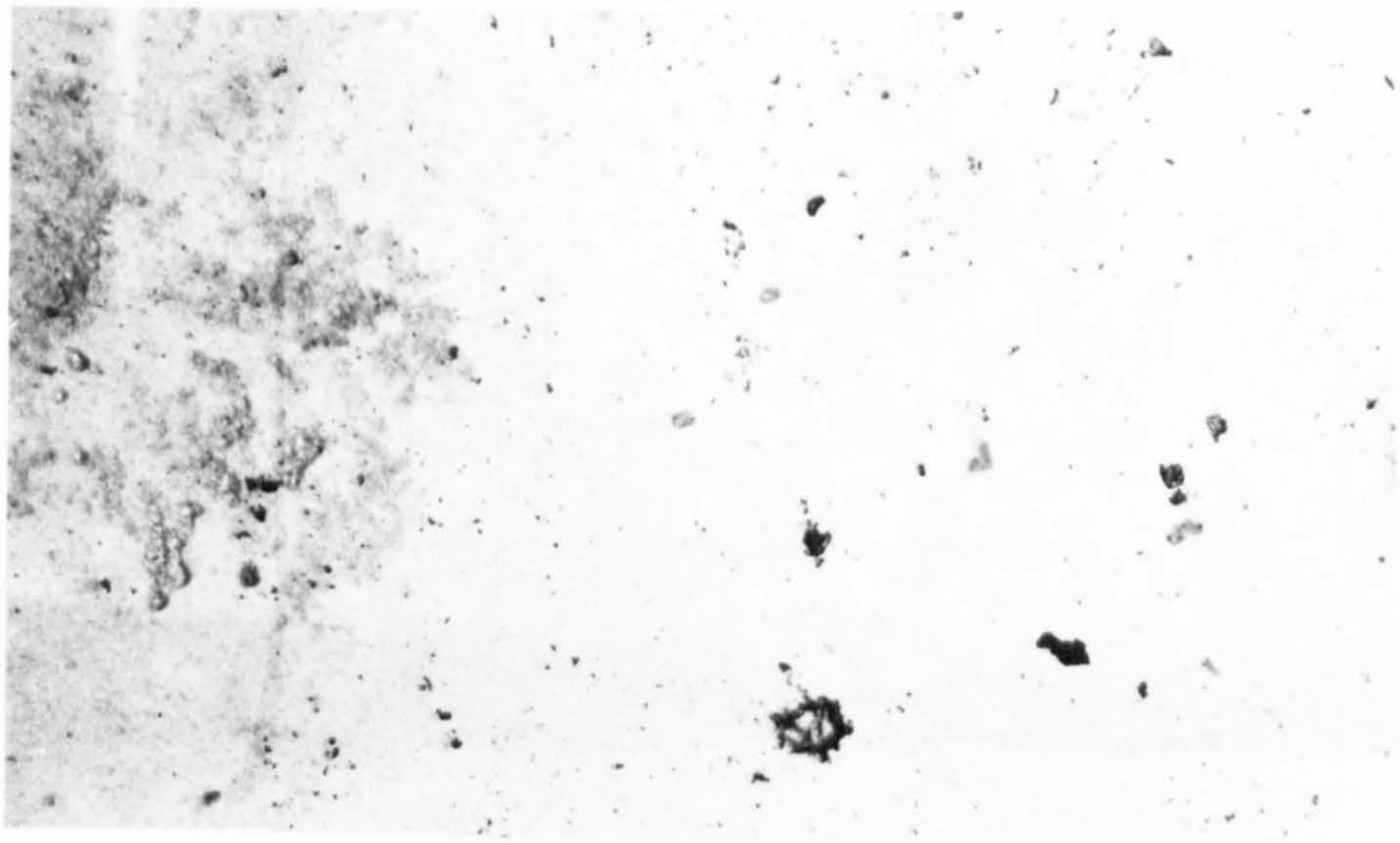


Fig 23. Milk. Deposition near stagnation point in standard chamber, at 38°C after 1 minute. Magnification 135x.

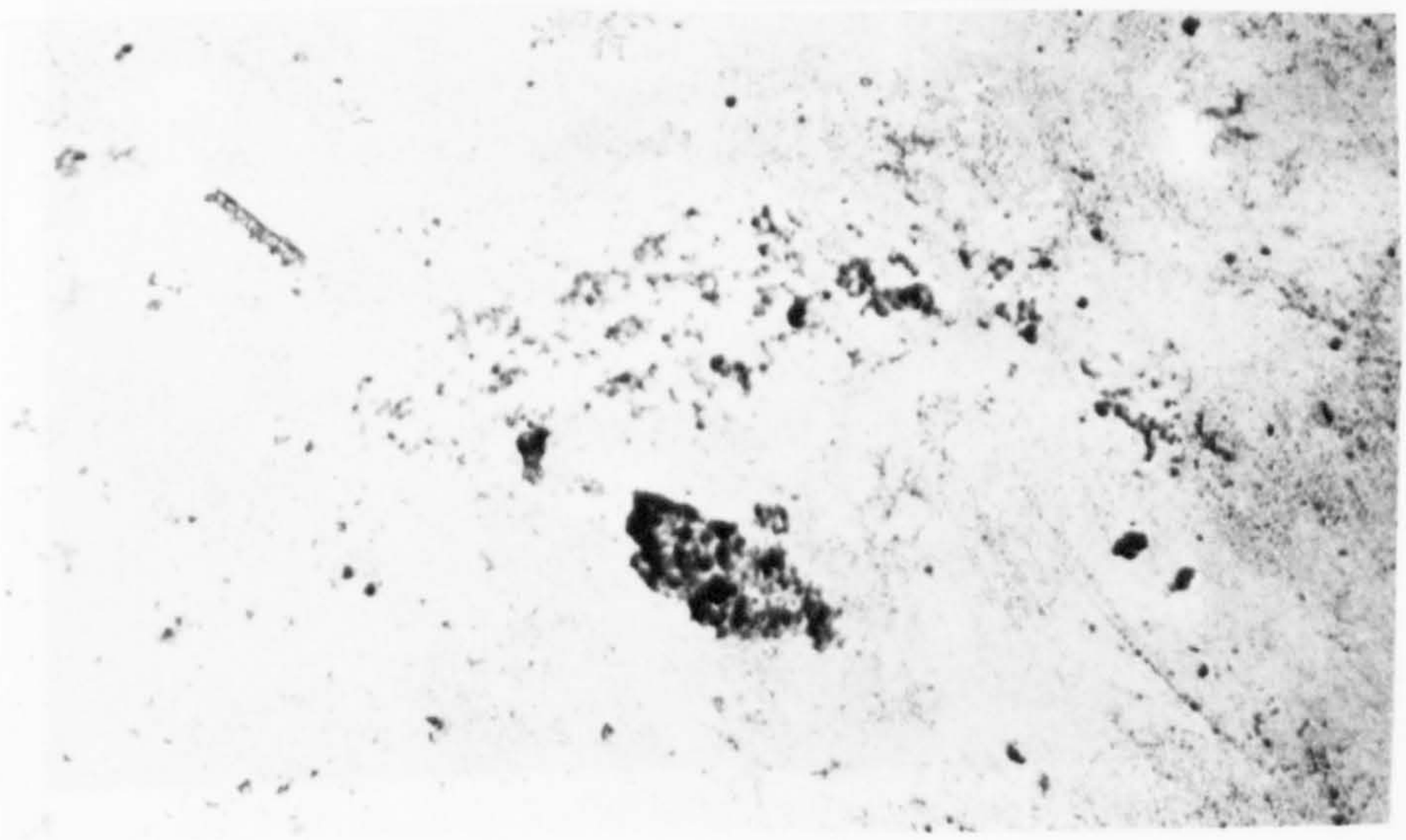


Fig 24. Milk. Wedge type depositions in stagnation point region at 30°C after 30 minutes in standard chamber. Magnification 135x.

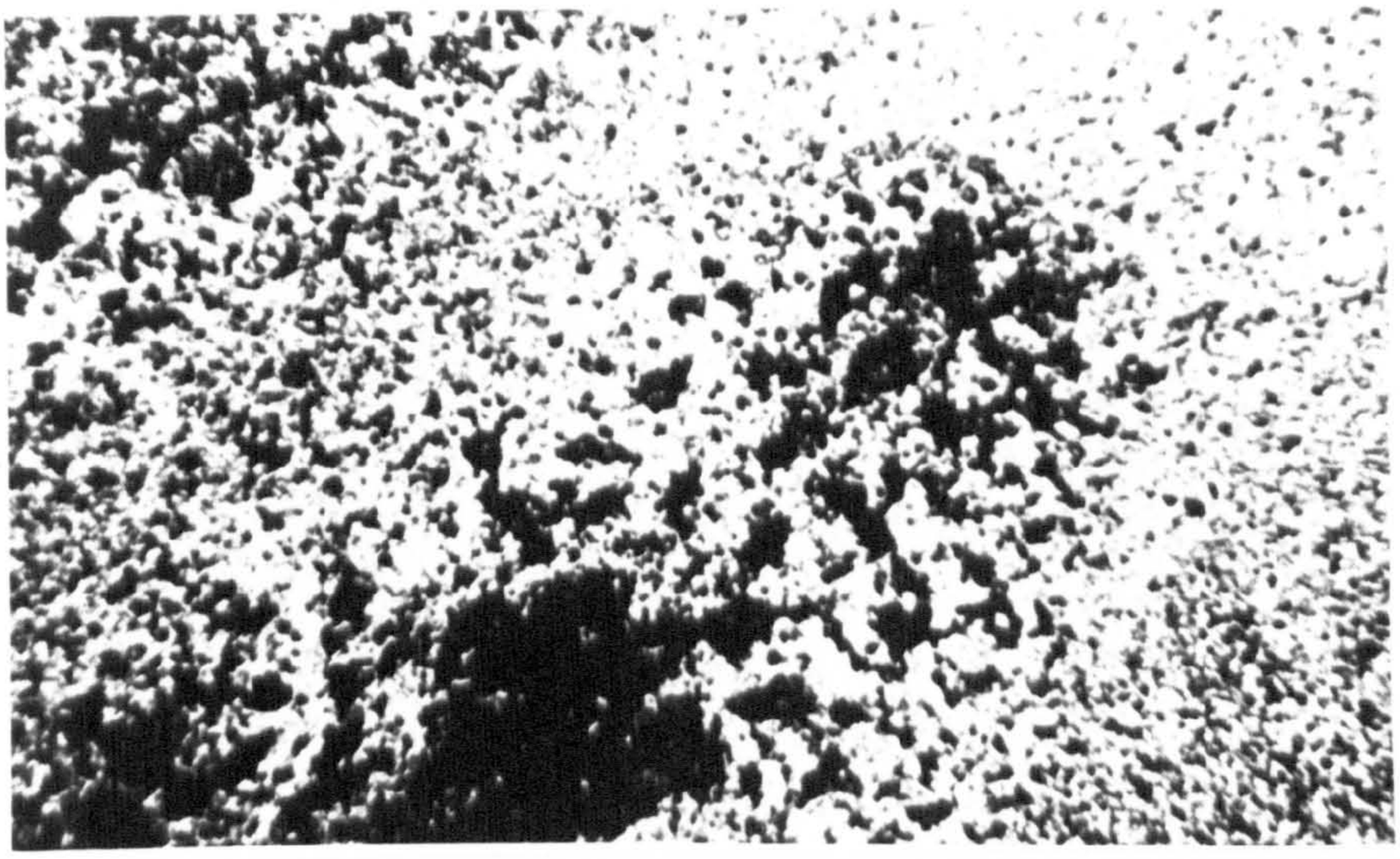


Fig 25. Blood. (Avco Everett Research Laboratory) Wedge thrombus on an ion-plated carbon slide in a chamber of β value 11 $(mm\ sec)^{-1}$ after 30 minutes. Magnification 120x.

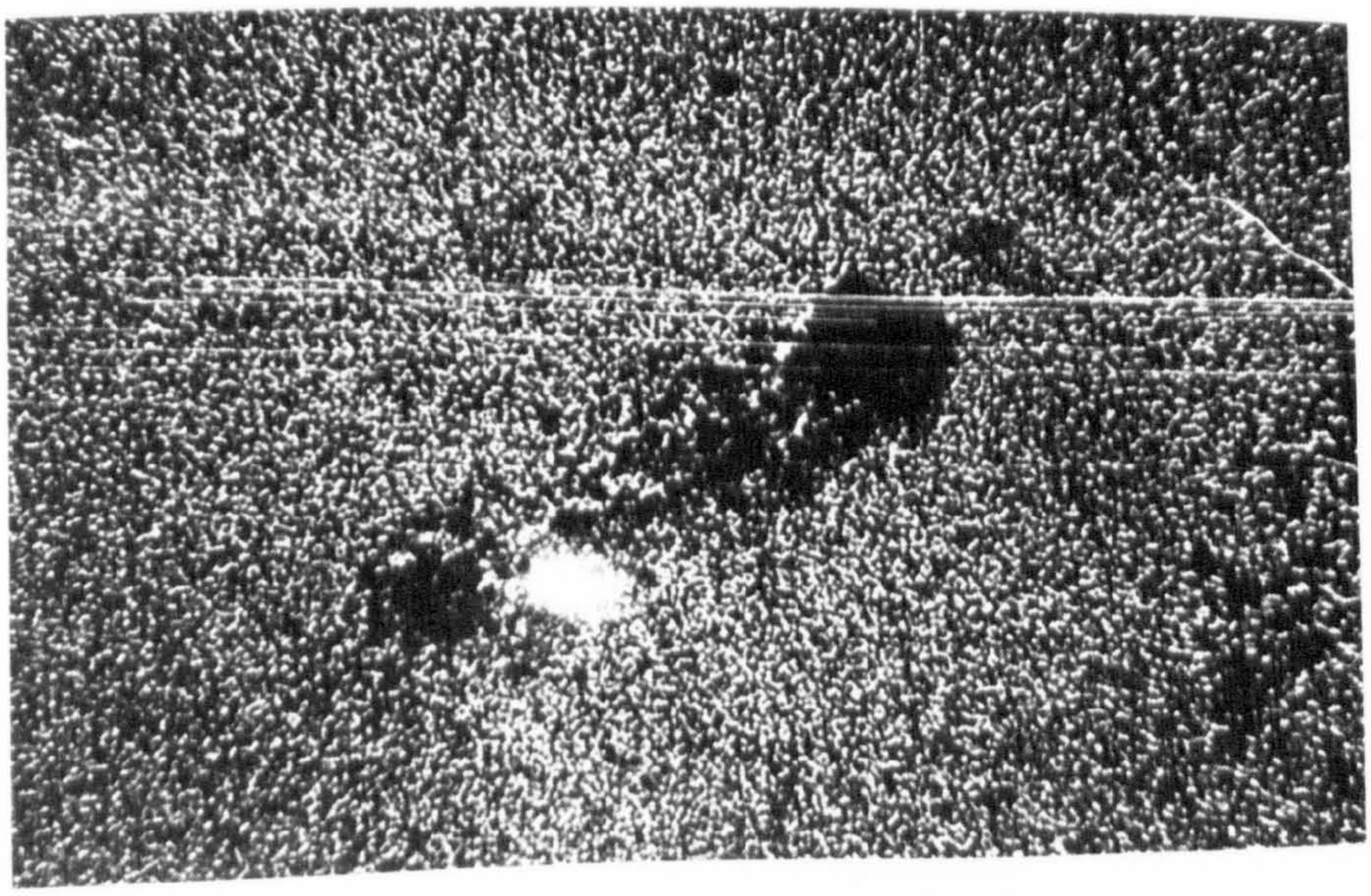


Fig 26. Blood. (Avco Everett Research Laboratory). Wedge thrombus near stagnation point on $C_2H_2/N_2/H_2O$ glow discharge polymer. β- Value 175 $(mm\ sec)^{-1}$ after 30 minutes. Magnification 150x.

With favourable conditions a calcium growth is possible forming a clot.

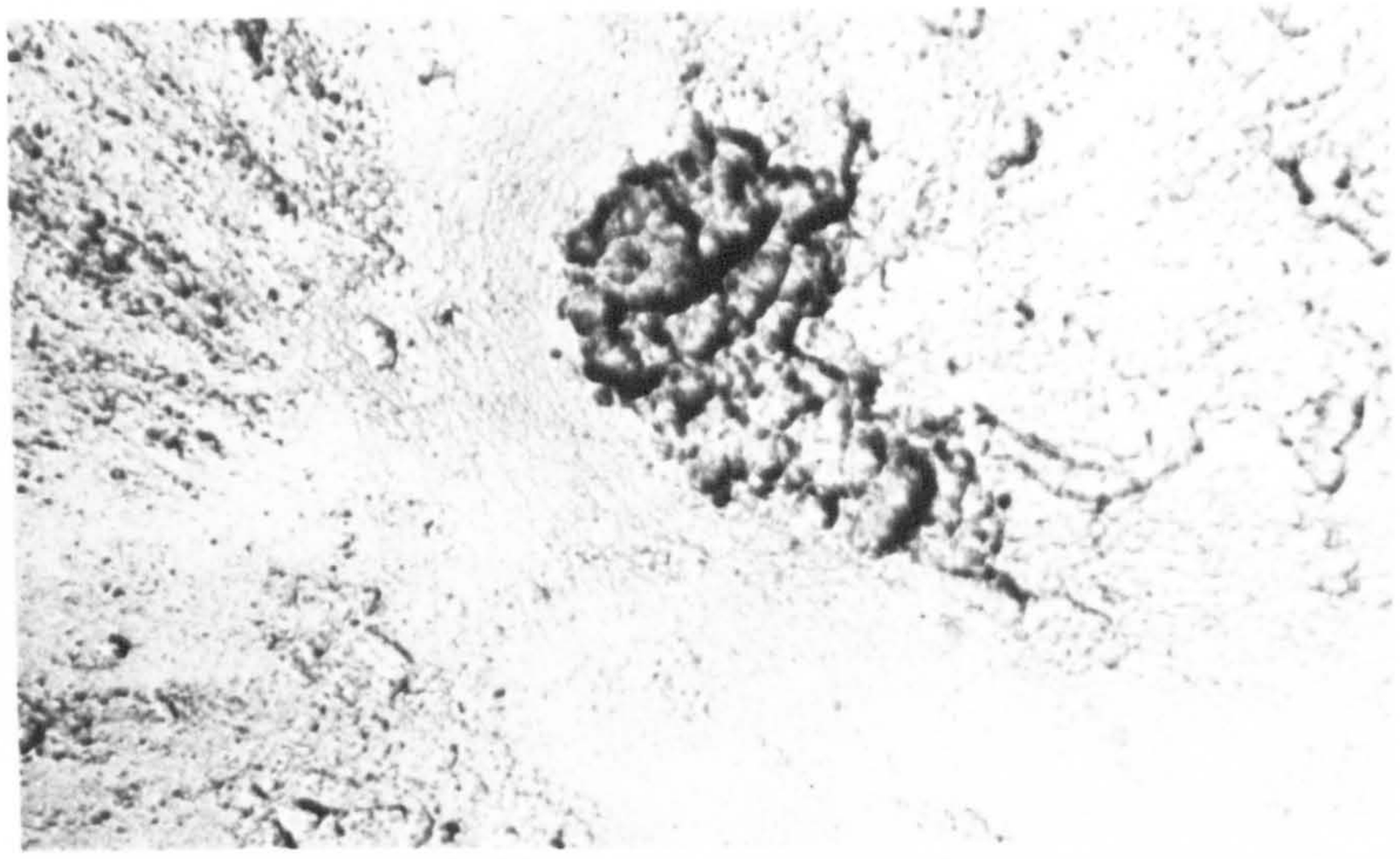


Fig 27. Milk. Stagnation point clot after 5 minute run at 38°C in standard chamber. Magnification 135x.

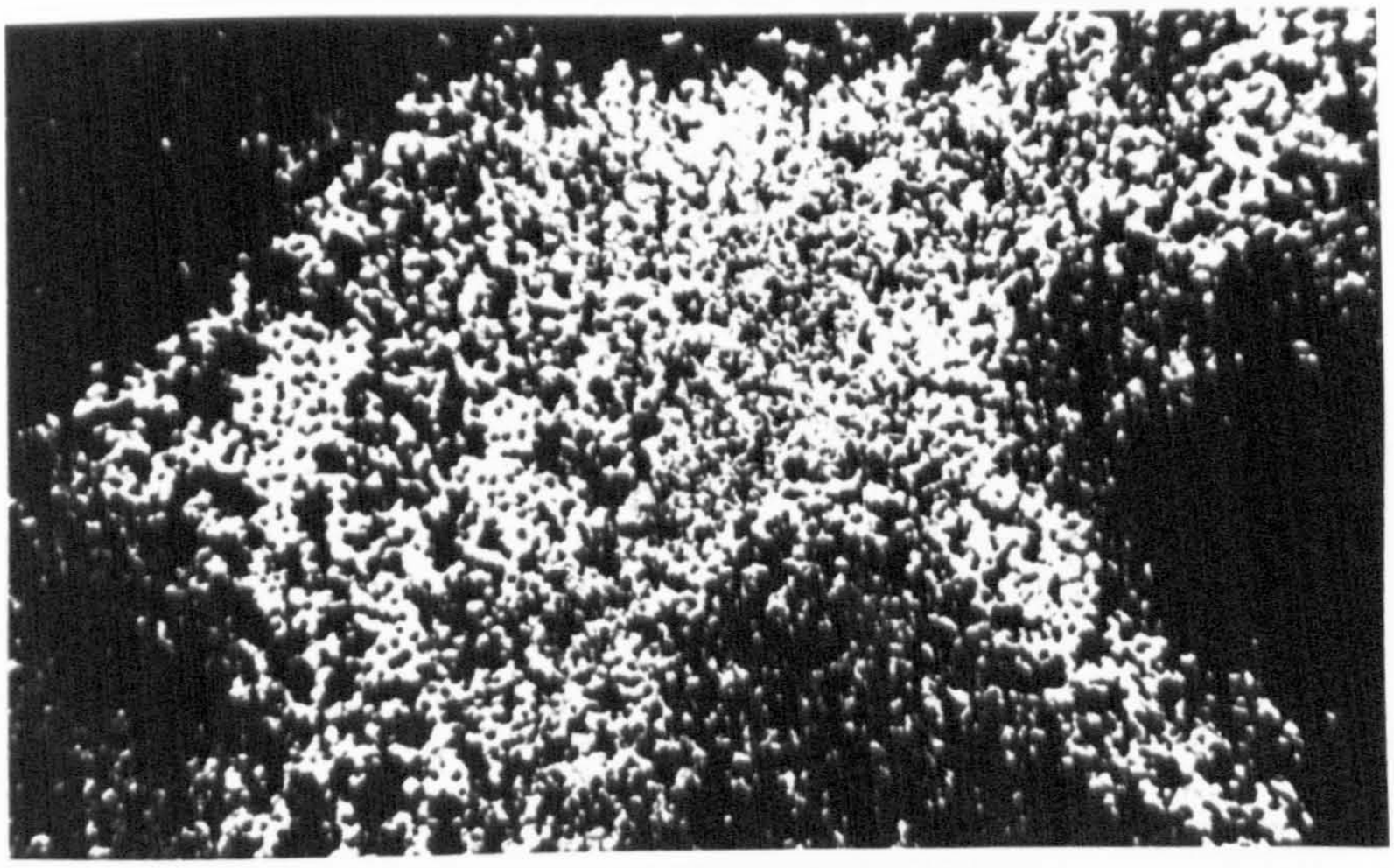


Fig 28. Blood. (Avco Everett Research Laboratory). Well developed asymetric thrombus around stagnation point, on LT1 carbon disc in a flow chamber of β-value 2.8 $(mm\ sec)^{-1}$ after 30 minutes. Magnification 120x.

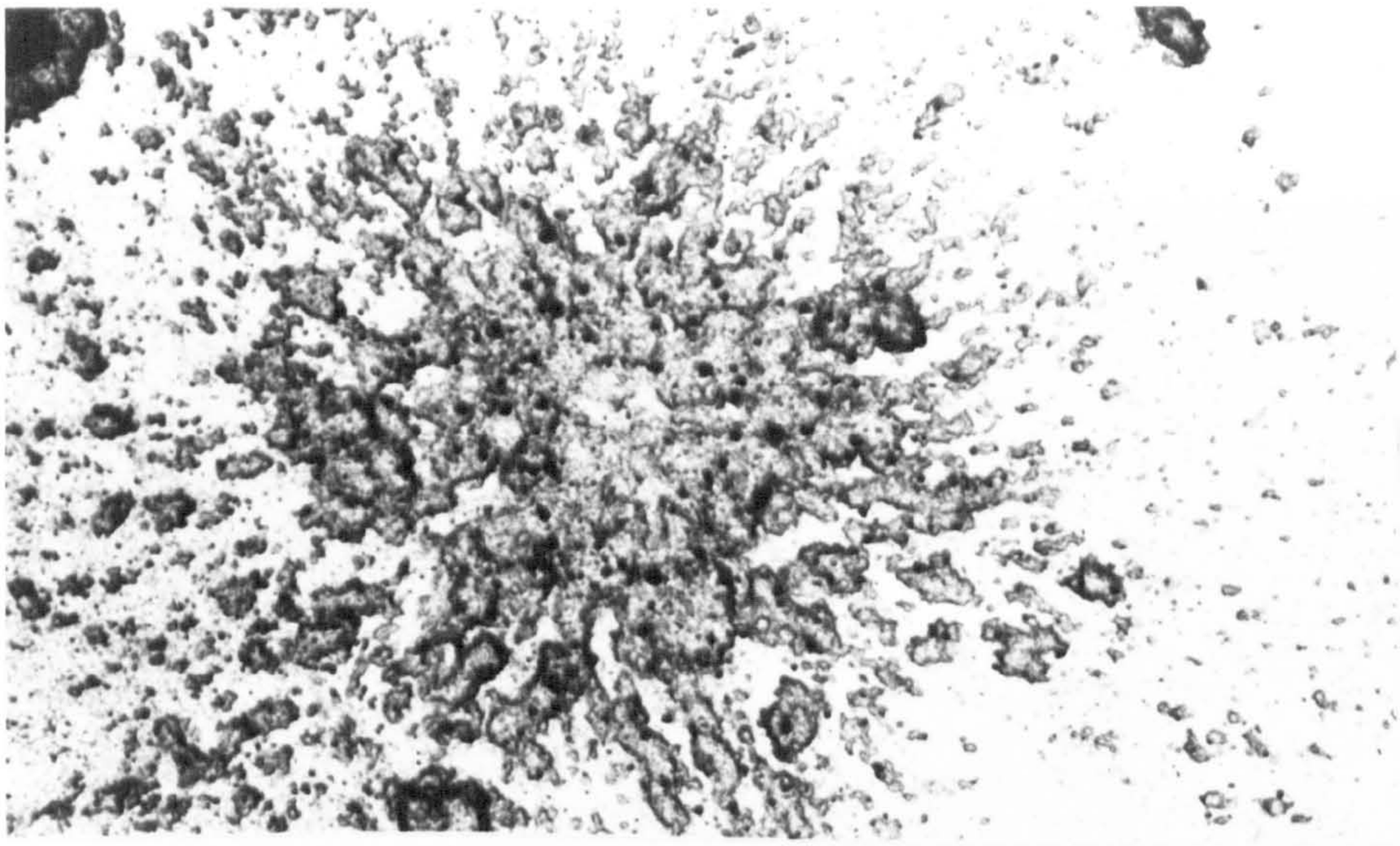


Fig 29. Milk. Stagnation point clot at 30°C after 30 minutes in standard chamber. Magnification 40x.

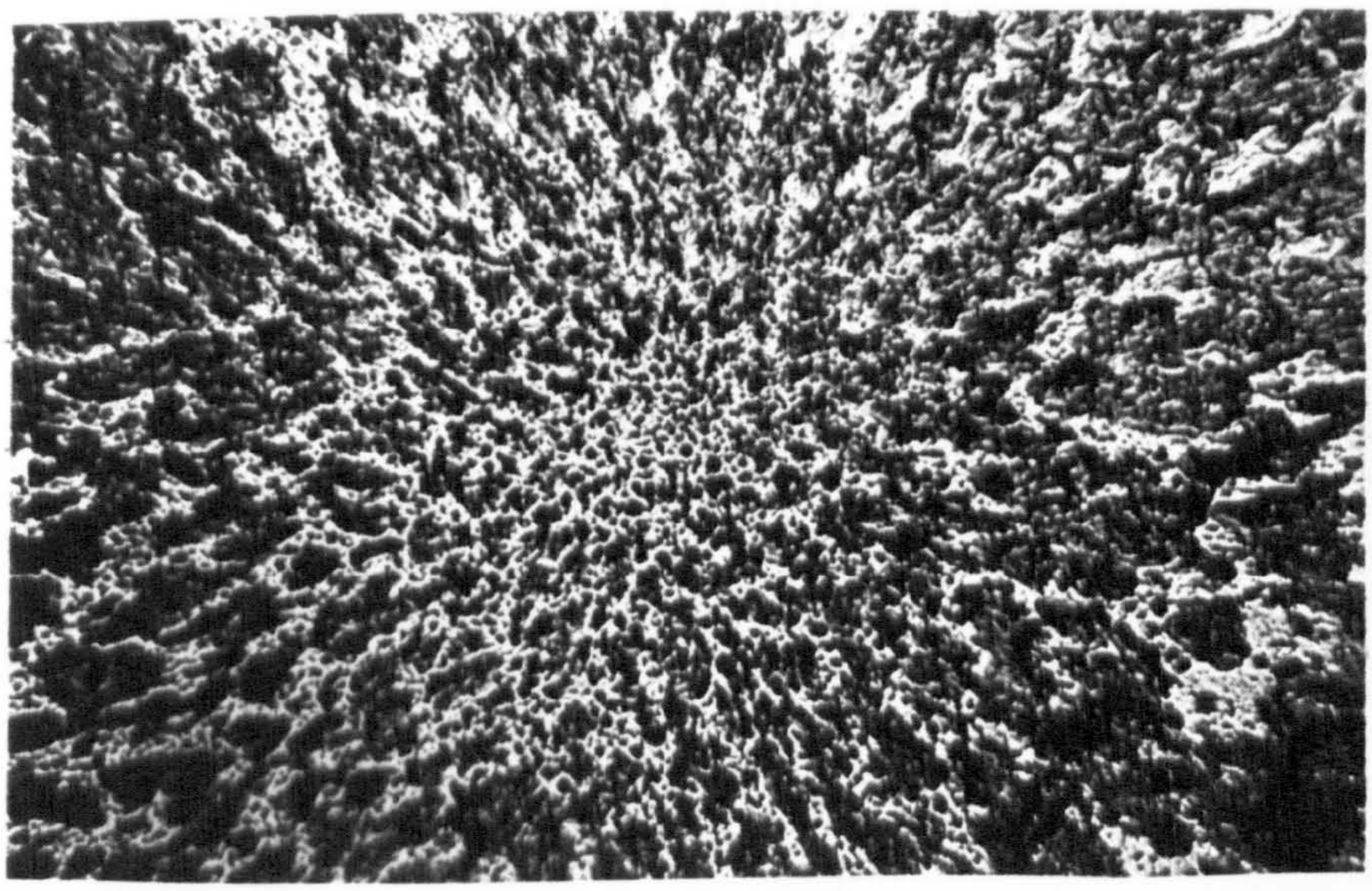


Fig 30. Blood. (Avco Everett Research Laboratory). Symmetric thrombus around stagnation point on segmented polyurethane after 20 minutes. Magnification 65x.

There are two distinct shapes of final clot possible. The symmetric clot

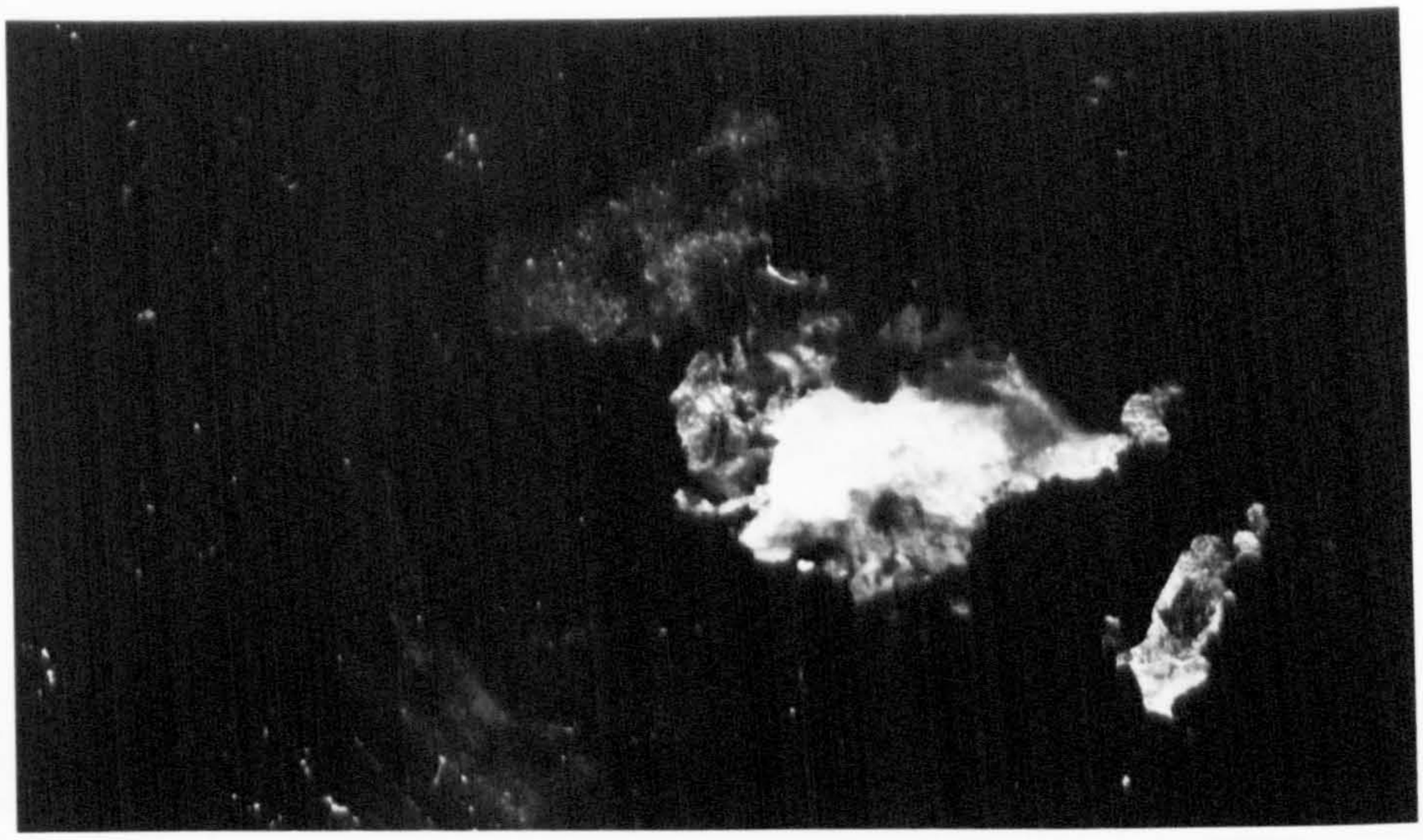


Fig 31. Milk. Large stagnation point clot in standard chamber at 38°C after 5 minutes. Magnification 10x.

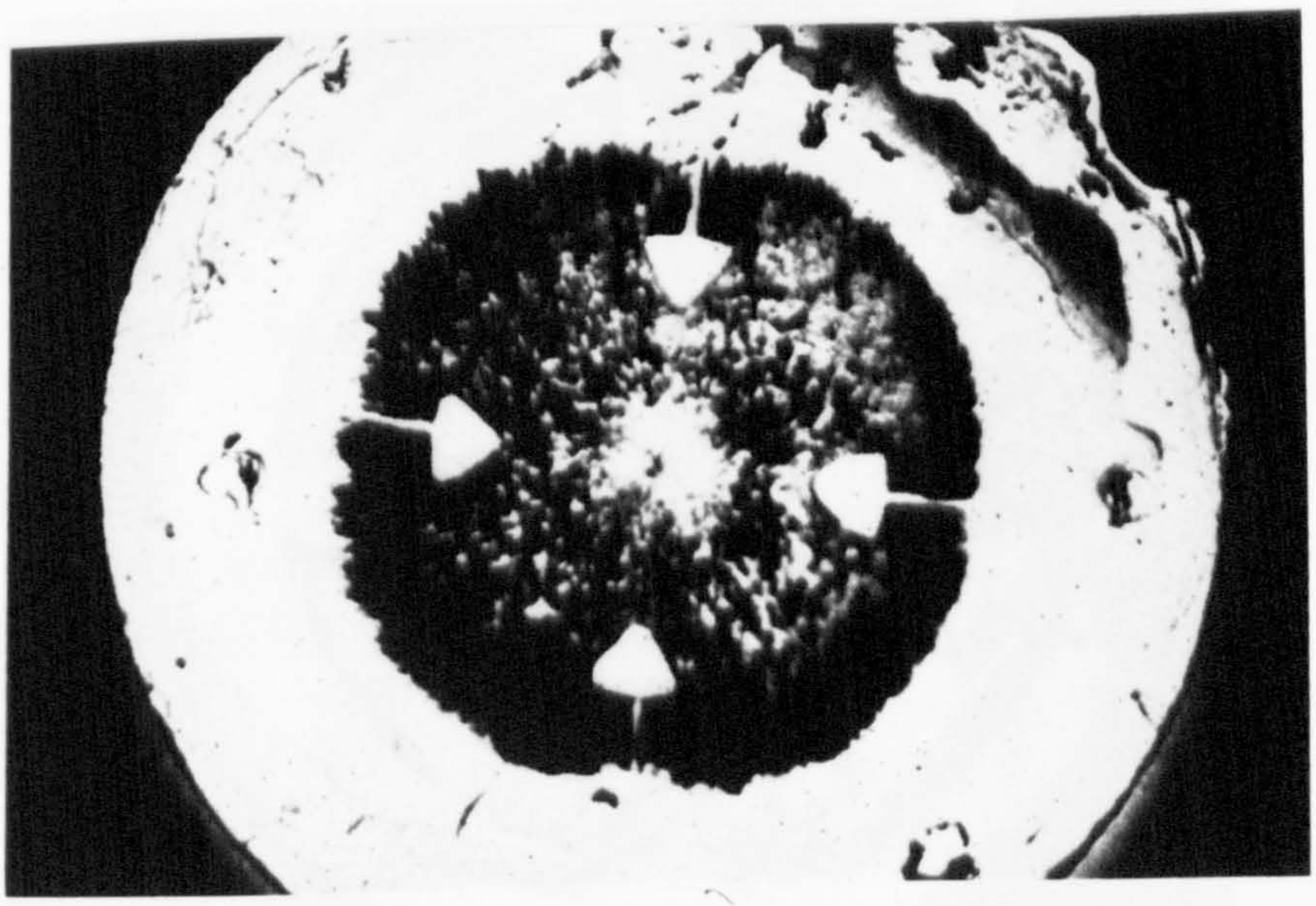


Fig 31 (b). Blood. Large symmetric clot from Petschek (11).

and the wedge type clot.

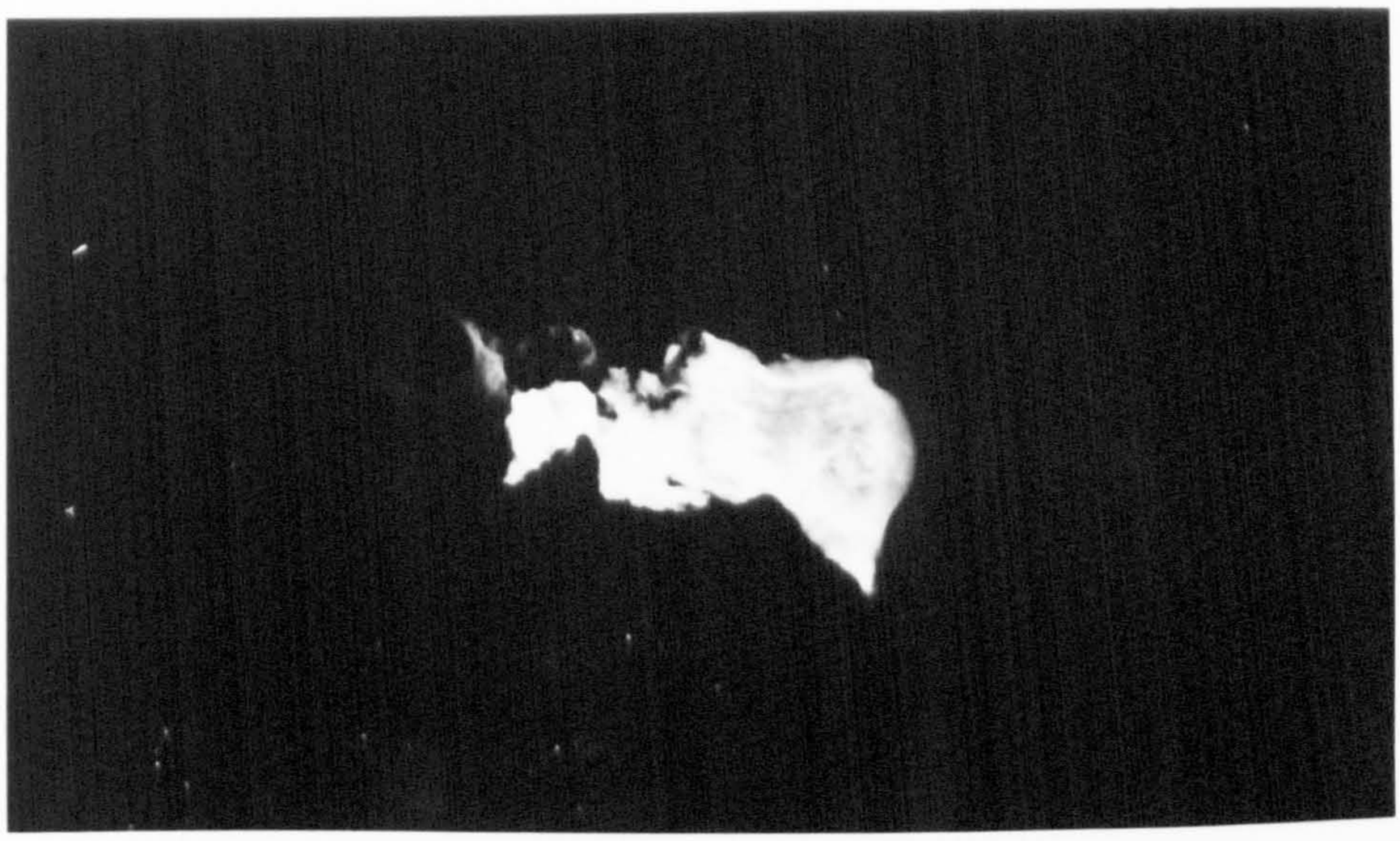


Fig 32. Milk. Large wedge type clot in standard chamber at 38°C after 20 minutes. Magnification 7x.

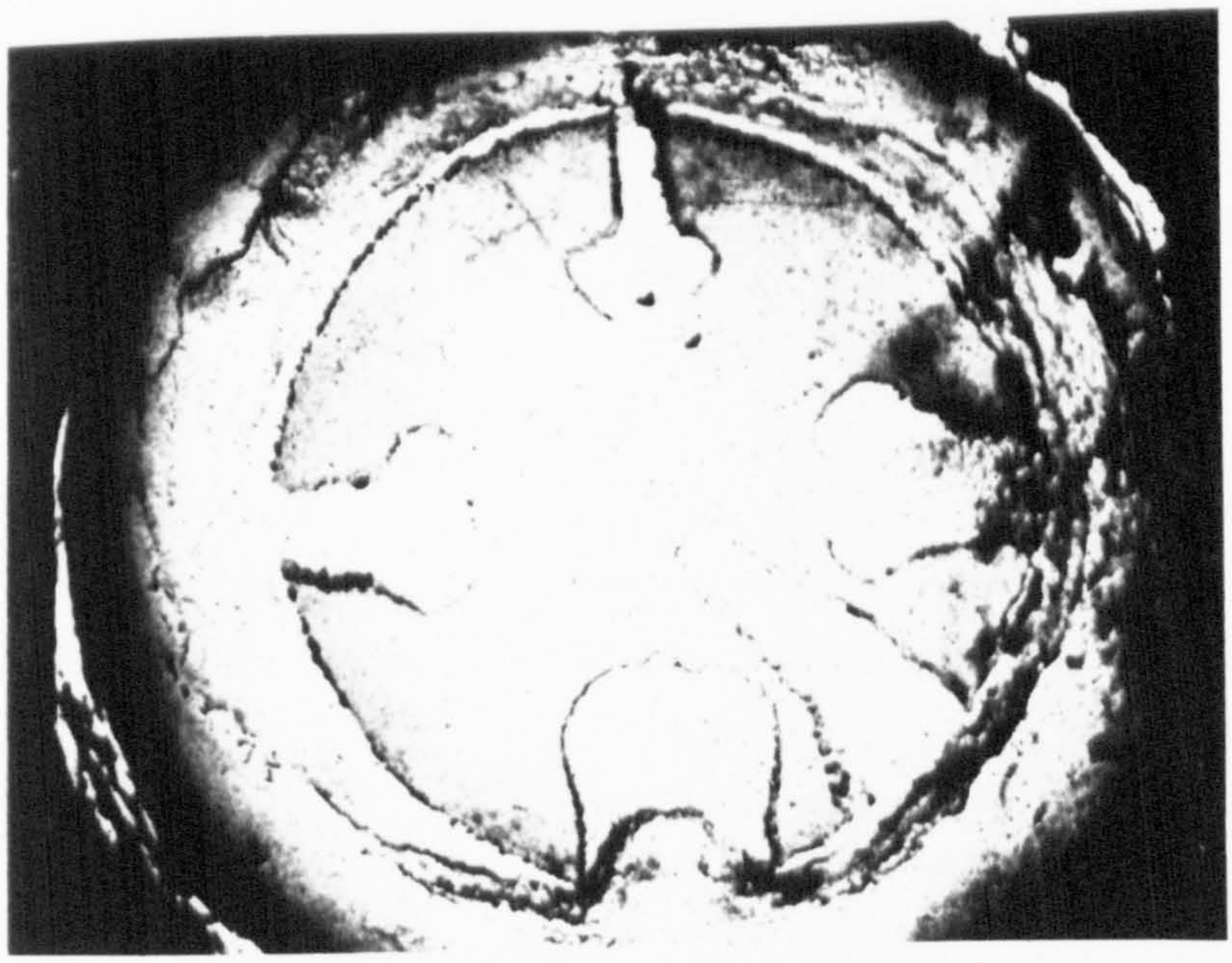


Fig 32 (b). Blood. Large wedge clot from Petschek (11).

A histological examination of the deposit done by Dr A Busuttil, a consultant pathologist at the Western General Hospital, Edinburgh has shownthat, for milk, in all cases of deposition, fat is present, but that only in the final case is calcium involved.

Effect of Shear Stress

During the experiments, it was found that with the low shear chamber any clot tended to form on the part of the chamber surrounding the entrance hole, rather than on the glass slides. With the high shear chamber very little deposition was found, paralleling the observations of Petschek et al.

The best chamber for deposition and the chamber used for most experiments was the medium shear chamber.

2.5 Discussion

It is remarkable that rennetized milk should give rise to very similar microscopic effects to those observed with blood. The sequence of events in deposition from the wall jet in Petschek's flow chamber would appear to be the same for both; the smallest particles deposit first, followed by the larger ones and by a calcium growth if the conditions are favourable.

General explanations of such phenomena may be given in very simple fluid mechanical terms. Thus, in the boundary layer flow in the region of a wall, it is easy to show that small rigid particles adhering to a wall require a higher mainstream fluid velocity for detachment than do larger particles of similar shape and the same force of adhesion per unit area of wall contact. It is likewise plausible to suppose that small particles, once deposited on the way, may modify the boundary layer downstream in such a way as to permit progressive retention or aggregation of increasingly larger adherent solid masses in their wake. In support of such a direct physical account, there is a broad correspondence, as Petschek and his co-workers pointed out, between the distribution and type of deposit formed on the foreign surfaces over much of the flow

field in his experiments, and the shear stress distribution in the fluid stream. The same correspondence is revealed in this experiment with rennetized milk in similar apparatus. Whilst it may be argued that such crude mechanical explanations are unduly simplistic in relation to the clotting behaviour of a fluid as complex and biologically active as blood, it must be pointed out that those formed elements of blood which are apparently principally concerned with the early stages of clotting - the platelets (10, 39, 13, 14) are not, in the strict biological sense, cells, having no nuclei; and, indeed, the same is true of the red corpuscles of mammalian blood. To that extent, their interactions and functional behaviour in forming thrombi in a flowing stream are likely to be simpler, and more fully describable in terms of external mechanical influences, than is common in biological systems.

Nevertheless, it is clear that clot deposition is not related to fluid shear stress effects in a suspension of minute rigid particles in a simple fashion; in particular it has been shown (56) that the pattern of deposition in Petschek's experiments is not exactly related to the distribution of local shear stress in all parts of the field of observation. This consideration, however, does not bear directly on the present question; viz whether, or to what extent, the same local fluid flow factors promote clotting on foreign surfaces exposed either to blood or rennetized milk. It must be borne in mind that milk, like blood, is a complex biological system composed of structured and differentiated functional elements, part, at least, of whose role is apparently to form a coagulum, when brought into the presence of the appropriate enzyme; i.e. the rennet in the natural recipient's stomach. Whatever the factors which complicate and modify the relationship between shear stress and clot deposition in Petschek's blood chamber experiments, it is noteworthy that the identical distribution of deposition is found in the flow chamber experiments with milk.

The constraints governing the design of the experiments by Petschek were different from those with milk. They designed the chamber to have a very low flow rate (2ml/min) in order to maintain each run for an acceptable period (30 minutes) before the loss of blood began to affect the animal. This low flow rate makes the use of the chambers with milk very unsatisfactory because of the problem of mixing three streams, two of which have only 1% of the flow of the other, immediately prior to the chamber. It was decided that in order to mimic Petschek's work the same size of chamber and flows must be used to avoid diverse effects which were not present in his experiments, but unfortunately no real answer to the mixing problem was found. The tubes involved were 3mm diameter and the inclusion of narrowings in the tube tended merely to cause clogging. It was decided that the next experiment tried should be on a larger scale so as to remove the problems of dealing with flows of only 0.02 ml/min and should be an experiment which would yield results on the macroscopic level to complement the excellent results already obtained using the stagnation point flow experiment. The most promising published experiment seemed to be Hladovec's Net Experiment (140).

CHAPTER 3

THE NET EXPERIMENT (140)

3.1 Hladovec's Experiments

The purpose of this experiment was to simulate the development of a thrombus in vitro, based on the assumption that it is necessary to form a favoured site where thrombus is produced from circulating blood. It was also desired to continuously register the process of its formation.

The experimental arrangement is shown in figure 33.

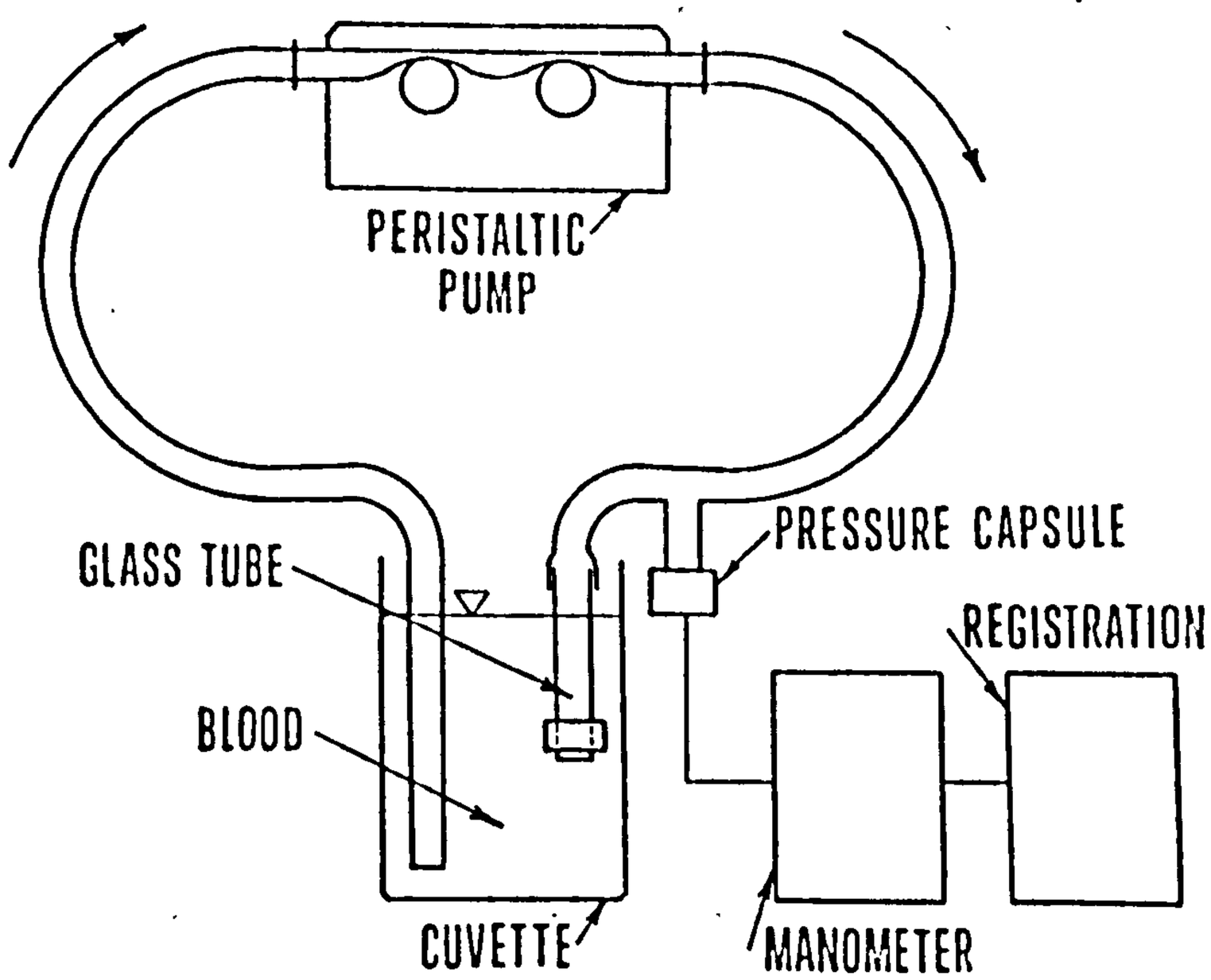


Fig 33. Experimental Arrangement from Hladovec (140)

A 50cm length of 3mm i.d. polythene tubing was connected to a small peristaltic pump and onto one end of the tube a 0.25, mesh nylon net was attached. Both the ends of

the tube were placed into a 3.5ml siliconized glass cuvette and the pressure upstream of the net was continuously recorded. The thrombus formation took place downstream of the net and it was this growth of thrombus which caused the pressure rise.

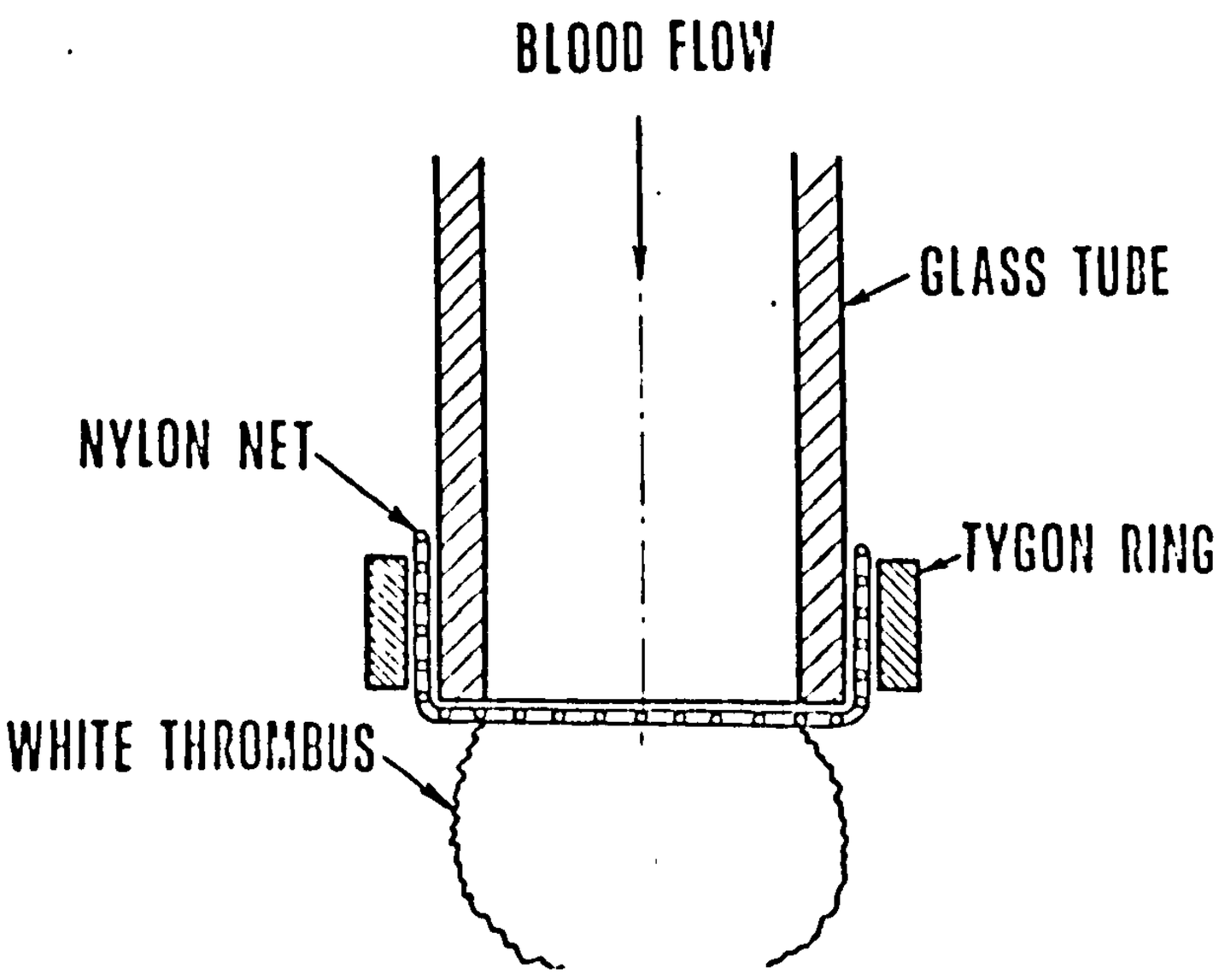


Fig 34. Net Fixation and Location of Thrombus (140).

The flow rate round the system was 55ml/min and the total volume of fluid in the system was 7.5ml. Human or rat blood was collected and was citrated to the extent of 1ml disodium citrate 3.8% to 9ml blood. At the beginning of each experiment the blood or plasma was recalcified using $CaCl_2$. Whole blood, platelet poor plasma and platelet rich plasma were used in the experiments, which were conducted at room temperature.

Hladovec found the following:-

1) The thrombus formation took place behind the net instead of in front of it as would be normal in filtration.
2) The time to formation of thrombus shortened with rising temperature.
3) Thrombus formation times were not affected by mesh size and diameter of tubing.
4) In the proximity of the mesh he found scattered islets of granular platelet masses with an increasing amount of fibrin and erythrocytes in the direction of flow.
5) There were three phases of thrombus formation: no change of pressure was observed in the first phase, a gradual increase in the second and an abrupt increase in the third.

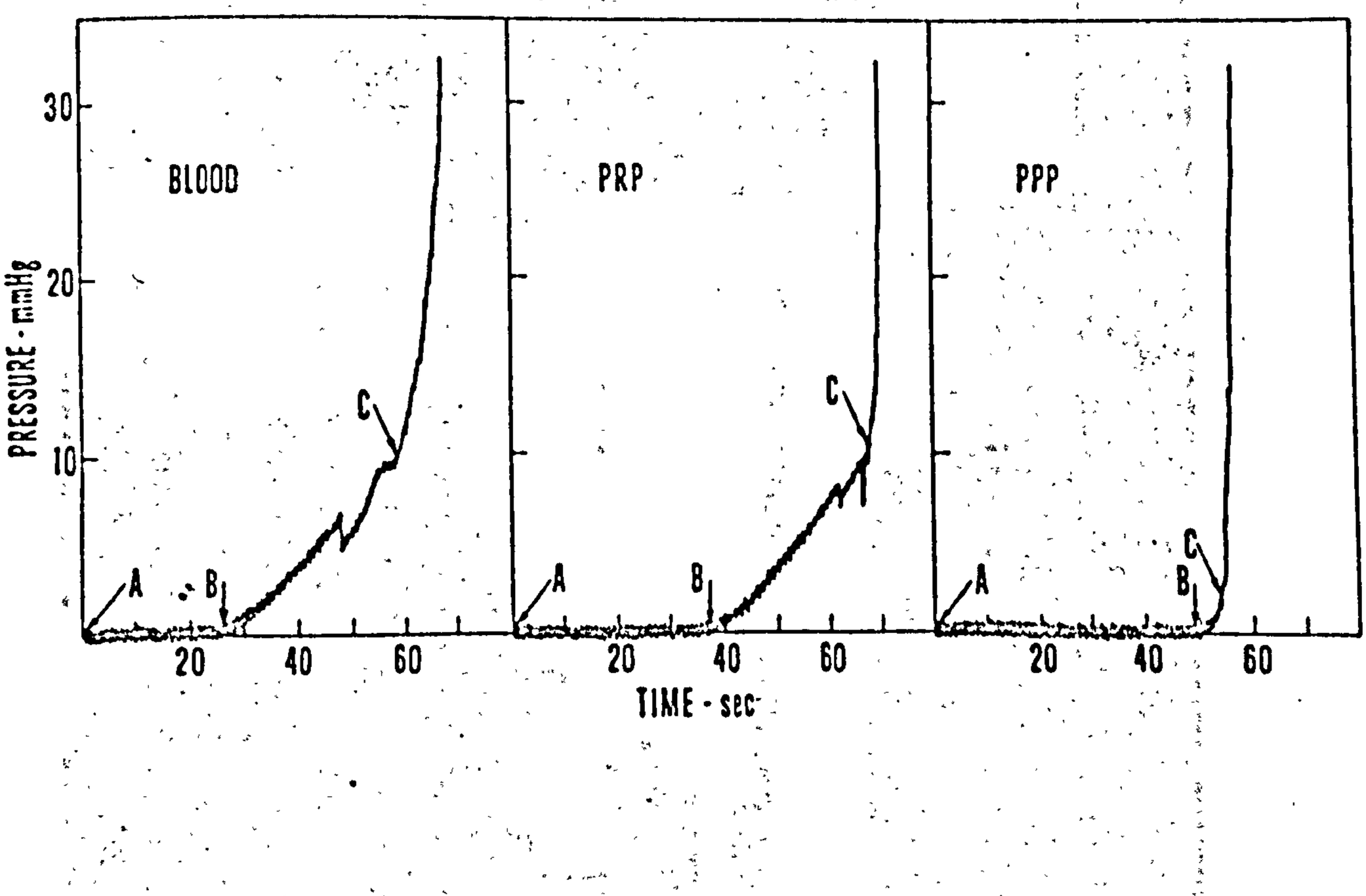


Fig 35. Pressure Curves found by Hladovec. A.B.C. represent Separate Phases of Thrombus Formation.

In order to duplicate Haldovec's work the following apparatus was used.

3.2 Milk Experimental Procedure

The equipment used is shown below:

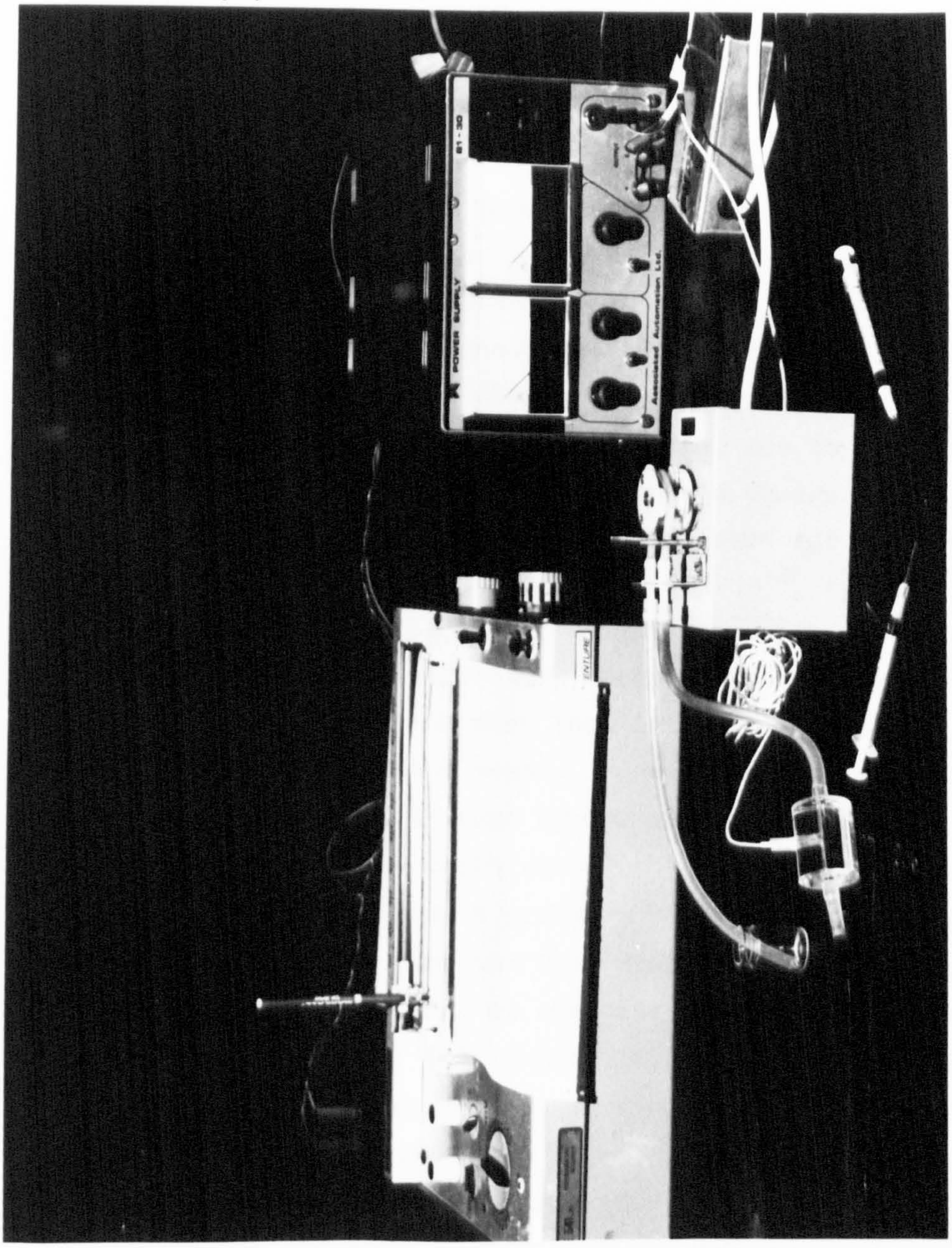


Fig 36. Apparatus Used for Milk Experiments.

A Shuco pericyclic pump was used to pump milk around the circuit at 75ml/min. The circuit comprised: a 10ml glass cuvette, a length of 3mm i.d. silicone tubing and a perspex T piece into the side arm of which a Gaeltech series 3EA minature strain gauge pressure transducer was attached. The pressure transducer was connected to a servoscribe chart recorder so that continuous recording of the process was possible. Attached to the end of the T piece was a short piece of silicone tubing with the mesh attached.

Metallic mesh which was donated by United Wire was normally used; a circular piece was obtained using a $\frac{1}{4}$" punch and was then glued to the end of the tubing using evo-stick. This was found to be very satisfactory as a good seal could be obtained which would, however, break before the pressure in the system, due to the clot in the mesh, became sufficiently high to damage the pressure transducer. A new filter was used for each run.

10cc of milk was filled into the glass cuvette, which was then heated to the temperature required for the experiment. Both ends of the tubing were then introduced into the cuvette and circulation began. Rennet essence and $CaCl_2$ solution were added through hypodermic needles from 1cc syringes and the chart recorder switched on. The endpoint was taken as being when a pressure rise of roughly 30mm Hg had occurred. The pump was then stopped and the system cleaned using Tergazyme, an enzymatic detergent, before the next run began. The cleaning fluid at 50^{o}C was pumped round the circuit for roughly 15 minutes followed by 3 separate washings with clean water. Saturated calcium chloride solution was used throughout and the rennet essence was the same as that used for the previous experiment.

3.3 Results

1) Clot formation took place behind the net as Hladovec had found with blood.

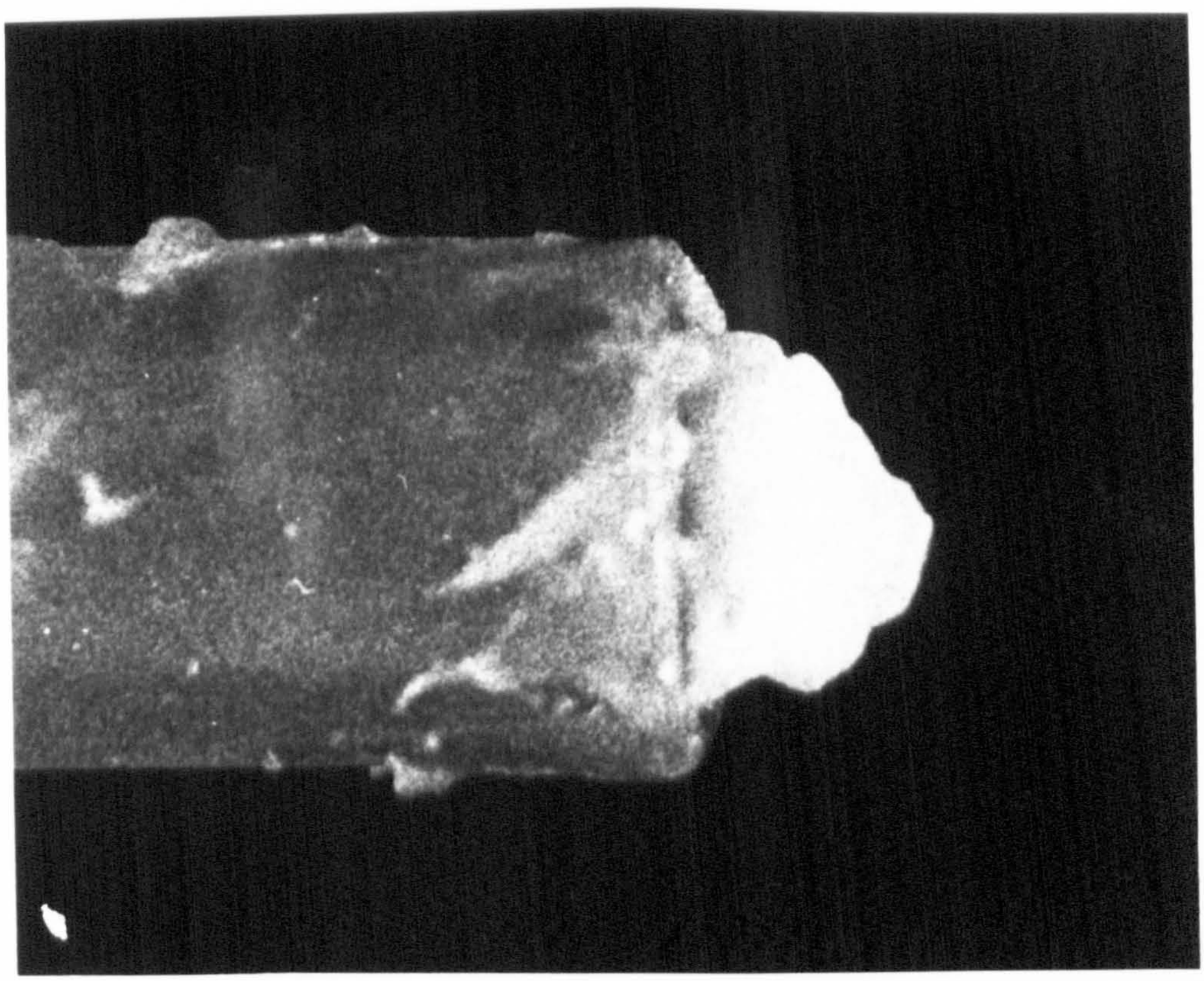


Fig 37. Location of Clot found with Milk

2) The time of formation of clot shortened with rising temperature.

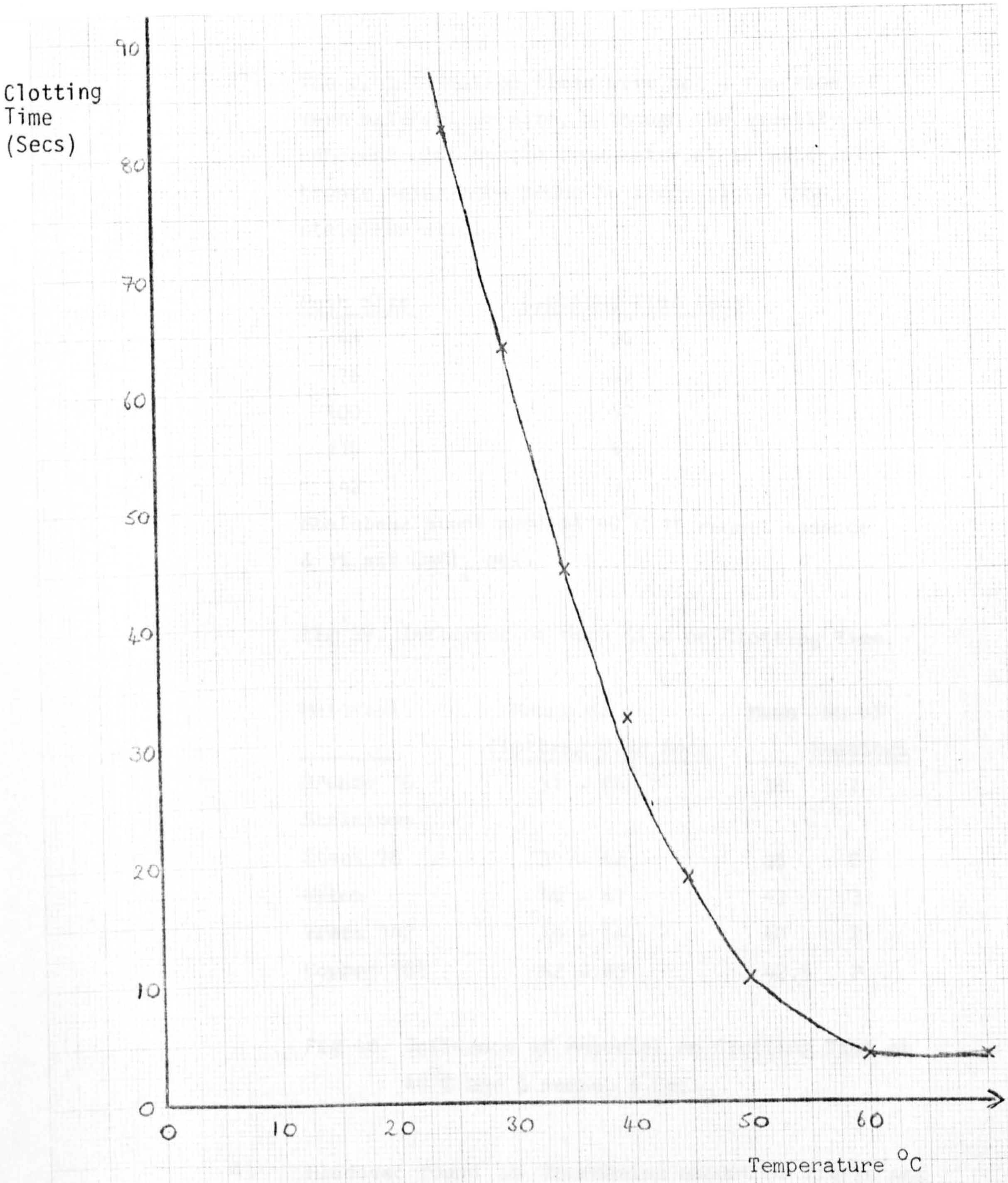


Fig 38. Clot Formation Time vs. Temperature.

3) The clot formation times were not a function of mesh material or size, although the quantity of adherent clot varied from material to material; bronze being more prone to large clots than stainless steel.

Mesh Size	Clotting Time Secs
48	36
76	33
100	42
119	45
142	31

Stainless steel mesh at $40^{o}C$ 1% rennet essence & 1% sat $CaCl_2$ sol.

Fig 39. Influence of Mesh Size on Clotting Time.

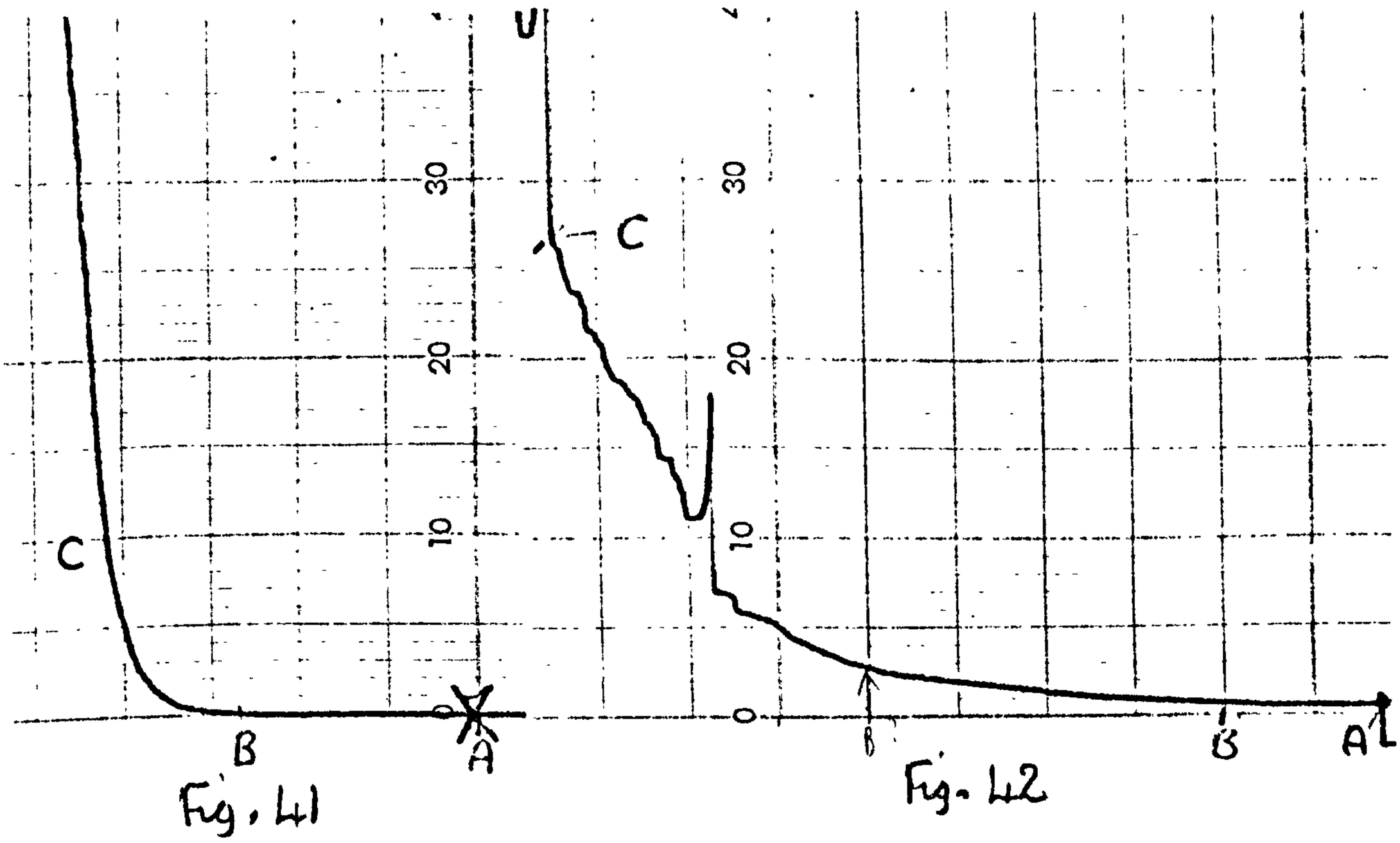
Material	Range of Clotting Time Secs	Mean	No of Readings
Bronze 76	31 - 44	38	7
Stainless Steel 76	31 - 42	35	8
Nylon	40 - 47	43	3
Brass 142	36 - 44	40	2
Copper 185	42 - 43	42.5	2

Fig 40. Influence of Material on Clotting Time at $40^{o}C$ and % rennet & $CaCl_2$

4) Hladovec found an increasing amount of fibrin and larger cells in the direction of flow. Histological examination of the deposit on the mesh revealed that in the whole area there was a protein meshwork with trapped fat globules but towards the periphery

there were globules 4 times the size of those near the mesh and large clumps of a calcium deposit, which is similar to Hladovec's findings.

5) The shape of the pressure rise with milk was very similar to that found with blood. Increasing the concentration of calcium chloride affected the shape of the curve (see Fig 42).



Figs 41 & 42. Influence of $CaCl_2$ conc on Shape of Pressure Rise (41, 1%, 42, 0.5%).

Haldovec identifies three phases and the equivalents for milk are marked on the figures.

As well as being used to duplicate Hladovec's work the apparatus was also used to obtain the effect of temperature, rennet concentration and calcium chloride concentration on the clotting time. These are shown in figures 38, 43, and 44 respecitvely.

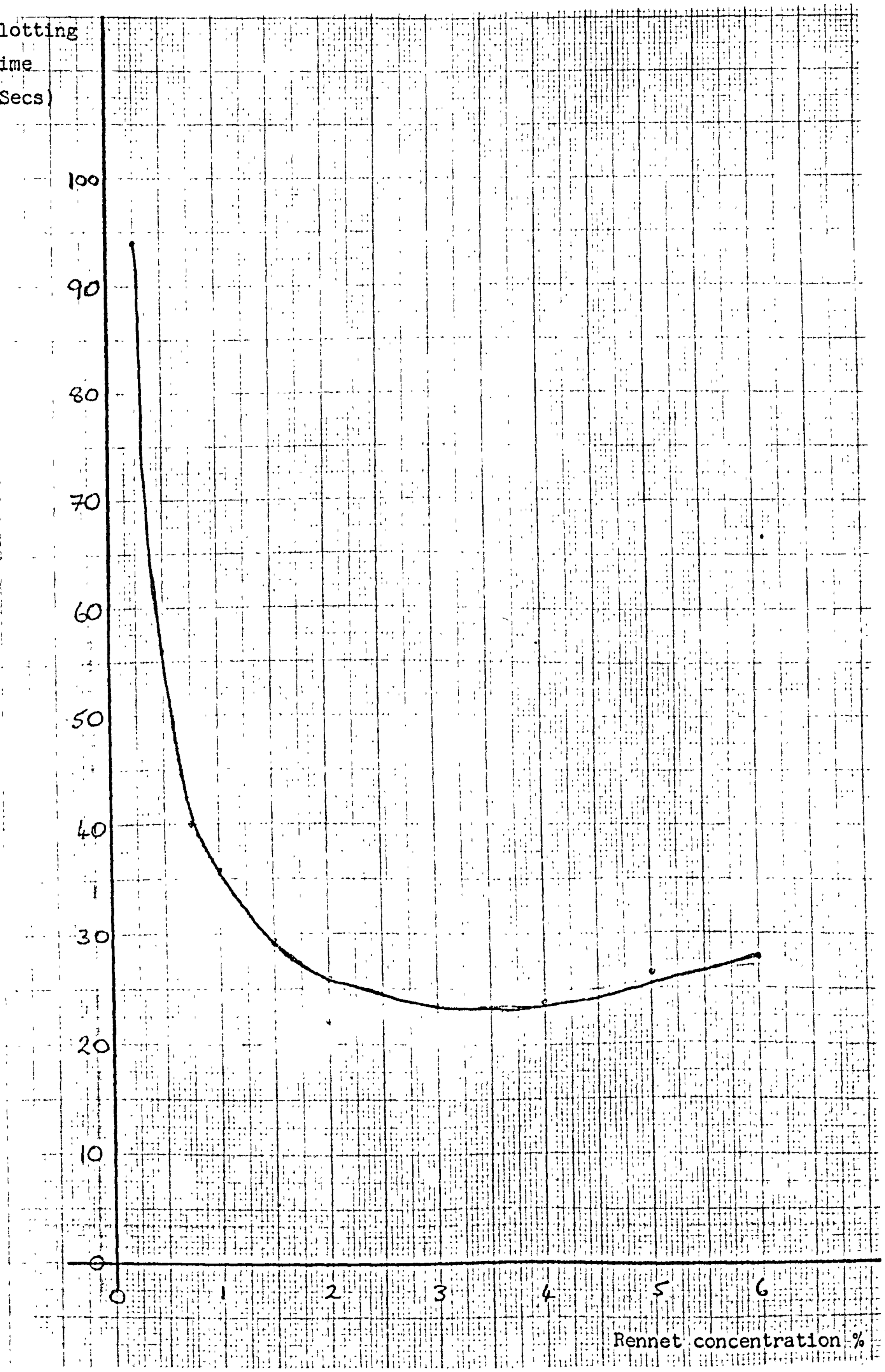


Fig 43. Clotting Time vs Rennet Conc. at 1% $CaCl_2$ Conc & 40°C.

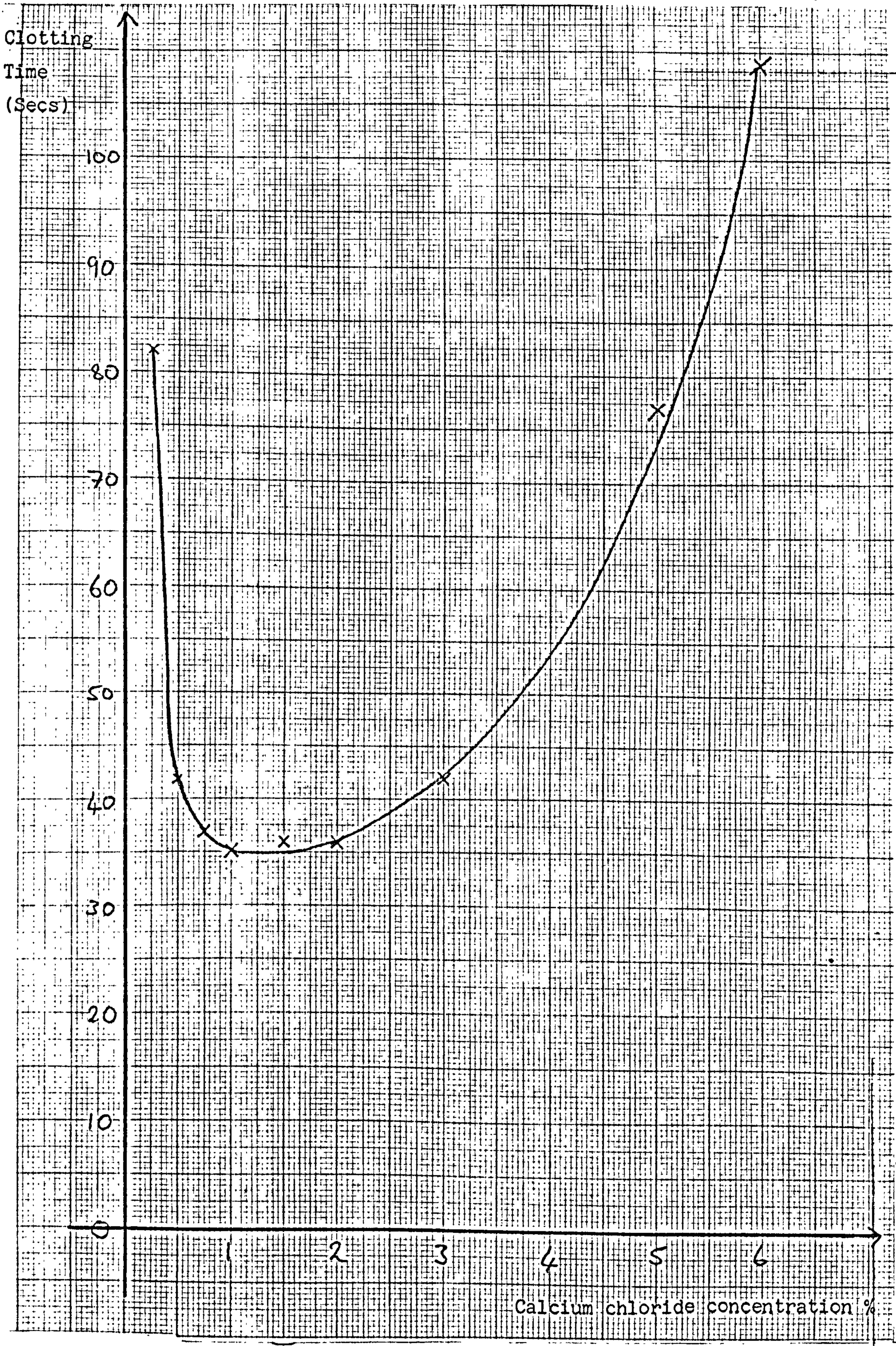


Fig 44 Clotting Time vs $CaCl_2$ Conc. at 1% Rennet Conc. & 40°C.

3.4 Discussion

This proved to be a very useful and successful experiment; the results highlighted remarkable similarities between the clotting and deposition behaviour of rennetized milk and blood and the functional relationships between the clotting time and the temperature, rennet concentration and calcium chloride concentration were obtained. These relationships are very important and make the design of a large scale testing ring very much simpler.

The observation that the clot forms behind the net for both milk and blood is very interesting. Normally, during filtration, the cake forms on top of the filter, therefore, one can assume that the phenomenon occurring here is not a filtration. The milk or blood does not simply circulate until aggregates are formed which are larger than the holes in the mesh and then become trapped onto the filter. If, instead of considering the filter mesh as a whole, one considers a single wire, then there will be a stagnation area immediately downstream of the wire onto which small particles will adhere. Once particles have begun to adhere to the underside of the mesh the stagnation area is increased so that larger particles or aggregates of small particles can deposit "sheltered" from the flow. When the enzymatic phase of coagulation is completed the rate of deposition will begin to increase as the stickiness of the particles is increased and as the stagnation area is increased the effect will be further enhanced.

If this argument is applied to the variation in pressure immediately upstream of the mesh one would expect to

observe; little or no pressure rise until the enzymatic phase is completed, for the particles will not be protuding into the path of the flow and thereby causing any restriction.

Once the enzymatic phase is over the pressure would begin to rise. If the flow area available was reduced linearly with time, the pressure rise would be parabolic as the pressure $\propto$ area^2. However, the rate of reduction of area is probably not linear but has increasing order as the bulk fluid coagulates so one would expect the pressure to begin to rise slowly as the particles begin to aggregate and then rise very steeply once bulk coagulation occurs. This would explain the three phases which Hladovec found and which can be identified with milk.

If the above theory were true one would expect to find a variation in composition of the clot downstream of the mesh with the larger particles and fibrin or calcium deposit towards the outside of the clot, assuming the deposition sequence to be the same as that found in the Stagnation Point Flow Experiment. This is indeed the case as Hladovec found an increasing amount of fibrin and erythrocytes in the direction of flow and a histological examination of the milk clot found the fat globules at the periphery were 4 times the size of those near the mesh and large clumps of a calcium deposit were also present at the periphery.

The clot formation times would not be expected to be highly dependent on material or mesh size, but, the value of the extended pressure rise and the extent to which the clot remains adherent to the mesh would be as Berridge (79) has shown. This was found but the range of materials tried was limited and no conclusions other than that the material affects the amount of clot can be drawn.

The influence of temperature on clotting time (Fig 38) was very marked and unfortunately there is no point on the graph at which a fluctuation in temperature could be said to have little effect. The temperature will, therefore, have to be carefully controlled in the full scale testing. The rennet and calcium chloride graphs (Figs 43, 44) show that provided a conc. of 1% or above is used small fluctuations in concentration will have no great effect.

After the success of this work it was felt that proceeding to the fabrication of a full scale rig for testing artificial heart valves was justified, as it had been shown that milk behaved similarly to blood microscopically in the Stagnation Point Flow Experiment and macroscopically in Hladouec's system. This work on the Net Experiment thus complimented the results with Petschek's apparatus and confirmed the analogy on the macroscopic level.

CHAPTER 4

THE USE OF RENNETIZED MILK FOR TESTING THE THROMBOGENICITY OF HEART VALVES

4.1 Introduction

The use of prosthetic heart valves first began in 1952 when Hufnagel implanted a caged ball prosthesis in the descending aorta (143). This prosthesis had limited success, but it was not until the early nineteen sixties that any real advance in valve replacement was made, although the ball valve was still the favoured design (3, 144, 145). The early models of the Starr-Edwards valve were beset by thromboembolic complications (146) and reports of ball variance (147). These complications led to the covering of the valve annulus and subsequently the cage, and the replacement of the silastic ball with a stellite ball. However, these modifications failed to solve the problems (38, 148 - 154) even when patients were undergoing anticoagulant therapy.

Caged disc prostheses were developed to overcome the problems of poppet inertia, and ventricular-prosthetic disproportion by reducing the length of the cage, but all central occluder prostheses were characterized by moderate to marked transvalvular gradients (155 - 160), which led to the development of tilting disc valves (62, 161 - 167). The Bjork-Shiley and Lillehei Kaster valves have been widely used and the transvalvular gradients greatly reduced, but the problem of thrombosis and thromboemolism still exists (20 - 23, 168 - 175). The

Edinburgh valve, designed to operate without anticoagulants, is as yet untried clinically although in vivo trials have been very promising (52, 51, 176, 177). The assumption on which the design of the Edinburgh valve is based is that, if the valve has the optimum hydrodynamics and is made of the most athrombogenic materials, it will not cause thrombosis. Unlike the Lillehei Kaster and Bjork-Shiley valves which only open to 60^{o}, the Edinburgh valve is made to align fully with the flow by employing an aerofoil shaped pivoted occluder and is designed to be moulded in vitreous carbon. The absence of a technique for evaluation of the thrombogenicity of a valve has, among other things, delayed its production.

Another solution to the problem of thrombosis and thromboembolism is the use of Tissue valves. Several types have been tried: the homograft (178, 179, 180), fasia lata (181), pericardial Xenograft (182, 183) and the porcine Xenograft (184 - 198). These valves are usually associated with a low incidence of thrombosis and thromboembolism, but the long-term durability is suspect; the expected life of the valves is only 5-7 years. Some centres also report a fairly high incidence of thromboembolism (199).

Although heart valves have been in use for nearly 30 years the ultimate valve, with negligible thrombosis, no thromboembolic complications, and which will last longer than the normal human life span, whilst allowing the recipient to lead a normal healthy life, has yet to be found. One of the main problems the designer has had to face is the difficulty of ascertaining the thrombogenicity of a valve without extensive clinical trials, and the

present work is an attempt to provide a means of doing this in vitro using rennetized milk as an analogue of blood.

4.2 Design Criteria

It was decided that a single pass system was required to reduce the problem of circulating aggregates/clots. A run time of 1 hour was felt to be the minimum acceptable, although other experiments have used shorter times for testing the thrombogenicity of materials (200). It was necessary to heat the milk to say 40°C before the artifical heart; and it was felt best not to heat the bulk fluid, as maintaining milk at 40°C for one hour would cause reactions to take place which might affect the results. As the temperature of the milk was to be raised the dissolved gases which were in equilibrium with the milk at room temperature would evolve from the liquid and somewhere these would have to be removed before the artificial heart. Rennet and calcium chloride would have to be added to the milk immediately before the heart and good mixing obtained. Since each experiment was clearly to be fairly expensive it was decided to use a two test valve artificial heart instead of the one test valve heart available.

4.3 The Apparatus

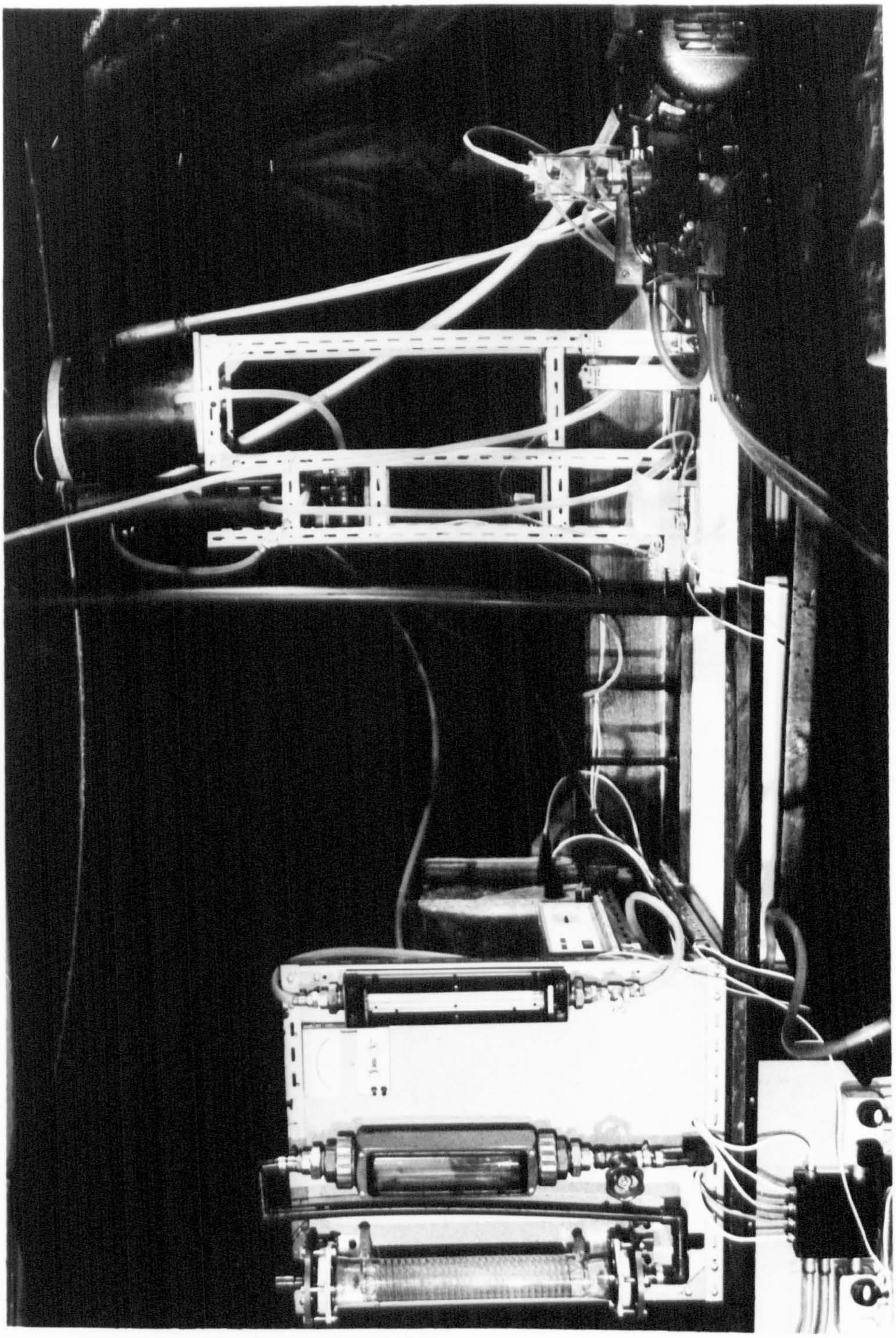


Fig 45. Apparatus Used for Valve Testing.

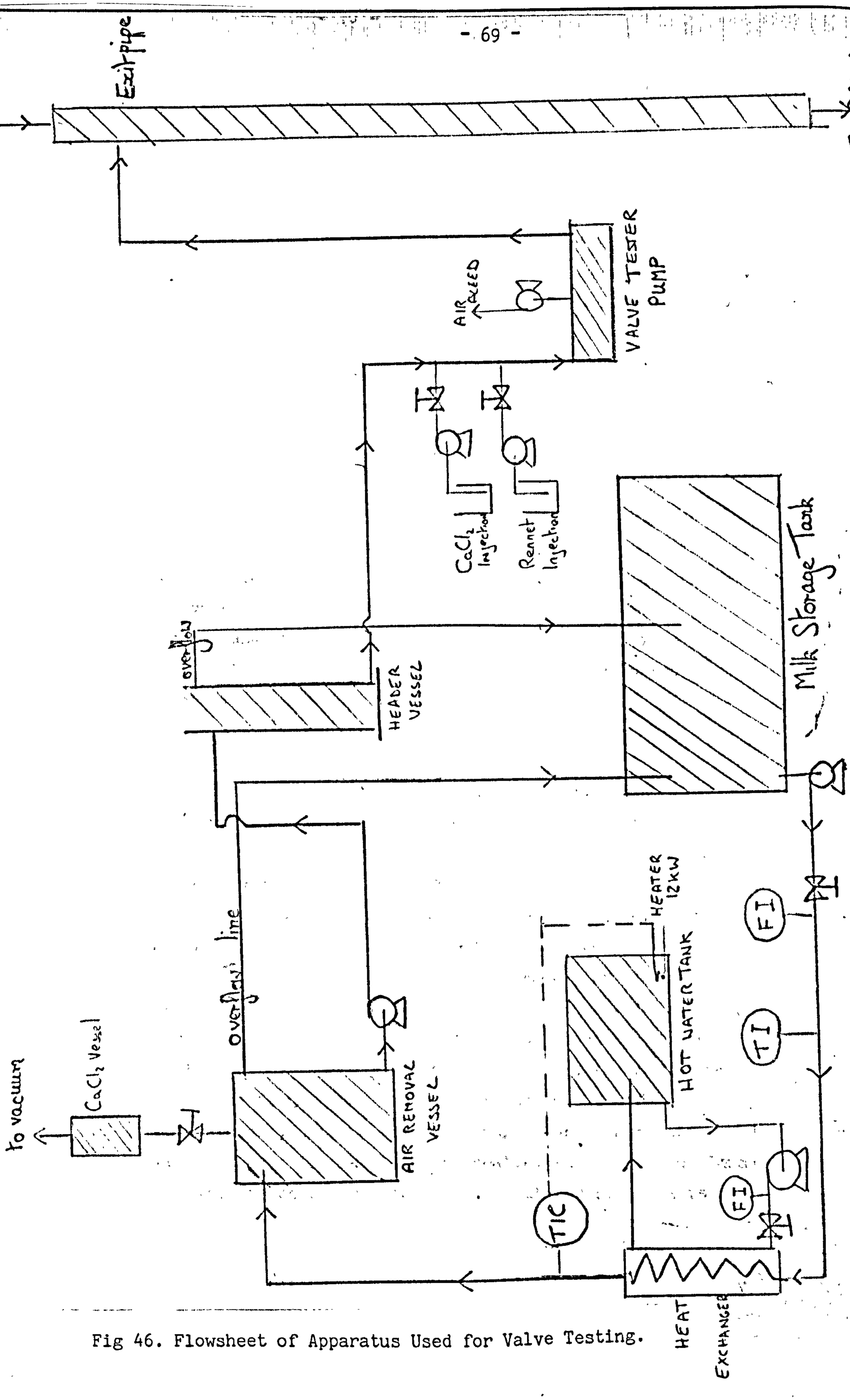


Fig 46. Flowsheet of Apparatus Used for Valve Testing.

4.3.1 Milk Storage Tank

The milk arrives in 3 x 10 gallon drums and is poured into a 50 gallon polypropylene tank. The tank is mounted in a frame on wheel to facilitate cleaning and moving if it becomes necessary. At one end of the tank is a plug for draining and at the other the outlet to the pump and the main apparatus. The pump is a Stuart Turner No 10 with a maximum flow rate of 130 gallons per hour, and is mounted into the frame with a power cable sufficiently long so as to enable the tank to be wheeled to the drain in the laboratory. The pump is designed to pump the milk to the vacumm tank through a metric size 14 rotameter with a valve attached and the heat exchanger. The valve is included so that the overflow from the vacumm tank and header tank can be kept to a minimum so that the main milk storage will not be heated too rapidly.

4.3.2 The Heat Exchanger

A Q.V.F. glass condenser type heat exchanger No HE3 is used with a heat transfer area of $0.3m^2$. The milk is inside the coil to facilitate cleaning and hot water is pumped through the shell. To avoid any risk of heat coagulation the heating water temperature has to be kept below $60^{o}C$. The water is heated in a covered tank containing 4 x 3kw kettle heater elements, as heating even a stream of 3 litres/min of milk from $5^{o}C$ to $40^{o}C$ requires 7.5kw. The temperature in the heating tank is controlled by the temperature on the inlet to the vacumm tank by switching one of the elements on and off automatically using a servomex temperature controller type TC 201. The level in the heating tank is the minimum possible to cover the elements so that the response is

fairly rapid. It is controlled by means of an overflow into the sink. A thermostat is also attached so that if the temperature in the tank exceeds a certain preset level all the elements would trip. This is in case the elements are accidentally left on. The pump used for circulating the heating water is a Stuart-Turner No 12 centrifugal pump with a metric No 35 Rotameter.

4.3.3 Vacuum Tank

From the heat exchanger the milk flows into the vacuum reservoir which is designed to remove the excess gases from the milk stream. The vessel has a diameter of approximately 10" and is 1ft high and in order to reduce the volume of fluid and improve the surface area/volume ratio is filled with ½" ceramic balls. Approximately 6ft of water vacuum is available and the reservoir is raised so that that back up in the overflow tube caused by this vacuum can be accommodated. The vessel is made of polypropylene and has a flanged top so that if necessary it could be opened. The vacuum line is protected by a calcium chloride filled cylinder to avoid excessive water vapour damaging the vacuum pump.

4.3.4 The Header Tank

From the vacuum tank the milk is pumped through a flow adjustment valve into a header tank. The pump used is a Stuart-Turner No 10. The header tank ensures that any gas which has not evolved in the vacuum reservoir will not create problems in the valve tester as the pressure there is approximately 4ft water. The vessel is a piece of 3" polypropylene tubing approximately 18" in height. The milk flows in near the top of the vessel and out to the valve

tester from the bottom. There is an overflow from near the top to the main milk tank. The overflow from the vessel is restricted to a minimum by the adjustable valve between it and the vacuum reservoir.

4.3.5 Rennet Essence and Calcium Chloride Addition

The milk is sucked from the header tank by the heart and is split into two streams by use of a Y piece. Rennet and calcium chloride are added separately into the different streams by use of Shuco pericyclic pumps. The addition circuits each comprise; a 1 litre flask in which the rennet or calcium chloride was kept, a pericyclic pump, a flow control valve, a rotameter and a Portex disposable manometer line. The pericyclic pumps give a maximum flow rate of 100cc/min. By having the flow cut back by this valve the effects of the pulsatile flow from the pericyclic pump and the heart pump are reduced, and steady flow can be obtained. The manometer line was fitted into the side arm of a T piece inthe main milk line.

After the rennet and calcium chloride had been added the flows are split again before entering the heart chambers.

4.3.6 The Heart Chambers

The perspex chambers which housed the valves under test were built to simulate the variation in flow area of the approaches to the valve in the human heart. (Fig 47).

The equivalent diameter cross-sectional flow areas were obtained from Weiting (201) who injected silicone rubber into human hearts during necropsy, subsequently slicing the silicone impression every 2.5mm parallel to the valve annuli for 30mm either side of the valves, and expressing

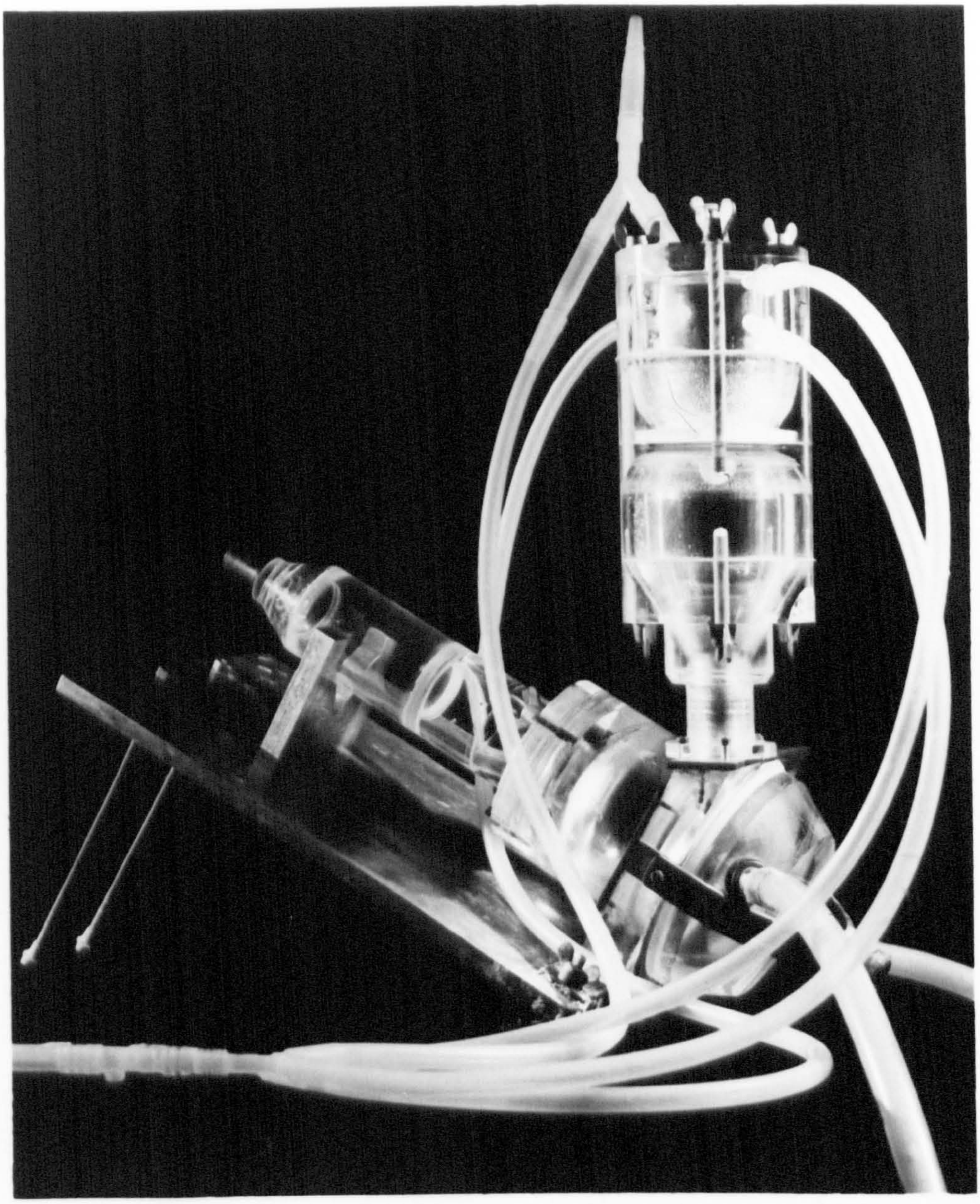


Fig 47. Photo of Chambers

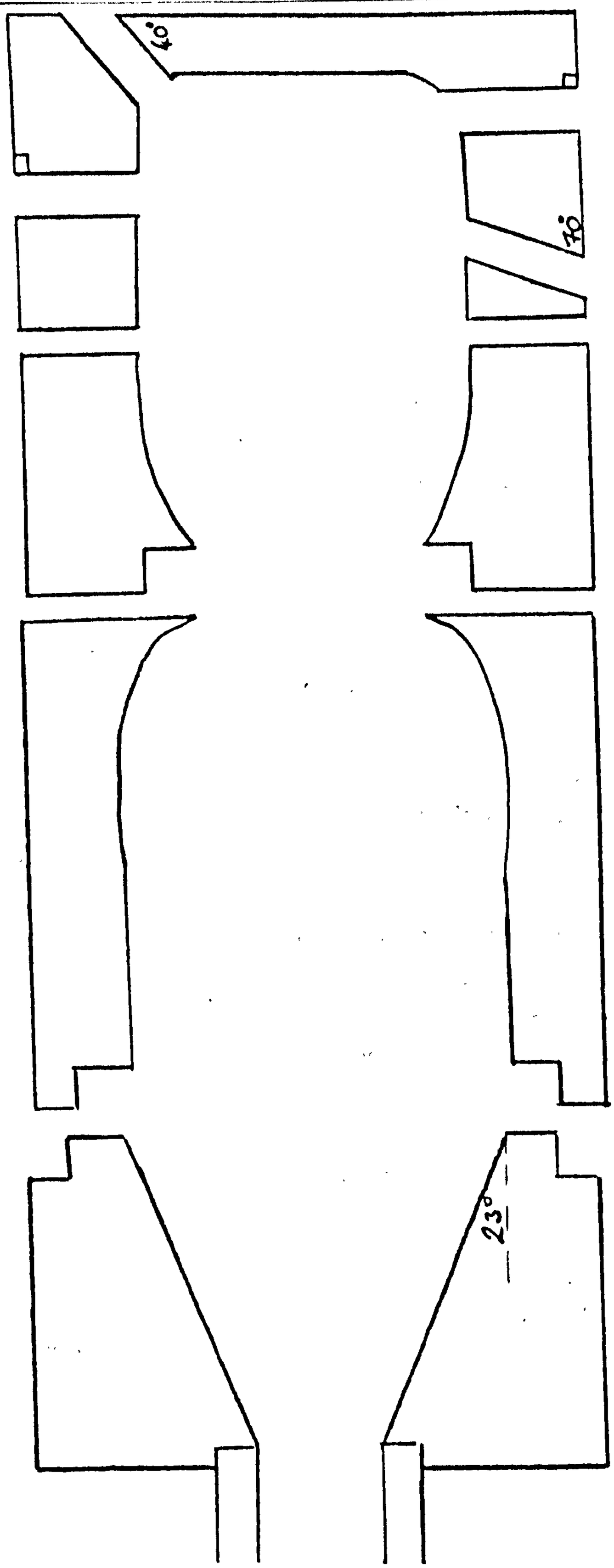


Fig 48. Actual Size Drawing of Mitral Chamber.

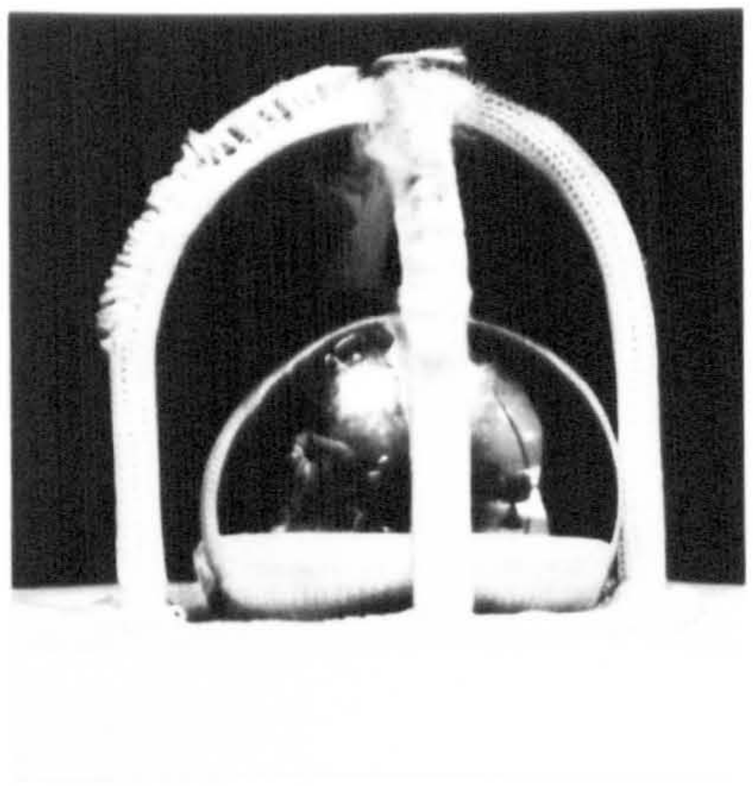
the area of each slice as an equivalent circular diameter.

The aortic chamber is described by Taylor (55) and the mitral chamber was made for this particular piece of work. A drawing of the chamber is shown in Fig 48.

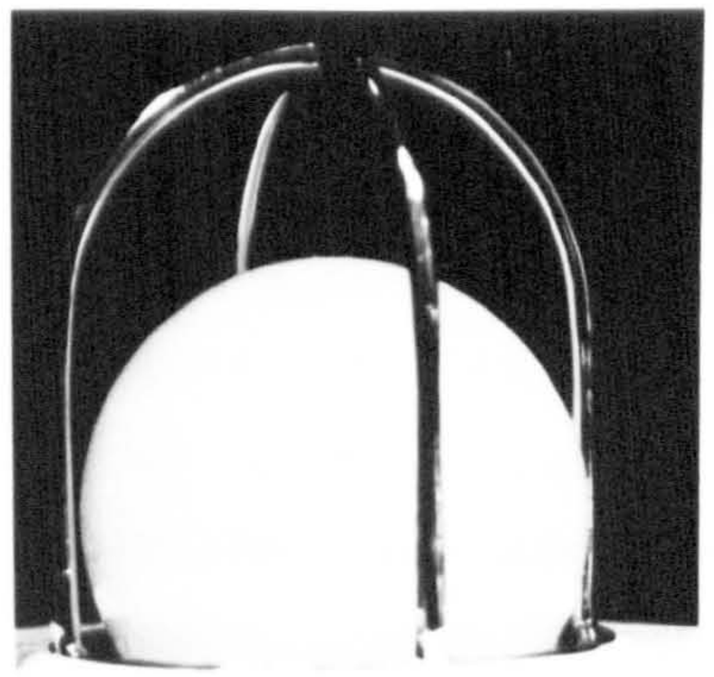
The positions and angles of the inlets are based on physiological data obtained from Professor G J Romanes of the Anatomy Department, University of Edinburgh and mimic those present in the heart. Having four inlets also helps achieve good mixing of the reactants in the chamber. A continuous air bleed is included at the top of the chamber so that any air which gets into the system can be removed.

The pump used with the chambers to obtain pulsatile flow is Macleod's positive displacement diaphragm pump, which has independently variable frequency, stroke volume and systolic/diastolic ratio (202).

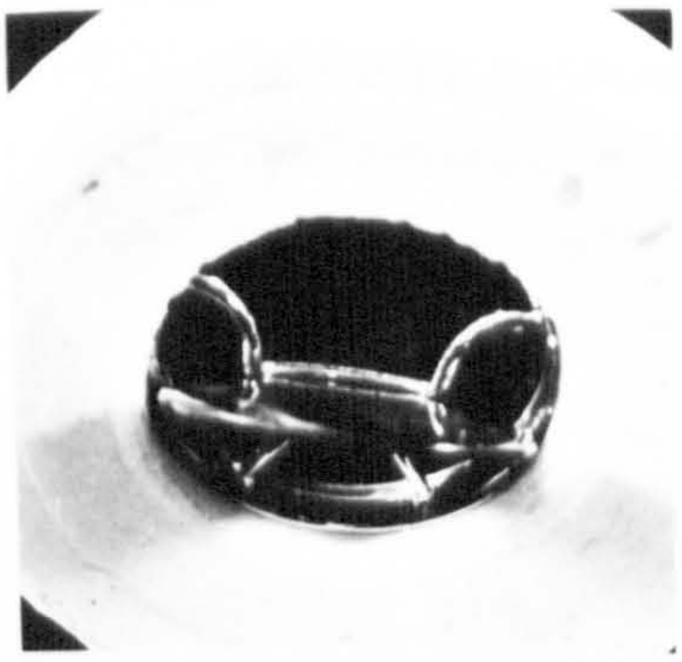
The valves are mounted into flexible rubber mouldings to simulate to some extent the flexibility of the real heart and as a mean of sealing the chambers. By using wing nuts on bolts quick extraction of the valves was possible. Some of the valves used in their mountings are shown in Fig 49.



Cloth covered
Starr-Edwards



Silicone Ball
Starr-Edwards



Bjork-Shiley

Edinburgh

Stainless Steel Flat Disc

Carbon Flat Disc

Fig 49. Valves Used for Experiments.

Outflow

From the chambers the milk is pumped to a down pipe at a height roughly 1ft above the height in the header tank. This is necessary to obtain a back pressure to close the valves. A stream of cold water is also introduced at the top of the down pipe to minimize fouling. The milk flows into a sink where the flow rate can be measured using a flask and stopwatch and thence to drain.

4.3.7 Control

All controls are manual apart from the automatic temperature controller. This has been found to be fairly satisfactory, although computer control using a PET would be desirable. The temperatures on the inlet to the heat exchanger on both the water and milk sides are measured as are the temperature on the outlet side (water), the inlet to the vacuum reservoir and the inlet to the header tank. Thermocouples attached to a Comark electric thermometer are used for all of these. The flow rates of the milk from the storage tank, the circulating water and the rennet and calcium chloride are all measured using rotameters. The flow rate through the heart is measured using a measuring flask and stopwatch, because the pulsatile nature of the flow does not allow the use of a rotameter.

4.4 Experimental Procedure

Before any run the equipment is thoroughly cleaned using a chlorine releasing detergent (TAP) and then rinsed thoroughly with clean water. Pump and valve operation are checked and the pericyclic pumps are checked to ensure that they are functioning properly as there are frequently problems with these.

The heating elements and the water circulation pump are switched on checking that there is sufficient water in the tank to cover the heating elements and avoid air entrainment. The water is heated to 60°C and then elements are switched off.

Meanwhile, the 30 gallons of milk are poured from the churns into the milk storage tank adding a dye if it is so desired. The pump from the storage tank and the pump between the vacuum reservoir and the header tank are switched on and the flows adjusted so that there is only minute flow down the overflow from the vacuum reservoir and sufficient to allow for the flow through the heart pump, down the overflow from the header tank. The heaters are turned on and the temperature allowed to settle at that required for the run.

The rennet and calcium chloride are filled into their containers and warmed to reduce condensation on the tubes of the pericyclic pumps which would impair their operation.

When the temperature in the vacuum reservoir has reached the desired level and the flows are settled the vacuum is switched on. When everything is settled and ready, the heart pump is started and the flow adjusted to the desired level. The pericyclic pumps are then switched on and the valves opened to give the desired flows. The valves are normally kept closed to stop beackflow through the lines when the pumps are not working.

A constant check is kept on the temperatures and flows.

When the milk storage tank is empty, after about an hour, the pump at the storage tank is switched off as are the heater elements, the water circulation pump and the vacuum pump. When the vacuum reservoir is empty, the pump between it and the header tank is switched off and the rennet and calcium chloride flows are stopped. The heart pump is then swicthed off and the overflow line removed from the high pipe and lowered into the sink to drain the system. The valves are now carefully removed from the chambers and placed in the freezer.

Cleaning

Two other valves are placed in the chambers and the whole system is rinsed through with water. When all the milk has been rinsed out the circuit is separated into two: a circuit which has had no rennet through and a circuit which has.

The cleaning of the circuit which has had no rennet added is achieved by adding T.A.P., a chlorine liberating detergent, to the main storage tank which is ½ filled with water. The tank is washed by hand and the rest of the circuit by pumping using the pumps already present. The overflow pipes are placed into the sink which has its plug in and is itself filled, allowing the water to drain down the sink overflow. The outlet from the header tank, where the first circuit ends is blocked by a stoppered flexible tube which is opened occasionally to drain and then restoppered. Once the circuit is filled and the cleaning fluid has circulated for roughly ½ hour, the pumps are switched off and the system left overnight to sterilize.

The second circuit which has had rennet added begins at the outlet from the header tank. Once the circuit has been rinsed through and some of the large clots adherent to the walls of the chamber have been removed, both the inlet and outlet tubes are placed in an elevated flask. Tegazyme, an enzymatic detergent, is added and the actuator is switched on to circulate the fluid. After it has been on for roughly ½ hour the pump is switched off and the circuit is allowed to clean overnight.

The following morning the circuit is connected and run normally and the fluid flows out through the overflow. When the main storage tank is empty it is filled with hot clean water and the circuit is run to clean.

Recording Results

All valves are photographed so that a permanent record is obtained. The milk is dyed using standard vegetable dyes so that if a white valve is used the clots are clearly visible.

Unless otherwise stated a concentration of 1% calcium chloride and rennet, 30 gallons of milk and a pulse rate of 60 - 70 were used throughout.

4.5 Illustrations of the Reproducibility of the Clotting Patterns Found Using Rennetized Milk

4.5.1 Results

Starr-Edwards Valve Upstream

Fig 50. Starr-Edwards Aortic Upstream. 43°C. 0.6% $CaCl_2$ and rennet. 1.7L/min milk. Pulse 50. Run Duration 10 mins.

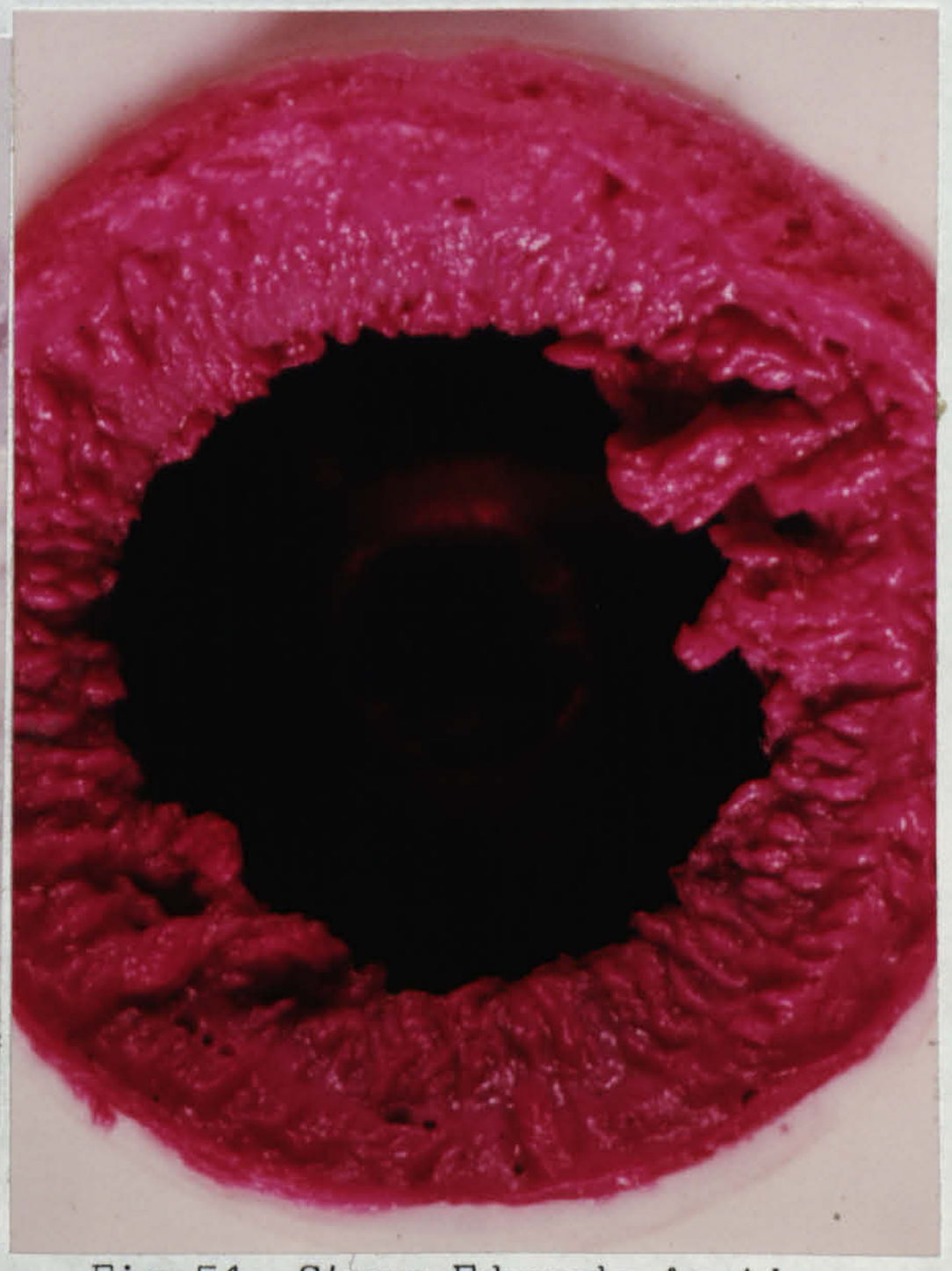


Fig 51. Starr-Edwards Aortic Upstream. 40°C. 2L/min milk.

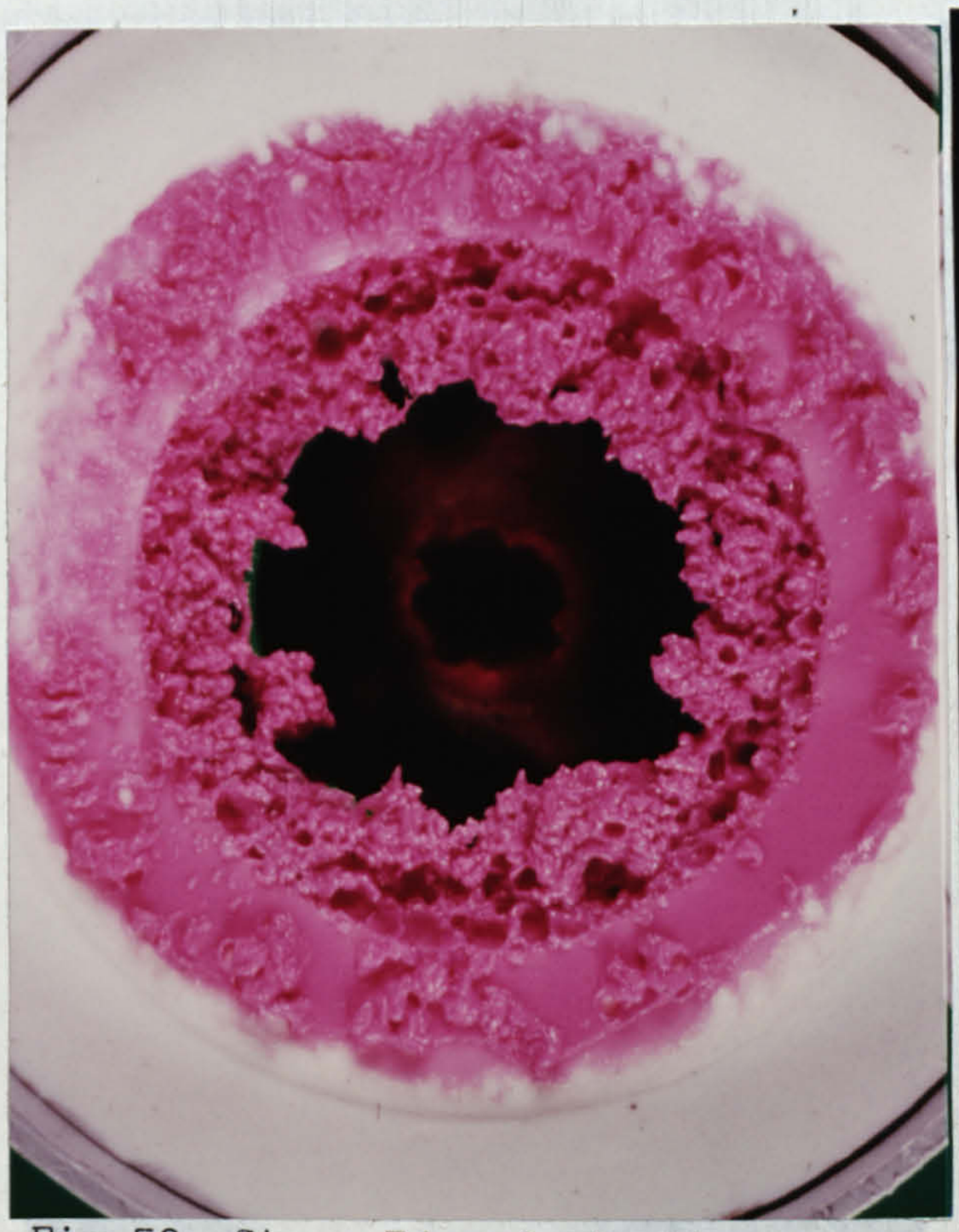


Fig 52. Starr-Edwards Aortic Upstream. 40°C. 2L/min milk

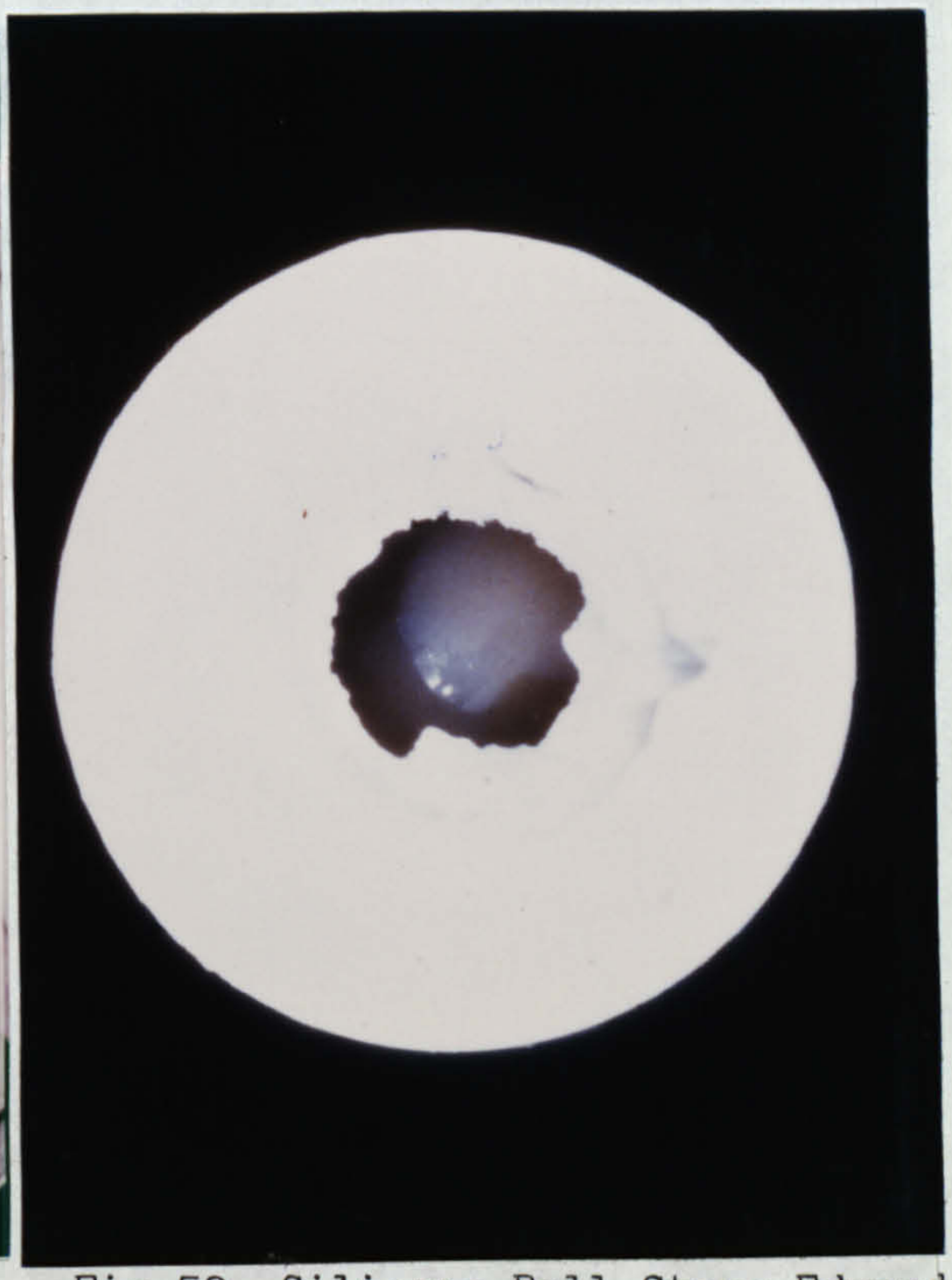


Fig 53. Silicone Ball Starr-Edwards Aortic Upstream. 38°C. 2L/min milk.

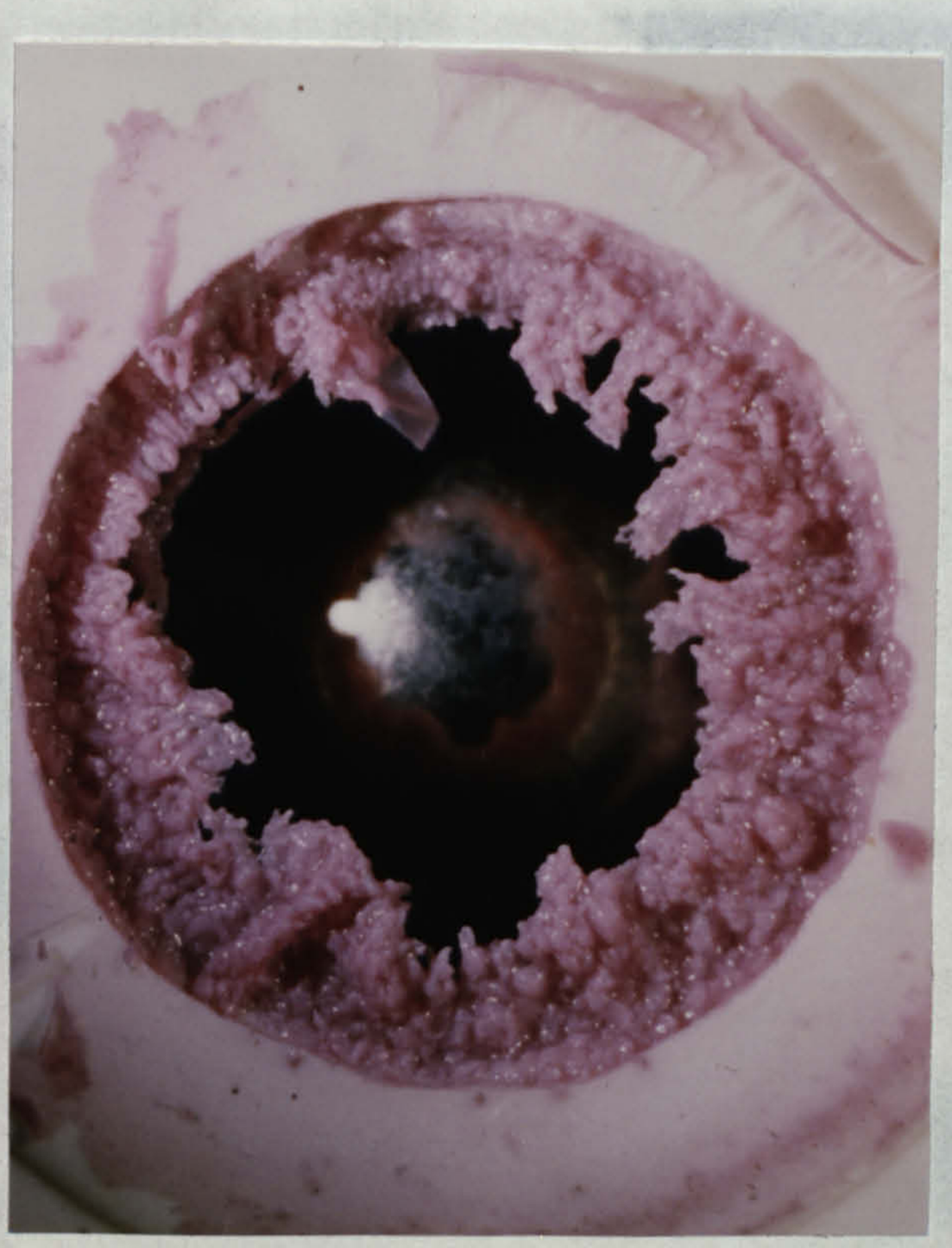


Fig 54. Starr Edwards Mitral Upstream. 38°C. 3L/min milk.

Starr-Edwards Valve Downstream

Fig 55. Starr-Edwards Aortic Downstream. Conditions as Fig 50.

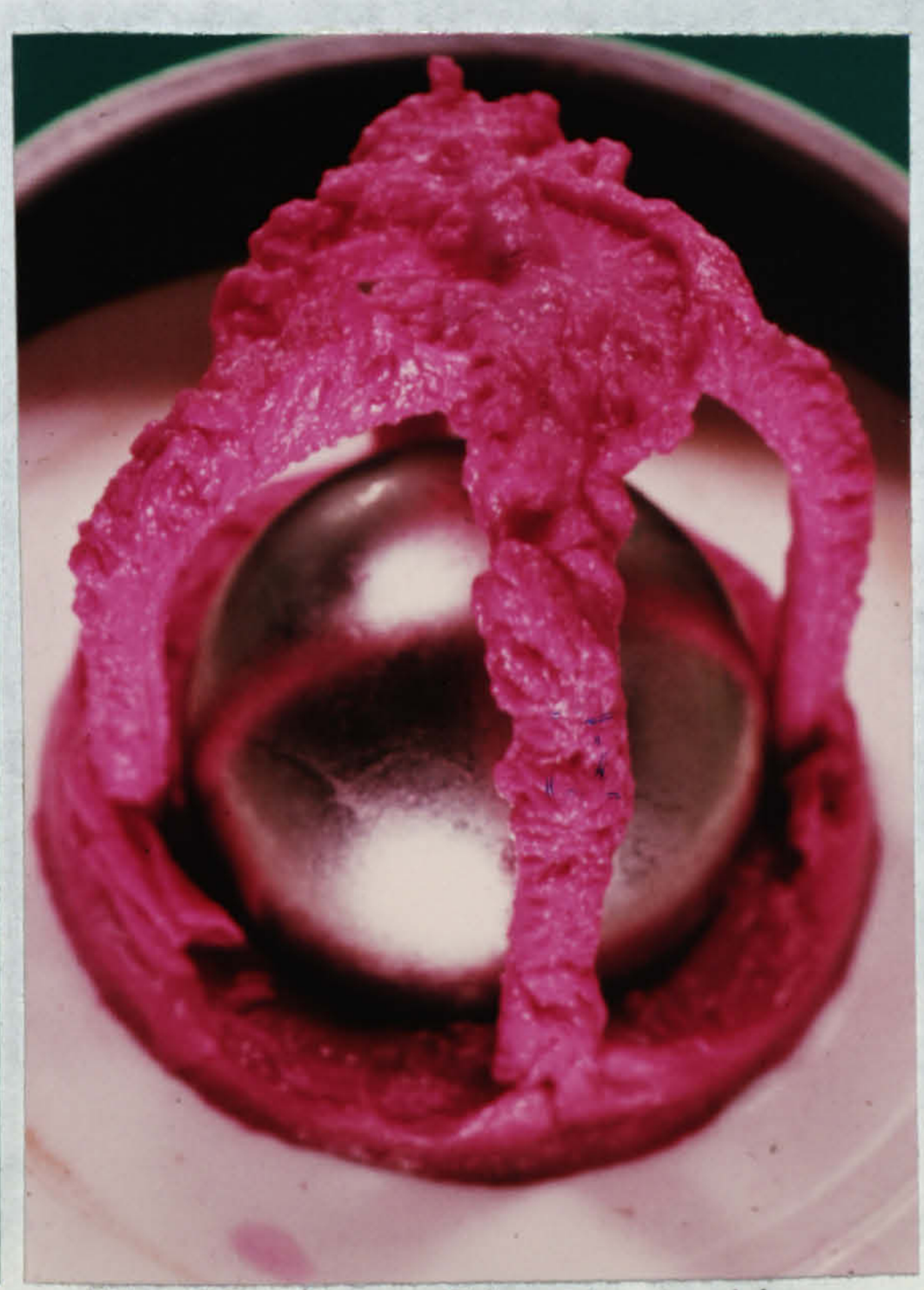


Fig 56. Starr-Edwards Aortic Downstream. 40°C. 2L/min milk.

Fig 57. Starr-Edwards Mitral Downstream. 40°C. 2L/min milk.

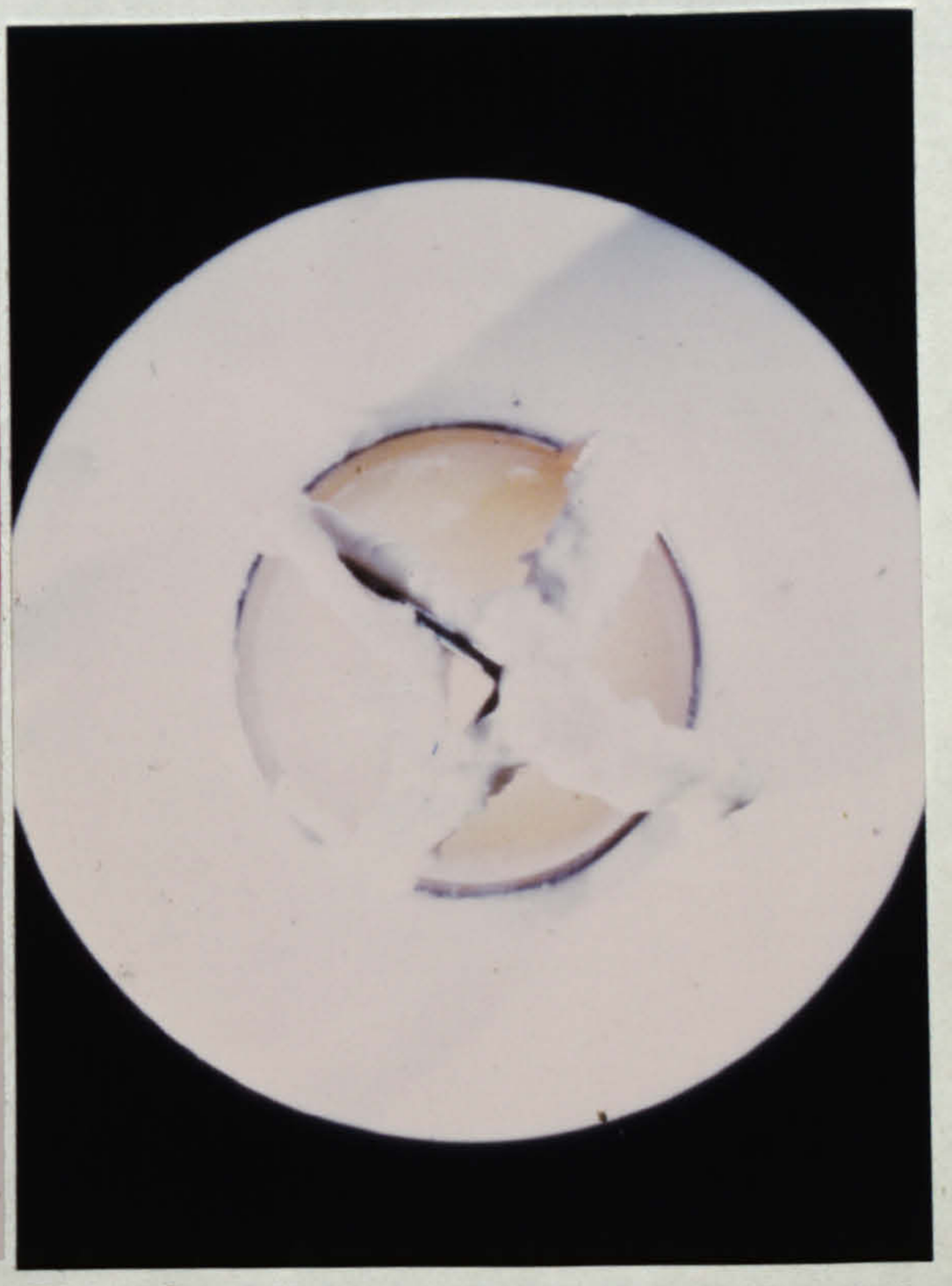


Fig 58. Silicone Ball Starr-Edwards Aortic Downstream. 38°C. 2L/min milk.

Fig 59. Starr-Edwards Aortic Downstream. 38°C. 2L/min milk

Fig 60. Starr-Edwards Mitral Downstream. 38°C. 3L/min milk.

Bjork-Shiley Valve Upstream

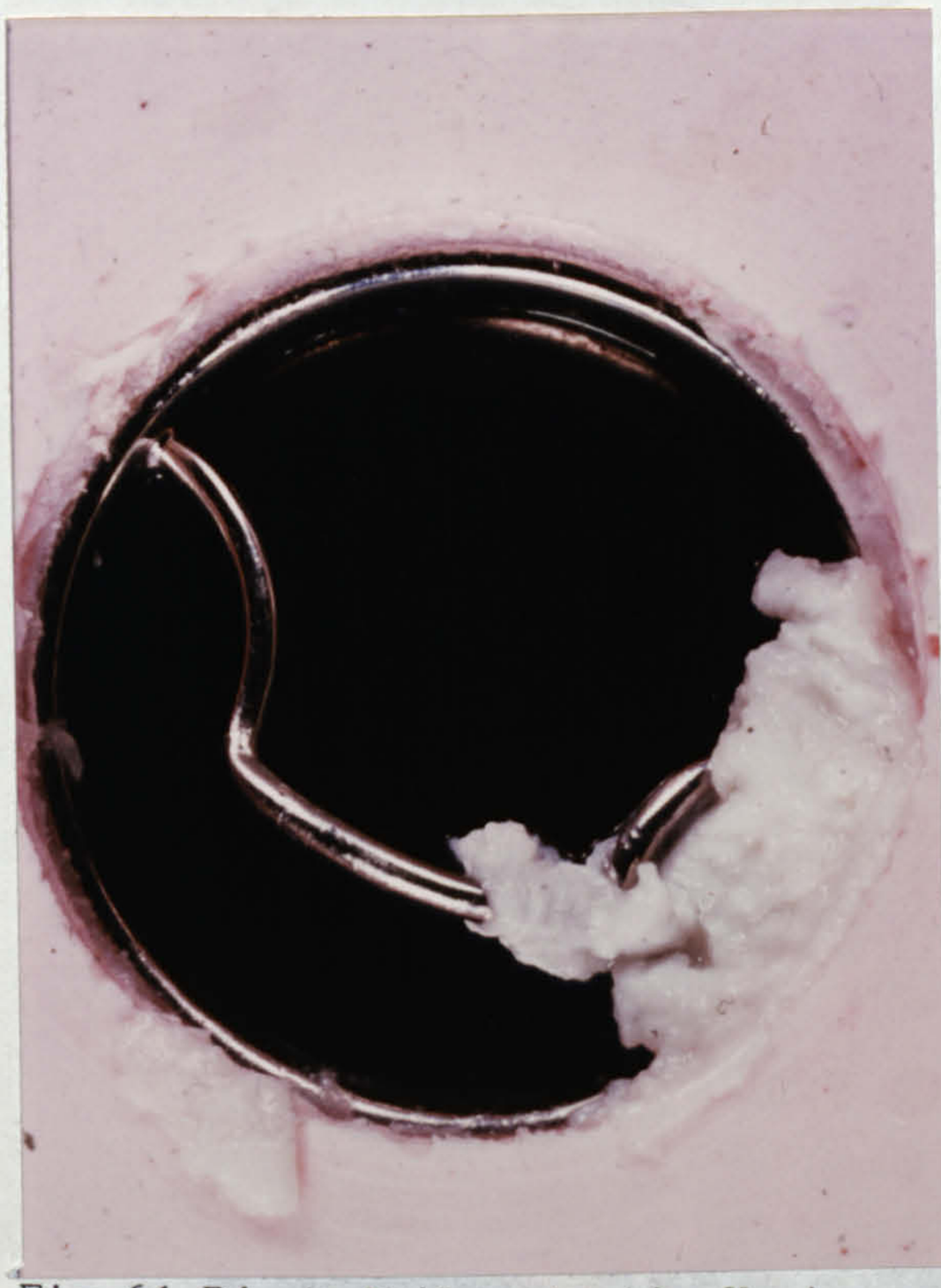


Fig 61.Bjork-Shiley Aortic Upstream
40°C. 0.6% $CaCl_2$ & rennet. 2L/min milk. Valve albumen coated.

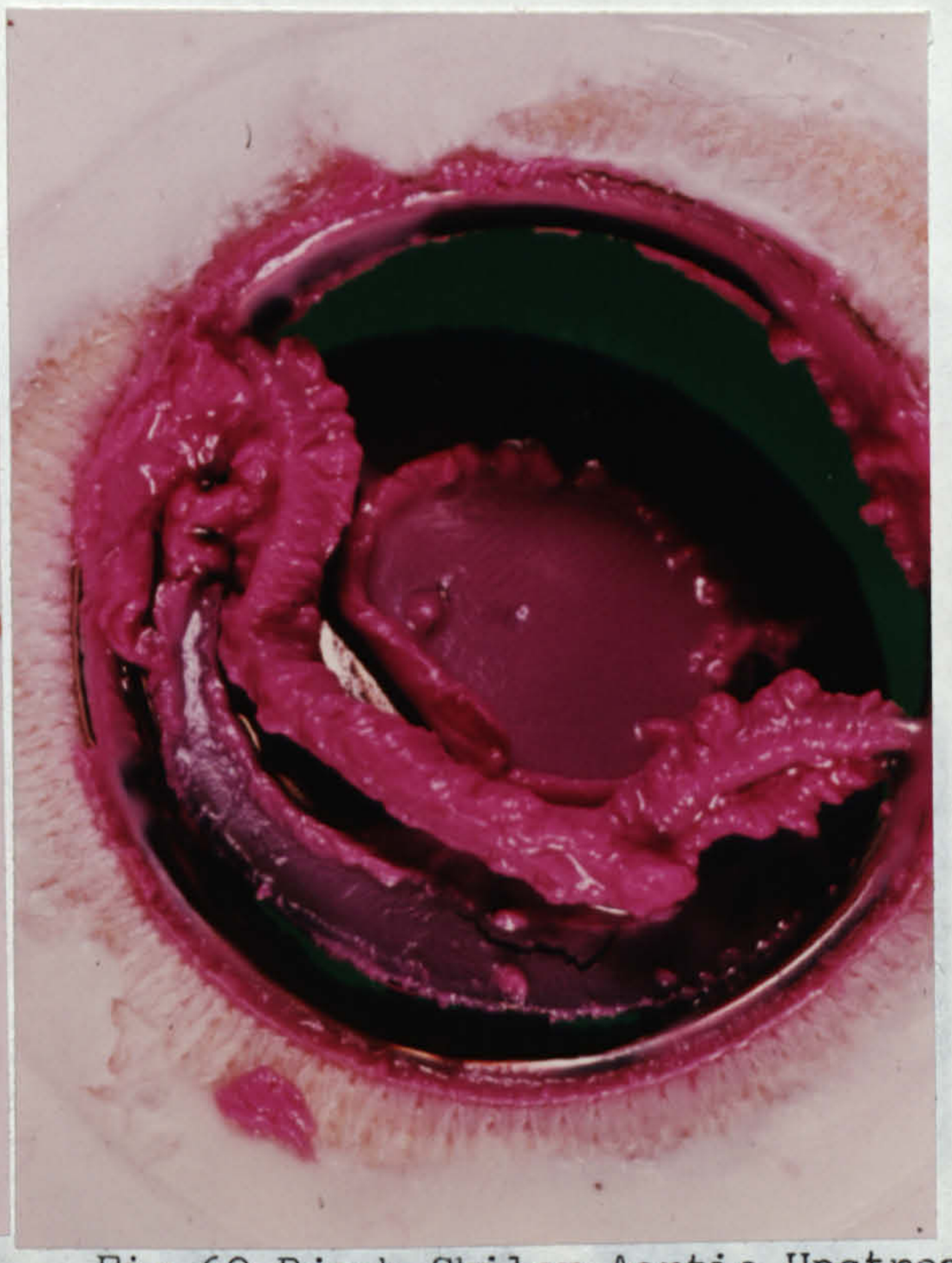


Fig 62.Bjork-Shiley Aortic Upstream
40°C. 2L/min milk.

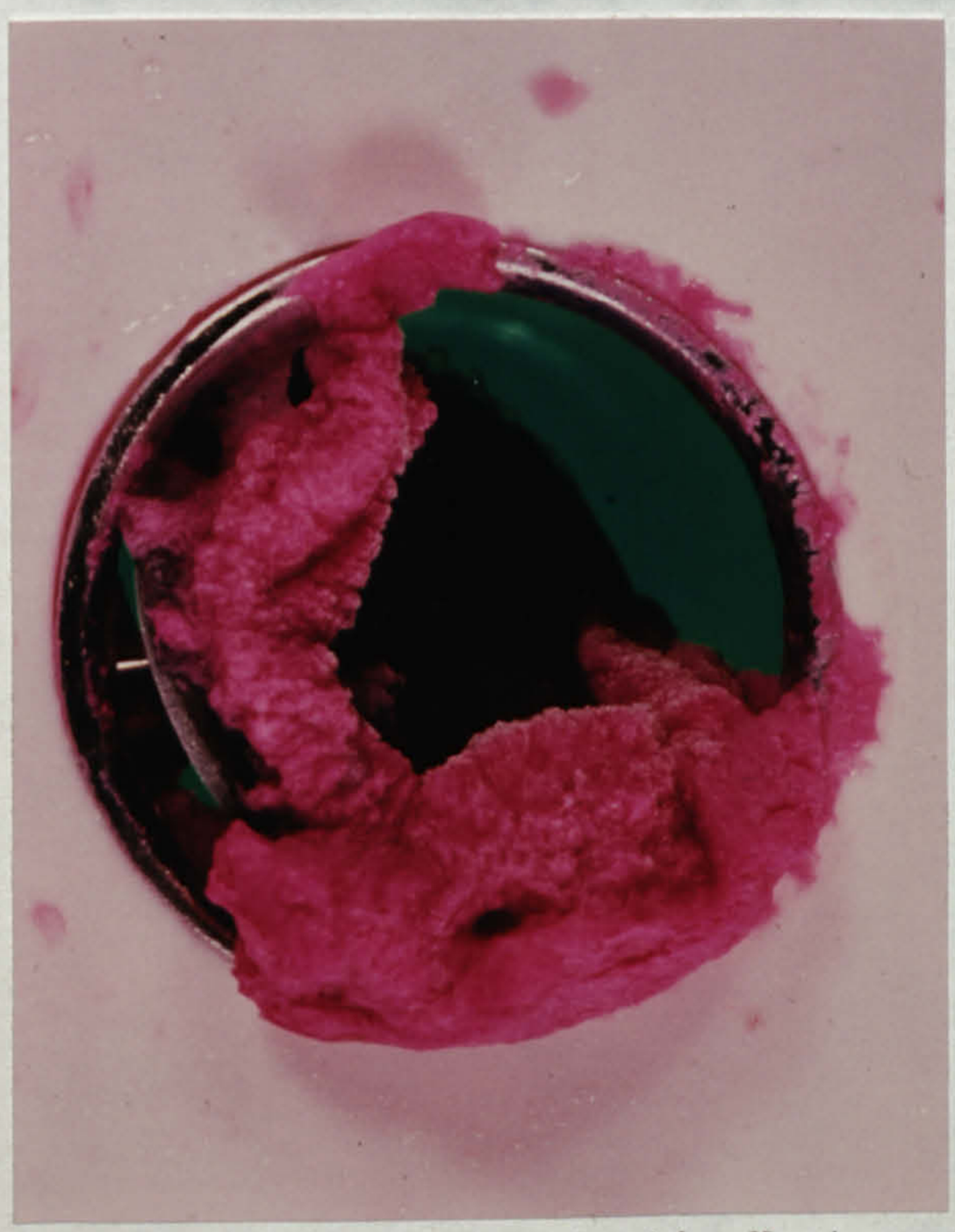


Fig 63. Bjork-Shiley Aortic Upstream
40°C. 2L/min milk.

Fig 64. Bjork-Shiley Aortic Upstream
40°C. 3L/min milk.

Fig 65. Bjork-Shiley Aortic Upstream 39°C. 3L/min milk.

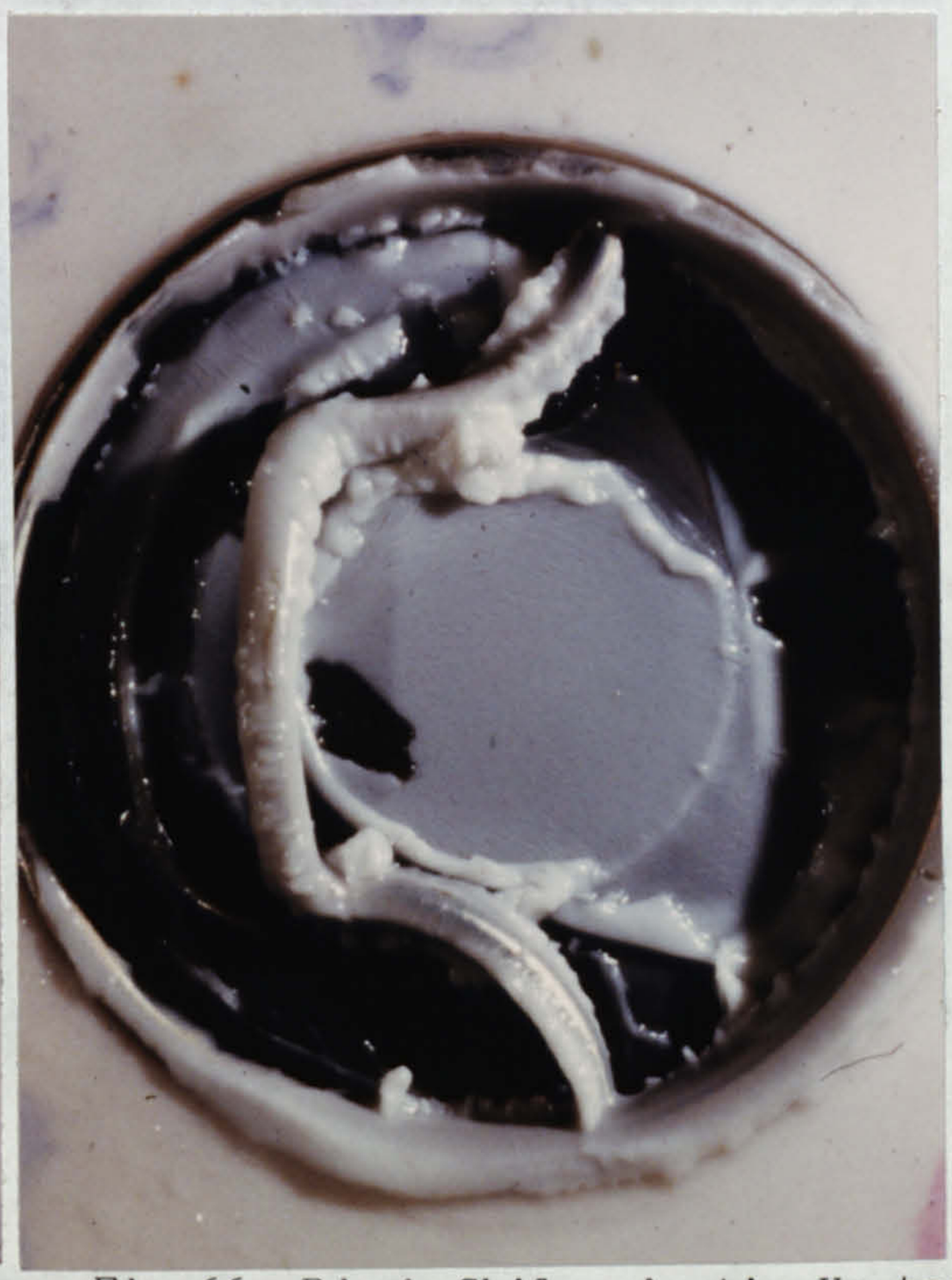


Fig 66. Bjork-Shiley Aortic Upstream 38°C. 3L/min milk.

Bjork-Shiley Valve Downstream

Fig 67. Bjork-Shiley Aortic Downstream. Conditions as Fig 61.

Fig 68. Bjork-Shiley Aortic Downstream. 40°C. 2L/min milk.

Fig 69. Bjork-Shiley Aortic Downstream. 40°C. 2L/min milk.

Fig 70. Bjork-Shiley Aortic Downstream. 40°C. 3L/min milk.

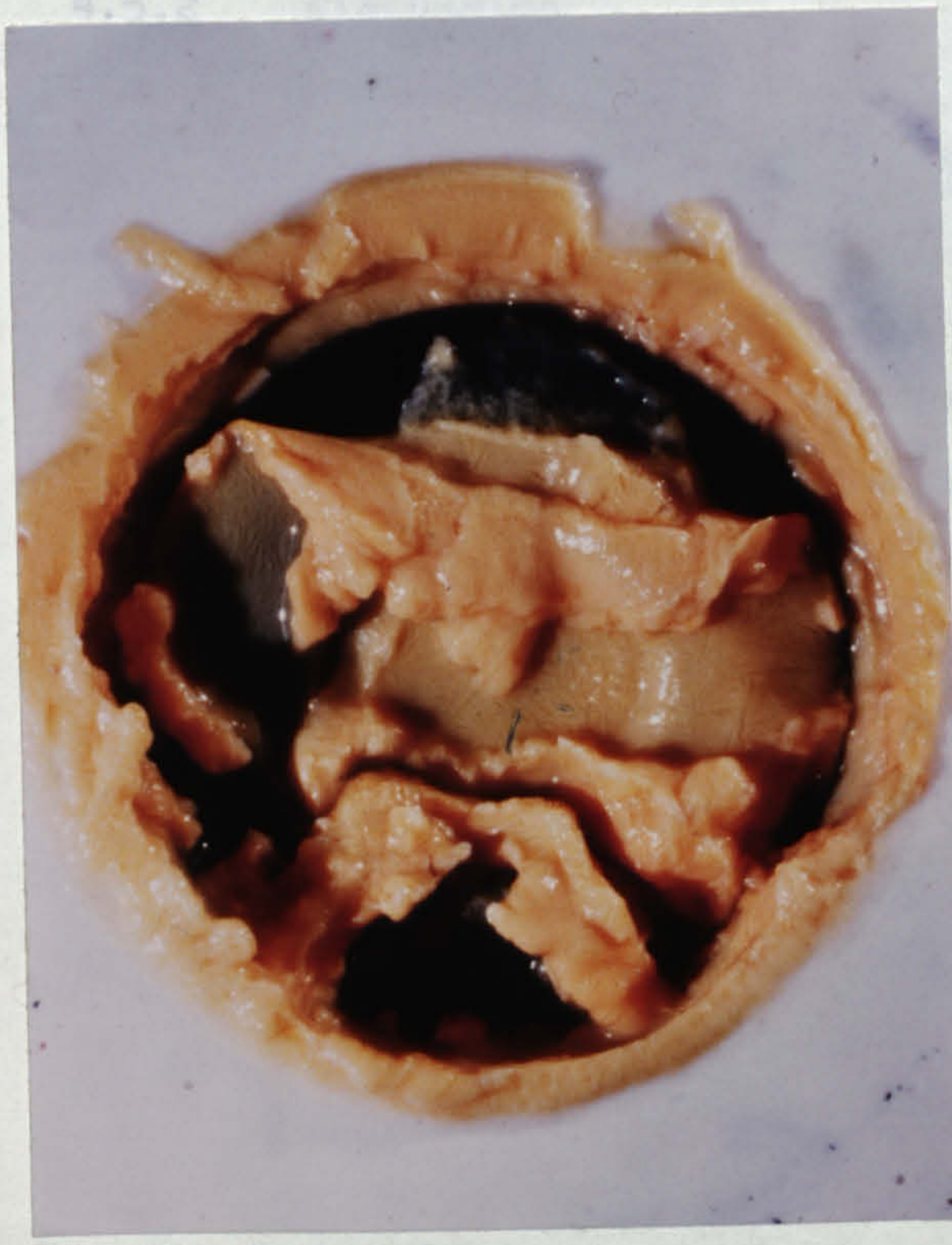


Fig 71. Bjork-Shiley Aortic
Downstream. 39°C. 3L/min milk.

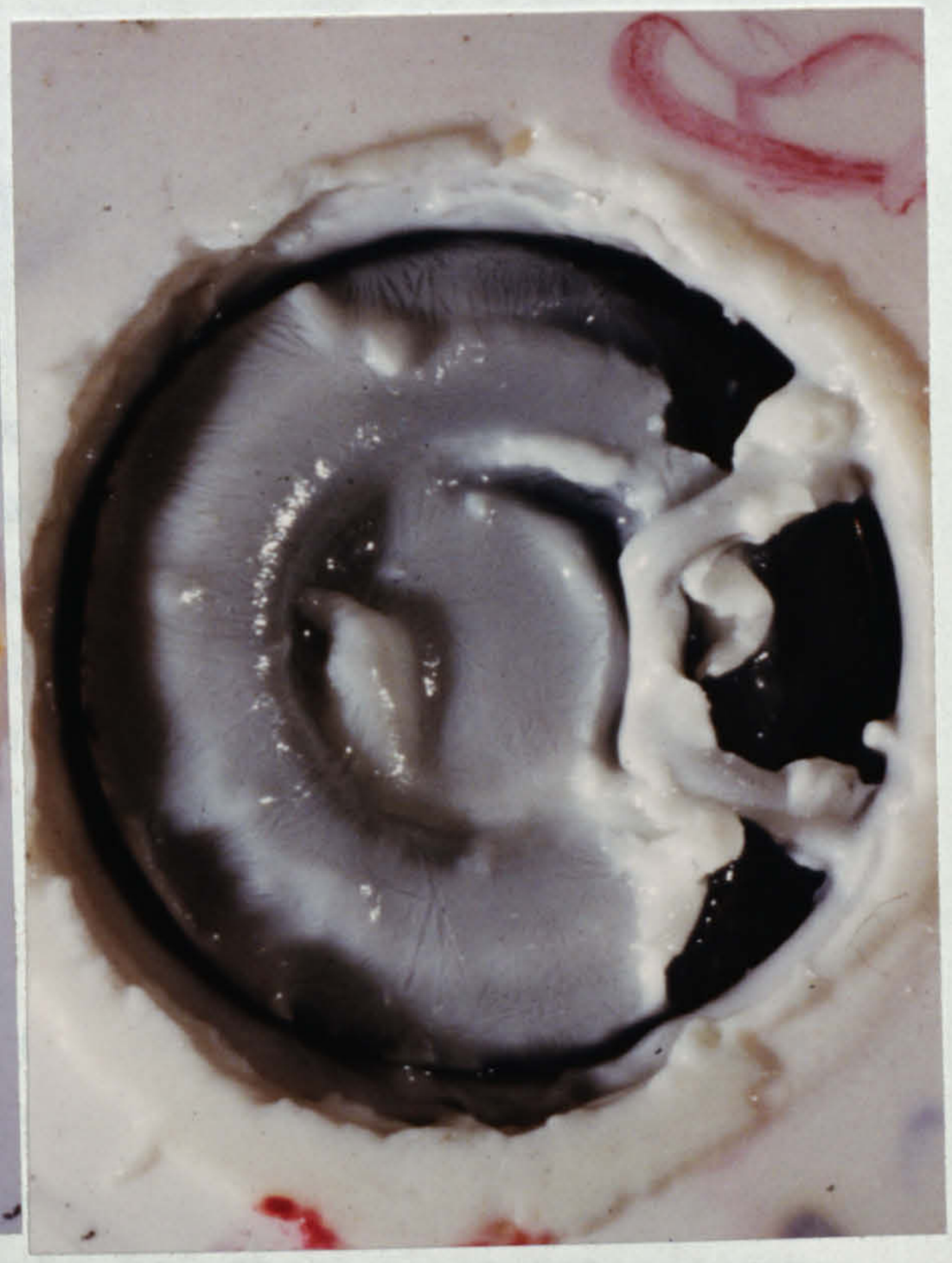


Fig 72. Bjork-Shiley Aortic
Downstream. 38°C. 3L/min milk.

4.5.2 Discussion

Starr-Edwards Inside Ring

The growth of a clot inside the ring of the Starr-Edwards ball valve occurs in all cases where any clotting is observed. This phenomenon is also found in the clinical situation as discussed earlier, and is presumably promoted by the presence of the ball inside the ring when the valve is closed. This illustrates the importants of transient effects in clot deposition and shows the need for pulsatile flow in the testing of heart valves.

Starr-Edwards on Struts

As discussed earlier, little or no clotting is observed on the struts of the Starr-Edwards ball valve in the clinical situation. However, when rennetized milk is used in vitro in all cases except Fig 60 gross clotting occurs on the struts, and the ball remains clear as it does clinically. For Fig 60 the flow rate was higher than in all the other cases (3l/min vs 2l/min) and, therefore, the duration of the trial was shortened, as 30 gallons of milk were used in all cases: clot has, however, clearly begun to form.

Bjork-Shiley on Upstream Side

Unlike the Starr-Edwards valve the Bjork-Shiley tilting disc valve appears to be prone to two distinct types of clot deposition as discussed earlier. The first type is a discrete deposit forming on the struts and not involving the disc, and the second is a more generalized deposit involving the

disc as well. Fig 61 illustrates the first type and is different from the other runs in that the valve was coated with albumen prior to the commencement of the run. The explanation for this curious behaviour is not immediately obvious. However, as the coagulability of the milk was low due to the low concentrations of rennet and $CaCl_2$ (0.5%) with the greater clot forming propensity of the valve surface due to the albumen coating, the clot forms at the most favourable site available, which may explain the discrete deposit. In the case of the clinical environment this is equivalent to a situation in which the blood has not become hypercoagulable, but there is some imperfection on the valve which makes a particular site preferentially thrombogenic.

In the other cases the patterns are largley similar with clot forming over the entire surface except for the region which is cleaned by the struts as the valve opens and closes. Larger deposits also appear on the struts presumably at the most favourable sites, similar to those discussed above, but the overall impression is of generalized clotting.

Bjork-Shiley on Downstream Side

The deposition on the downstream side of the Bjork-Shiley valve appears very similar to that occurring on the upstream side.

4.5.3 Conclusions

These results show that the clotting phenomena observed on the heart valve used and in the experiental system described earlier, are sufficiently reproducible that

inordinately large numbers of runs are not required to test the thrombogenicity or clot fomring propensity of a given heart valve.

4.6 Illustrations of the Similarities in Appearance Between Valvular Deposits Found with Rennetized Milk and Clinical Experience of Clotted Heart Valves

4.6.1 Results

Bjork-Shiley Valve

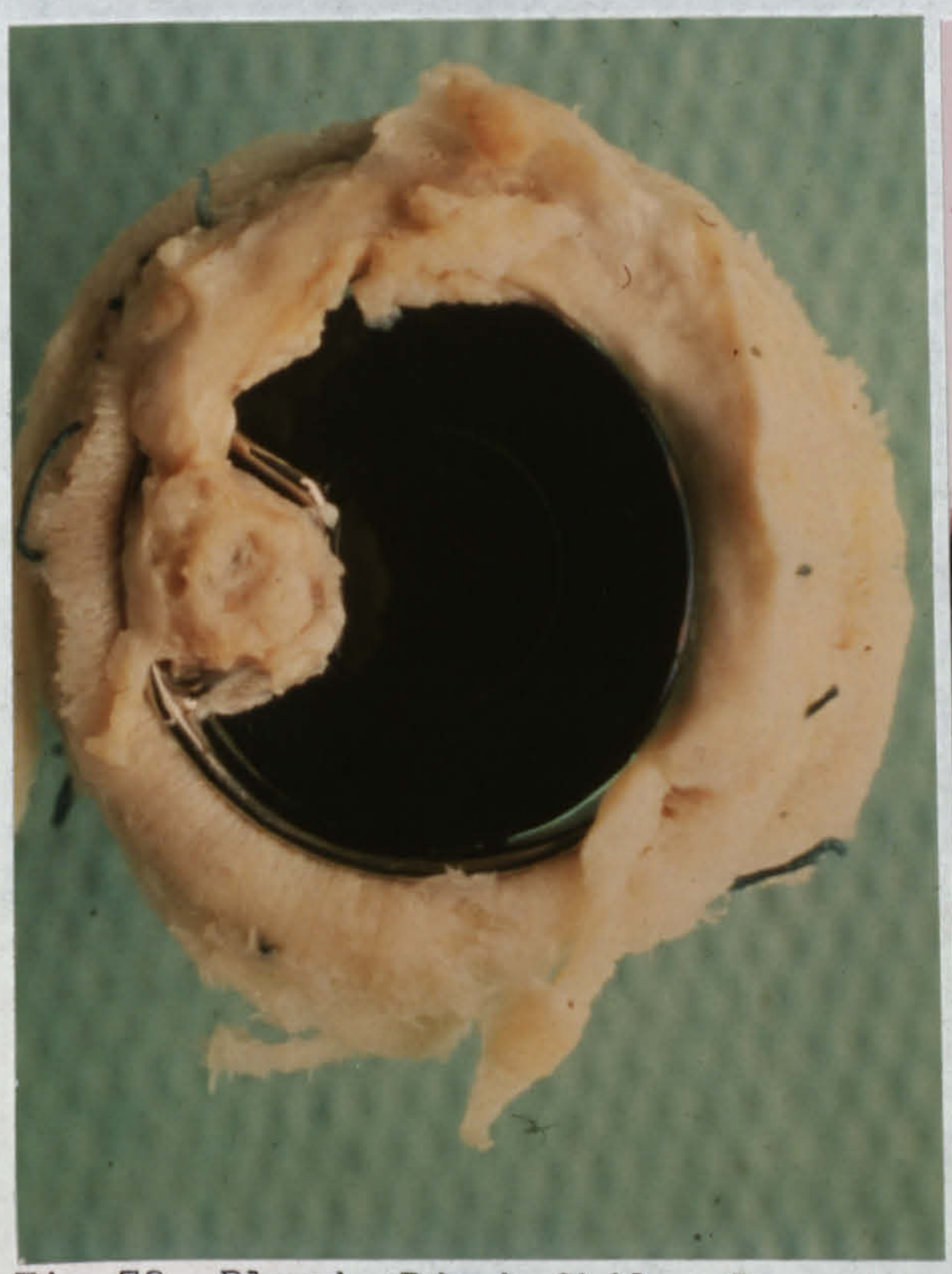


Fig 73. Blood. Bjork-Shiley Downstream After 2 Years. Courtesy MacGregor.

Fig 74. Milk. Bjork-Shiley Aortic Downstream. 40°C. 0.6% $CaCl_2$ & rennet. 2L/min milk. Albumen Coated.

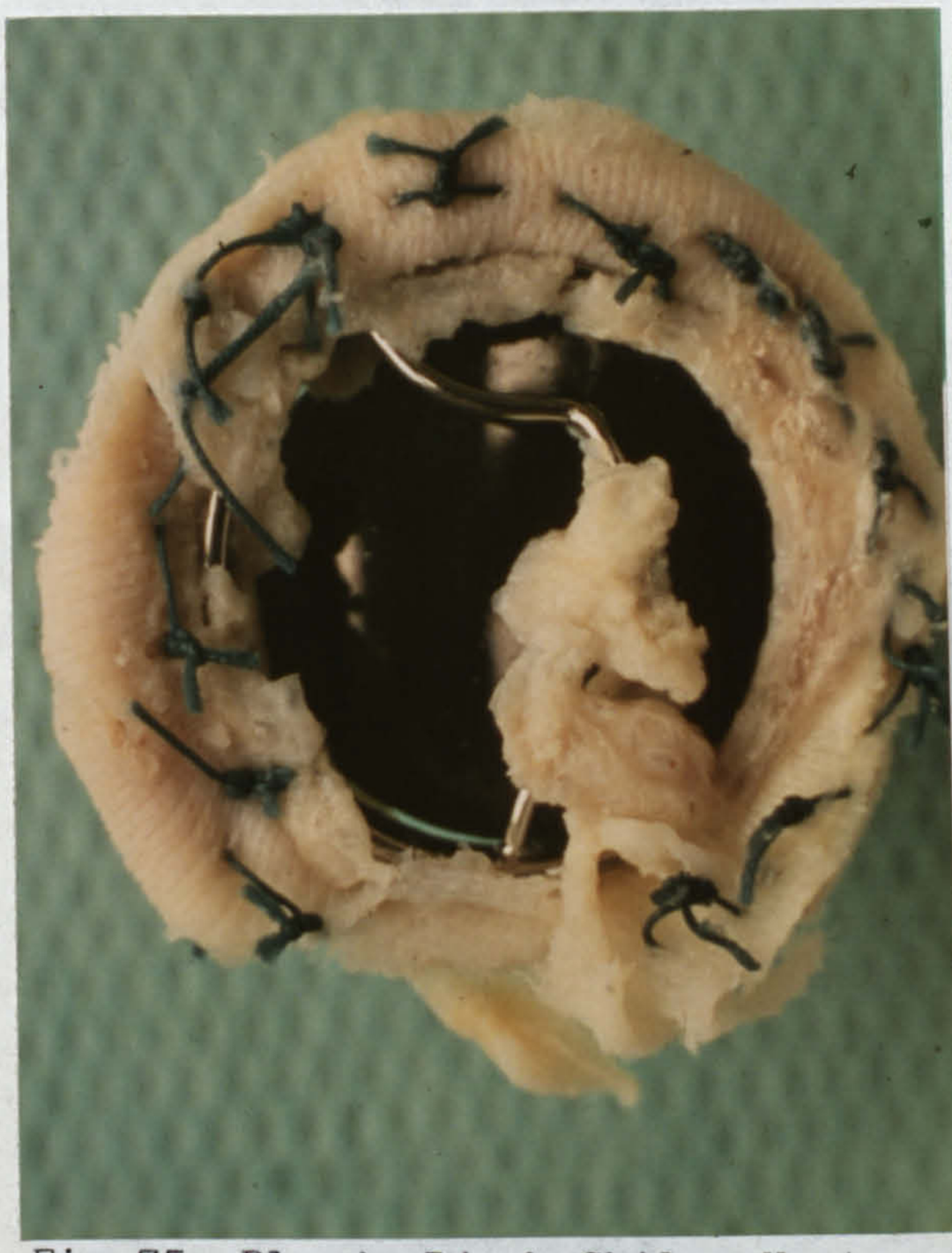


Fig 75. Blood. Bjork-Shiley Upstream
Other side of Fig 73.

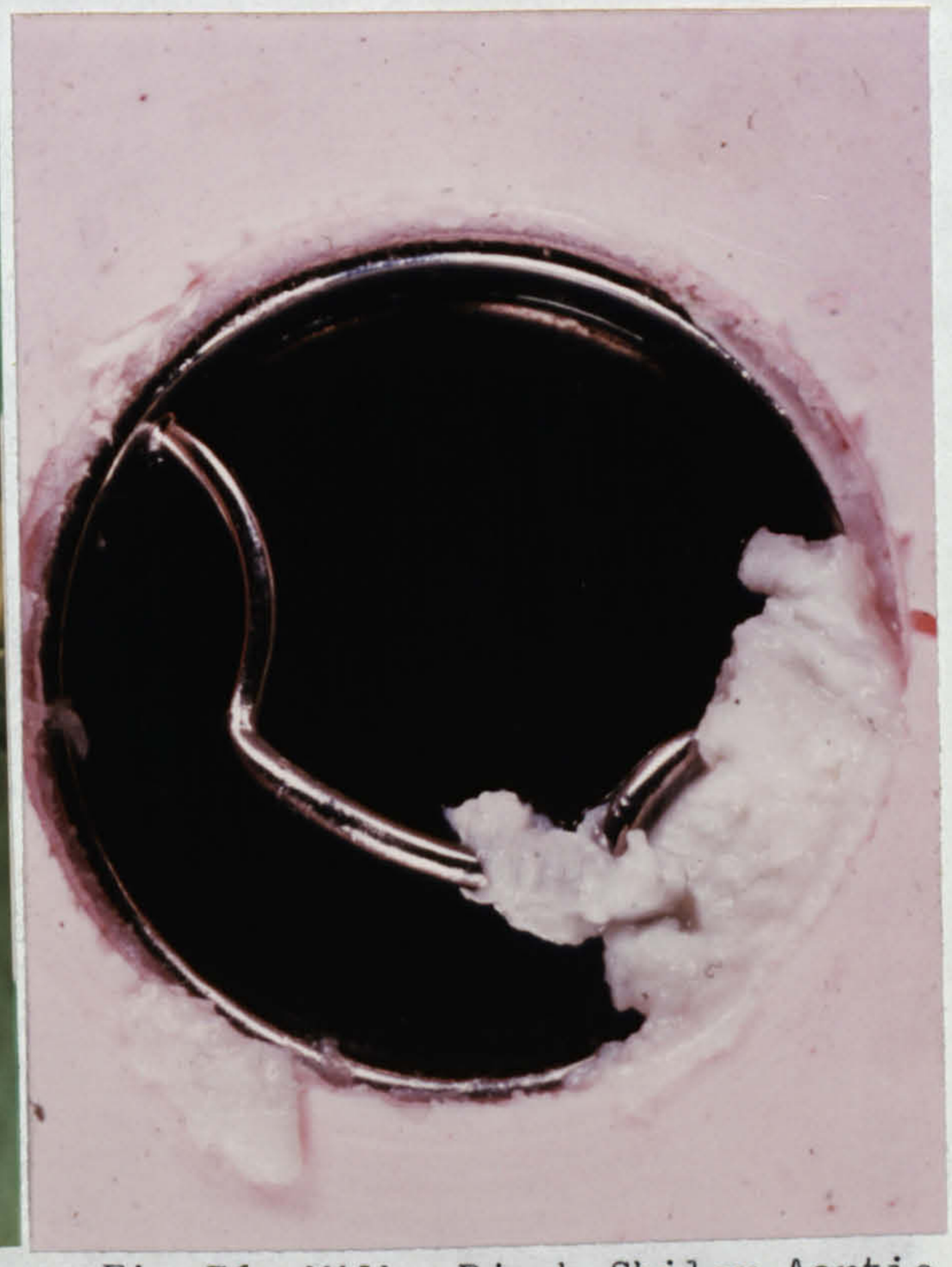


Fig 76. Milk. Bjork-Shiley Aortic
Upstream. Other side of Fig 74.

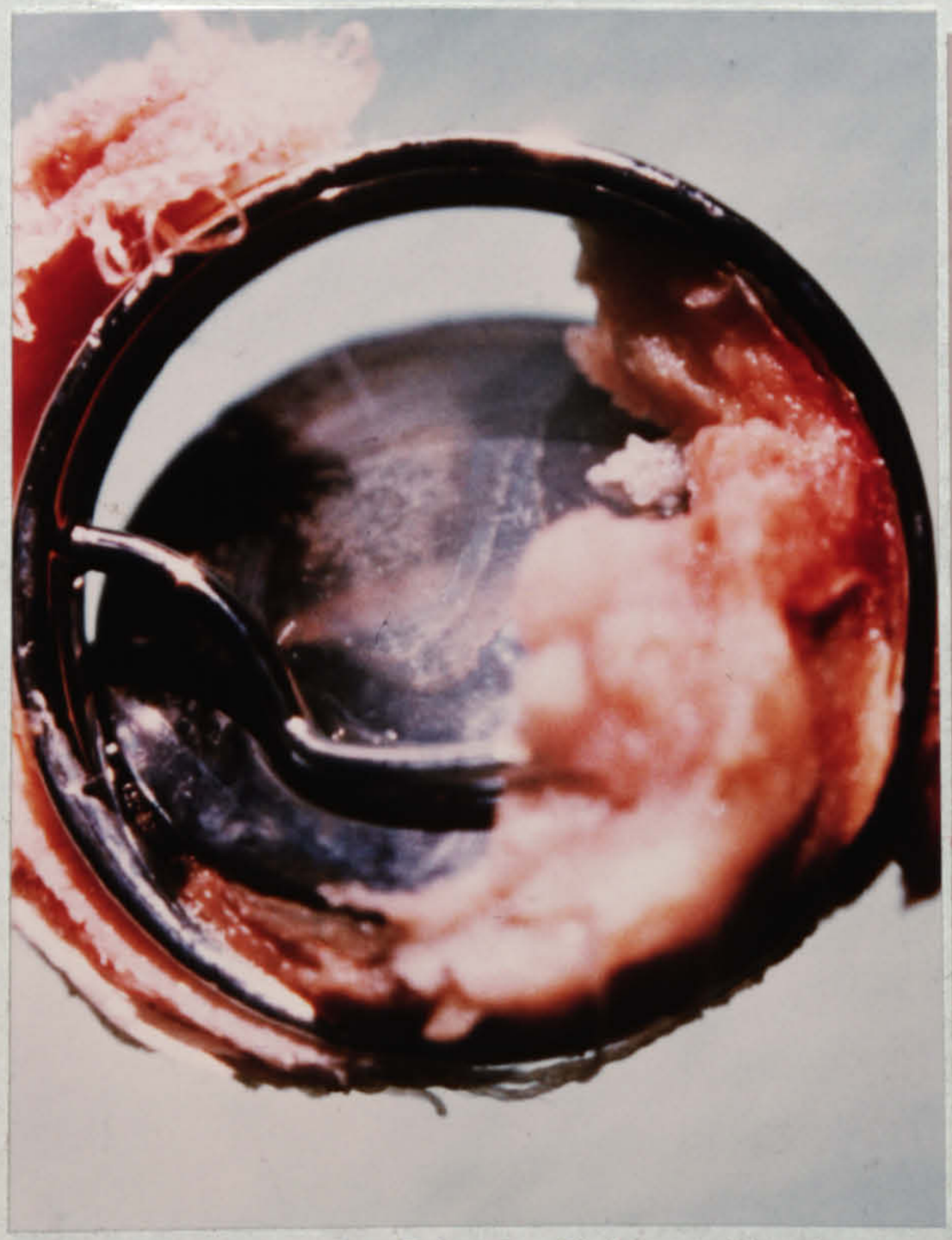


Fig 77. Blood. Bjork-Shiley Upstream
After 6 Months.

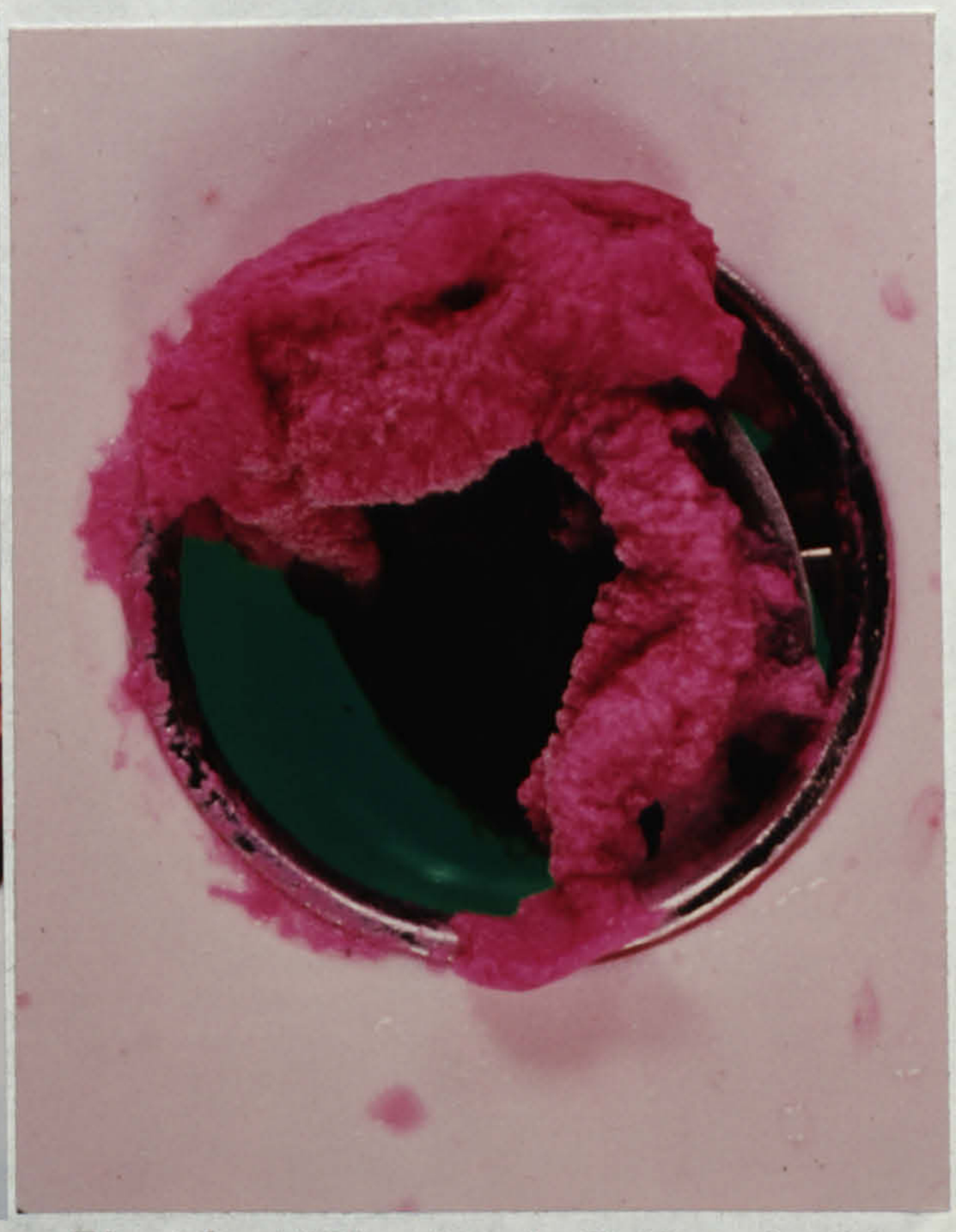


Fig 78. Milk. Bjork-Shiley Aortic
Upstream. 40^{o}C 2L/min milk.

PAGE NUMBERING AS ORIGINAL

Fig 79. Blood. Bjork-Shiley Downstream Other Side of Fig 77.

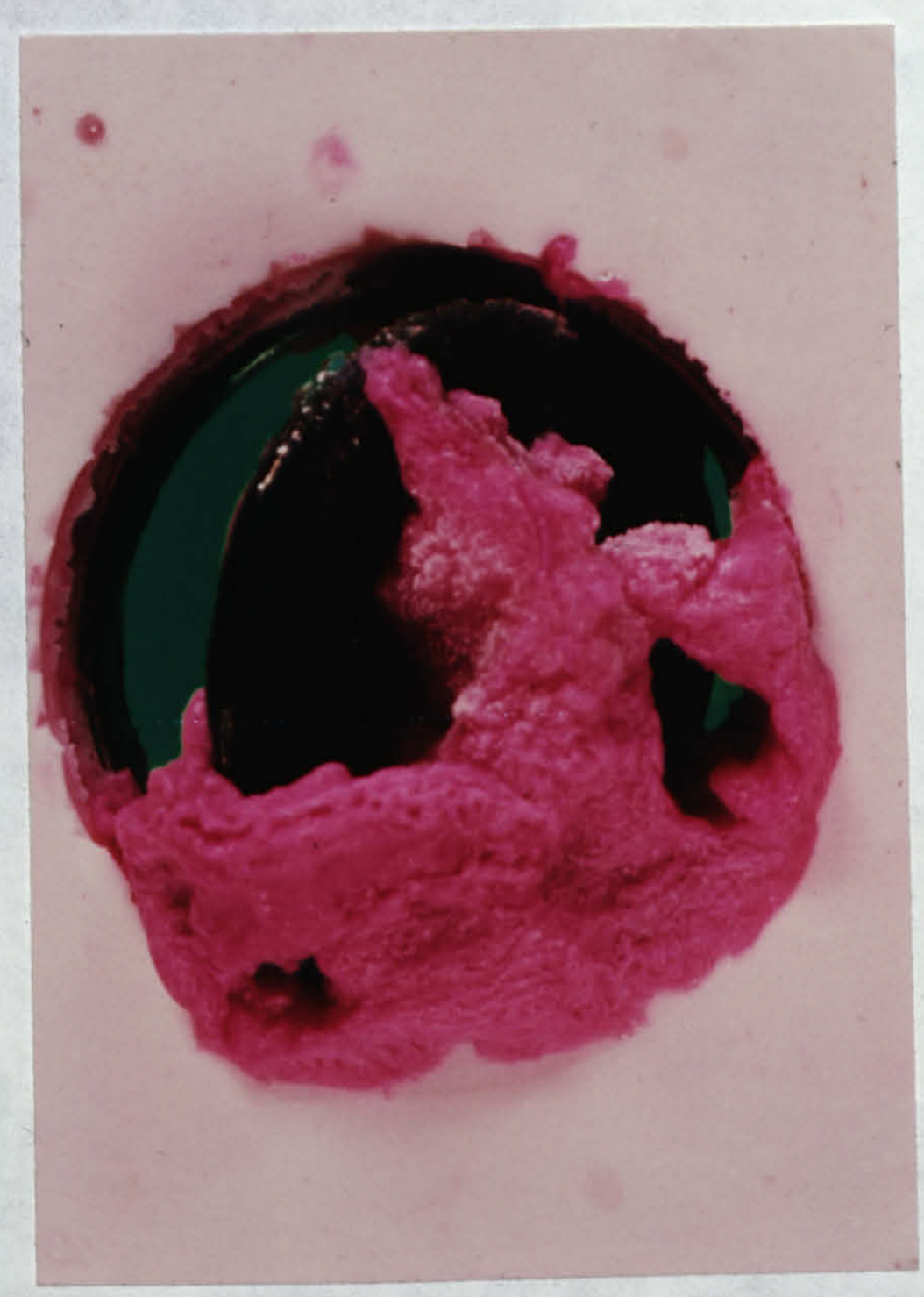


Fig 80. Milk. Bjork-Shiley Aortic Downstream. 40°C. 2L/min milk.

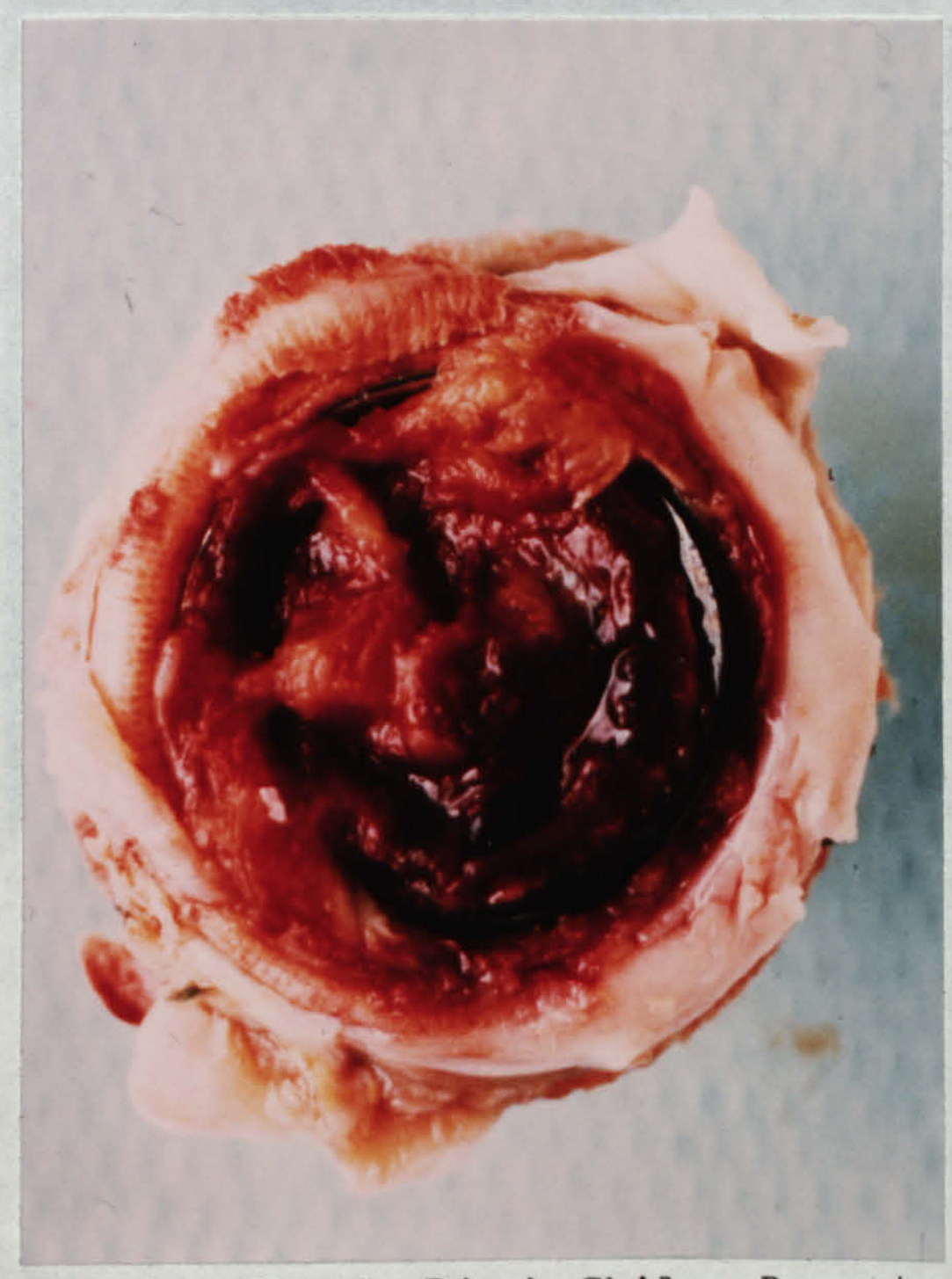


Fig 81. Blood. Bjork-Shiley Downstream After 6 Months. Courtesy Busuttil.

Fig 82. Milk. Bjork-Shiley Aortic Downstream. 40°C. 3L/min milk

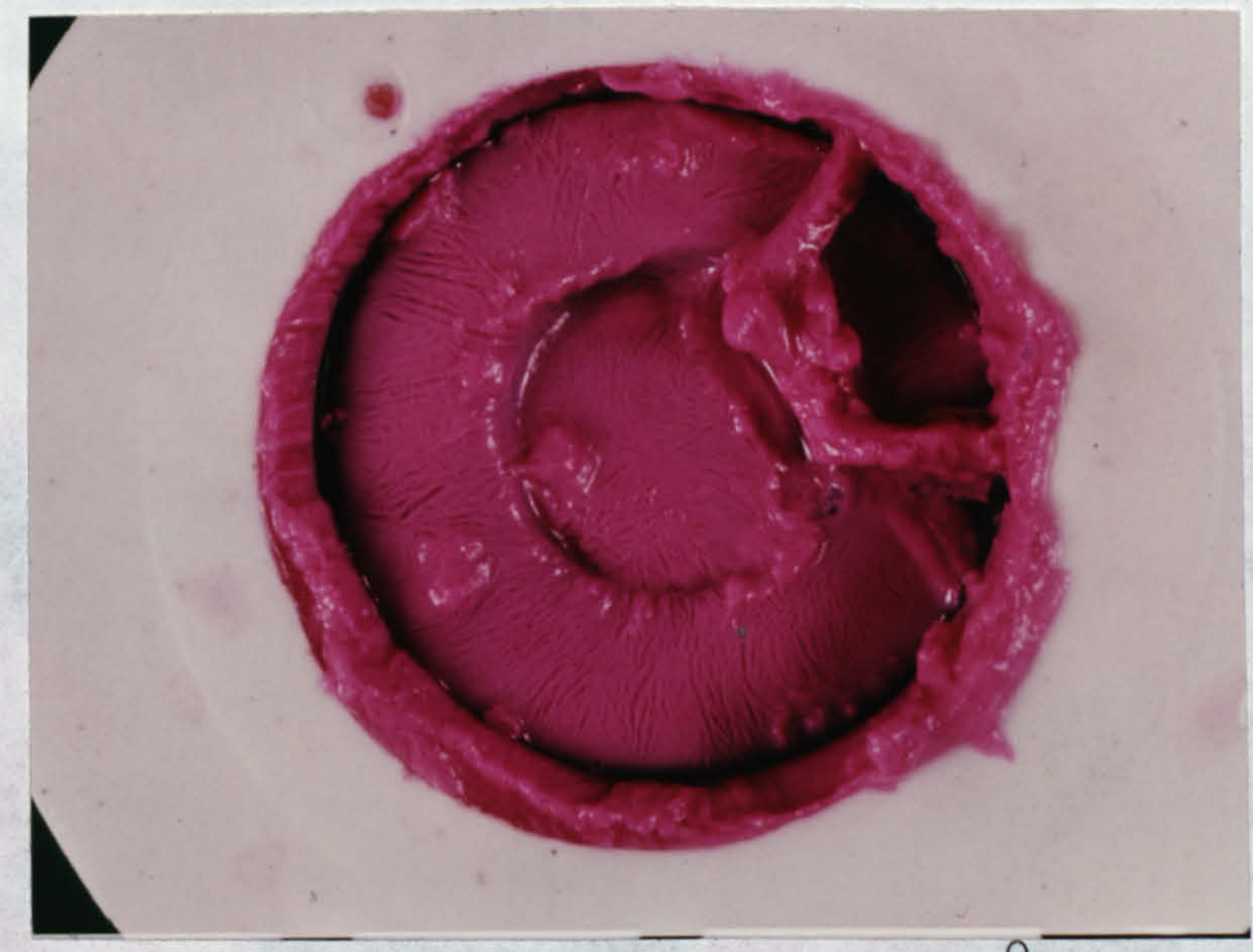


Fig 83. Milk. Bjork-Shiley Aortic Downstream. 40°C. 2L/min milk.

Starr-Edwards Valve

Fig 84. Blood. Starr-Edwards Aortic Upstream. After 5 Years.

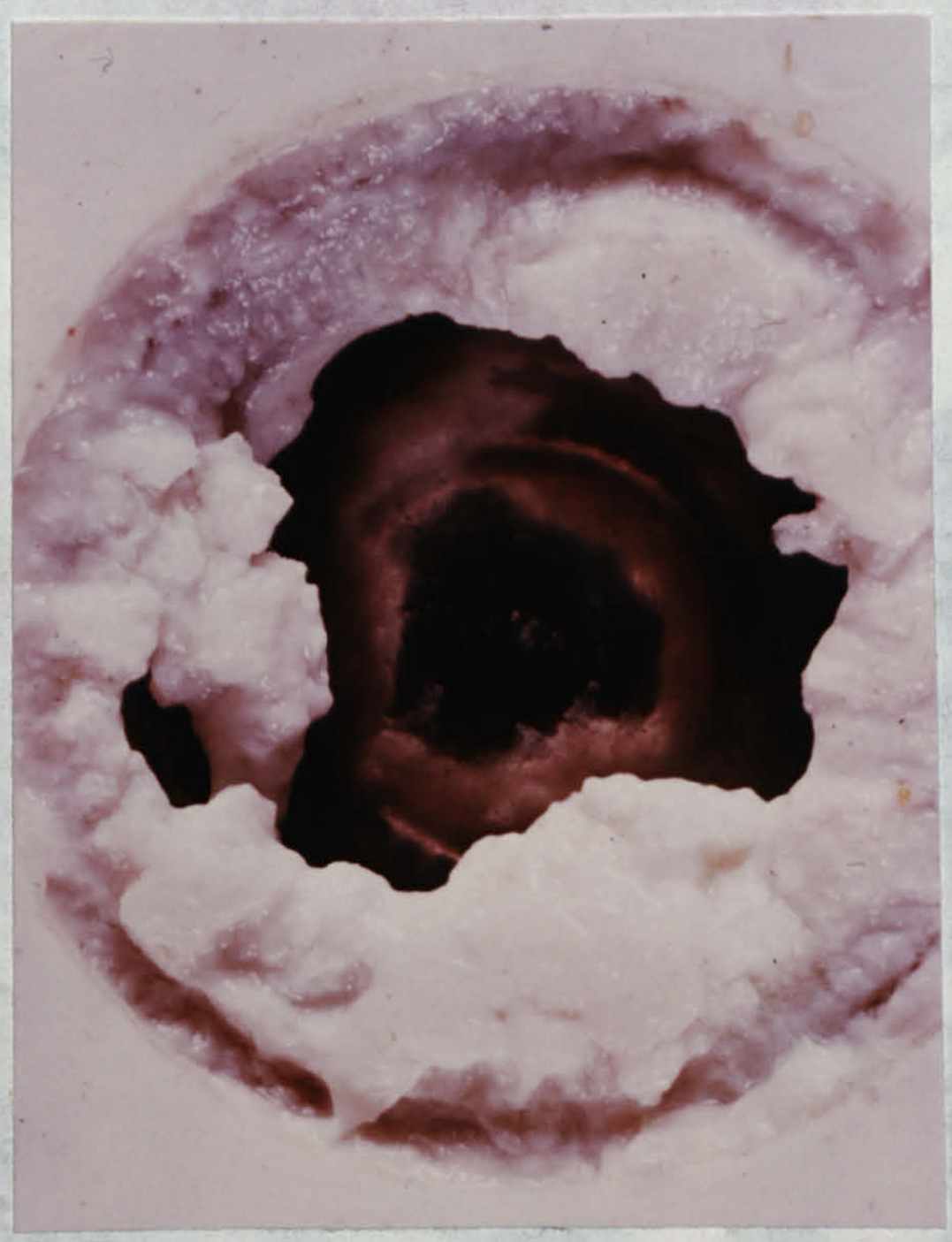


Fig 85. Milk. Starr-Edwards Aortic Upstream. 43°C. 0.6% $CaCl_2$ & rennet. 1.7L/min milk. After 10 Mins.

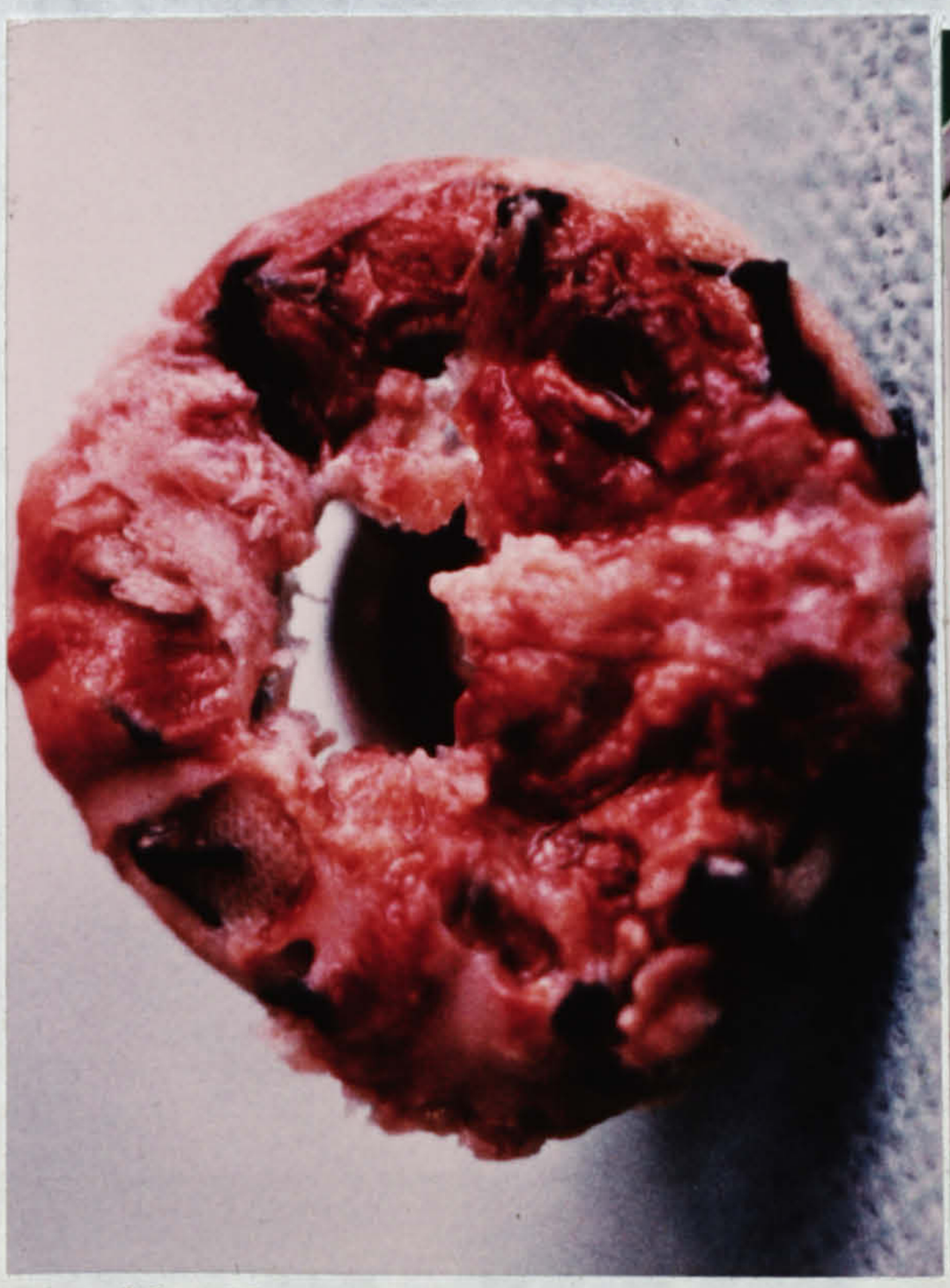


Fig 86. Blood. Starr-Edwards. After 2 Years. Courtesy Busuttil.

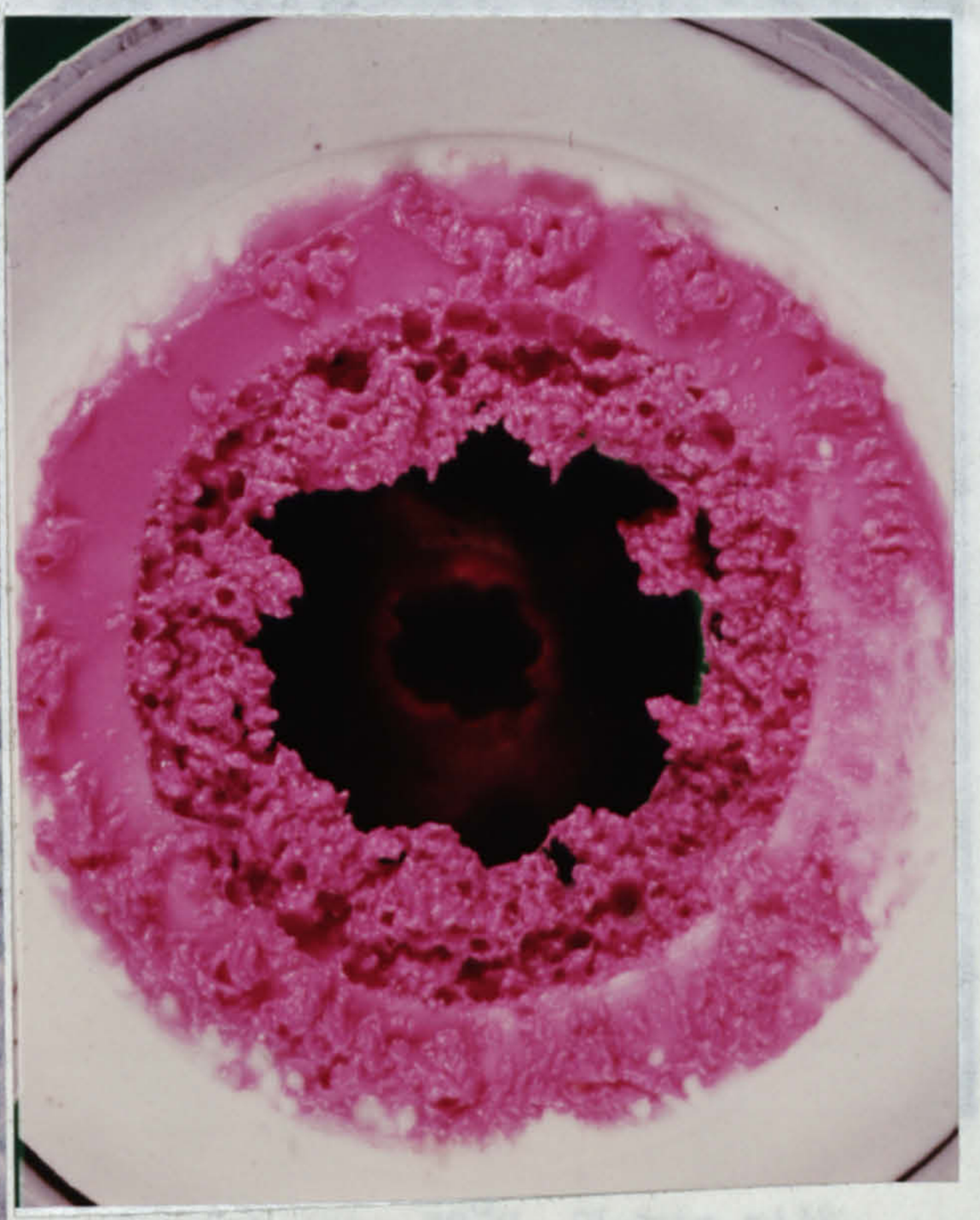


Fig 87. Milk. Starr-Edwards Mitral Upstream. 40°C. 2L/min milk.

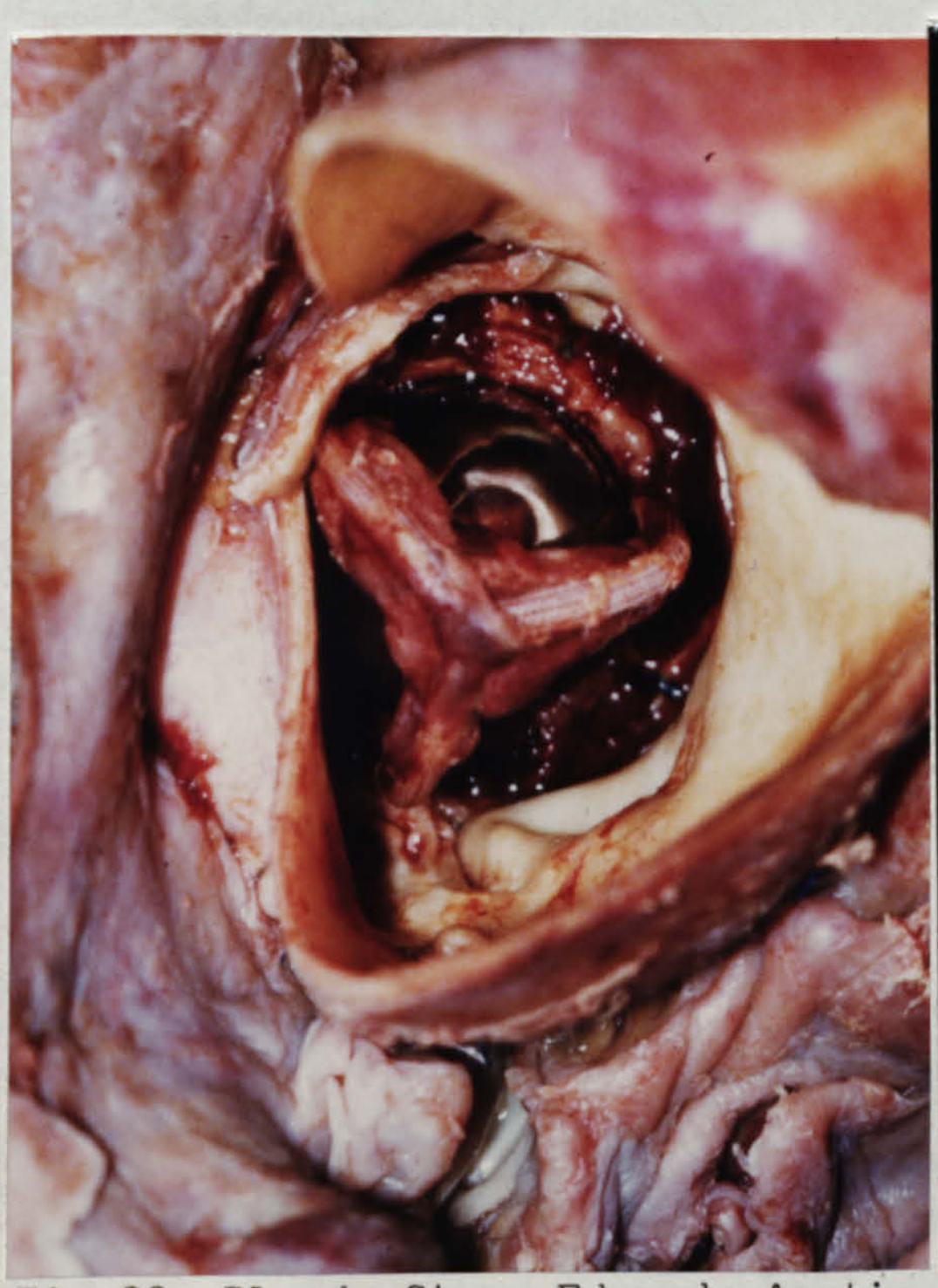


Fig 88. Blood. Starr-Edwards Aortic After 1 Month. Courtesy Stovin.

Fig 89. Milk. Starr-Edwards Mitral Downstream. 38°C. 3L/min milk.

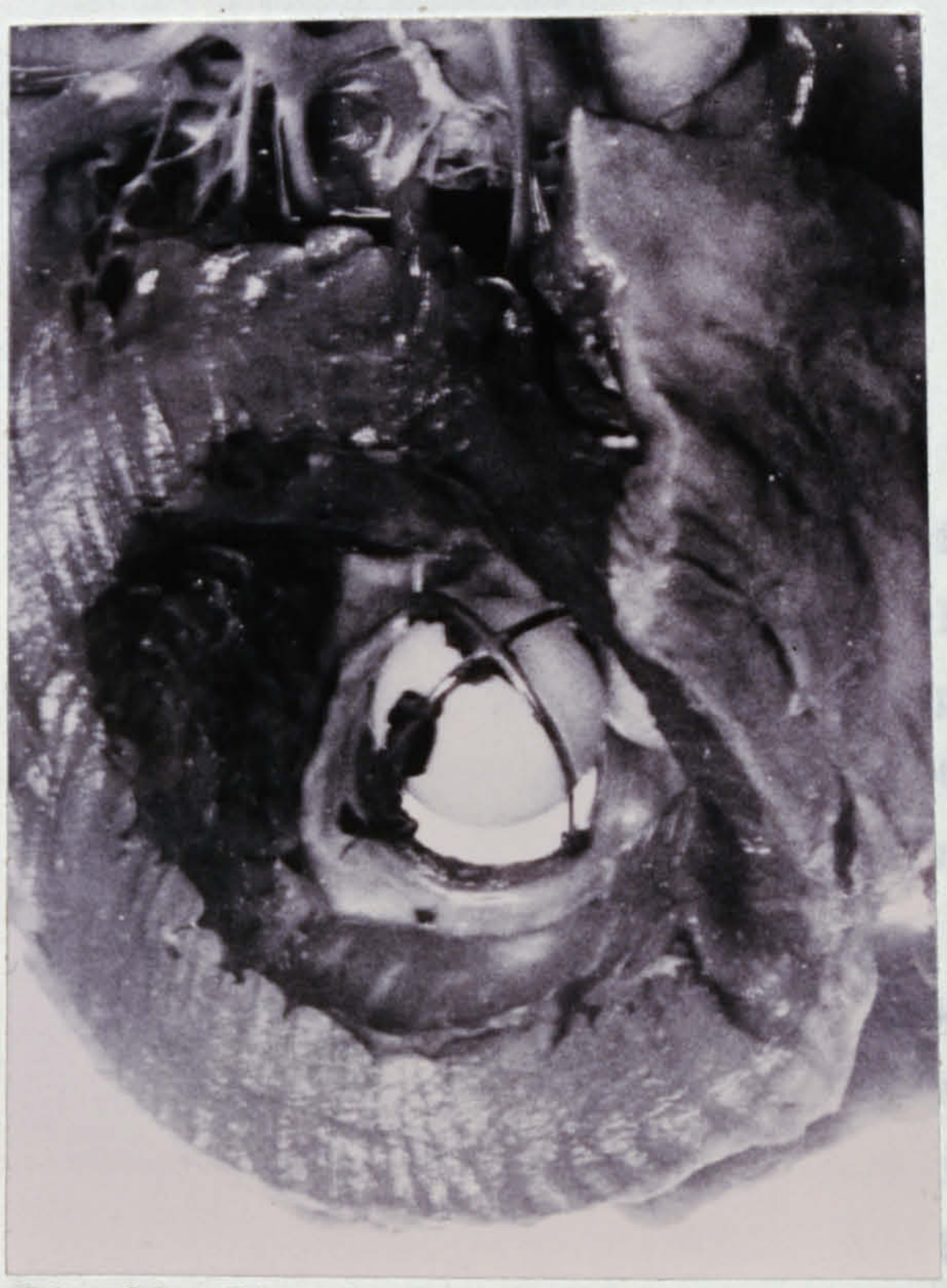


Fig 90. Blood. Starr-Edwards Mitral After 5 Years . Courtesy Stovin.

Fig 91. Milk. Starr-Edwards Aortic Downstream. 38°C. 2L/min milk.

4.6.2 Discussion

Bjork-Shiley Valve

The ability of the rennetized milk system to mimic the clinical situation is well illustrated by the above photographs. Clinical experience of the Bjork-Shiley valve reveals two distinct patterns of deposition. The first of these involved primarily the struts and the sewing ring (Figs 73, 75) and the second involves the disc, particularly the downstream side, as well (Figs 77, 79, 81). The results obtained using milk, as well as confirming the validity of the analogue technique, demonstrate that two distinct patterns of deposition occur in this case also.

Virchow (5) stated that the three predominant factors important in coagulation are surface chemistry, blood chemistry and hydrodynamics. The materials of construction of the valves and their preparation prior to implantation are the same, apart from an albumen coating on the valve used in the first example, which one would expect to lead to a more general deposit, so it seems unlikely that surface chemistry will be the key factor in causing these diverse deposition patterns. Since both milk and blood are prone to the same duality of results and the chemistry of the two fluids, although similar, is still very different, it would seem logical to assume that Virchow's blood chemistry is not the prime determining factor in this anomoly. However, the hydrodynamics of the situation for the two different milk results (Figs 74, 76, 78, 80) are identical, the results being obtained in the same pulse duplicator at the same flow rate and the same pulse rate. It, therefore, seems improbable that hydrodynamics can be of

prime importance in this instance. The main difference between the two milk runs appears to be in the fluid chemistry. The rennet and calcium chloride concentration of figures 74 and 76 were much lower than those for figures 78 and 80, whilst on the other hand the temperature for figures 74 and 76 was higher than that for figures 78 and 80 so that although the clotting times were comparable in both cases, the chemistry of the coagulation may have been rather different. In the case of the blood clots Effler (68) would suggest that the differences observed between the deposits is attributable to a difference in their cause. On the one hand the deposit covering the whole valve would be caused by a chemical mechanism due to the chemcials used in bleaching the sewing ring and the deposit adhering to the struts alone would be a thrombus. This may be the case, but it seems improbable that if it were, it would be possible to mimic it using rennetized milk.

It has been proposed (8) that activation of the intrinsic pathway of blood coagulation results in a "hypercoagulable state" which leads to the formation of venous thrombi but does not cause disseminated intravascular coagulation. For example, if, in the case of the Bjork-Shiley valves, the intrinsic pathway had been activated in one instance, but not the other, this might explain the different phenomena occurring. If the blood has not become hypercoagulable, one would expect clot formation, if any, to be on the most thrombogenic surface present and since pyrolytic carbon is known to be less thrombogenic than stainless steel one would expect any clot to form on the

stainless steel struts as has been found. Stainless steel has also been shown to be more prone than most other materials to curd adhesion when coagulating milk is passed over it (79) and one would, therefore, expect any clot formation with milk to occur on the stationary steel struts rather than the carbon disc. It is possible, therefore, that a hypercoagulable state may occur in some heart valve implantees, and that this leads to a different form of deposit from that which would normally be associated with thrombosis. It may also be possible to mimic these two distinct situations using rennetized milk by varying the reactant concentrations and temperatures but this needs further investigation.

Starr-Edwards

The main feature of the clotting found on the Starr-Edwards ball valve in the clinical situation is the tendency for clot formation inside the sewing ring (Figs 84, 86). This phenomenon is also found using rennetized milk (Figs 85, 87).

Clinical experience of the clots forming on the downstream side of the valve is that (a) no clots form on the ball and (b) some deposit is found on the struts, but appears to "grow" from the sewing ring (Figs 88, 90). With milk as with blood no clot forms on the ball, but clot does form onthe struts (Figs 89,91).

The deposits on the silicone ball Starr-Edwards valve found in the clinical situation and in vitro using rennetized milk are very similar in nature. In both cases it appears that clots form on the struts and are easily knocked off suggesting that embolization would be

a problem with the valve. The deposit on the cloth covered struts in the clinical situation appears to be different in nature. The cloth covered valve with a hollow metal ball was introduced to reduce the incidence of thromboembolism compared with that found with the silicone ball valve with bare metal struts (149, 203). The cloth covering encourages strut encapsulation with autogenous tissue which apparently decreases the clotting propensity and explains why the degree of clot deposition on the struts is far greater using milk than is generally found in the clinical situation. However, in the period immediately following implantation, and before any encapsulation has been possible, thrombus can form, similar in nature to that found with milk (Fig 88). Another possible explanation, for the general lack of large scale clots forming on the struts in the clinical situation is that during the cardiac cycle the struts may come into contact with the walls of the ventricle or aorta dislodging any clot which has started to form, and leading to thromboembolism which is known to be a problem with these valves (148, 204, 205). In the case of the mitral valve, the small end-systolic volume of the left ventricle of the healthy heart means that this must generally be true. With the aortic valve it is also feasible that, due to the flexibility of the heart, the valve comes into contact with the wall of the aorta. Support for this hypothesis is obtained from observation of photos (Figs 88, 90) above. Although there are clots on the struts of these valves, they are situated on the inside of the struts, which if the hypothesis is not true is very suprising, as one would expect the moving ball to dislodge them. This indicates that the effect of the ball is less than that of the wall.

4.6.3 Conclusion

The clots found on the Bjork-Shiley valves with milk are very similar to those found with blood with two patterns of deposition being possible in each case. (a) a discrete deposit involving the struts and the sewing ring and (b) a more general deposit covering the disc, primarily the downstream side.

The results of the experiments with the Starr-Edwards valve are less conclusive. With both milk and blood a deposit is found inside the sewing ring, but, whereas with milk large-scale deposits are found on the struts, in vivo the struts remain relatively clear. This, however, may be attributable to the inadequacy of the heart simulator to mimic the heart, and it is probable that in vivo clots form on the struts but are dislodged.

4.7 Tests on the Clotting Propensities of Different Valves

4.7.1 Results at 2 litres/min.

Fig 92. Starr-Edwards Aortic Upstream 40°C.

Fig 93. Starr-Edwards Aortic Downstream 40°C.

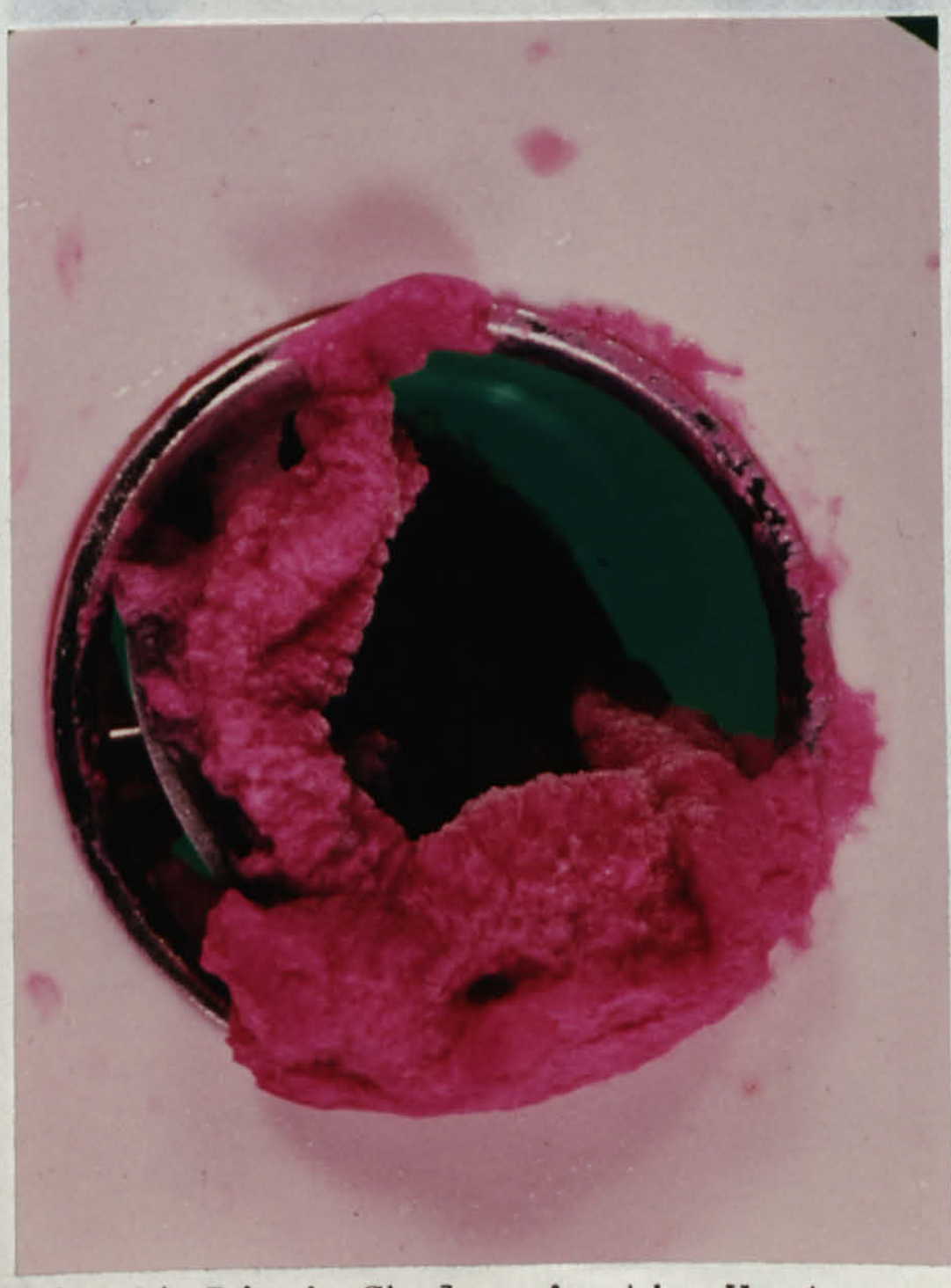


Fig 94.Bjork-Shiley Aortic Upstream 40°C

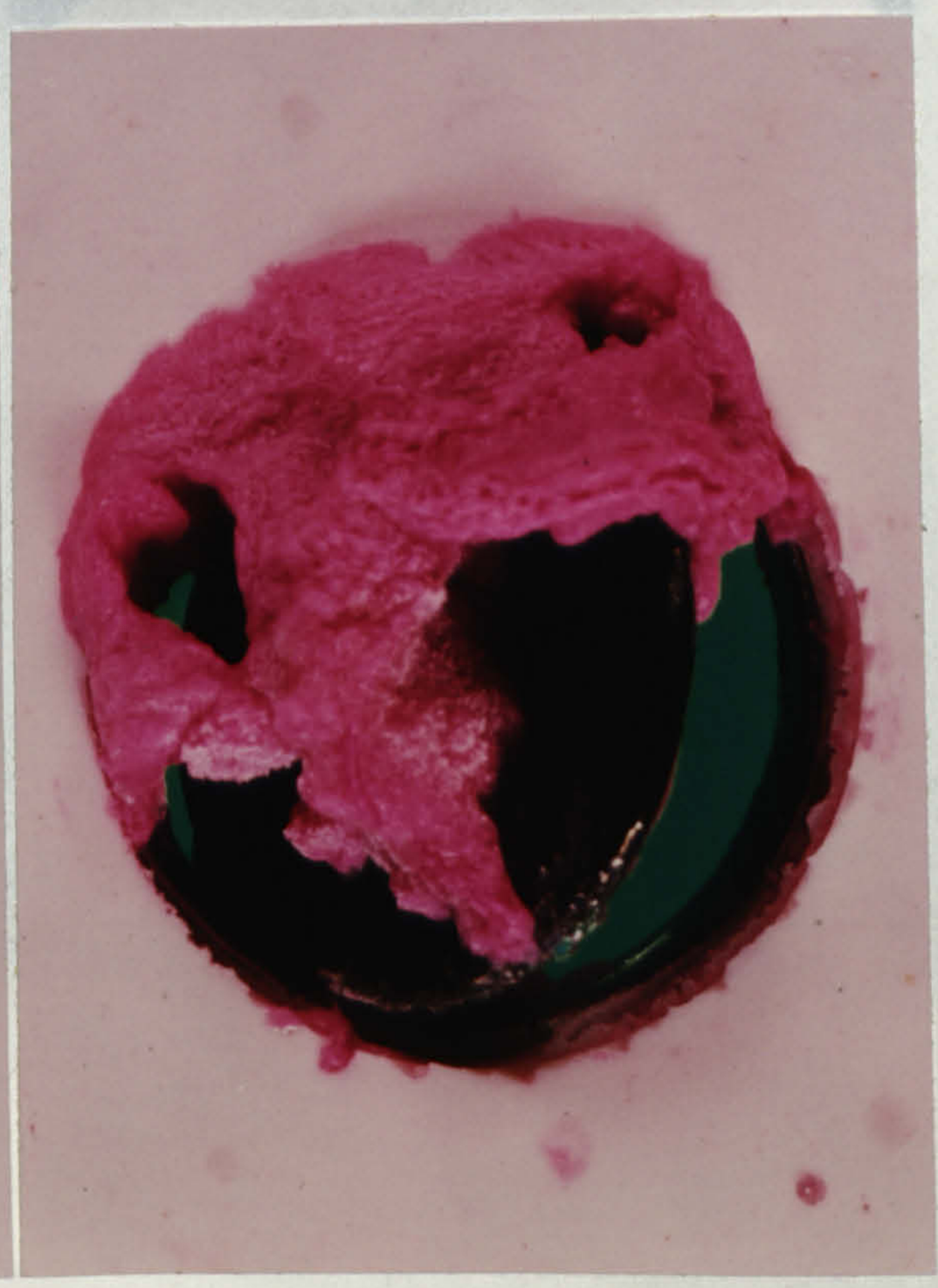


Fig 95. Bjork-Shiley Aortic Downstream 40°C

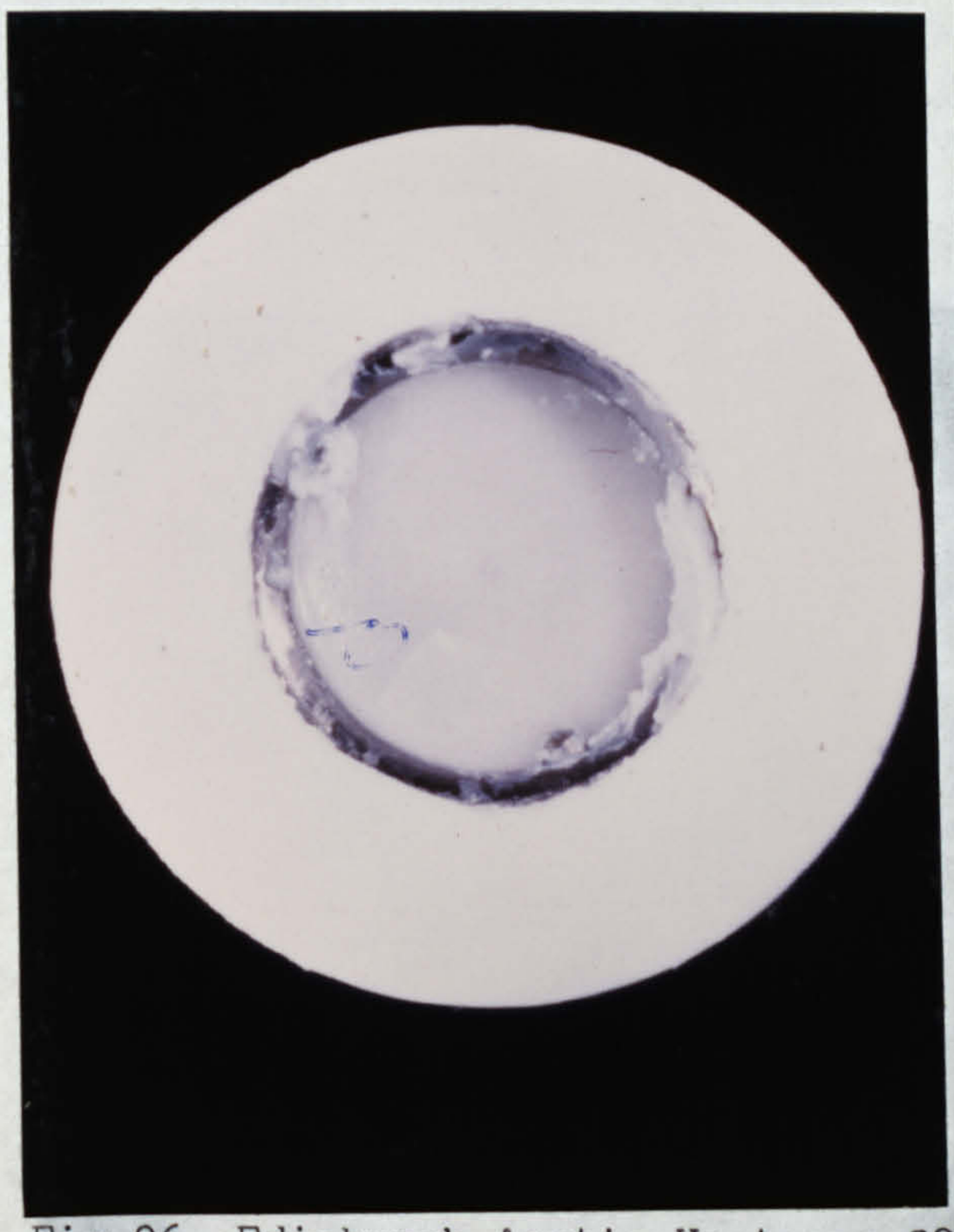


Fig 96. Edinburgh Aortic Upstream 38°C

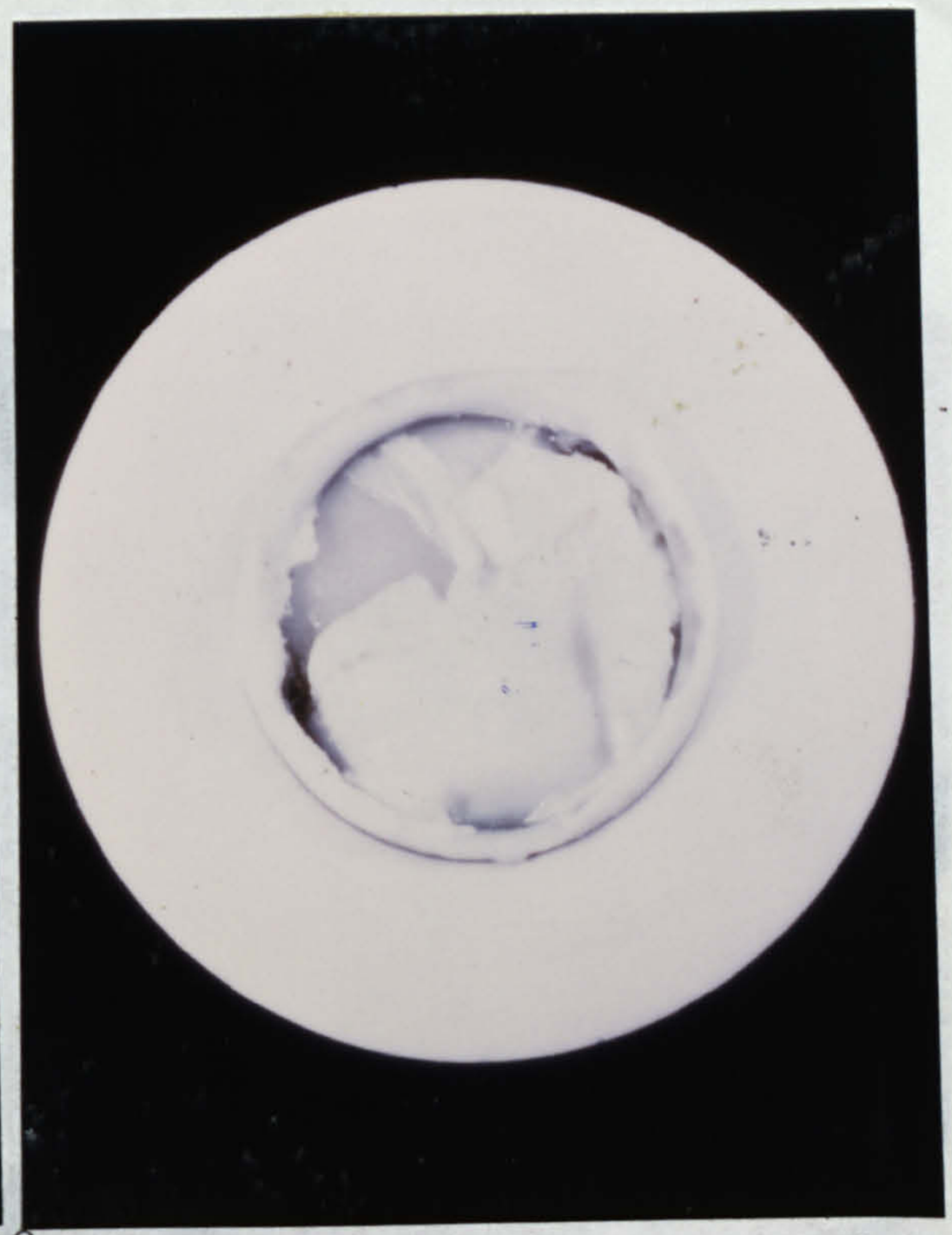


Fig 97. Edinburgh Aortic Downstream 38°C

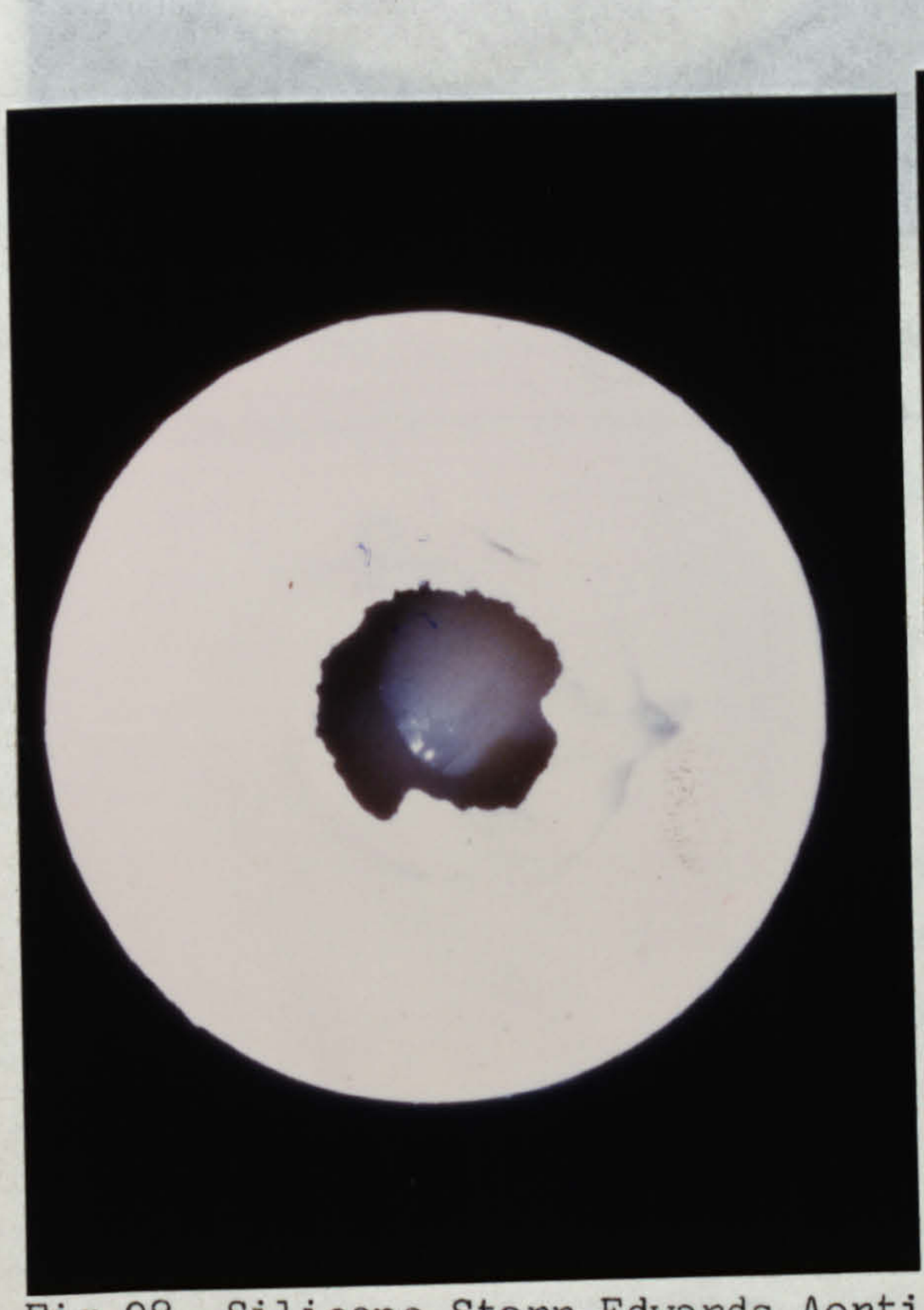


Fig 98. Silicone Starr-Edwards Aortic Upstream 38°C.

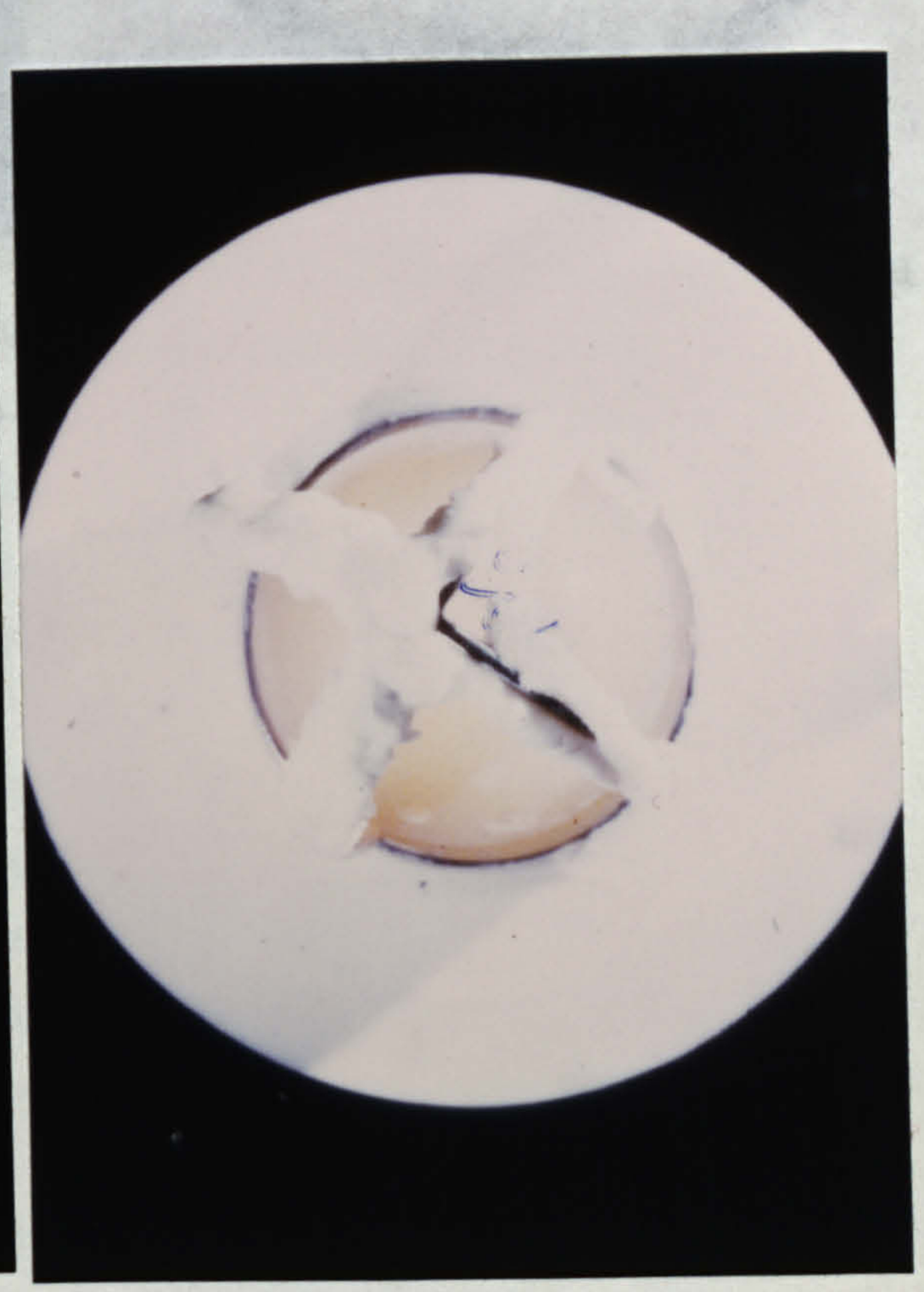


Fig 99. Silicone Starr-Edwards Aortic Downstream. 38°C.

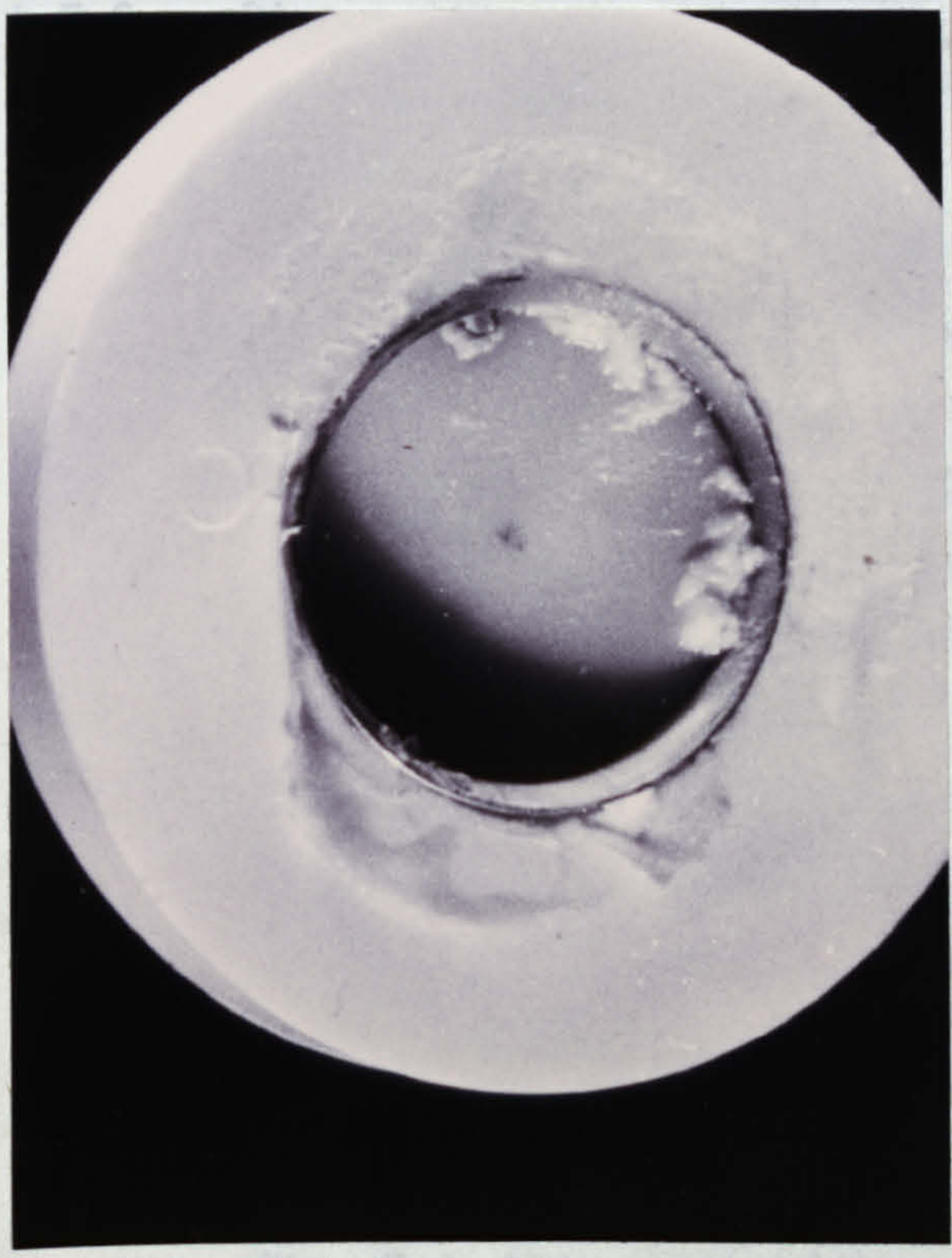


Fig 100. Flat Disc Edinburgh Mitral Upstream. 43°C. 0.6% $CaCl_2$ & rennet 1.7L/min milk After 10 Mins

Fig 101 Flat Disc Edinburgh Mitral Downstream. As Fig 100.

4.7.2 Discussion

Because of the differences between the chemistry of milk and blood, it is not necessarily true that those surfaces which are known to be athrombogenic will also be associated with a low incidence of milk clot deposition and vice-versa. Therefore, to compare the clotting propensities of valves of different designs, ideally they would be constructed of the same materials. As a preliminary investigation, however, it is of value to compare the valves as used in clinical practice, since these are more readily available.

All the valves tested showed tendency to clot formation particularly at the lower flow rate of 2 litres/min.

2 Litres/min Milk Flow

The flat disc valve with a pivoted occluder is a prototype which is not commercially available. Clot forms on the downstream side, whilst the upstream side remains relatively clear. This is due to a large stagnation region on the downstream side caused by the occluder not opening fully.

The Starr-Edwards ball valve was consistently found to be prone to gross clotting on the struts and inside the orifice. These clots were occasionally dislodged by the action of the ball, and in the clinical situation the clots on the outside of the struts may be dislodged by contact with the walls of the ventricle, in the case of a mitral valve, or the aorta, in the case of the aortic valve. The Starr-Edwards valve would, therefore, appear

to be potentially very dangerous due to its proneness to clotting and thromboembolism and very careful anticoagulation therapy is required. (148 - 150, 203, 206, 207).

The Bjork-Shiley valves shares with the flat disc valve described above the problem of stalling, and hence stagnation over a large region of the downstream side of the occluder (20). It has also the added problem of struts which are used to restrain the disc and these can act as sites for the inception and development of clots. The Bjork-Shiley valve was shown in these tests to be better than the Starr-Edwards valve or the flat disc valve, but the flat disc has an occluder made of delrin, whereas the Bjork-Shiley valve has a pyrolytic carbon occluder which is less prone to clot deposition. It is certainly possible for gross clotting to occur on the Bjork-Shiley valve when rennetized milk is used in vitro (Figs 94, 95) and it would be expected that anticoagulant drugs would be needed in the clinincal situation as is indeed the case (20 - 23, 169 - 173, 208, 209).

The Edinburgh Valve (52, 176, 177) is evolved from the flat disc valve and incorporates an aerofoil shaped occluder, which enables the valve to open very nearly 90^{o} with the pivot axis only slightly eccentric. The bore of the housing is hyperboloidal or flared, the walls diverging slightly in the direction of flow which helps minimize the flow disturbance. It was hoped that implantation of this design of valve should not require the recipient to undergo anticoagulant therapy. It is, therefore, interesting to see how this valve compares

in vitro with the Bjork-Shiley and Starr Edwards valves which are known to require anticoagulants in vivo. Unfortunately, due to circumstances beyond our control, no valves constructed in the original specified material, vitreous carbon, are available and the valves used are, therefore, made of delrin and stainless steel which are more prone to clot deposition than carbon (see later) (Figs 153, 154).

Despite the lack of the correct materials, the Edinburgh valve has been shown to be superior to the other valves tested; the typical pattern of deposition being an even covering over the entire delrin disc which did not lead to gross clotting in any case. Had vitreous carbon been used it is possible that the deposition might have been negligible.

In the experiment in which the valve had been coated with albumen, the result suggests that there may be some flow separation on the upstream side of the leading edge and on the downstream side of the trailing edge (Figs 155, 156).

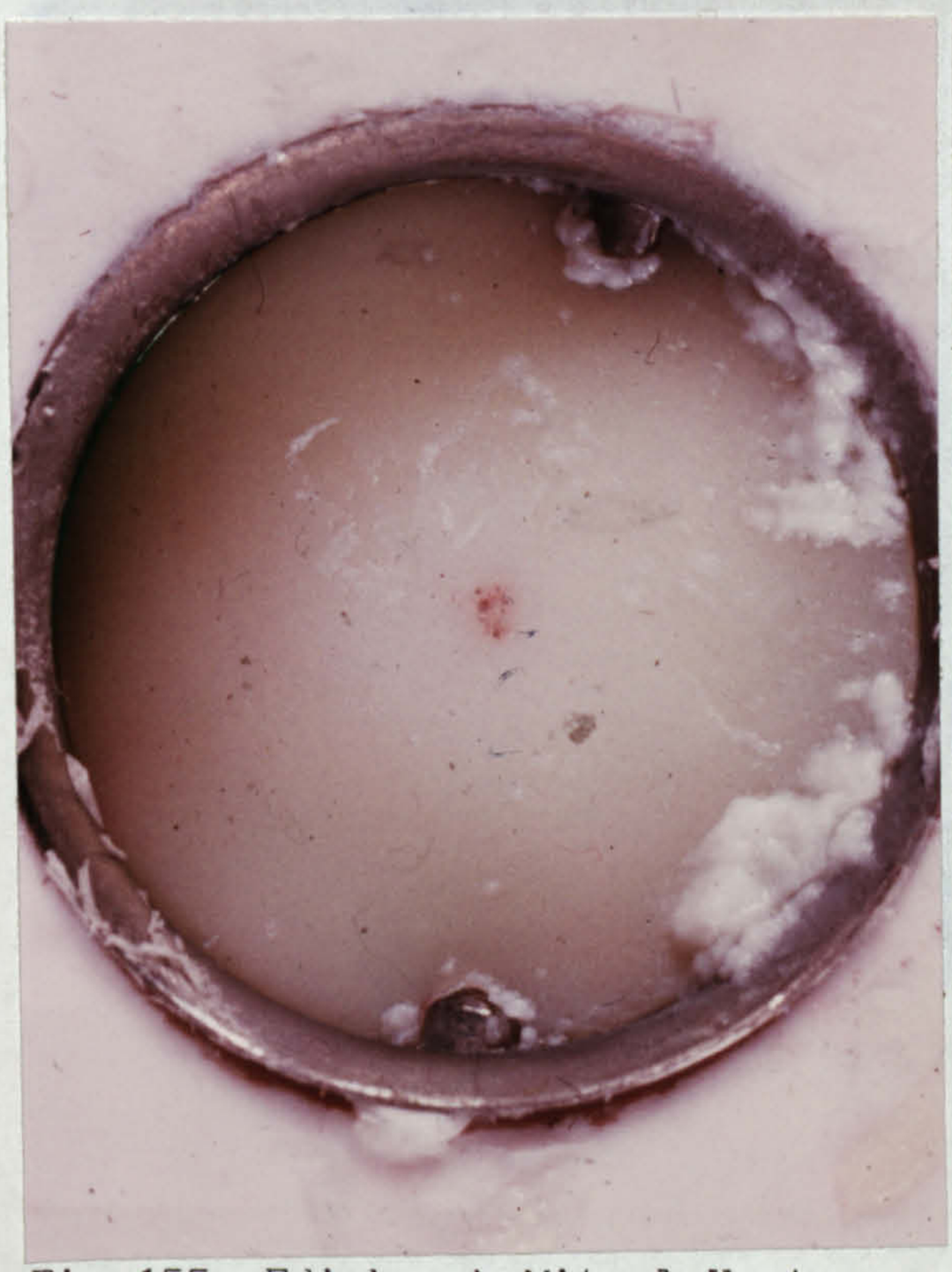


Fig 155. Edinburgh Mitral Upstream 40°C. 2L/min milk.

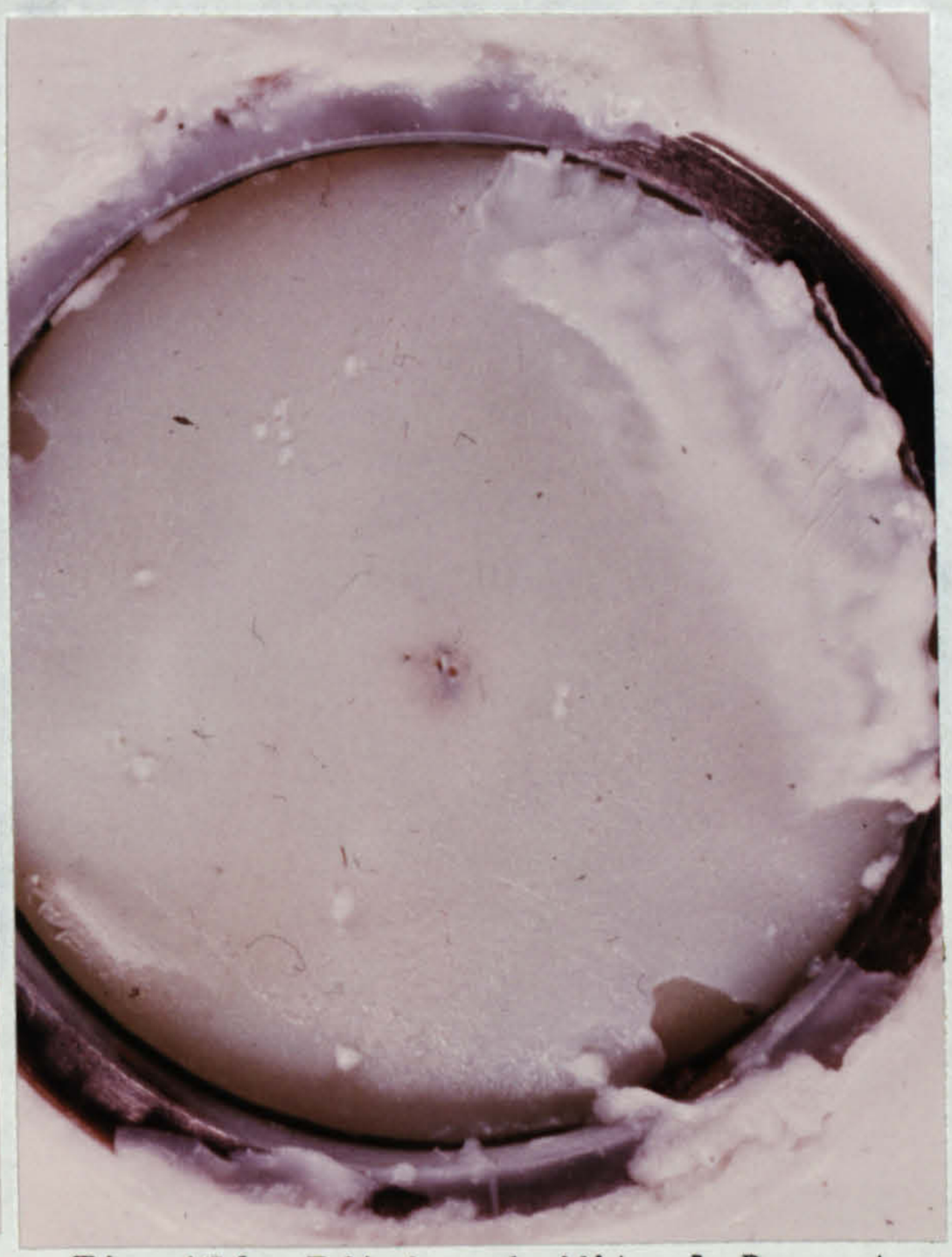


Fig 156. Edinburgh Mitral Downstream 40°C. 2L/min milk.

With the limited number of experiments performed to date, it is not possible to predict whether the Edinburgh valve will achieve its goal of not requiring the recipient to undergo anticoagulant therapy, but it is possible to state that on the basis of these experiments its in vivo performance, in terms of thrombogenicity, is likely to be better than the Bjork-Shiley or Starr-Edwards valves.

Comparison at 3 Litres Per Minute Milk Flow

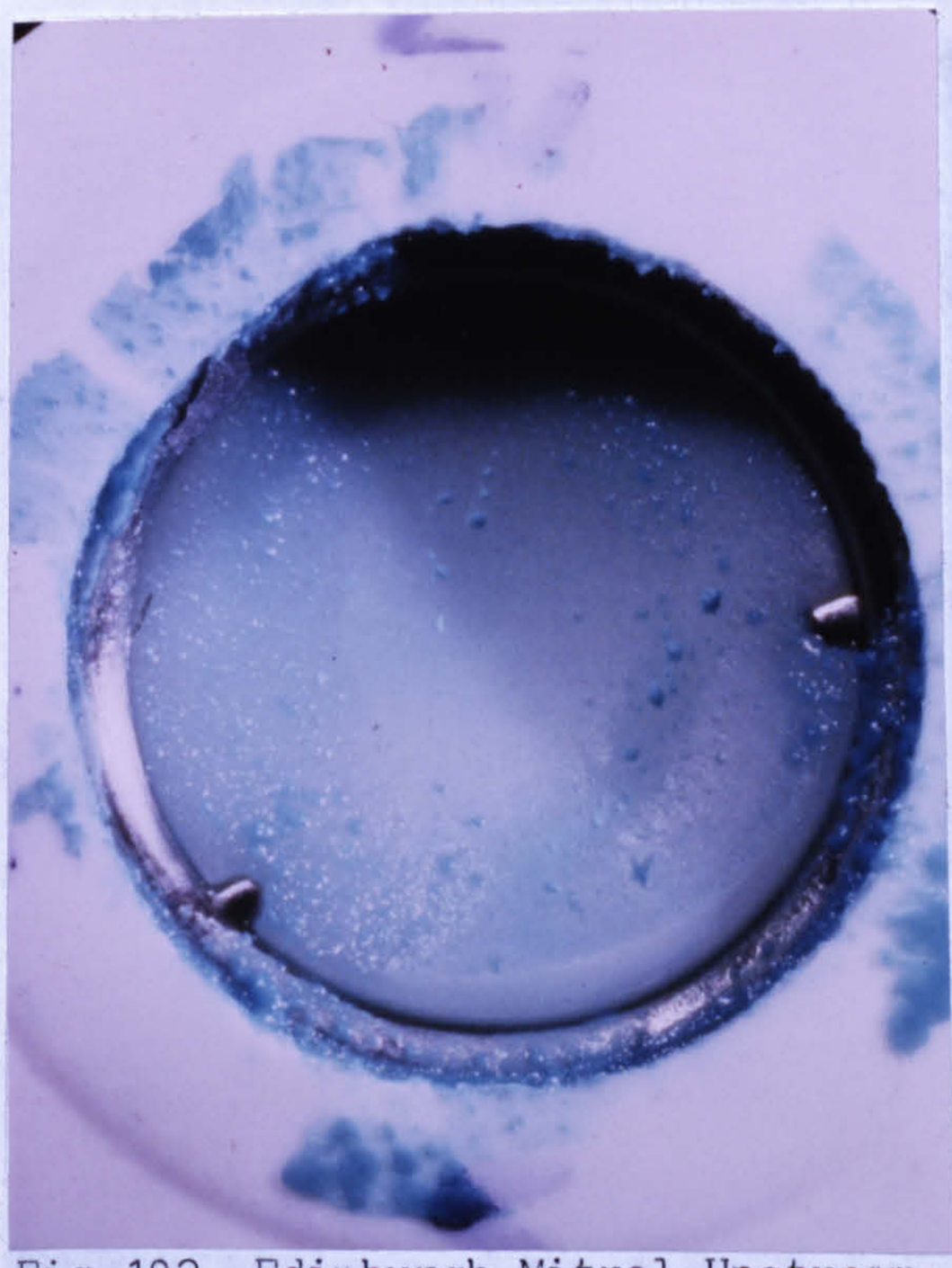


Fig 102. Edinburgh Mitral Upstream
38°C

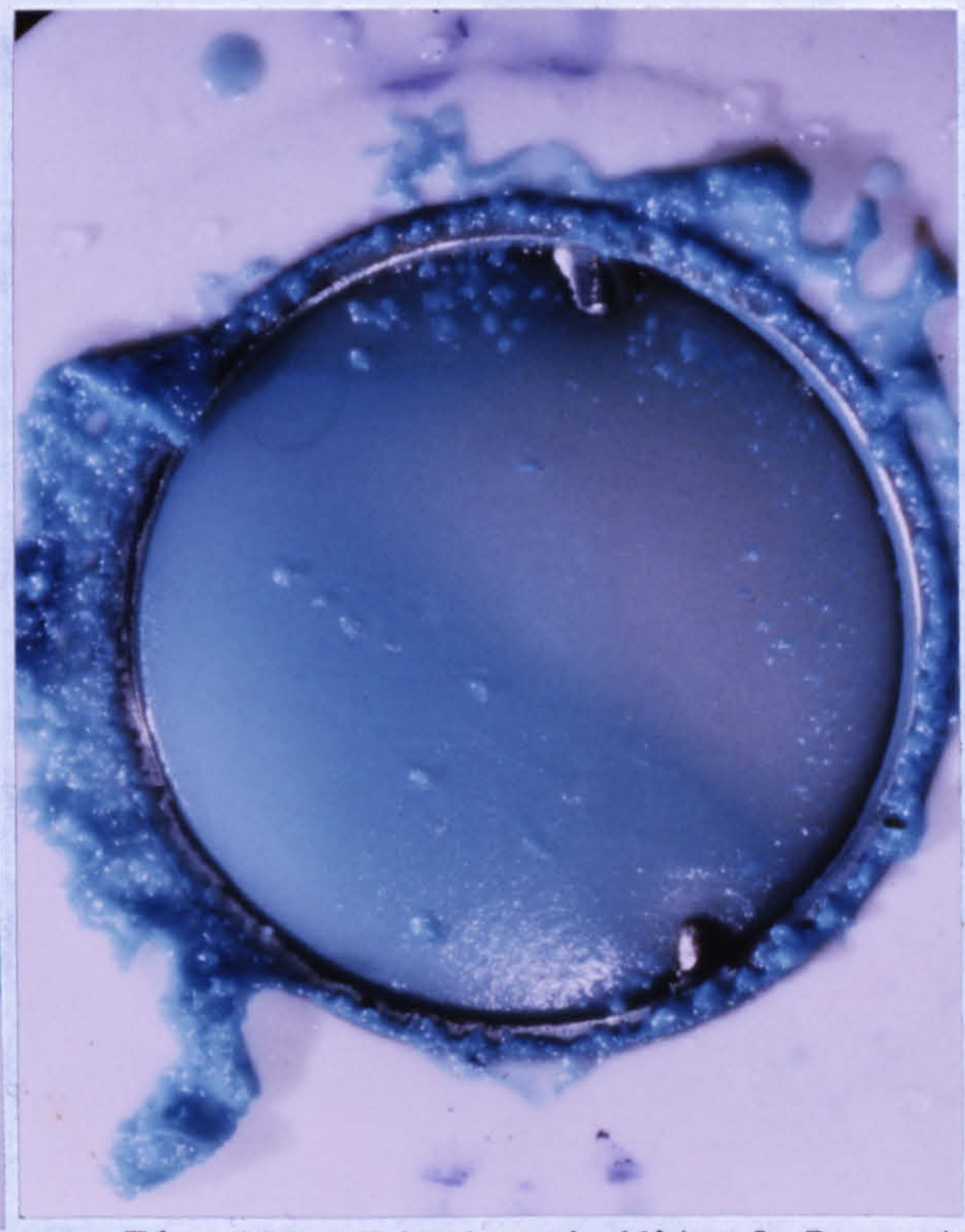


Fig 103. Edinburgh Mitral Downstream
38°C

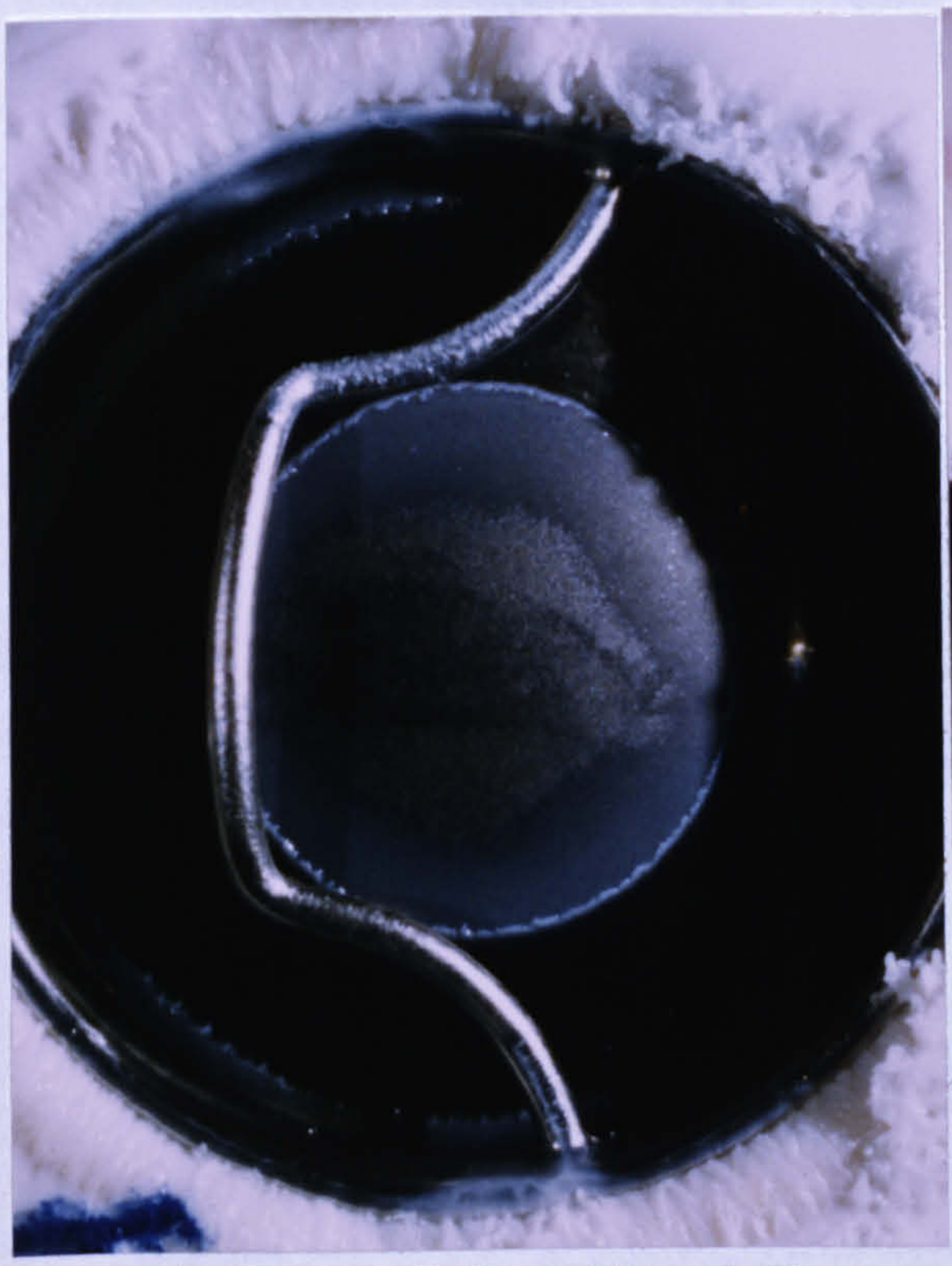


Fig 104. Bjork-Shiley Mitral Upstream
38°C

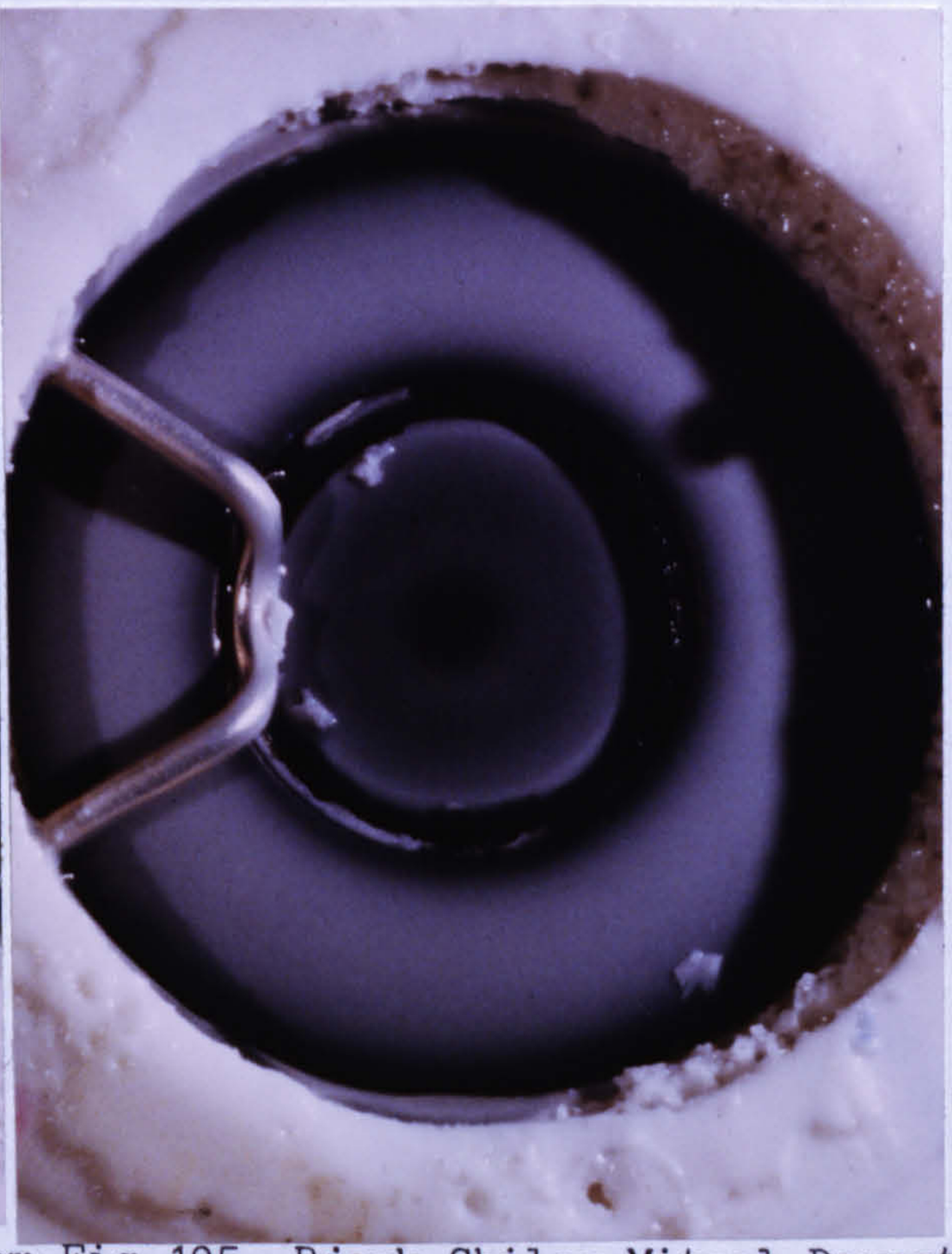


Fig 105. Bjork-Shiley Mitral Downstream
38°C

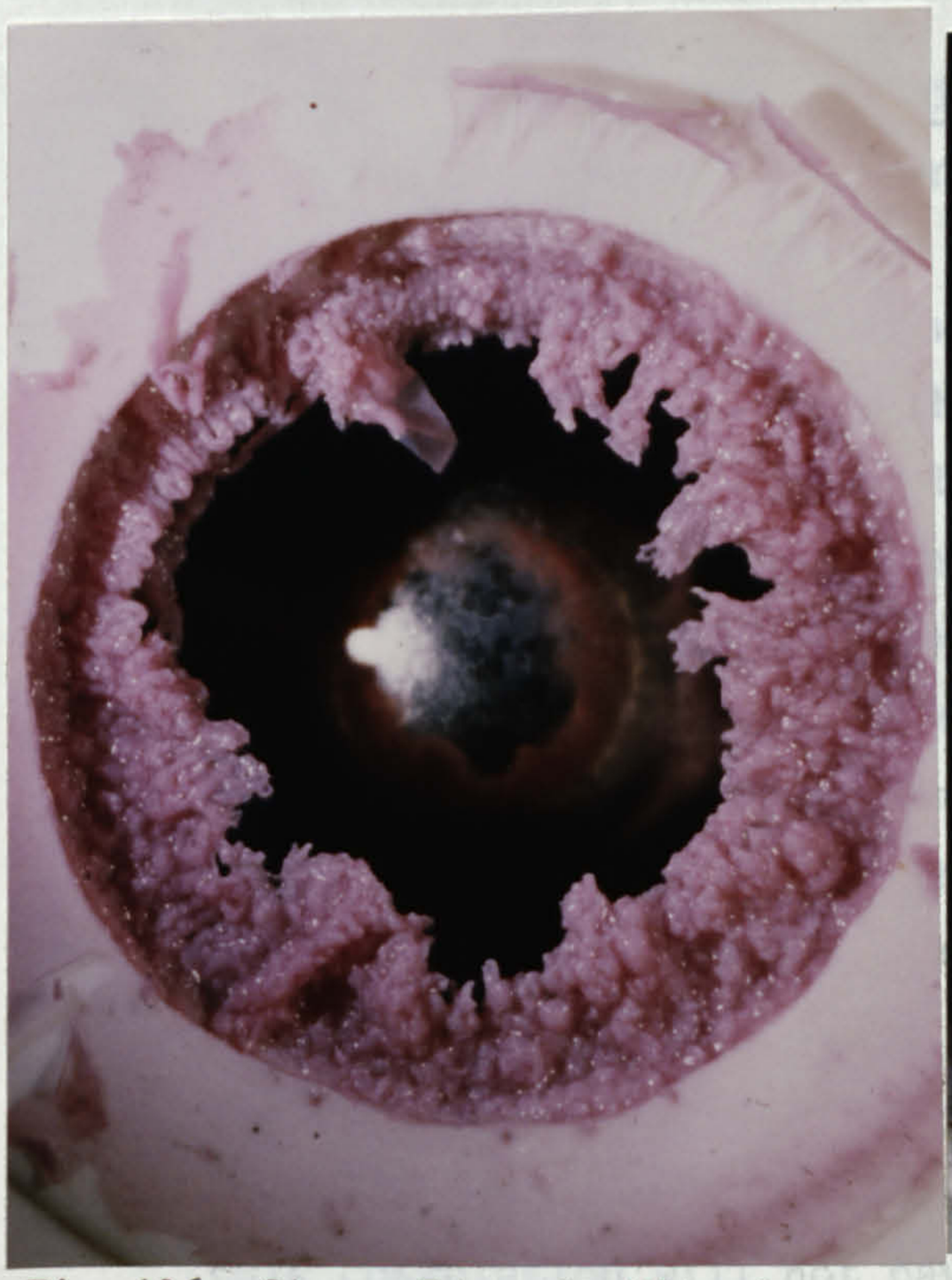


Fig 106. Starr-Edwards Mitral
Upstream 38°C

Fig 107. Starr-Edwards Mitral
Downstream 38°C

4.7.3 Comparison at 3 Litres/min Milk Flow

Having compared the performance of the valves at 2 litres/min, it is of interest to compare them at different conditions, and one of the main factors which may affect the clot deposition is the flow rate. Photographs showing typical deposition patterns found with the Edinburgh, the Starr-Edwards and the Bjork-Shiley valves are, therefore, shown above. Unfortunately no experiment using the Starr-Edwards valve in the aortic position at the flow rate has been successfully performed, and the photograph shown is of a valve used in the mitral site, which due to the nature of the rennet and $CaCl_2$ addition is a less severe test than the aortic site. This is because at the mitral site the rennet, $CaCl_2$ and milk are still mixing and the clotting reaction is only just beginning, whereas at the aortic site the milk is nearer to bulk coagulation, although bulk coagulation will not have occurred.

In order to facilitate comparison, the clots on the Bjork-Shiley and Edinburgh valves shown were also obtained in the mitral position at the same temperature as used for the Starr-Edwards. Whereas the amount of clot on the Bjork-Shiley and Starr-Edwards valves is much less than found at 2 litres/min in the aortic site, the amount of clot found on the Edinburgh valve is not markedly changed. Thus, whereas at a flow rate of 2 litres/min the Edinburgh valve appears very much better than the others, at 3 litres/min it is hard to choose between them. It may be that at the higher flow rate more milk is required to allow time for gross clots to form and that the Edinburgh valve with its lack of favoured sites will not be prone to this growth. However, the coarse nature of the delrin discs on the Edinburgh valves used makes them very susceptible to an initial deposition and retention of a film of clot, even at the higher flow rates.

4.7.4 Conclusions

The differences between the materials of construction of the various valves make a comparison between the different designs very difficult. None of the valves has shown itself to be totally free of clot deposition, but the Edinburgh valve with its lack of favoured sites appears to be the most promising, assuming a suitable athrombogenic material of construction, such as perhaps vitreous carbon, can be used.

4.8 The Sequence of Clot Deposition

4.8.1 Results

Bjork-Shiley Valve Downstream

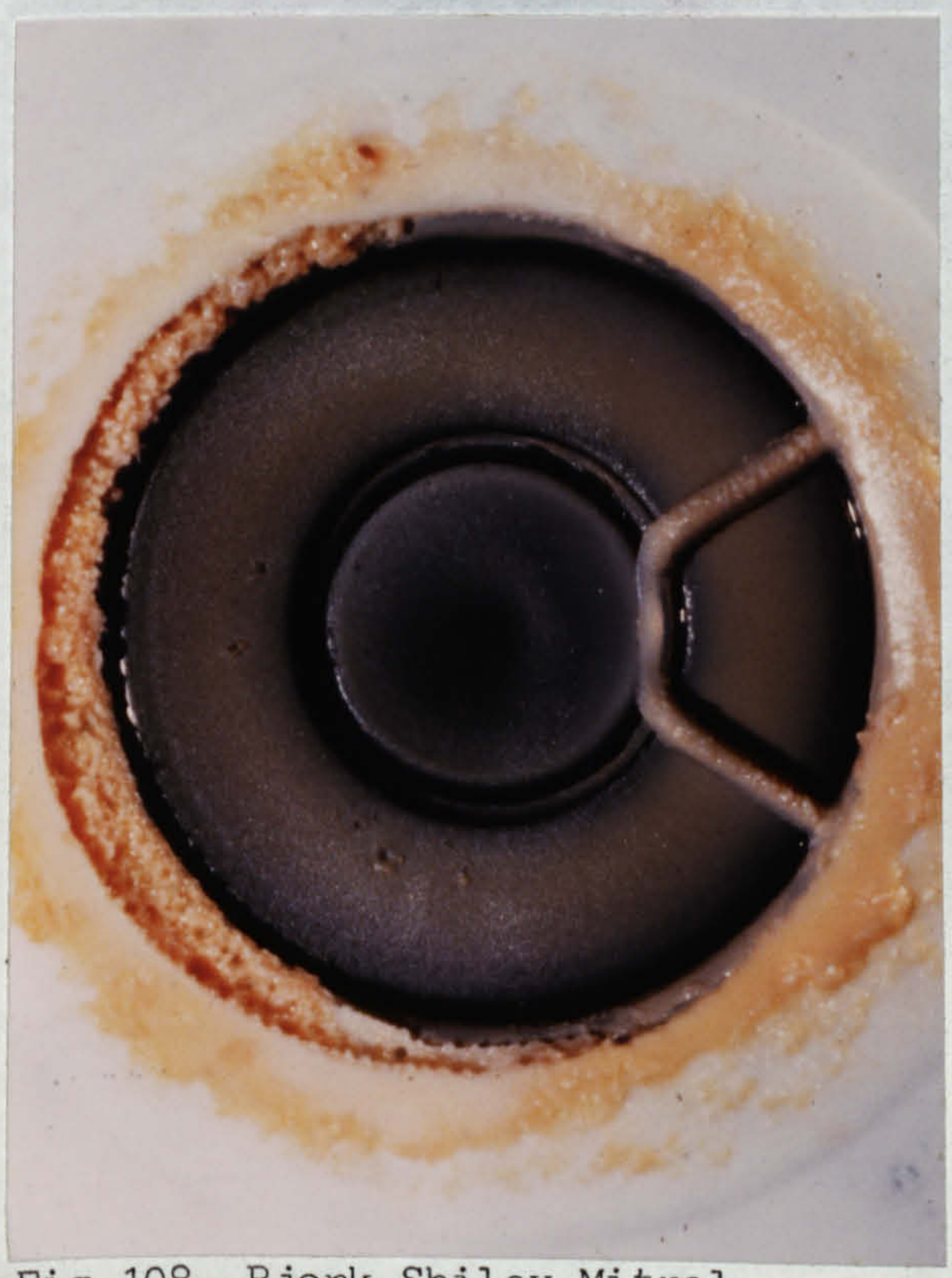


Fig 108. Bjork-Shiley Mitral
Downstream 39°C. 3L/min milk.

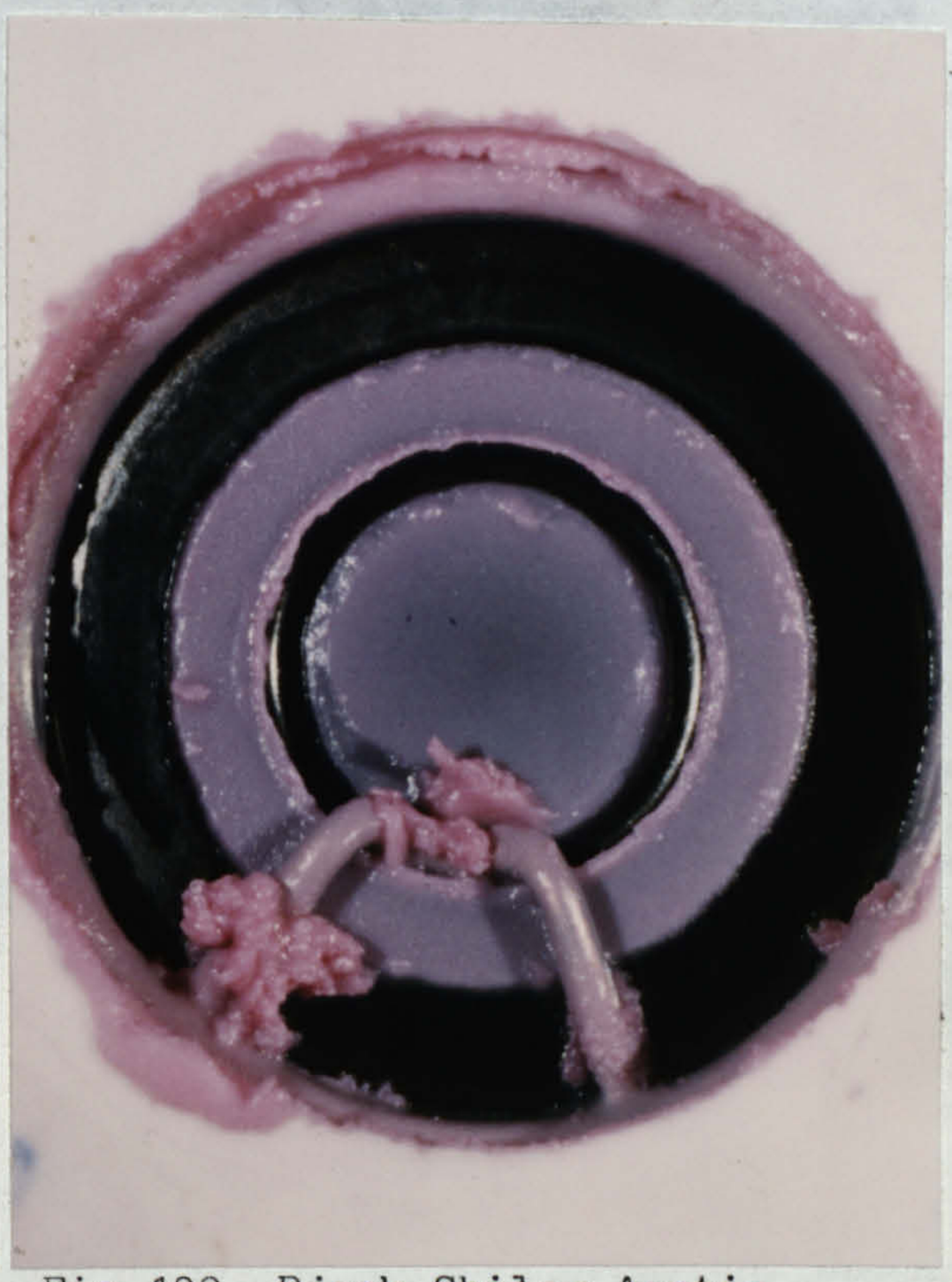


Fig 109. Bjork-Shiley Aortic
Downstream 38°C. 3L/min milk.

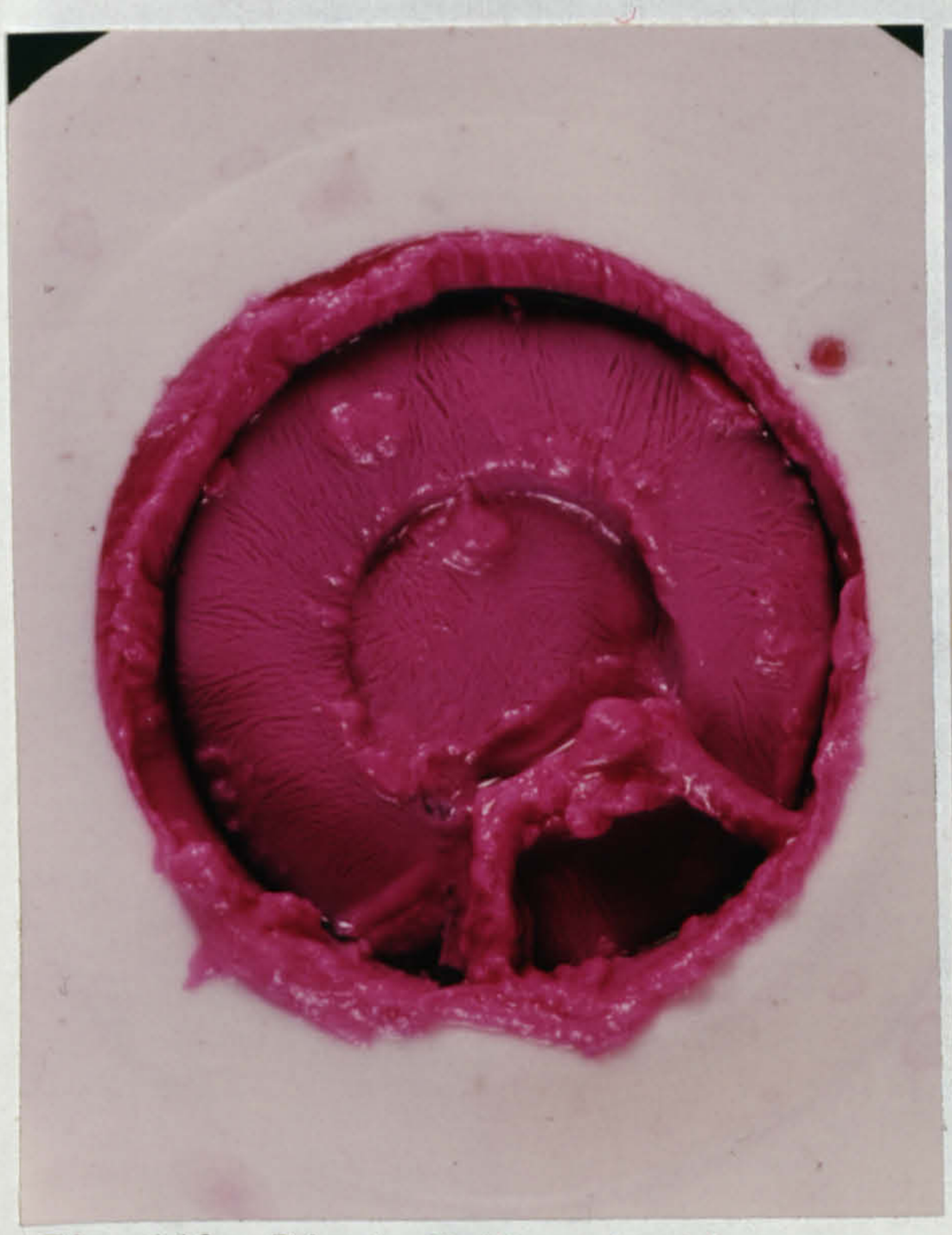


Fig 110. Bjork-Shiley Aortic Downstream 40°C. 2L/min milk.

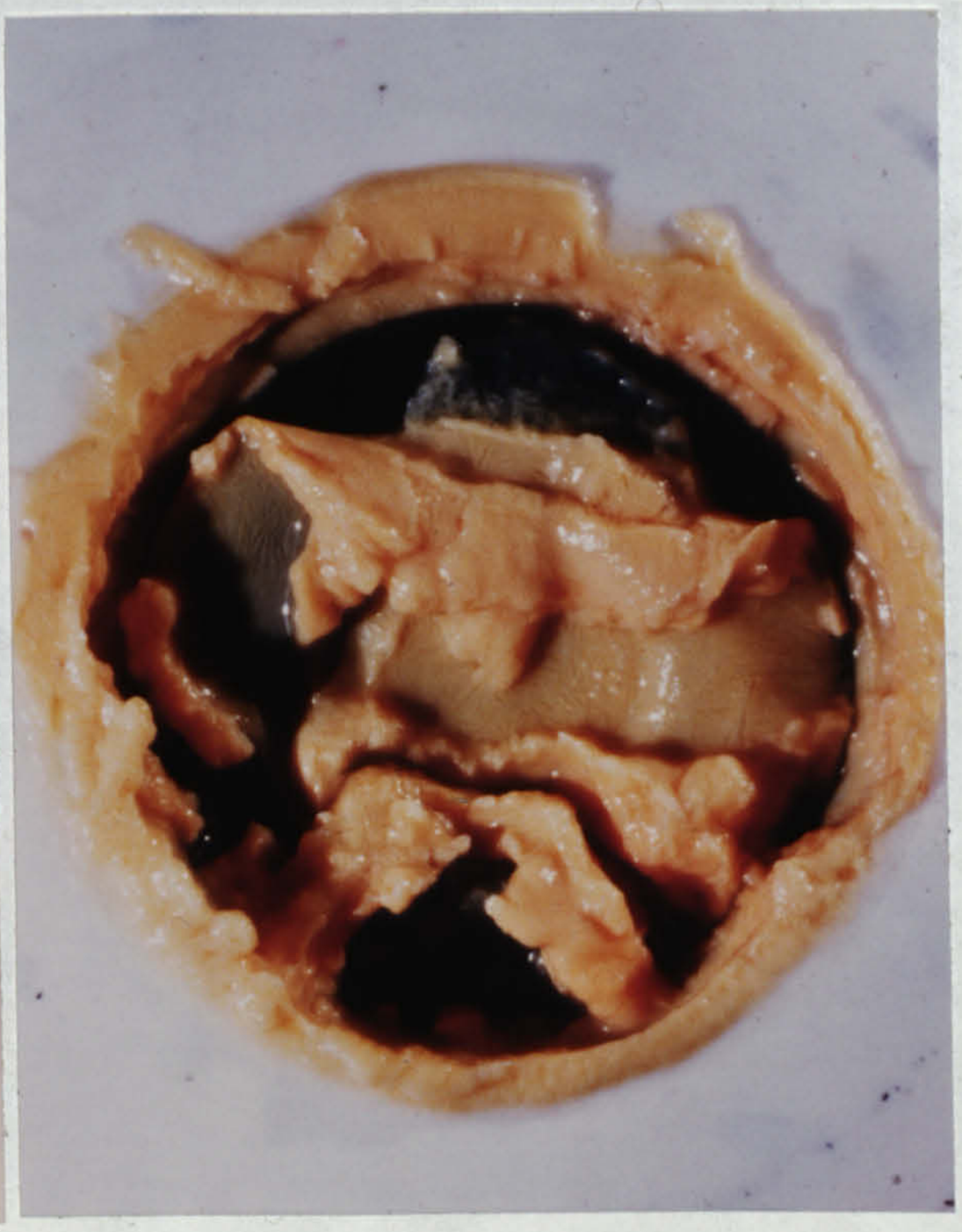


Fig 11. Bjork-Shiley Aortic Downstream 39°C. 3L/min milk.

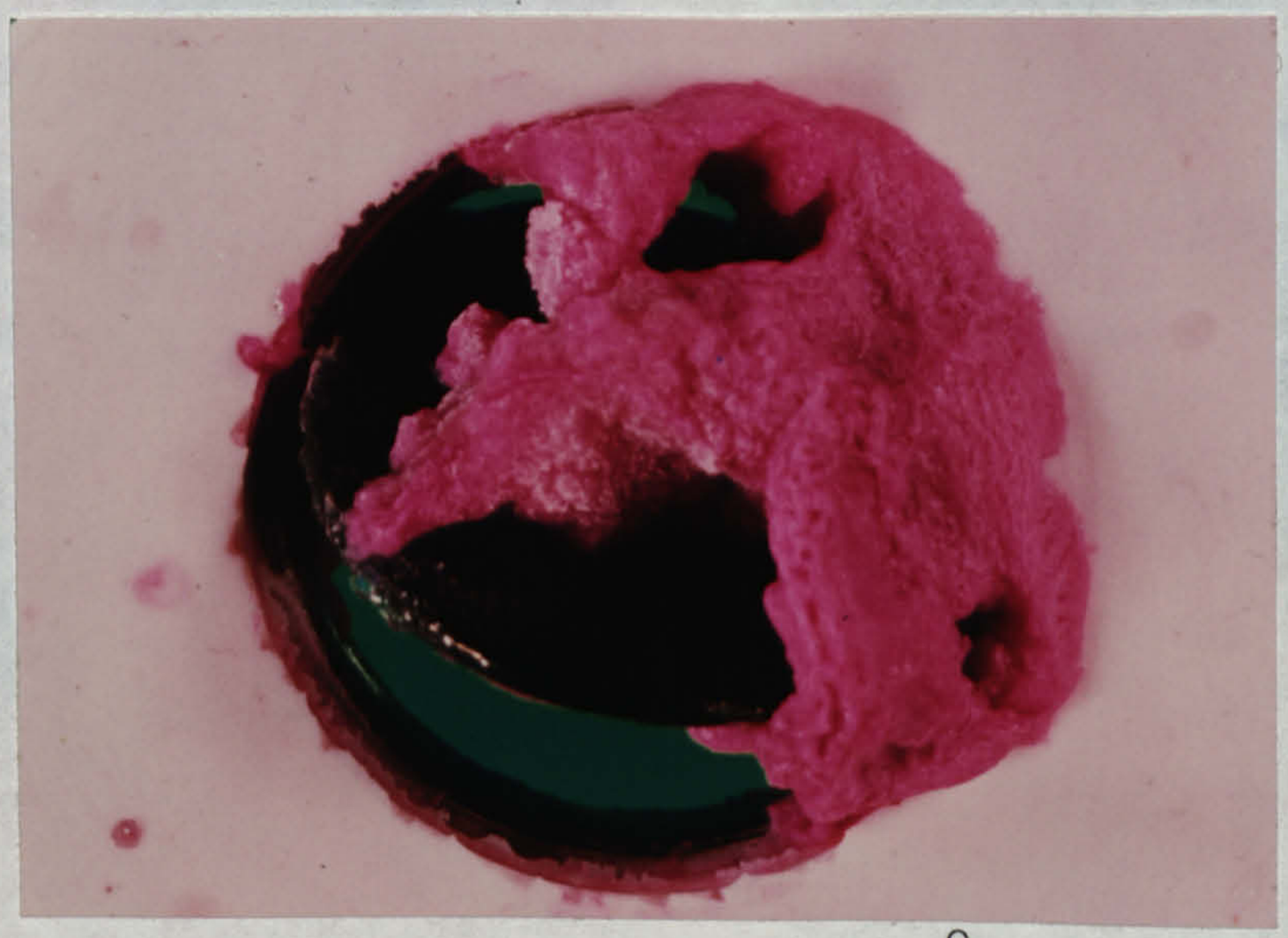


Fig 112. Bjork-Shiley Aortic Downstream 40°C 2L/min milk.

Bjork-Shiley Valve Upstream

Fig 113. Bjork-Shiley Mitral
Upstream 39°C. 3L/min milk.

Fig 114. Bjork-Shiley Aortic
Upstream 38°C. 3L/min milk.

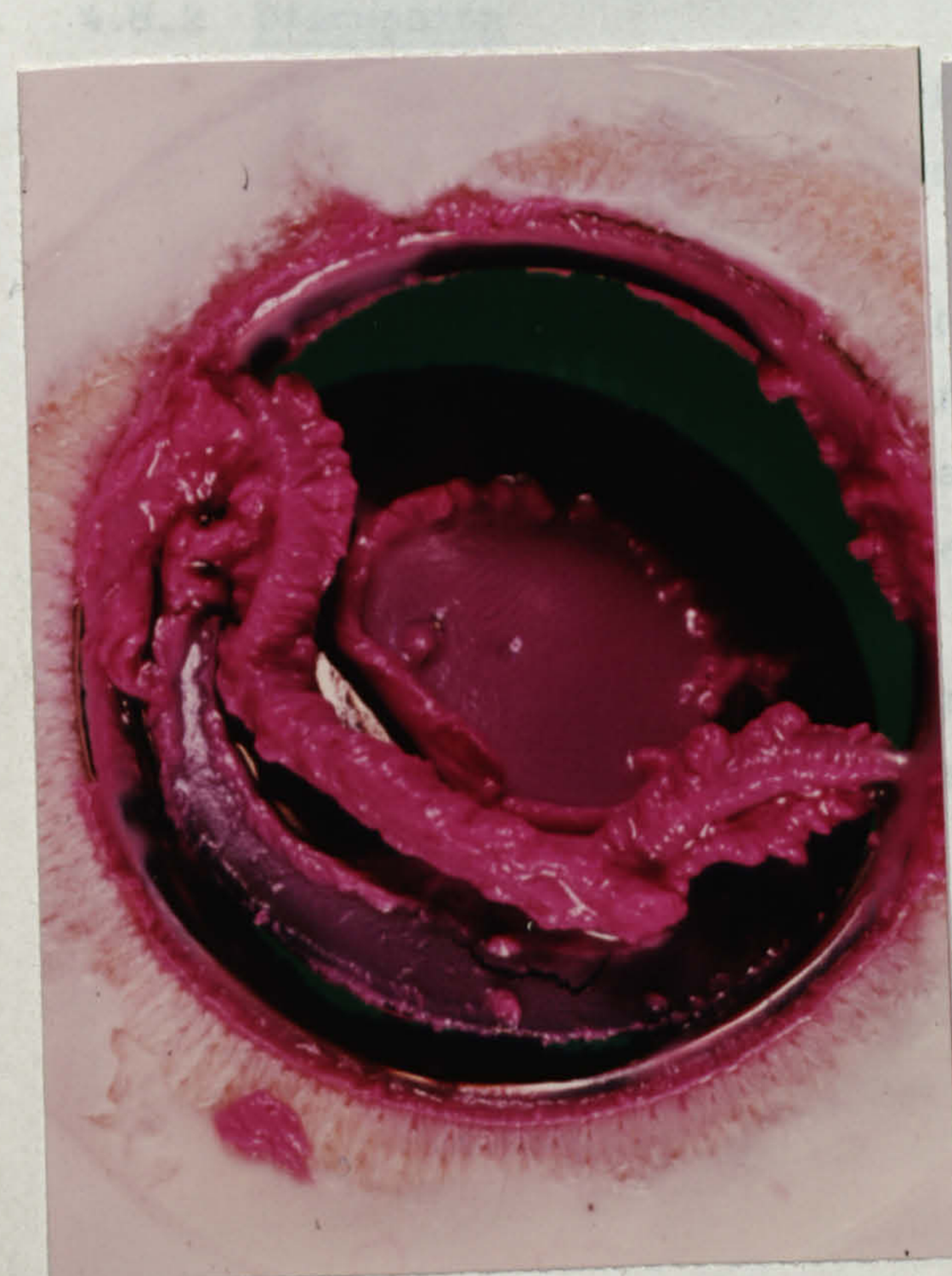


Fig 115. Bjork-Shiley Aortic
Upstream 40°C. 2L/min milk

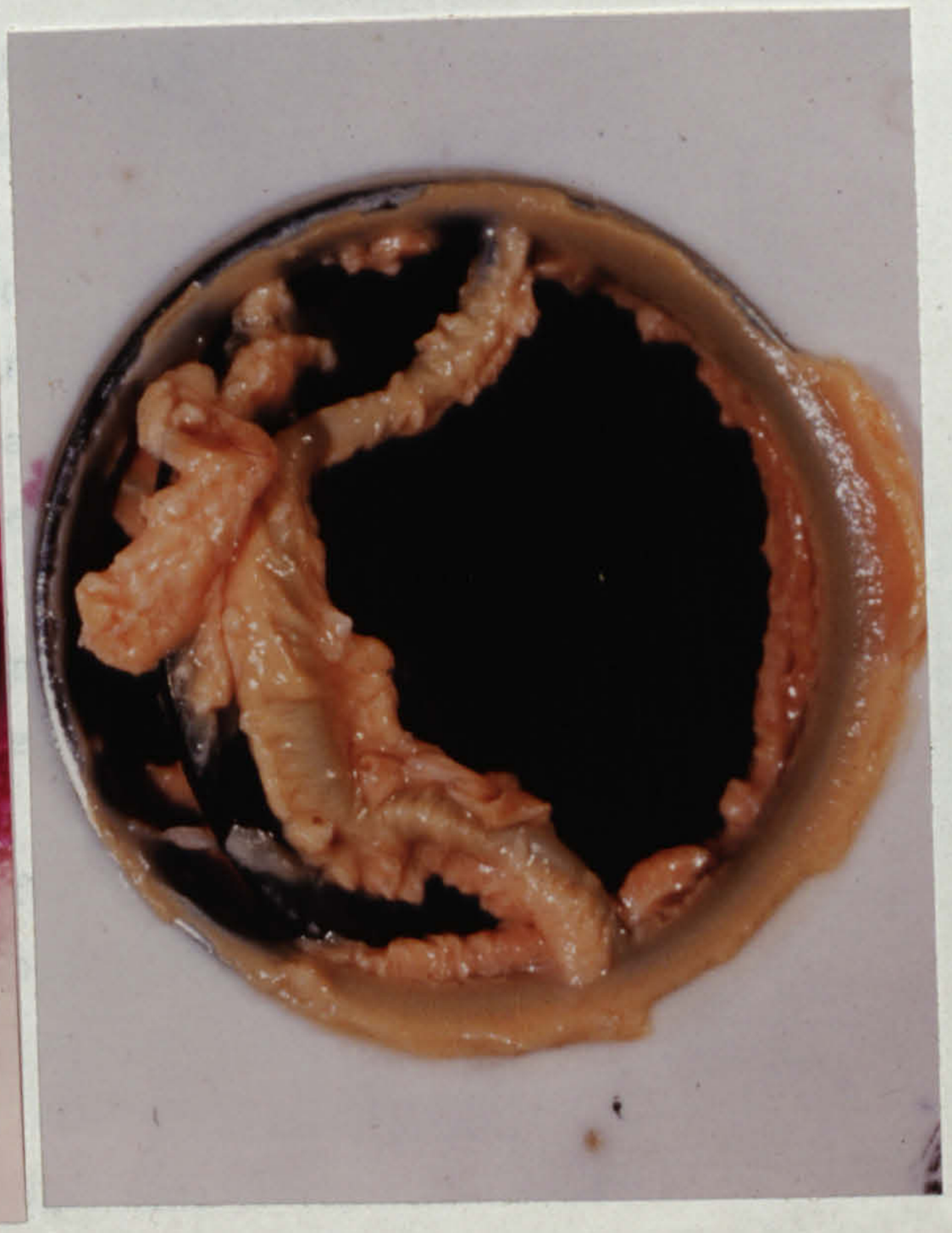


Fig 116. Bjork-Shiley Aortic
Upstream 39°C. 3L/min milk.

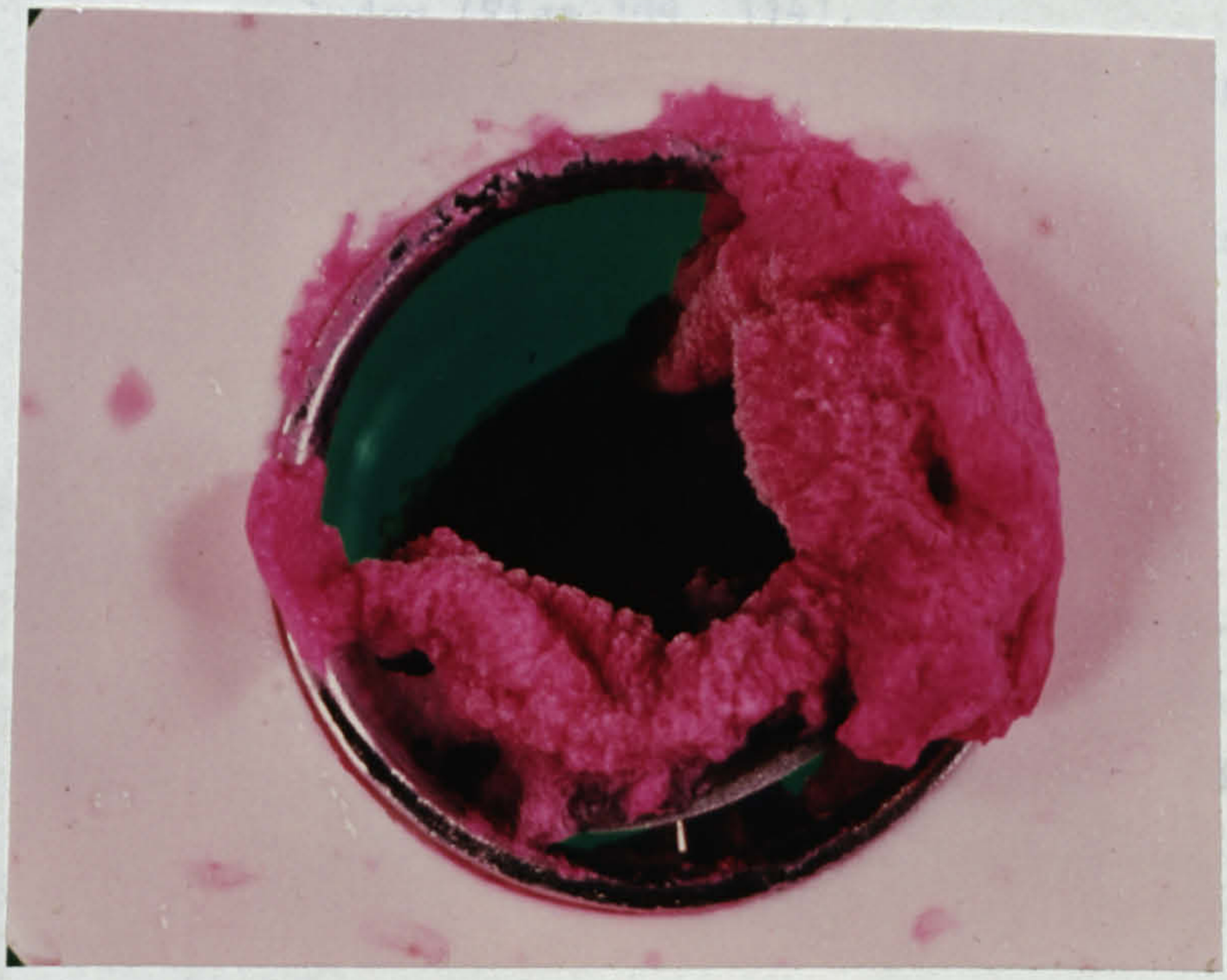


Fig 117. Bjork-Shiley Aortic Upstream 40°C. 2L/min milk.

4.8.2 Discussion

Using the Stagnation Point Flow Experiment described in Chapter 2, the sequence of milk deposition was found to be as follows:-

1) Protein layer adsorption.
2) Deposition of small particles evenly over the surface.
3) Deposition of larger particles where stagnation occurs.
4) Alternate to (3), larger particles can deposit at favoured sites.
5) The growth of a calcium clot symmetrically if (3) has occurred, or in a wedge type formation if (4) has occurred.

The sequence of deposition found when rennetized milk is passed over heart valves appears to be as follows:-

1) An even and thin layer is deposited over the entire surface apart from those areas where rubbing action keeps the surface clear (Figs 108, 113).

2) Larger scale clots form at favoured sites such as around the struts on the Bjork-Shiley and especially on the downstream side where the strut touchs the centre of the occluder (Figs 109, 114).
3) The thickness of the deposited clot increases (Figs 110, 115).
4) The deposits can become disrupted leading to greater fluid mechanical disturbances (Figs 111, 116).
5) Gross clot can grow affecting the performance of the valve and leading to obstruction (Figs 112, 117).

Presumably the first visible event of deposition on heart valves corresponds to (3) and (4) of the Stagnation Point Flow Experiment, and protein layer adsorption and deposition of small particles evenly over the entire surface has already occurred.

Where the overall surface is not conducive to the deposition of the thin layer of clot, it is still possible to find clot forming at favoured sites (Figs 118, 119).

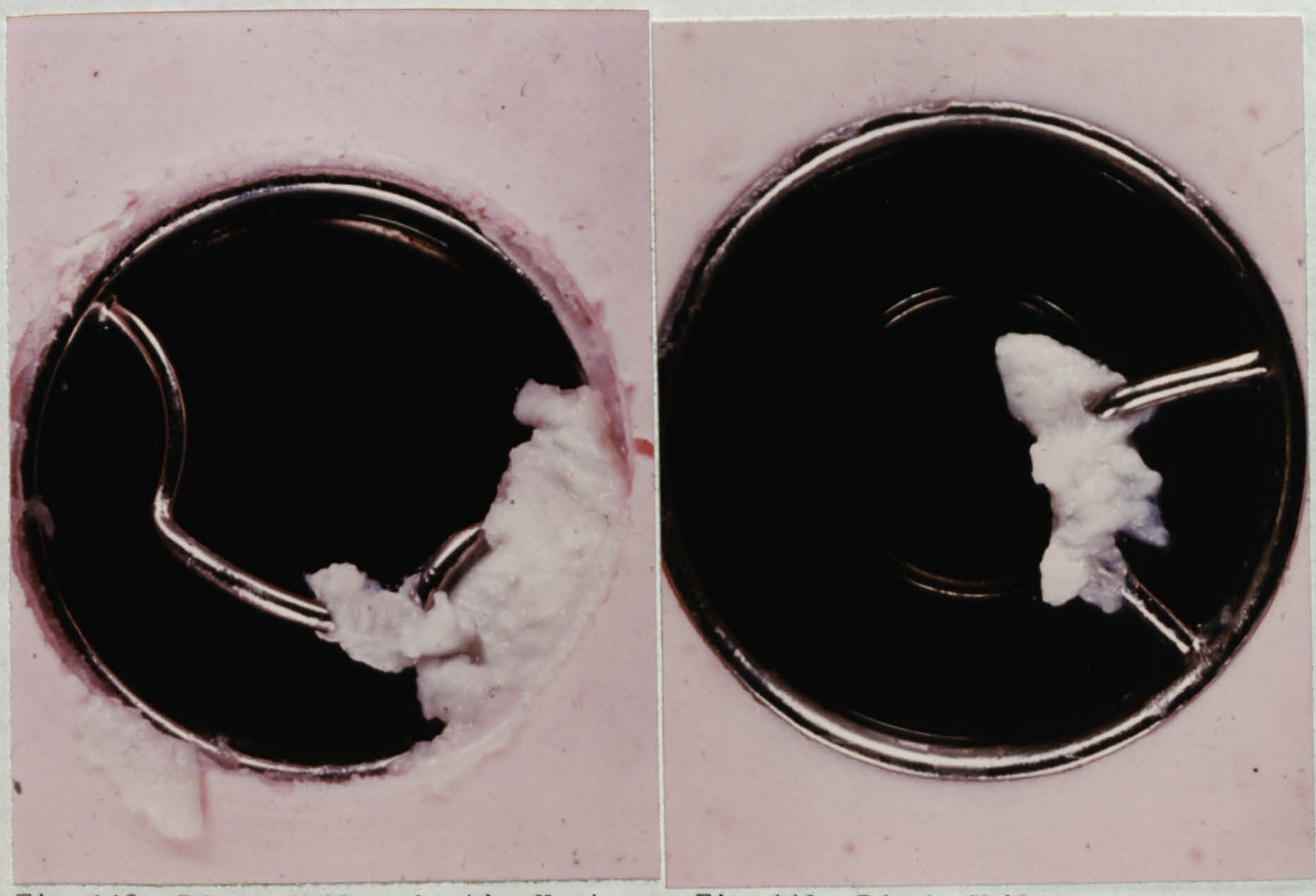


Fig 118. Bjork-Shiley Aortic Upstream Fig 119. Bjork-Shiley 0.6% $CaCl_2$ & rennet. 40°C 2L/min milk Downstream Conditions as Fig 118.

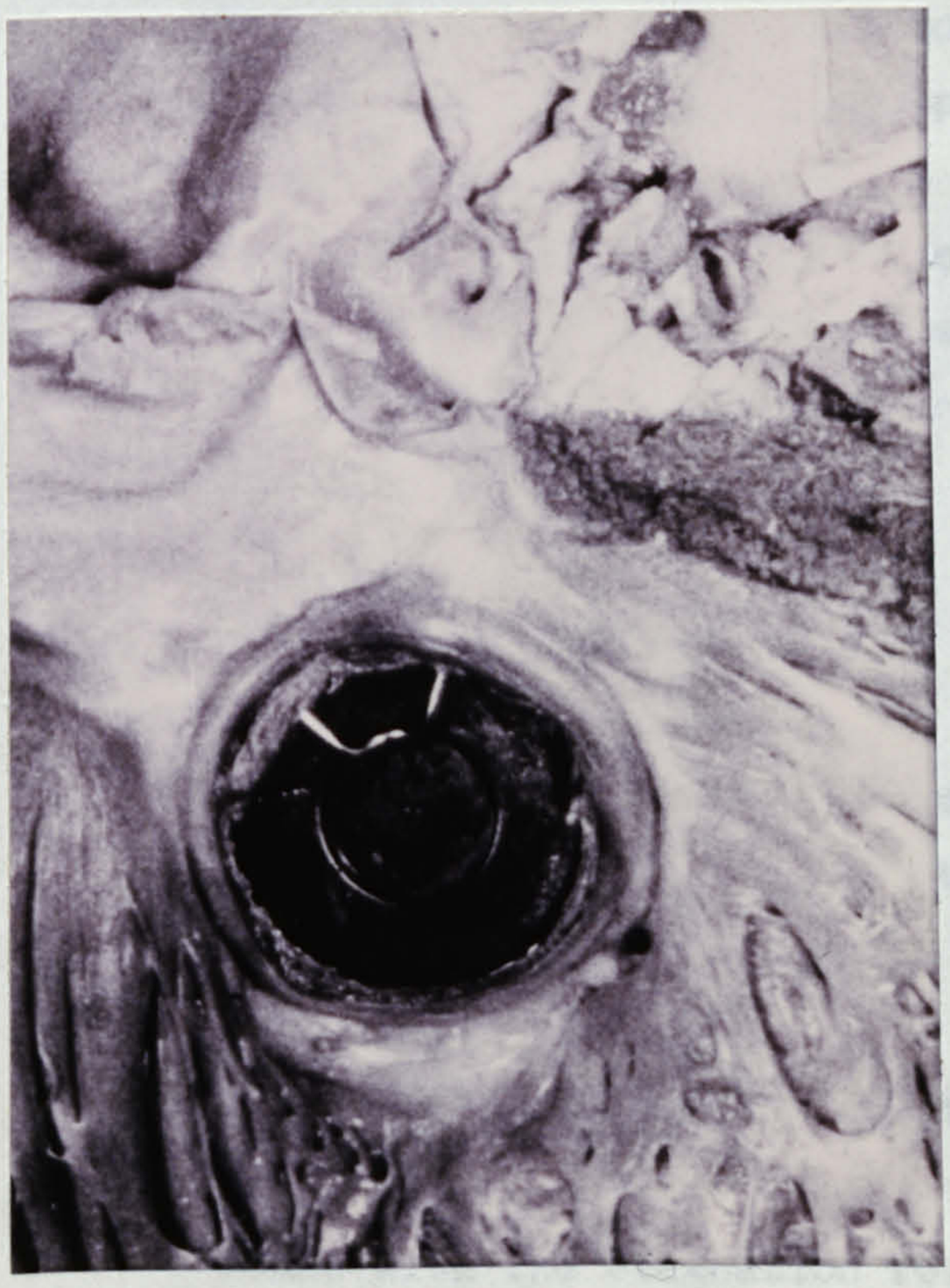


Fig 120. Bjork-Shiley After 2 Years
Courtesy Stovin

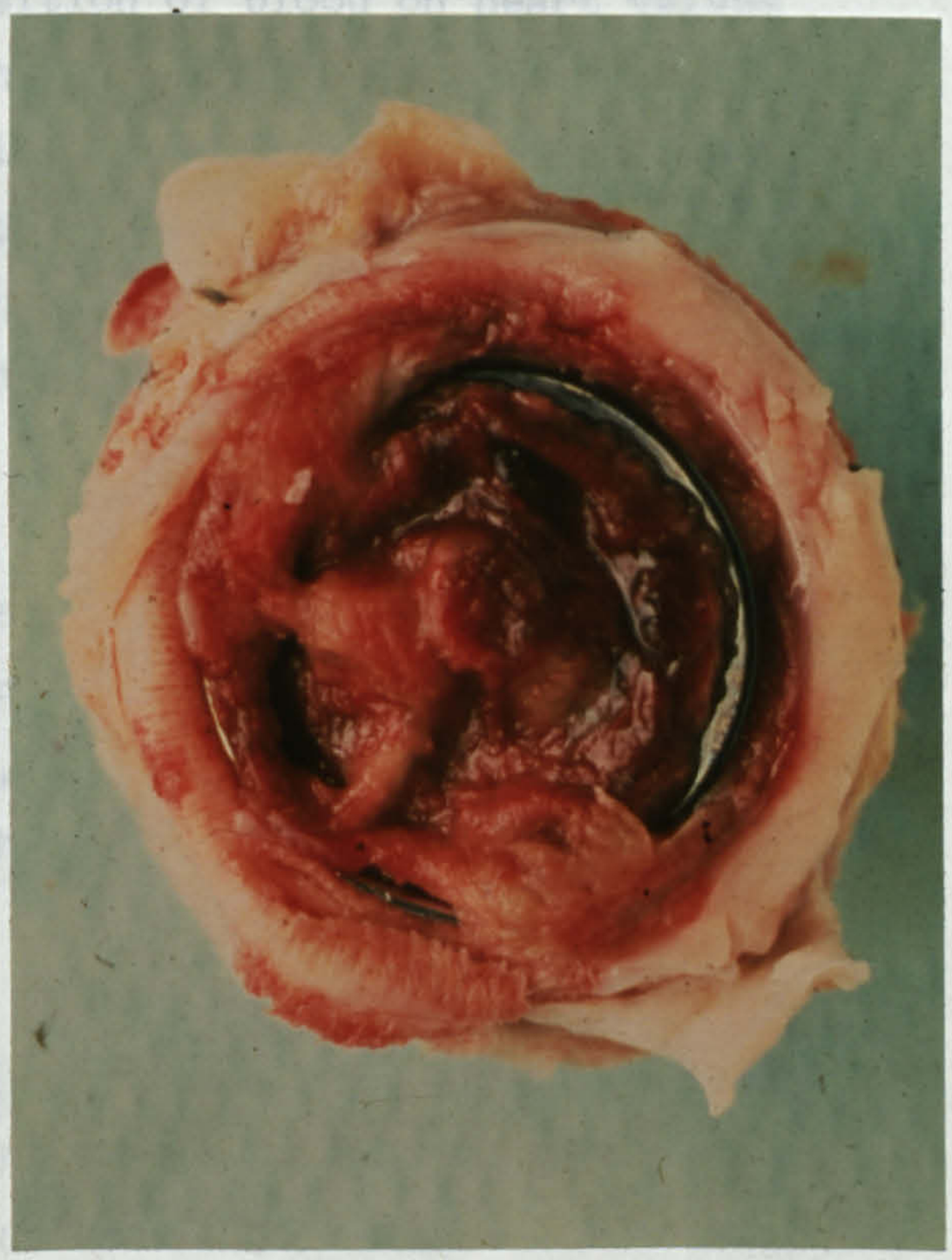


Fig 121. Bjork-Shiley After 6 Months
Courtesy MacGregor

Fig 122. Bjork-Shiley After 6 Months

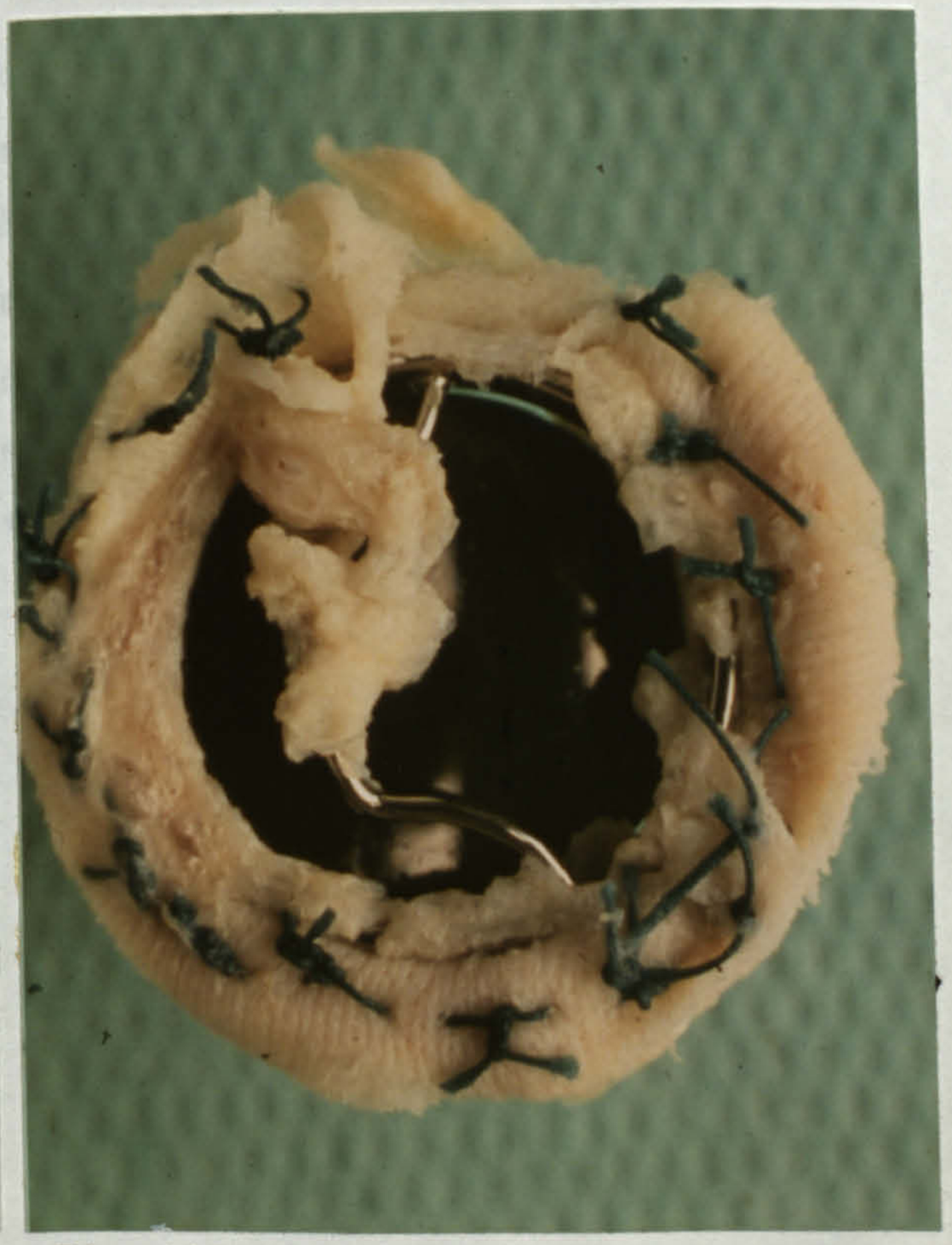


Fig 123. Bjork-Shiley After 2 Years.
Courtesy Busuttil

The probable sequence of deposition of blood on heart valves in vivo is shown on photographs 120 - 123. The first of these shows a valve on which there is no organized clot, but close examination reveals a thin deposit covering the entire surface with more clot at the point where the strut touchs the centre of the occluder, and the third photograph shows a gross clot covering the entire surface.

With milk it was found that, even where there is no overall clot deposition, it is still possible to find deposit forming discretely at a favoured site, and an example of this found in vivo is shown in the fourth photograph.

4.8.3 Conclusions

The apparent sequence of clot deposition found on heart valves with milk seems to be in accord with the sequence of deposition found with the Stagnation Point Flow Experiment. Furthermore, it is probable that the sequence of deposition is very similar to that found in the clinical situation.

It must be noted that these proposed sequences of deposition on heart valves are based on results obtained in vivo at the end of experiments and each photograph belongs to a different trial. However since it is unlikely that large scale clot appears on the valves spontaneously, there must be a sequence of deposition, and the proposed sequence which is supported by the photographs is the most probable.

4.9 The Effect of Altering Conditions on the Clotting Behaviour

4.9.1 Flowrate

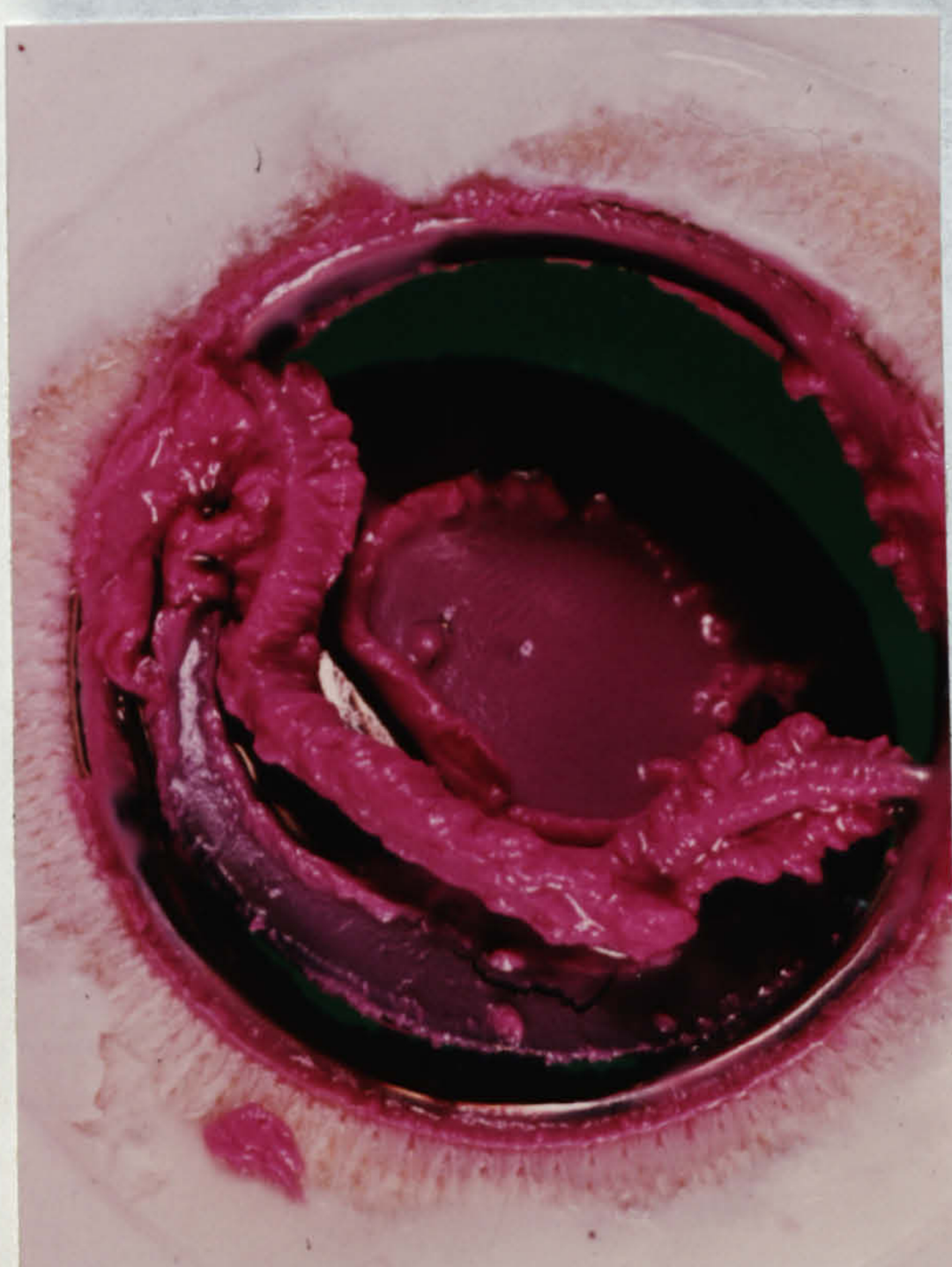


Fig 124. Bjork-Shiley Aortic
Upstream 40°C. 2L/min milk

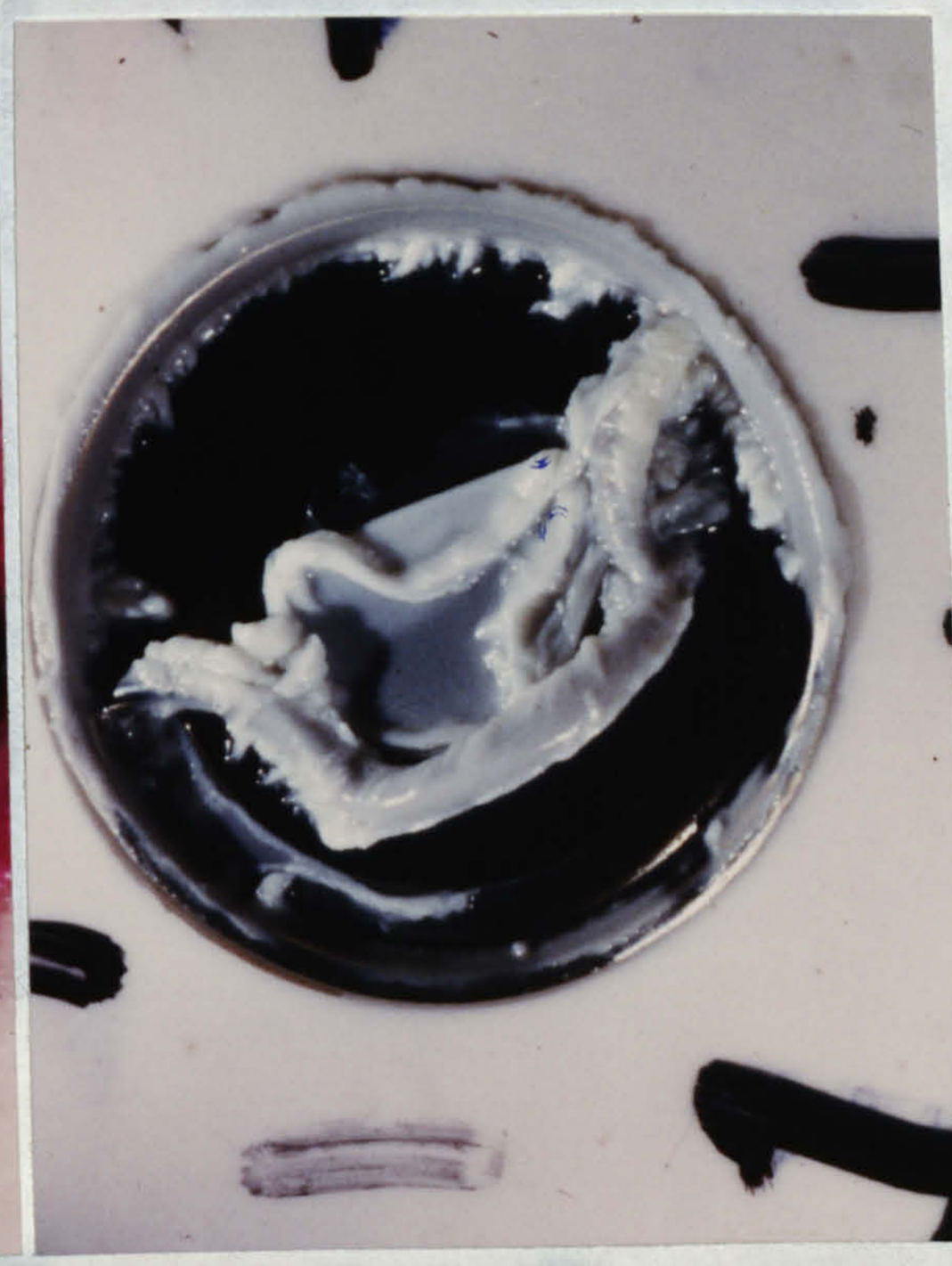


Fig 125. Bjork-Shiley
Upstream 40°C. 3L/min milk

Fig 126. Bjork-Shiley Aortic
Downstream 40°C. 2L/min milk

Fig 127. Bjork-Shiley Aortic
Downstream 40°C. 3L/min milk

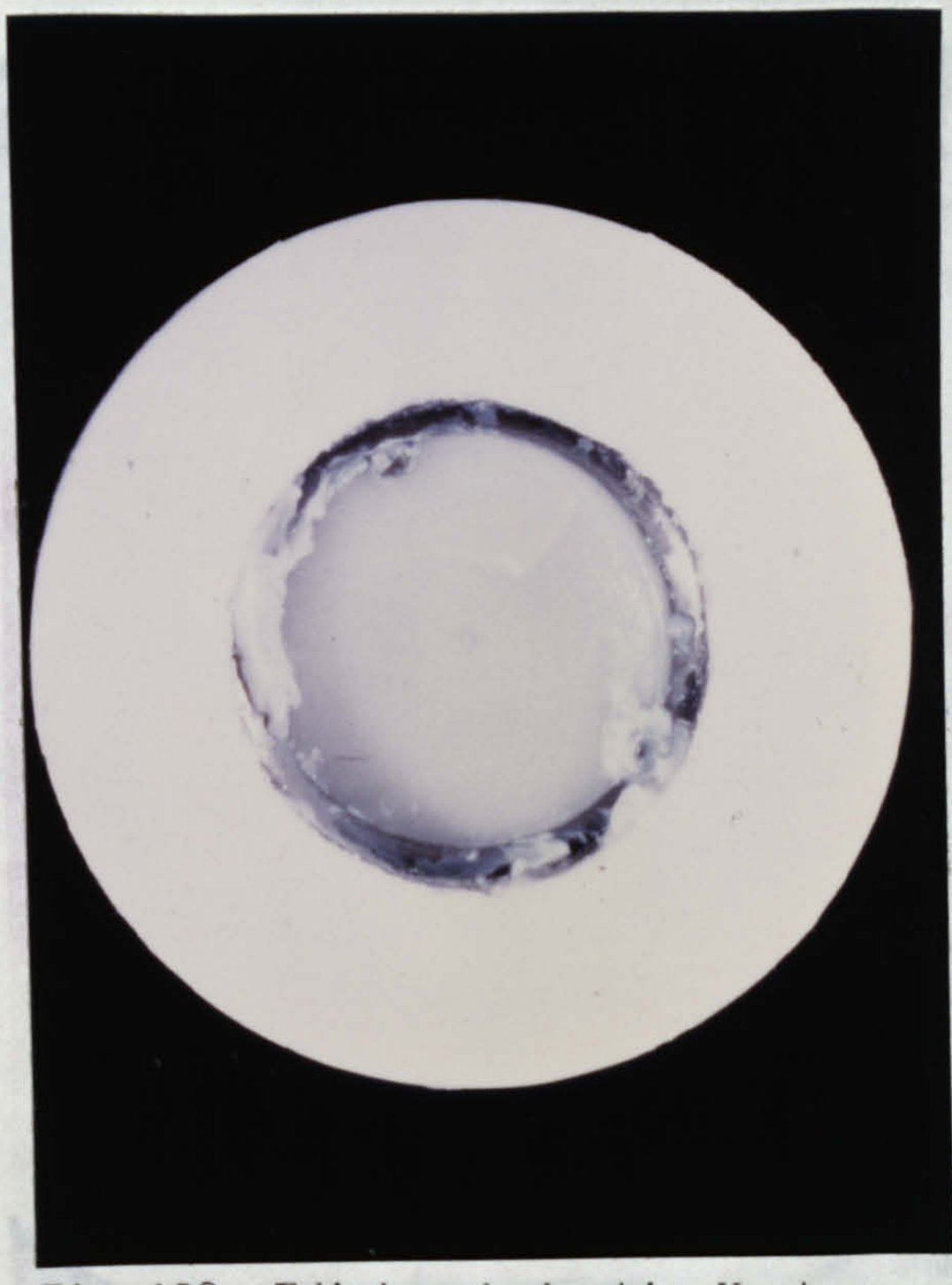


Fig 128. Edinburgh Aortic Upstream 38°C. 2L/min milk

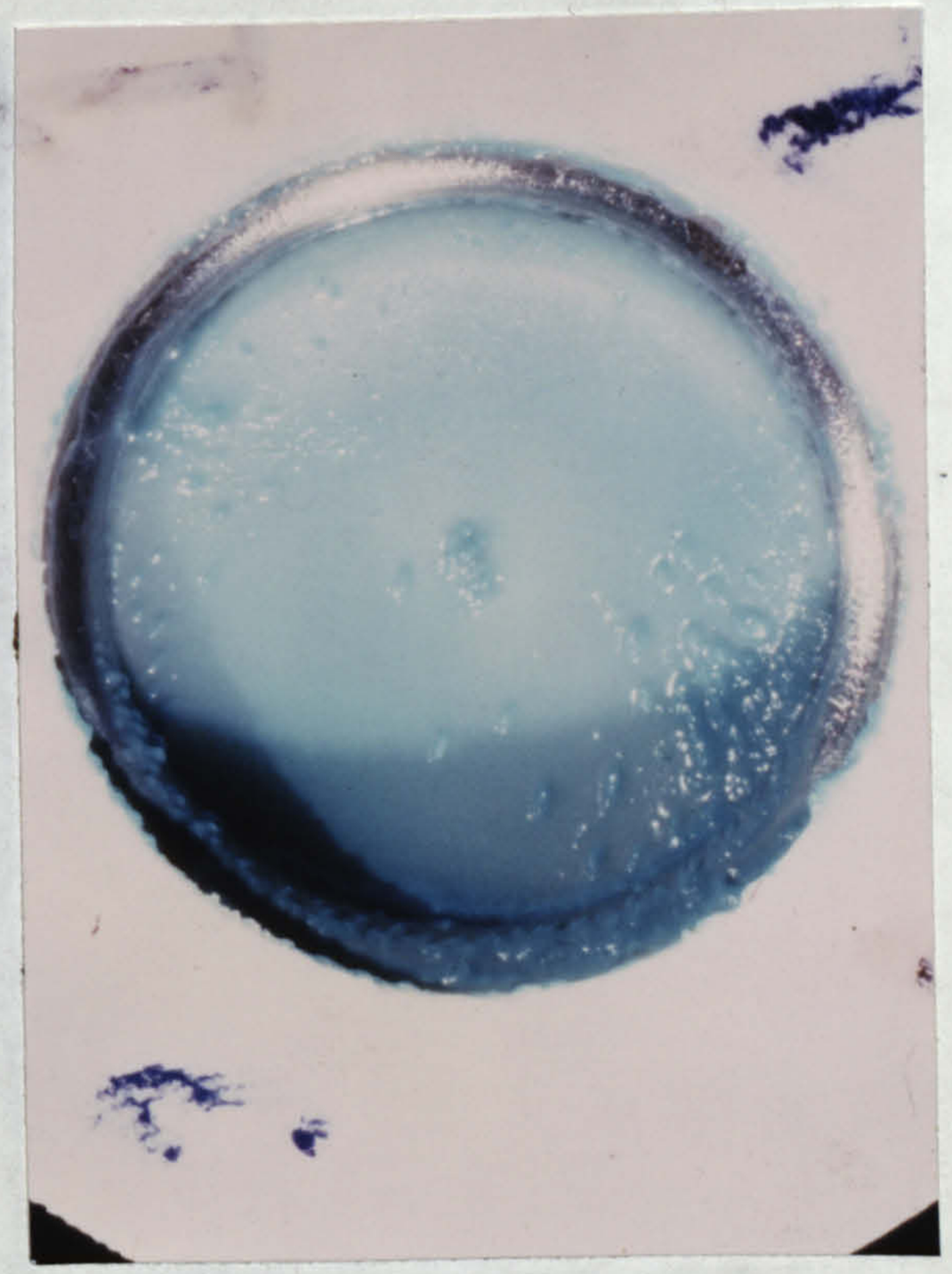


Fig 129. Edinburgh Aortic Upstream 38°C. 2L/min milk

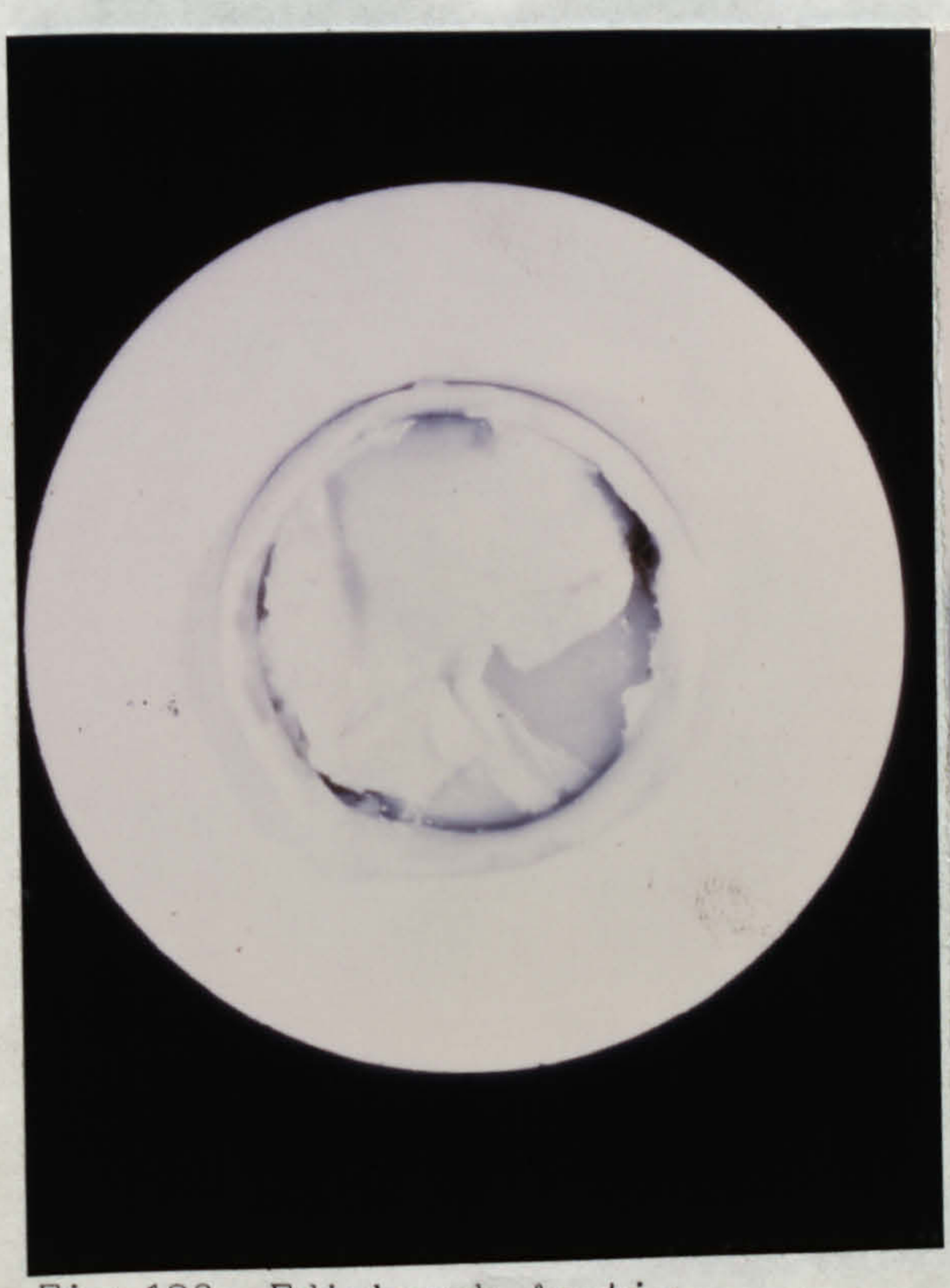


Fig 130. Edinburgh Aortic Downstream 38°C. 2L/min milk

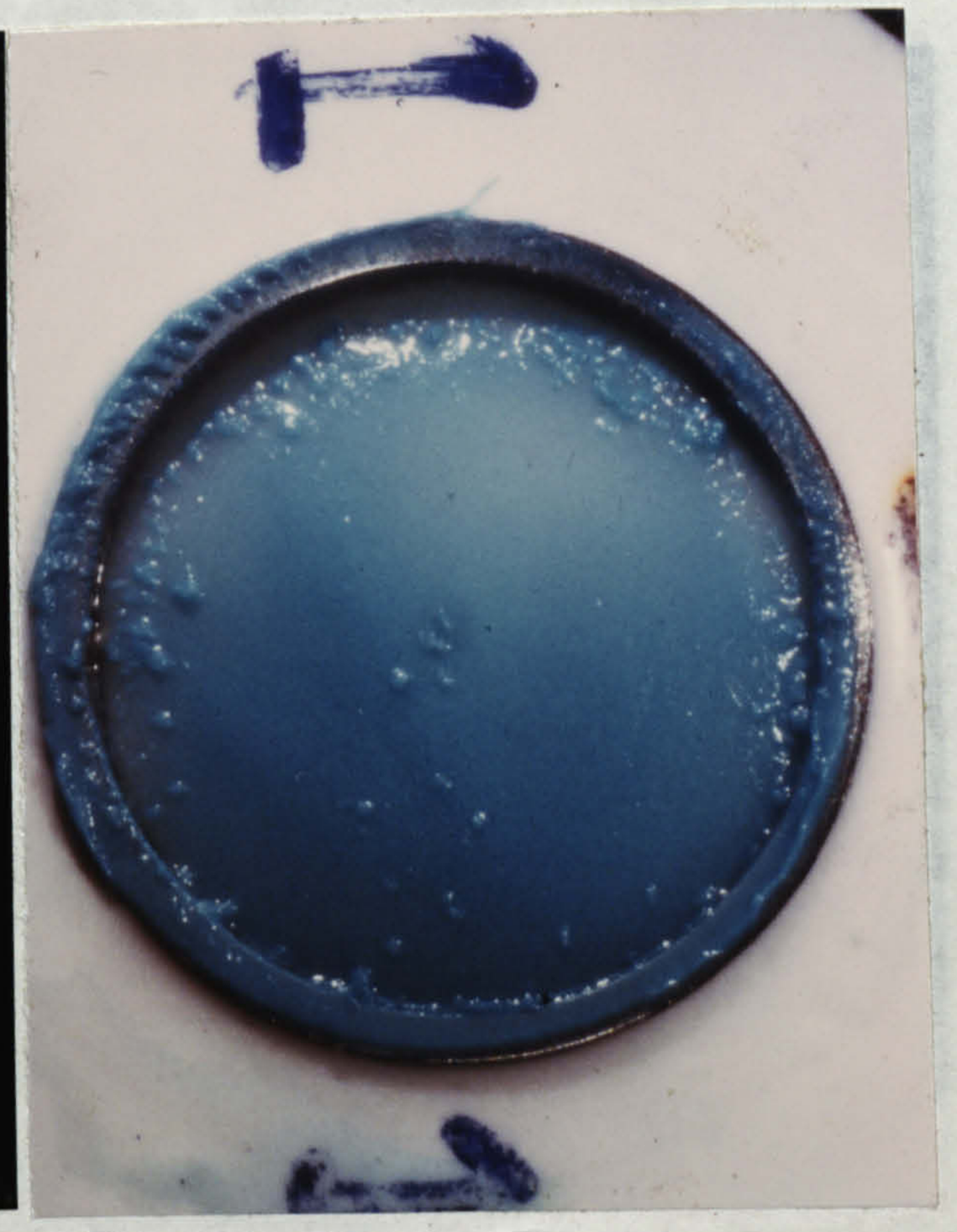


Fig 131. Edinburgh Aortic Downstream 38°C. 3L/min milk

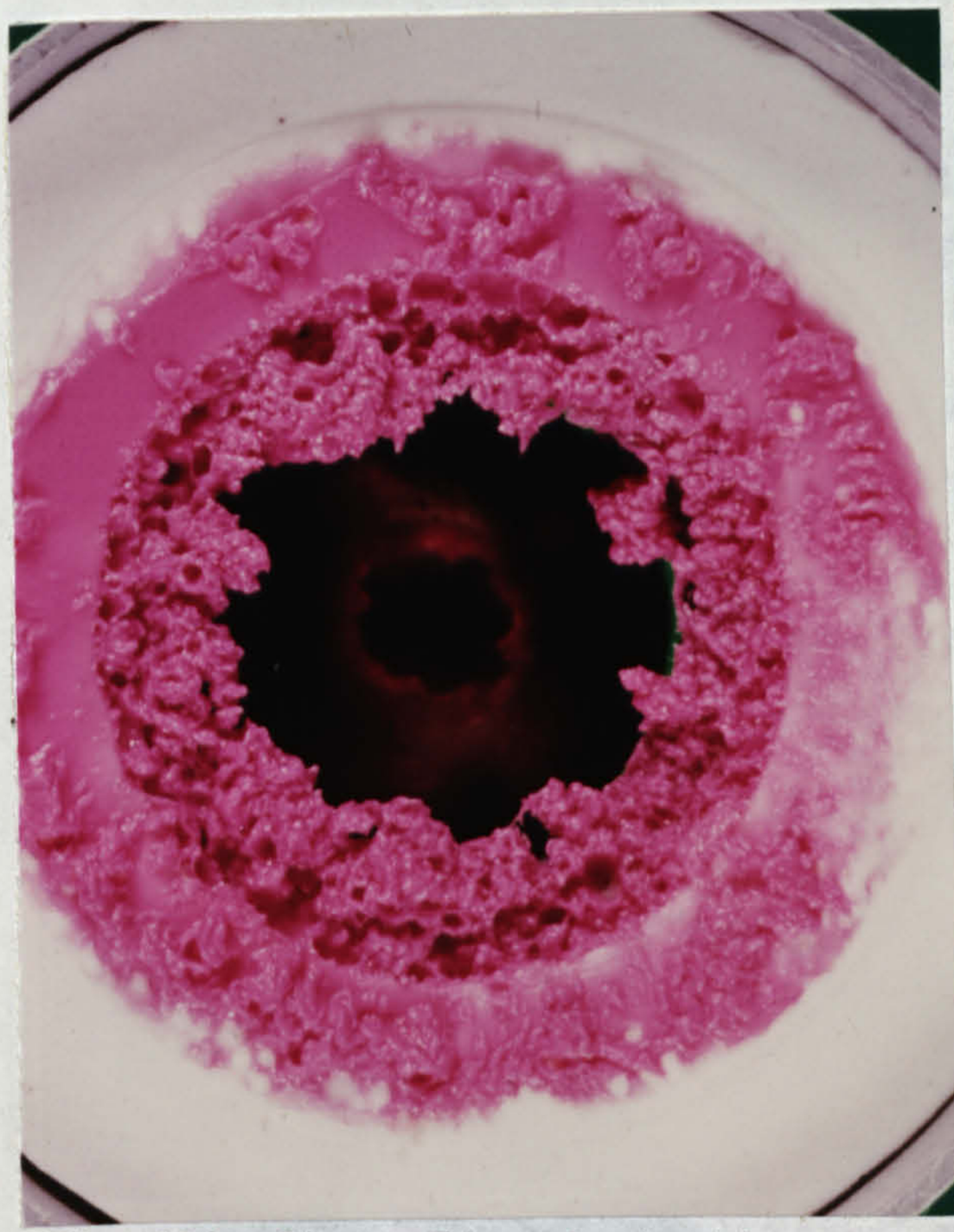


Fig 132. Starr-Edwards Aortic
Upstream 40°C. 2L/min milk

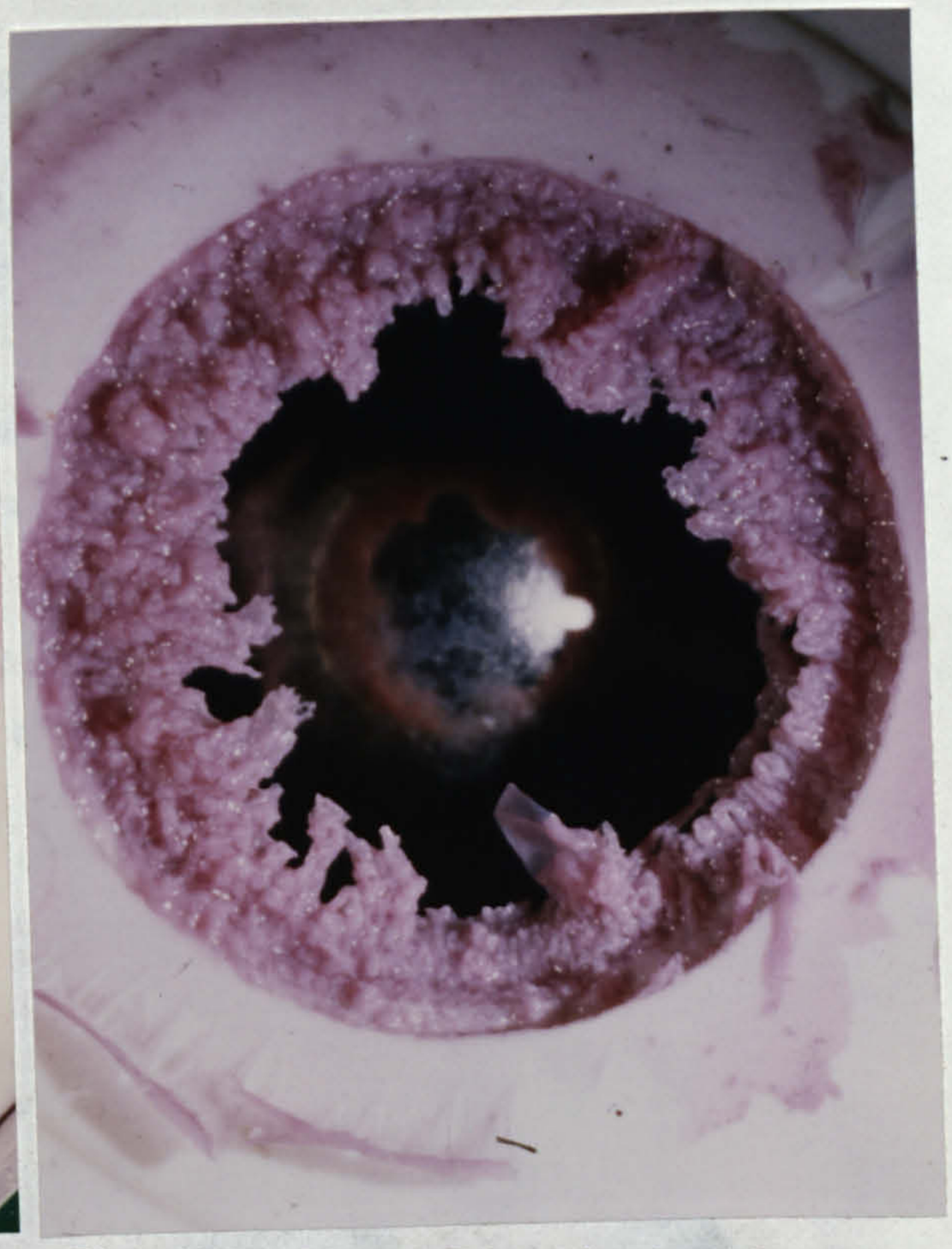


Fig 133. Starr-Edwards Aortic
Upstream 38°C. 3L/min milk

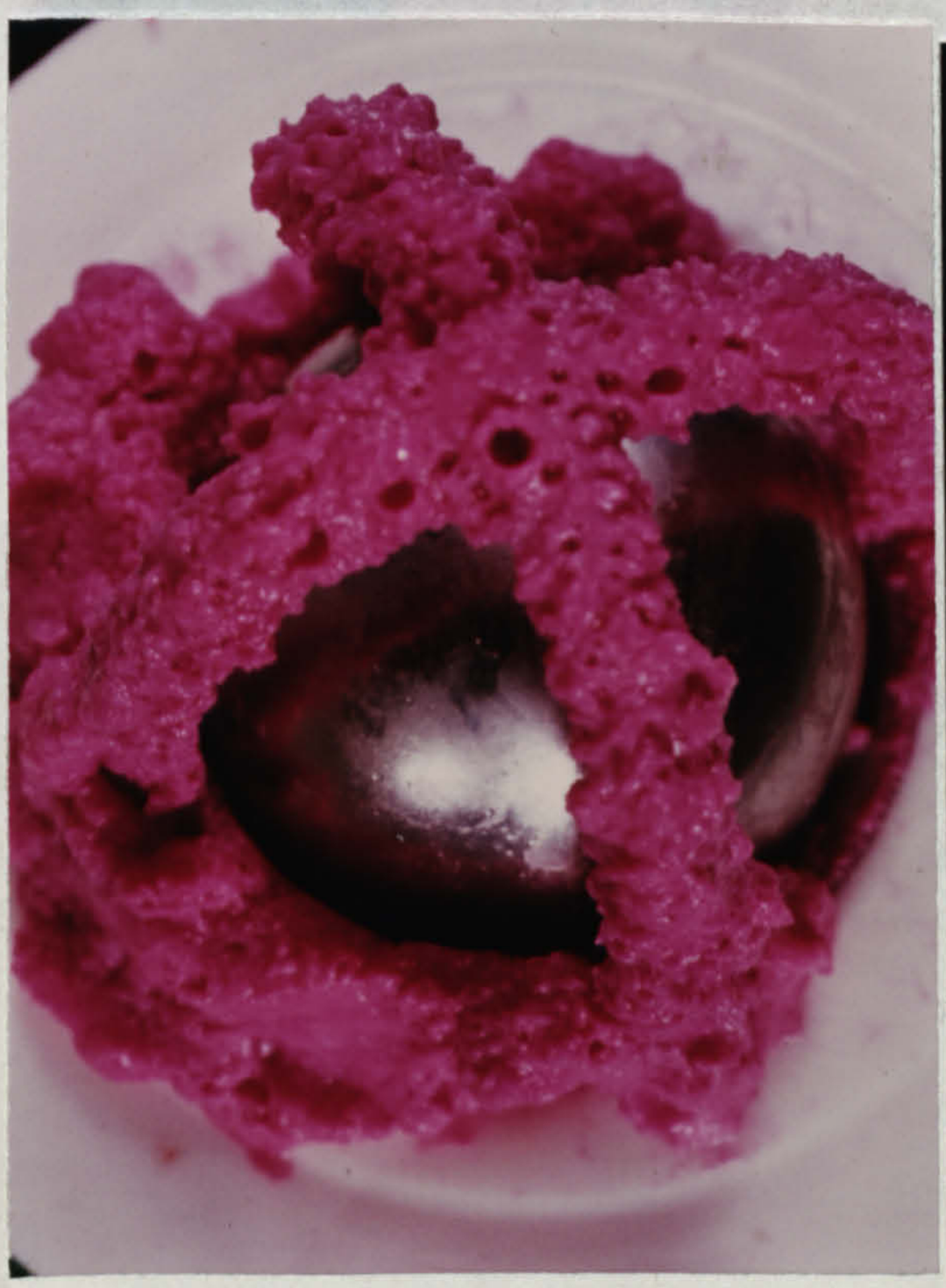


Fig 134. Starr-Edwards Aortic
Upstream 40°C. 2L/min milk

Fig 135. Starr-Edwards Aortic
Upstream 38°C. 3L/min

Discussion

The effect of altering the flowrate from 2 litres/min to 3 litres/min on the amount of clot deposition is not very great. In general more clot is deposited at the lower flow rate, but it could be that this is due to the longer run times associated with the lower flow rate.

Gross clotting, such that the valve function is prevented, only occurred in these tests at the lower flow rate (Figs 136 - 138).

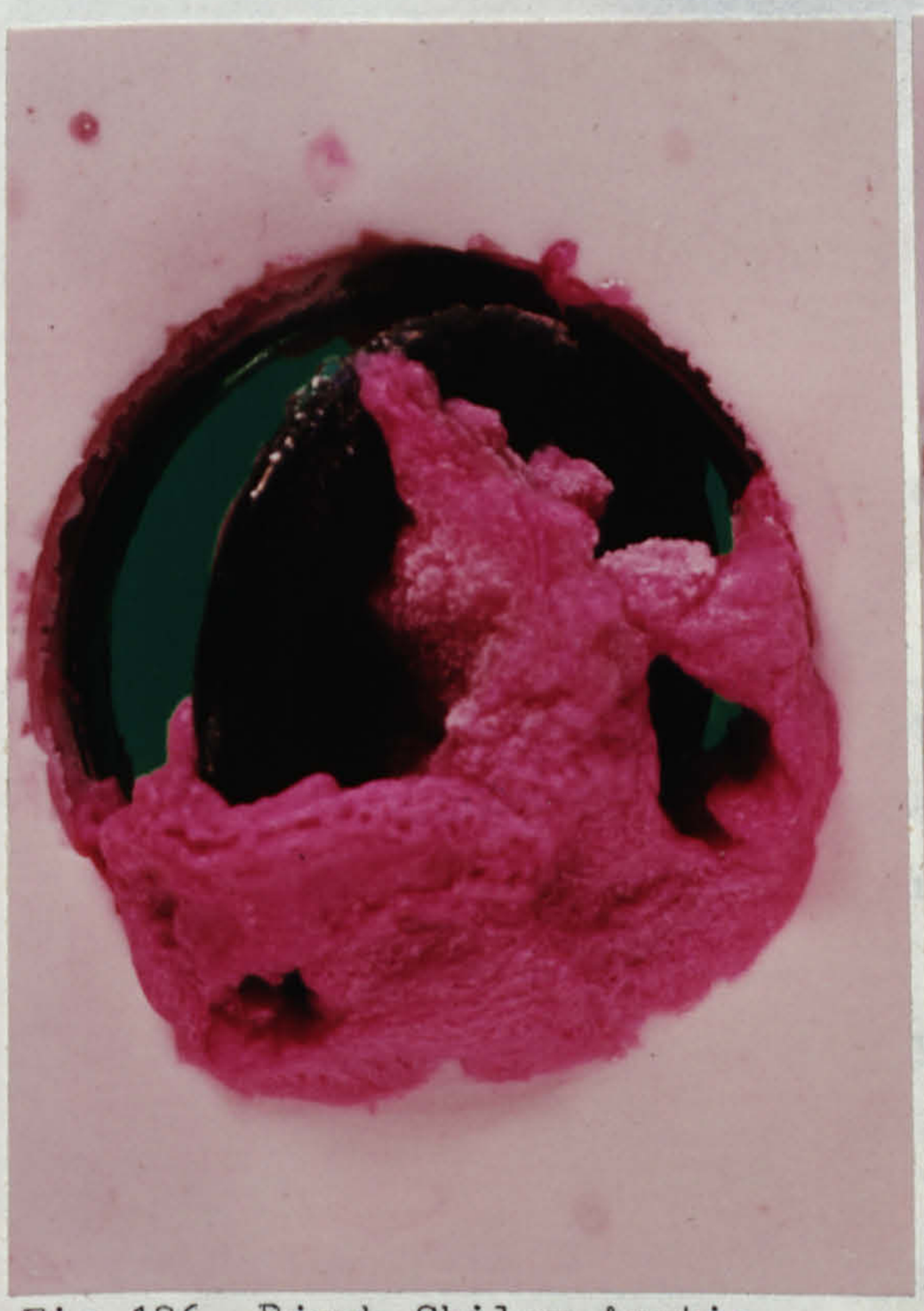


Fig 136. Bjork-Shiley Aortic
Downstream 40°C. 2L/min milk

Fig 137. Flat Disc Mitral
Downstream 43°C. 0.6% $CaCl_2$
and rennet. 1.7L/min milk.
Pulse 50

Fig 138. Starr-Edward Aortic Downstream. Conditions as 137.

This suggests that low flow on a valve is potentially very dangerous. The flow velocity through the valve, and hence the shear stress, can become very low if a large valve is used in a damaged heart, and a trade off between the pressure drop, which is related to the 4th power of the diameter of the valve, and the enhanced possibility of valve failure through massive clot deposition is required.

The flow rates used in all the experiments described are lower than that found physiologically, which is 70ml stroke x 70 pulse rate 5 litres/min, but it appears that this may not be critical. However, it must be borne in mind that the flow rates are low, and the maximum possible flow rates should be used in the tests. This is limited to 3 litres/min in the case of the apparatus used for these experiments.

4.9.2 Position of Valve in the Heart Simulation Chamber

Fig 139. Bjork-Shiley Mitral
Upstream 40°C. 3L/min milk

Fig 140. Bjork-Shiley Aortic
Upstream.Same run as (139)

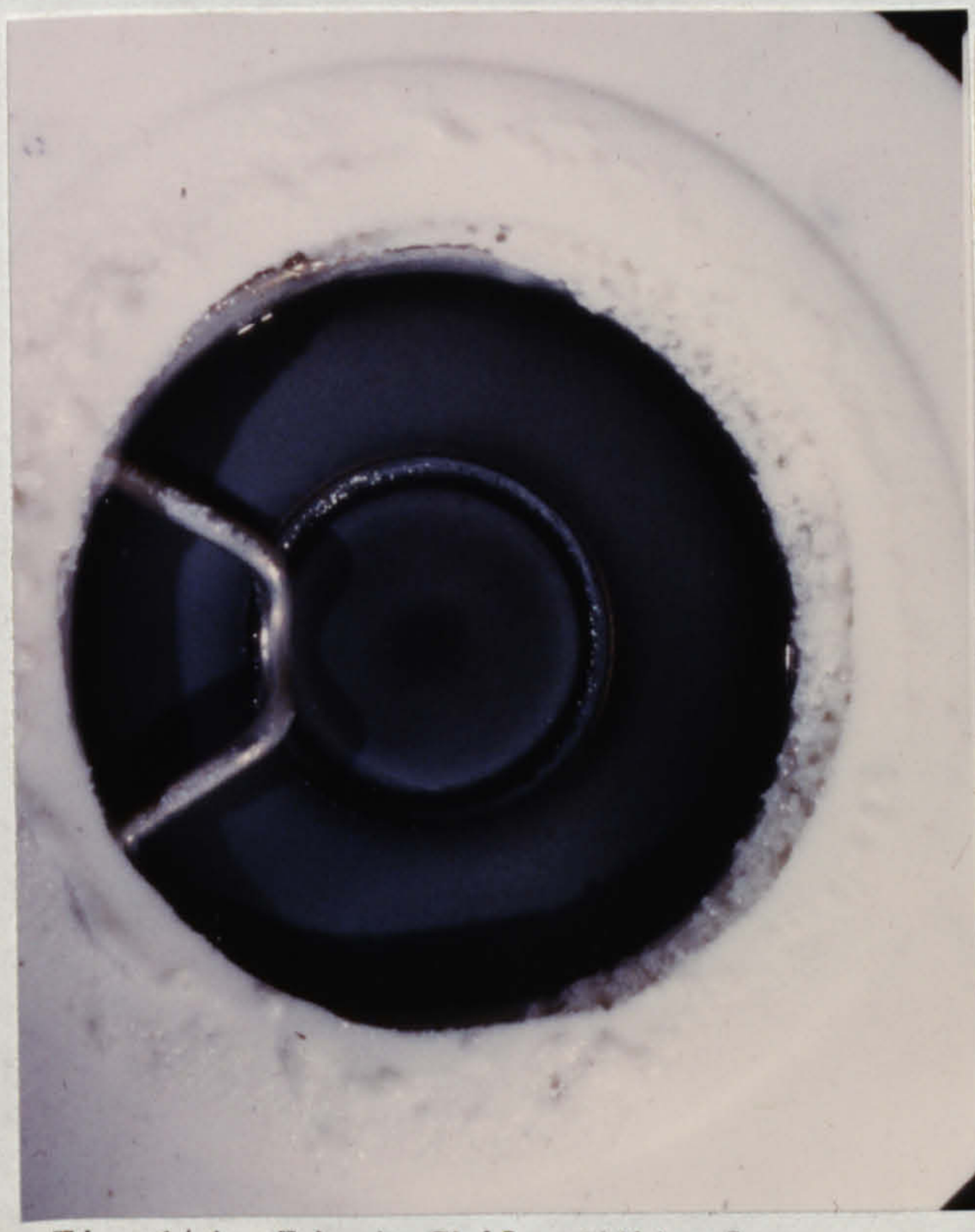


Fig 141. Bjork-Shiley Mitral
Downstream. Same run as (139)

Fig 142. Bjork-Shiley Aortic
Downstream. Same run as (139)

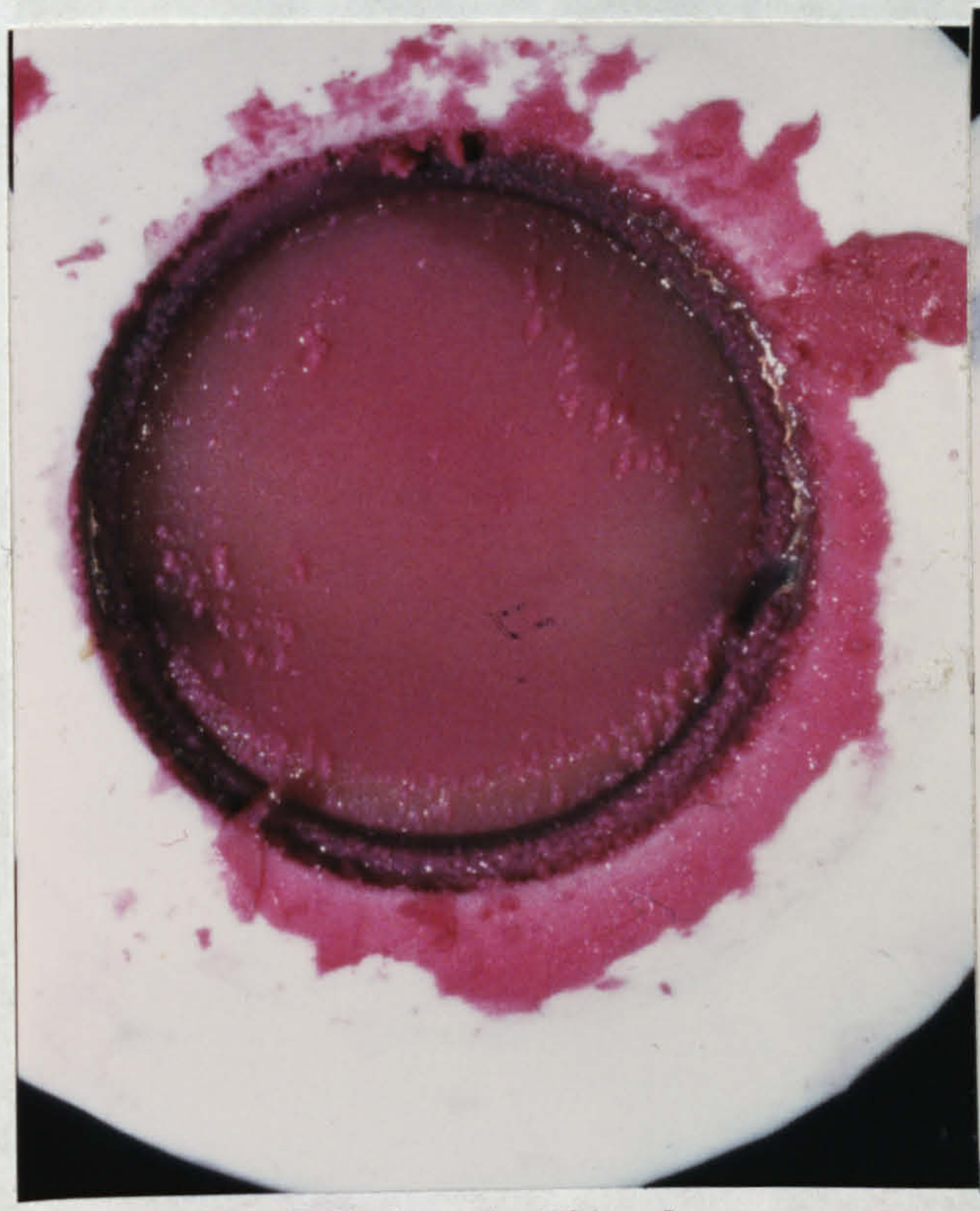


Fig 143. Edinburgh Mitral
Upstream 40°C. 3L/min milk

Fig 144. Edinburgh Aortic
Upstream. Same run as (143)

Fig 145. Starr-Edwards Mitral
Upstream 40°C. 2L/min milk

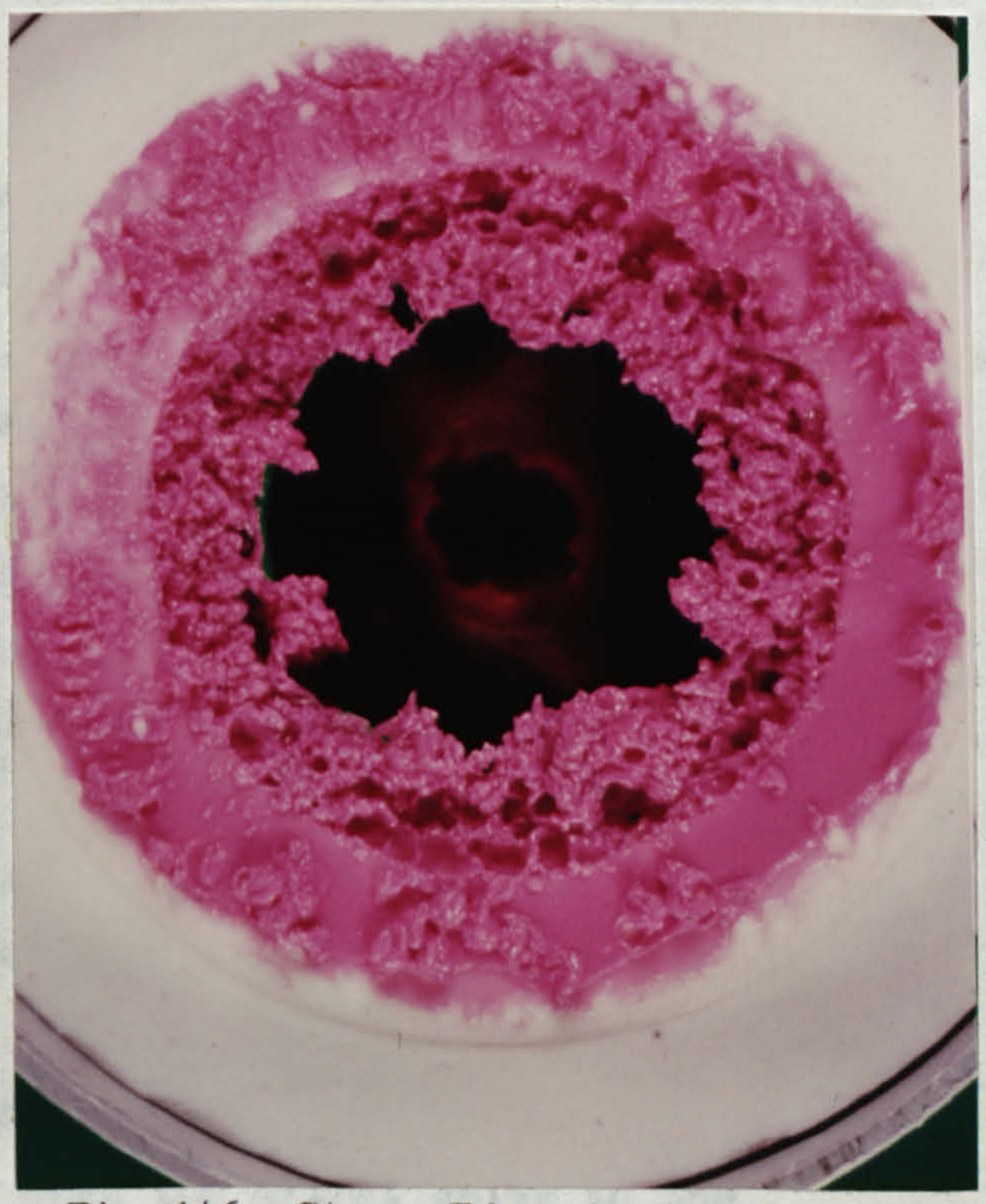


Fig 146. Starr-Edwards Aortic
Upstream 40°C. 2L/min milk

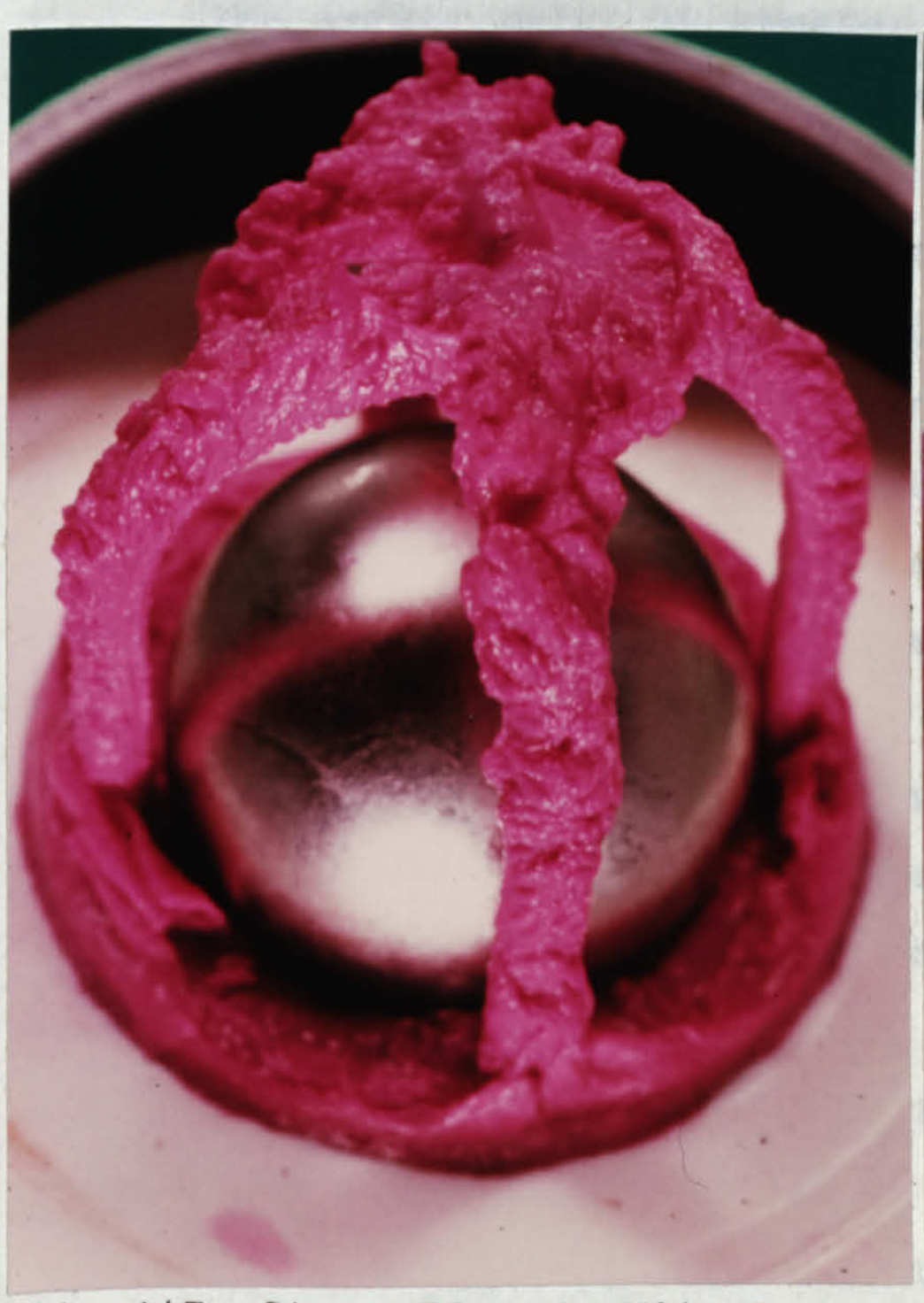


Fig 147. Starr-Edwards Mitral Downstream. Same run as (145)

Fig 148. Starr-Edwards Aortic Downstream. Same run as (146)

Discussion

The rennet, $CaCl_2$ and milk are not mixed together until they enter the perspex chambers of the artificial heart, just upstream of the mitral valve, at which time the clotting reaction is initiated. It takes 10 - 15 seconds for the bulk of the milk to reach the aortic valve so that bulk coagulation which takes 30 - 40 seconds at the temperature used is then more imminent. There is also a considerable residence time distribution and in some regions of the chambers where the fluid is relatively stagnant coagulation occurs on the walls. This clot appears to remain attached and does not interfere with the clotting on the valves.

A much smaller flow disturbance is therefore needed to cause clotting on the aortic valve than the mitral valve in the artificial heart used here, and the amount of clot found on

the aortic valve is greater than that associated with the mitral site. This is illustrated by the photographs of the Bjork-Shiley and the Edinburgh valves. The two photographs of each are taken of valves used in the same run, one in the mitral position and the other in the aortic position, and it can be seen that in each case there is more clot on the aortic valve than the mitral. Thus, the aortic position is a more severe test of the valves' clotting propensity in these experiments than the mitral position.

In the case of the Starr-Edwards valve the importance of position is not quite so clear, and although the results shown are obtained from consecutive runs the conditions pertaining were identical. The quantity of deposit appears similar in both cases, but the nature of the deposit appears very different; the deposit on the mitral valve appears very much more grainy than that on the aortic valve. On closer examination, it can be seen that where clotting does occur on valves in the mitral position this granular appearance is normal (Figs 157, 158), but that does not occur on valves in the aortic position.

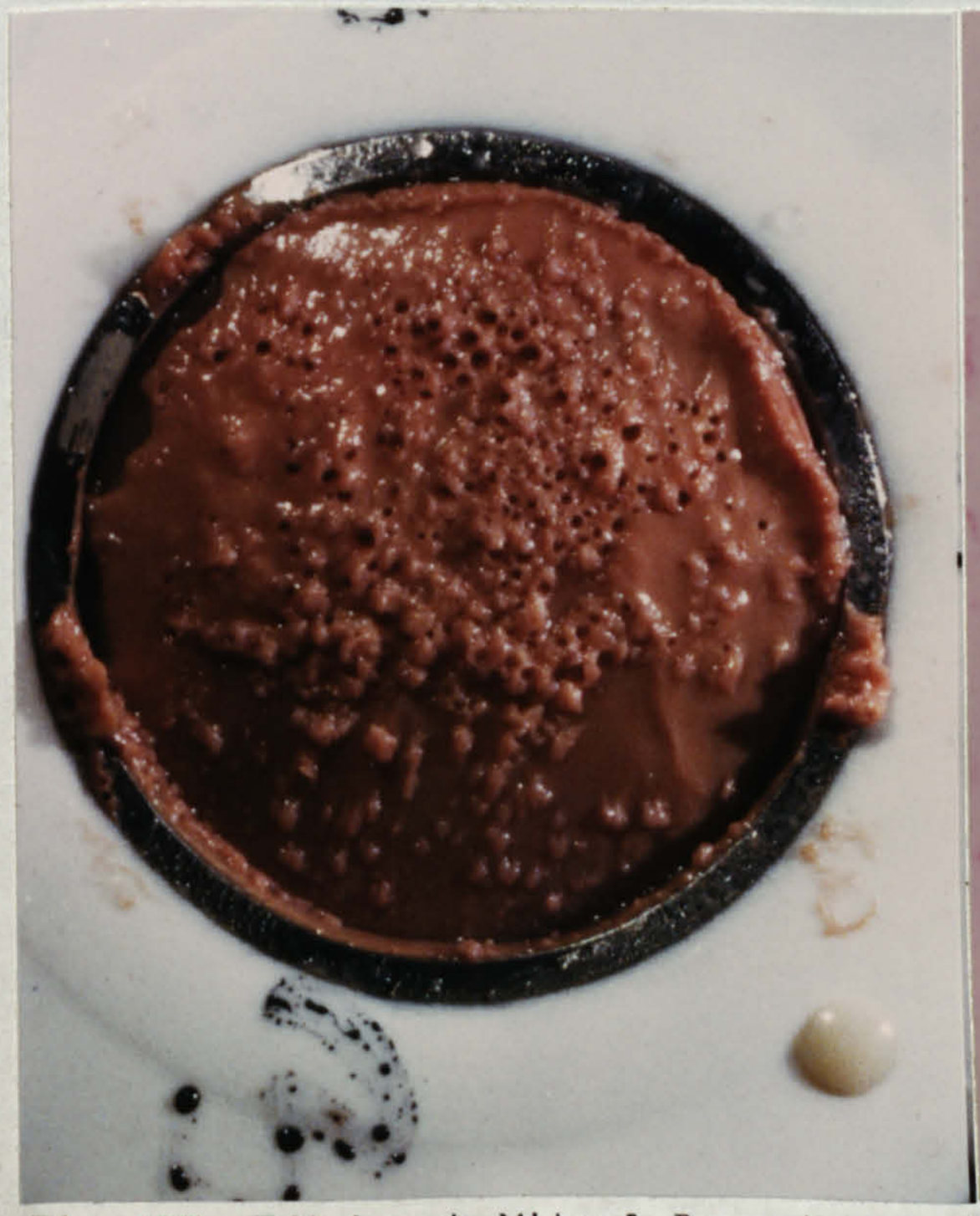


Fig 157. Edinburgh Mitral Downstream
40°C. 3L/min milk

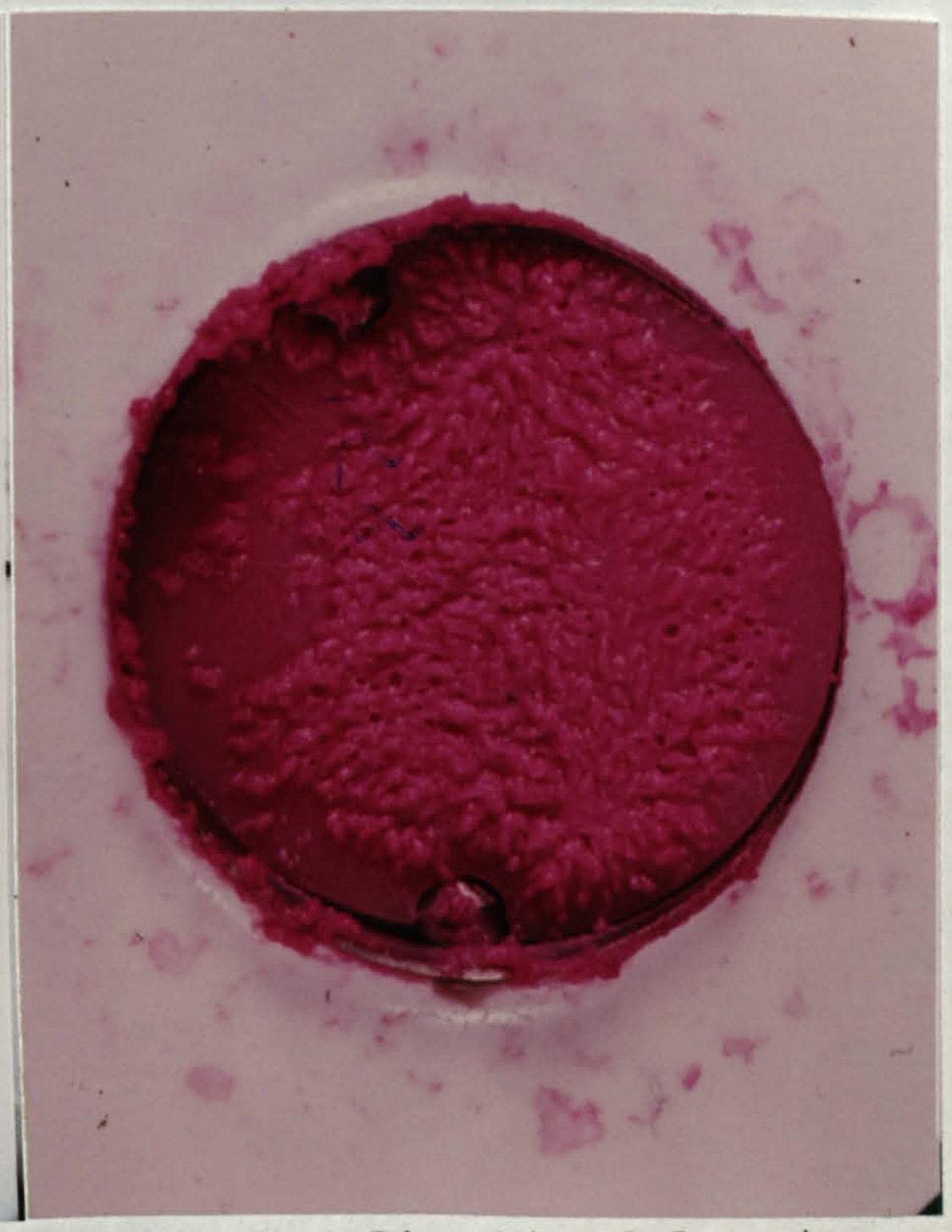


Fig 158. Flat Disc Mitral Downstream
40°C. 2L/min milk

The explanation for this diversity in the nature of the deposit is probably that despite the precautions taken to exclude air from the system it is still present, albeit in small volumes, in the mitral chamber and this air becomes involved in the deposition. Air is known to promote embolus formation in vivo (61, 210, 60, 211, 212, 213) and, as previously discussed transient negative pressures have been observed during the valve cycle (52). It may be that the presence of air dissociated from the blood by this negative pressure leads to enhanced clotting and yields a clot of more granular nature than usual. There is, however, no direct evidence for this.

Conclusions

The position of the valve in the artificial heart used is a very important factor in determining the quantity of deposited clot. It does not appear to affect the position of the clots, but can affect the appearance of the deposit due to the presence of air in the mitral chamber.

4.9.3 The Effect of Temperature

Fig 149. Flat Disc Mitral
Downstream 43°C. 2L/min milk

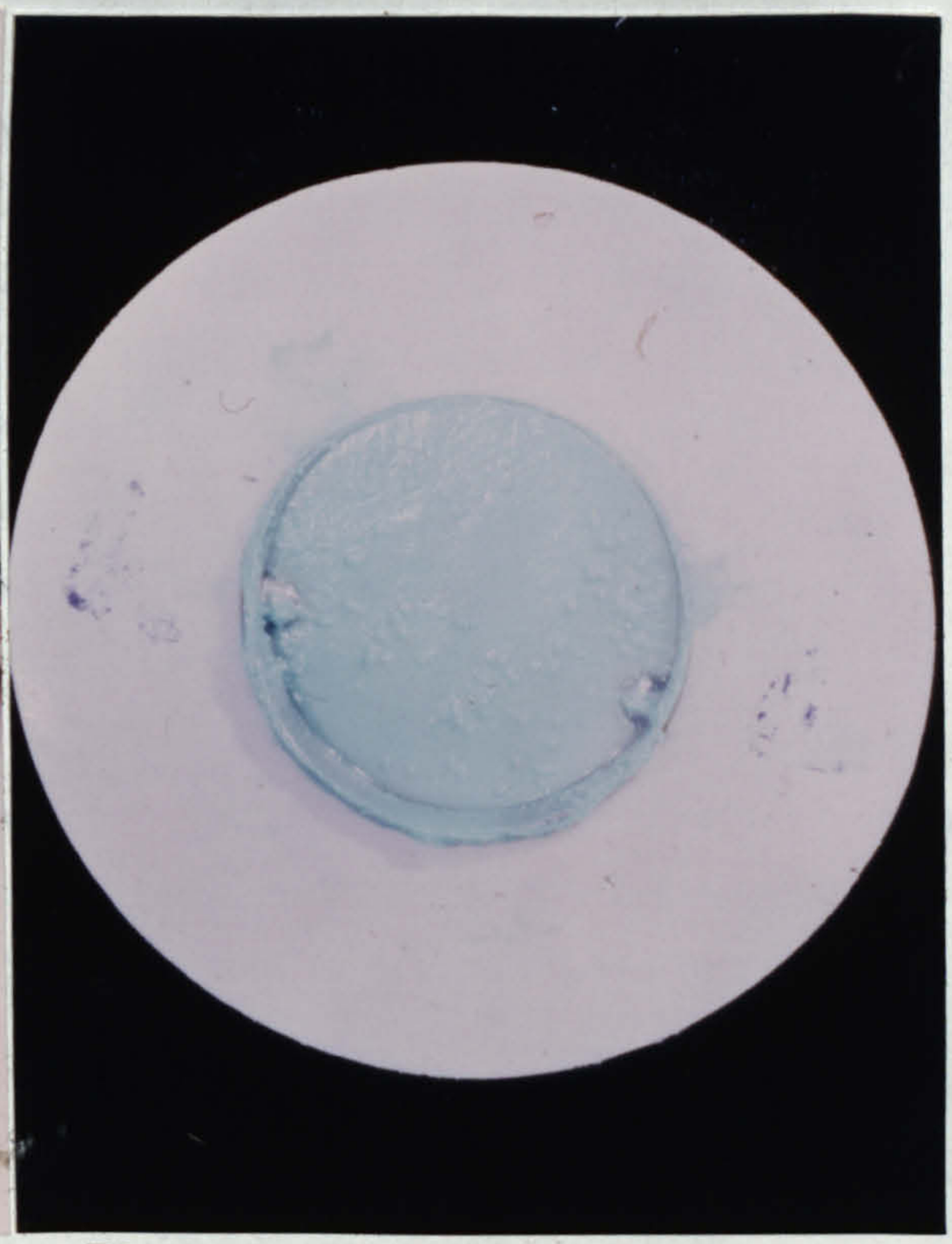


Fig 150. Flat Disc Mitral
Downstream 36°C. 2L/min milk

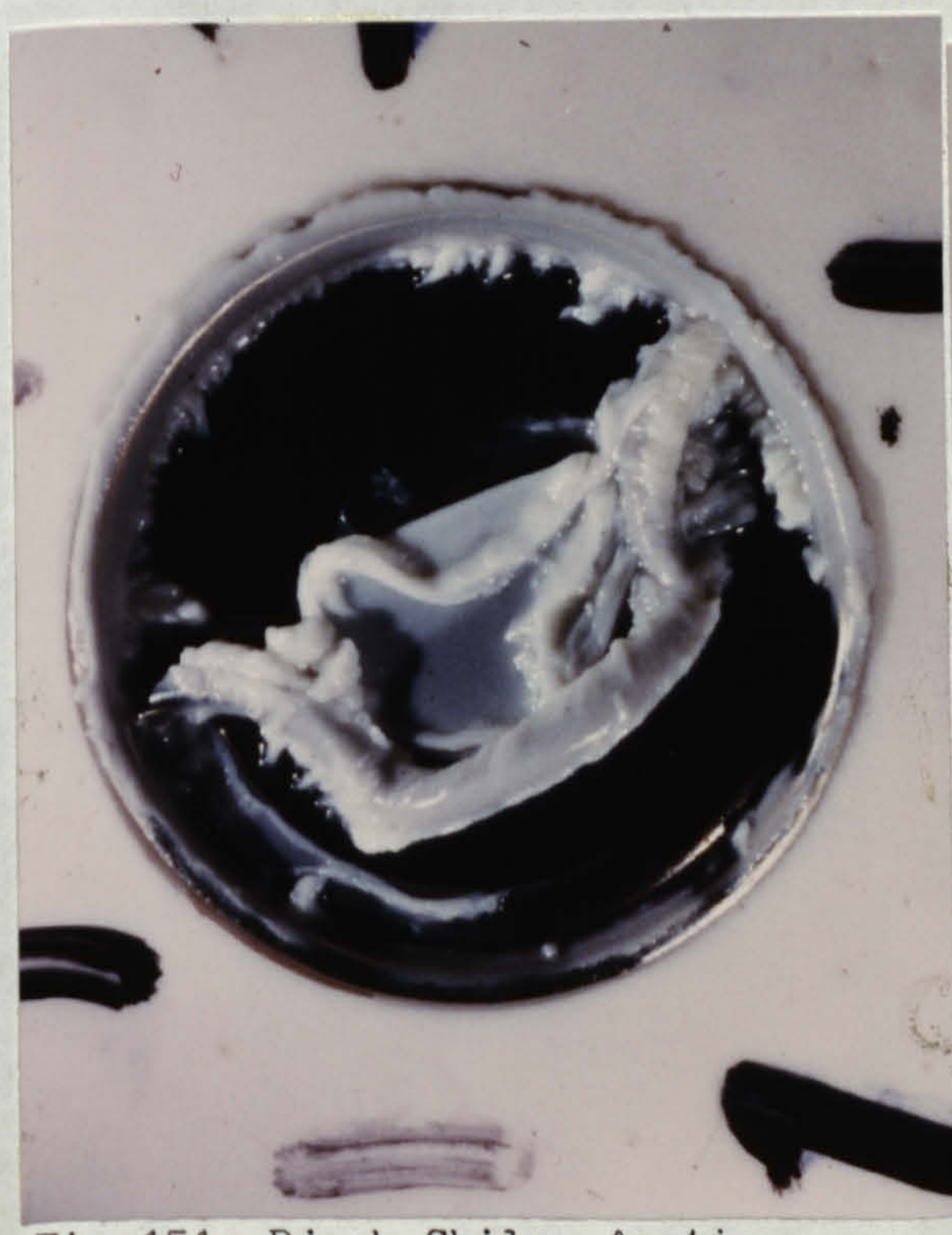


Fig 151. Bjork-Shiley Aortic
Upstream 40°C. 3L/min milk

Fig 152. Bjork-Shiley Aortic
Upstream 37°C. 3L/min milk

Discussion

Temperature was shown to affect the clotting time of milk in Chapter 3. The above photographs (Figs 149, 150) show the effect of a 7°C difference in temperature, despite the greater concentration of rennet and calcium chloride at the lower temperature case which would be expected to give greater clotting. The effect of a 1 - 2°C change in temperature is sometimes marked (Figs 151, 152) and the results suggest that the temperature needs to be controlled ± 0.5°C.

The effect of changing rennet and calcium chloride concentrations was found to be of less significance within fairly wide limits e.g. 0.8 - 1.2%. This was intentional and the concentrations were chosen to achieve this. A 2% concentration of rennet and calcium chloride would have given even greater stability, but due to the expense of these two chemicals this was not convenient.

4.9.4 The Effect of the Valve Materials and Surface

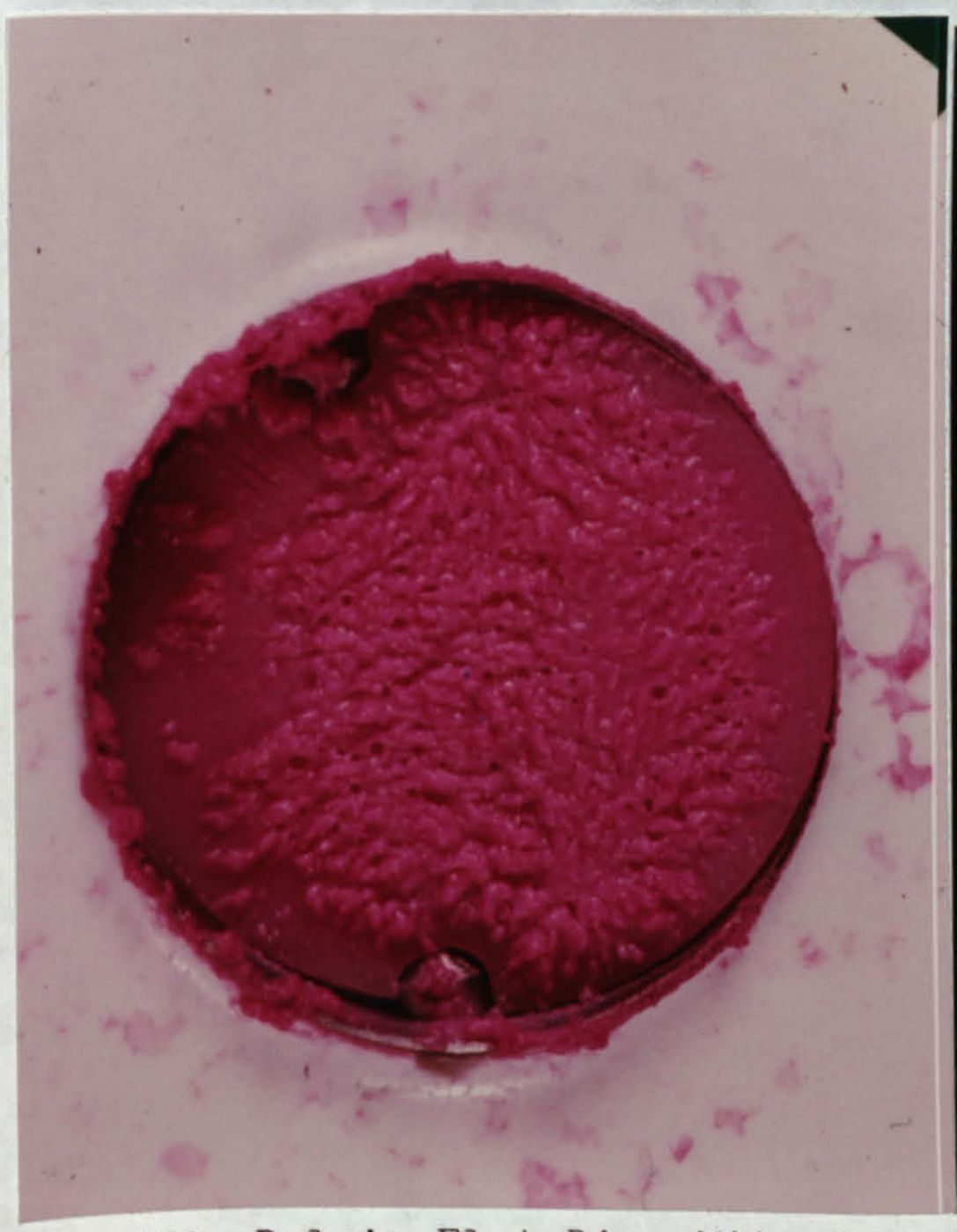


Fig 153. Delrin Flat Disc Mitral
Downstream 40°C.2L/min milk
1% rennet and $CaCl_2$

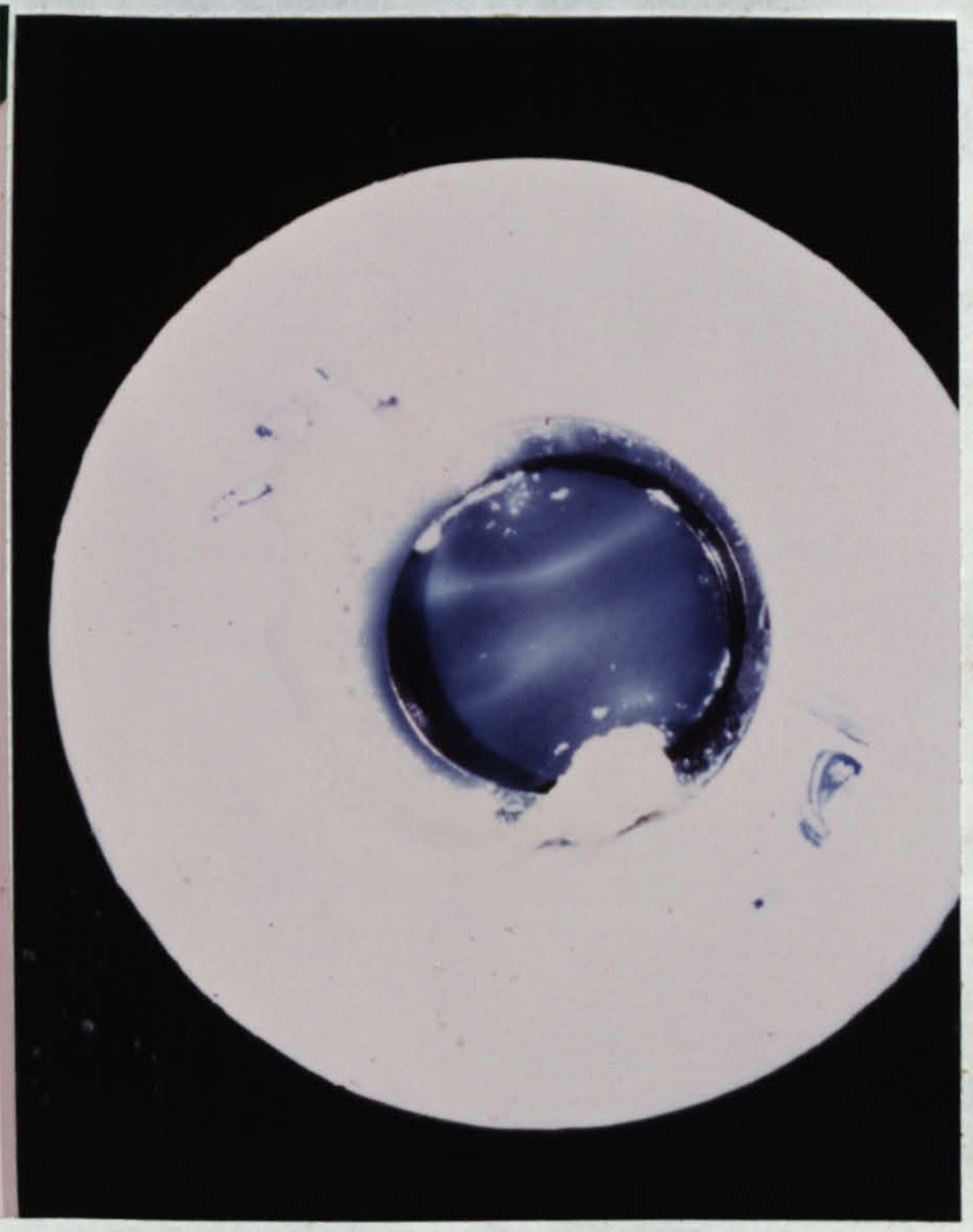


Fig 154. Carbon Flat Disc Mitral
Downstream 38°C. 2L/min milk
1% rennet and $CaCl_2$. NB Run stopped as flow reduced due to Aortic Valve clotting.

Discussion

The effect of the materials of which a valve is made and the cleanliness of the valve must be of paramount importance. If a valve has been cleaned in an enzyme detergent prior to a run and has not been sufficiently rinsed afterwards, clot deposition will be inhibited and if the valve has not been properly cleaned clot deposition will be promoted. The photographs show the difference in clot deposition when two valves of similar design are used under similar conditions but whose material of construction is different, one being made of carbon and the other delrin. In the case of the carbon valve there is a clot adherent to the ring but very little clot attached to the occluder, whereas in the case of the delrin valve there is clot over the entire surface of the occluder.

This disparity between the performance of different materials makes it very difficult to compare valves of different designs, as has previously been mentioned. One solution to this problem would be to find a substance which could be used to coat all the valves used thereby removing surface effects. Albumen was tried, but some surfaces adsorb it better than others, so no advantage was gained.

The importance of the surface poses another problem. If the analogue method is to be used for testing prototypes, it is vital that these prototypes should be polished and prepared uniformly before testing. The problem of surface has affected the results of the clotting trials on the Edinburgh valve, and an even covering is found over the entire surface in nearly all cases. This might not occur if carbon valves were available, as can be seen from the flat disc results above.

The surface properties are also important in the clinical situation or indeed in animal trials (48, 214, 215, 44, 216, 45, 46, 12, 41, 134, 217, 48, 133, 49, 50) so this disadvantage is not peculiar to the use of milk.

It would be very useful if work could be done comparing the effect of various materials and surfaces on blood and milk depositions to see if there is any relationship between them.

CHAPTER 5

DISCUSSION

5.1 Introduction

The notion that it might be possible to use the deposition of milk curds as an analogue for the deposition of blood seems at first glance somewhat far fetched. The deposition of solid constitutents from blood is extremely complex and as yet not fully understood. Milk is apparently a much simpler fluid, being a suspension of micells and fat globules which have little in common with the platelets, erythrocytes and leucocytes present in blood. The role of the coagulation of the two fluids is also very different; the clotting of blood occurs to heal wounds of the carrier animals, whereas the physiological purpose for the clotting of milk which occurs in the stomach of infant mammals, appears to be to educate the stomach for solid food. Another fundamental difference between the two is that all the chemical species required to initiate clotting in blood are present in the blood, whereas milk requires the addition of a coagulating agent, rennin, to initiate the clotting reaction.

However, there are also reasons for expecting the two fluids to behave in a similar fashion in respect of their essential clotting and deposition properties. Milk is largely dreived from food products in the blood by the action of a mammary gland, and all the ingredients of milk must have originated in the blood. Fibrinogen and k-casein, the main proteins involved in blood and milk coagulation respectively are genetically related (132).

Both coagulation reactions involve the proteolytic splitting of a protein by the action of an enzyme and, furthermore, the kinetics of the two reactions appear to be the same (113). Both fluids are a suspension of particles in a serum, the fat globules apparently behaving in a manner analogous to the behaviour of the platelets in blood which are known to be of great importance during the inception of thrombosis (10, 39, 125, 138, 218, 136, 219, 24, 220, 221, 222, 15, 16).

5.2 Stagnation Point Flow Experiment

The results of the work done using milk in the stagnation point flow chambers to compare the microscopic deposition of rennetized milk on a glass slide with that found by Petschek et al, using canine blood (11, 12, 33) support the hypothesis that there is a parallelism between milk and blood. The appearance of the deposits and the sequence of deposition appear to be very similar.

The stagnation point flow experiment is very difficult to perform using milk, as the flow rates are very low. If a milk flow of 2-4ml/min is used the flows of rennet essence and calcium chloride solution will be only .05ml/min and obtaining good mixing at these flow rates is very difficult. This experiment is of limited value and I would not recommend its further use.

5.3 Net Experiment

Hladovec's net experiment (140), on the other hand, proved to be very useful and simple. The pressure variation curve upstream of the filter mesh was found to be the same for

both milk and blood, and clot was found to form downstream of the mesh when rennetized milk was circulated, as had been found with blood. Although the time lapse before the pressure began to rise was not affected by the material or size of the mesh, the size of the clot adhering to the mesh after the run depended on the material used, which one would expect from the work on the adhesion of coagulating casein to various surfaces done by Berridge (79). The net experiment was also used for obtaining the relationships between clotting time and temperature, rennet concentration and calcium chloride concentration to ascertain the optimum conditions for use in a large scale heart valve testing apparatus.

5.4 The Testing of Heart Valves

The results of the full scale testing of the analogy between milk and blood deposition were as good as the results of the stagnation flow experiment and the net experiment had suggested they would be. The patterns of deposition on the various types of valve tried corresponded very closely to the deposits found on heart valves used in vivo with the exception of the Starr-Edwards valve struts. Very little clot is found in the clinical situation, whereas in the test rig extensive clots were found. This is apparently due to the inadequacy of the heart model to mimic the natural heart. The model heart is constructed in rigid perspex and does not, therefore, collapse during systole like the natural heart. The forming clot is not, therefore, dislodged in the experimental set up where it probably is in vivo.

This is probably desirable, for if the clot is forming and then being dislodged, the thromboemboli thus created can be very dangerous, and it is important to know if this is

happening. By allowing the formed clot to remain adhering to the struts it can be seen immediately that they are a site for thrombus initiation and propogation, which is very useful knowledge for a designer of a heart valve.

The sequence of deposition onto the valve was found to be the same as that found with the stagnation flow chamber and the net experiment, and the conditions prevailing were found to affect the deposition. Perhaps the most important observation is the effect of the material of construction of a valve on the amount of deposit adhering to it. This makes comparison between valves of different design and made of different materials very difficult. If a designer wishes to test the effect of a different design he must ensure that not only are the materials of different designs the same, but also the preparation of the surface, such as polishing and cleaning, is the same in all cases.

It must be stressed that bulk coagulation of the milk used for testing heart valves does not occur until it leaves the chambers and reaches the drain. Thus any clot forming on a valve is not due to bulk coagulation of the milk at that point, but is related to come local fluid mechanical disturbance; be it a region of stasis, impinging flow leading to stagnation or high mass transfer. The presence of a high level of shear stress, as for instance might be found on the ball of the Starr-Edwards valve, causes that surface to remain clear from clot. Further evidence that the phenomenon of deposition observed is not merely that the bulk milk has become destabilized and will deposit anywhere is the importance of the materials of the surfaces.

Certain parts of the chambers where the flow was particularly slow or areas of stasis occurred favoured clot deposition and large clots formed in these areas. These deposits did not dislodge during the runs so no action was deemed necessary to prevent them, however, they did prolonge the cleaning time needed between runs. The finding of deposits on the chambers illustrates the potential of the analogue technique for testing other implant devices such as artificial hearts.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER WORK

6.1 Conclusions

On the basis of the preliminary investigative work described here, rennetized milk can be used as an analogue fluid for testing the thrombogenicity of articifical implant devices. It must be borne in mind that the test cannot yield information on the effects a device might have on the blood chemistry, nor on the effects of a modification of the surface chemistry on the thrombogenicity. However, in circumstances where the clotting mechanism may be activated, which pertains to most cases of artificial device implantation, and where the deposition is flow related, the use of rennetized milk as an analogue of blood can yield rapid, cheap, reproducible results which do not necessitate the use of a surgical team.

Furthermore, the following conclusions have also been reached:-

1) In the design of artificial heart valves it is important that parts which intrude into the heart should be avoided, because, as the heart contracts any small clots forming on the impediment will be dislodged leading to thromboembolism.

2) The sequences of the deposition of rennetized milk and blood appear to be be similar and the fat globules in milk appear to mimic the behaviour of the platelets in blood.

3) The material of construction of a valve is important in determining the extent of the deposition onto it with milk as well as blood.

4) A minimum concentration of 1% rennet essence and 1% saturated calcium chloride and a temperature in the region of 40°C were found to yield satisfactory results.

5) The deposition phenomena observed are not due to the bulk coagulation of the milk but are related to local fluid mechanical disturbances, which would also give rise to similar deposition phenomena with blood.

6.2 Recommendations for Further Work

The present control system of the valve tester should be improved. The temperature needs to be controlled to $\pm$ 0.5°C which cannot easily be managed with the present feed-back system controlling only one element in the hot water tank. A feed-forward computer controlled or a more sophisticated feed-back system would be desirable. The rennet and calcium chloride addition has been improved by the use of a metering pump with a plastic pump head.

A method for negating the influence of materials used needs to be found, which might involve coating the device under tests with a substance of uniform chemical properties.

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