Coagulation-Resistant Coatings by Enzyme Inhibition

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ABSTRACT

The major approaches to the problem of coagulation on foreign surfaces have centered about heparin or heparin-like surfaces and charged surfaces in addition to low energy surfaces such as the silicones. These approaches do not prevent contact activation though they may delay it. Thus we proposed to prepare surfaces which might prevent or inhibit the contact activation process; we proposed to do this by preparing polymeric surfaces with enzyme-inhibitory activity.

INTRODUCTION:

The intrinsic blood coagulation mechanism is believed to be dependent on the modification of a plasma protein by surface denaturation and its subsequent reactions. In the classical Ratnoff & MacFarlane cascade theory, Factor XII is activated by the surface to XII*, an enzyme, which can then catalyze XI → XI*, and the cascade mechanism is initiated.

Though Factor XII has been isolated and purified, it is still a relatively uncharacterized protein and controversy as to its role and possibly even its existence continues. Seegers feels that a separate contact factor may be unnecessary as the prothrombin molecule has the capability of activating itself (1), though few studies exist on its interaction with solid surfaces (2).

It is generally accepted that the intrinsic clotting mechanism is dependent on a modification of some plasma protein at a solid surface, be it XII, XI, or prothrombin.

The logical question is how to prevent the surface modification from occurring, thus preventing the necessary activation. We are attacking this from two different approaches. The first is chemical. We assume that the denaturation process exposes certain reactive groups which are then capable of reacting with the next protein in the sequence or perhaps with other parts of the same molecule, leading to factor-like activity. The object is to tie-up the groups thus preventing the protein from reacting in the clotting mechanism. This may be possible with enzyme inhibitors. The problem is that the active groups necessary for clotting have not been isolated. Most inhibitors tend to be effective in inhibiting clotting activity in solution. We studied their effect on a solid surface by adsorbing aromatic enzyme reagents onto a graphite surface and then determining coagulation time. In all cases the time was shorter than for the non-inhibitor surface. This indicated that the protein is probably being more rapidly denatured at the surface by being non-specifically bound or adsorbed. Thus we were aiding the activation process rather than inhibiting it.

A new technology is evolving on the specific inhibition of the enzymic active-site (3). But we must know what the active site is before we can deduce compounds to inhibit it.

We have some clues as to the active site of prothrombin, but such information on Hageman Factor is virtually non-existent. Hageman Factor may be inhibited (4) by diisopropyl fluorophosphate, an esterase inhibitor, though there is also evidence that such treatment may inhibit its esterase activity without inhibiting its clotting activity (5). The work of Ray & Roy (6) and Caldwell & Seegers (7) showed that disulphide and free amino groups are essential for prothrombin activity. Inhibition of these groups leads to a loss in activity. The amino can be inhibited by dinitrofluorobenzene and phenylisocyanate. The sulfide by reducing agents.

Thus we have several avenues to follow in attempting the chemical inhibition of the surface-activated clotting proteins.

The second approach is steric or geometrical. If, rather than exposing a chemically reactive group on denaturation, the protein is forced to assume some specific geometric shape, then an enzyme-specific lock-and-key mechanism might be operating. This would be especially compatible with Vroman's model of clotting (2, 8, 9) as a sequence of hydrophilic-hydrophobic bonds. Thus if we could change the nature of the surface so that the sterically specific site could not develop, we might slow down or stop the process. We might be able to do this by roughening of the surface on the molecular level.

Margolis (10) has shown that clotting time decreases as the surface area of silica in contact with blood increases. But this relation breaks down when the particle size drops below 300 Angstroms. Even though the surface area may be quite large, the clot-promoting activity is low. Margolis explains this by means of a denaturation mechanism for the activation of Hageman Factor. When the surface area available for activation is appreciably smaller than that of the protein, surface activation simply cannot occur.

Thus if we could produce a surface that appeared rough or discontinuous on the molecular level, then contact activation might be strongly hindered. The dimensions of such a surface would be crucial. Also the side chains must be highly polar and rigid so that they are attracted out into the plasma solution. The length of the chains and the distance between them would be very important. If we use Margolis' 300 Angstrom figure, which is not unreasonable in light of the dimensions of proteins, then we might attempt to graft polar chains of 100-300 Angstroms length and of the order of 100 Angstroms apart to a smooth polymeric surface. (Fig. 1) Another problem is that the ends of the chain might just act as a new surface several hundred Angstroms out from the base, then the protein may just be activated as usual, as shown in Fig. 1.

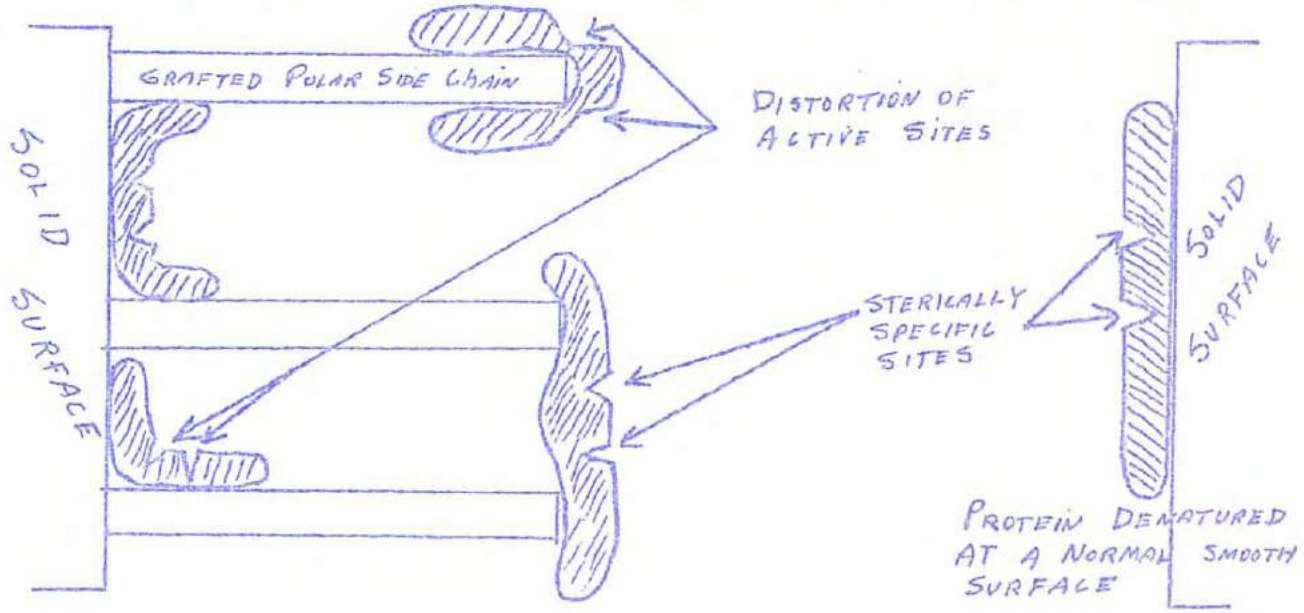


Fig. 1: Possible Action of a Molecularly "Rough" Surface.

Our efforts are completely based on the intrinsic system. We assume that denaturation and the resultant distortion of some plasma protein is necessary to start the process. We assume that the distortion leads to a chemical or structural reaction specificity (and most likely it is a combination of the two). We are also neglecting charge effects, which might be very important. These two models for the intrinsic process provide broad guide lines for selecting the types of surface which have coagulation-resistance potential.

SURFACES:

If polystyrene is nitrated and the nitro group then reduced to the amino, polyaminostyrene is prepared. The polyaminostyrene can then undergo several different reactions, as shown in Fig. 2.

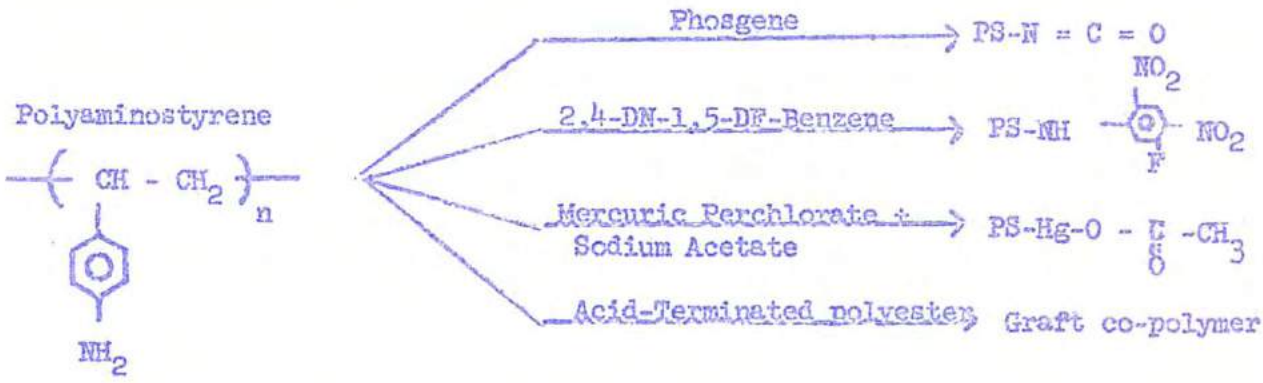


Fig. 2: Some Possible Reactions of Polyaminostyrene

Treatment with phosgene gas results in polyisocyanatostyrene, a polymer containing the reactive phenylisocyanate group. Manecke (11) has studied this polymer and finds that it is protein-reactive, but not nearly as reactive as some of the others we will discuss.

Another common reagent for amino groups and also for thiols is dinitrofluorobenzene (DNFB). If we react 2,4-dinitro-1,5-difluorobenzene with polyaminostyrene, a polymer is prepared containing the reactive DNFB grouping (12) (Fig. 3).

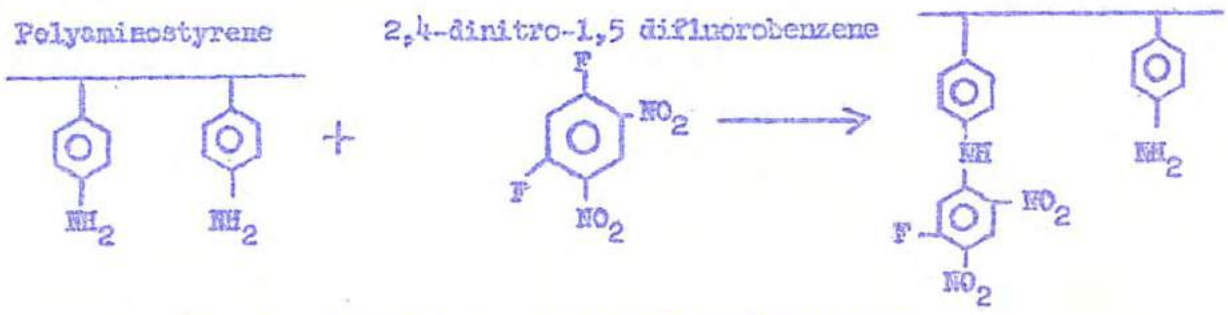


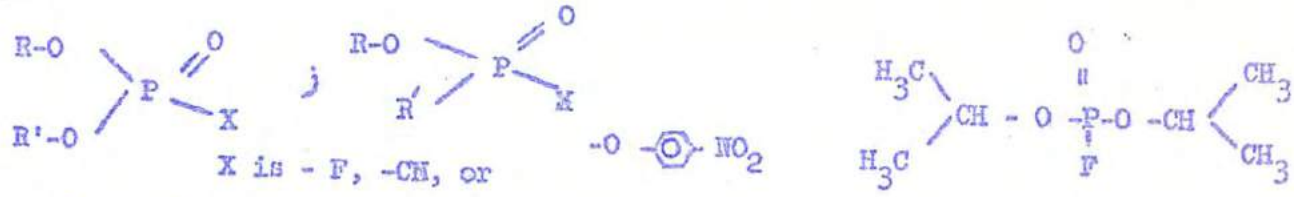
Fig. 3: The DNFB grouping on Polyaminostyrene.

Polystyrylmercuric acetate has been shown to react with sulthydral groups and thus bind protein (13). The graft co-polymer in Fig. 2 will be discussed later.

Thus polyaminostyrene has great potential for the preparation of protein-reactive surfaces. Polystyrene is available in test tube shape and tubing, in addition to sheets of various thicknesses. The nitration and reduction can be carried out on the surface; we have done this and verified it with attenuated total internal reflection (ATIR) infra-red spectroscopy. Thus we can produce a chemically reactive surface without significantly changing the mechanical properties of the base polymers, just as has been done for the heparinized polymers. If polystyrene is too brittle for implant applications, then butadiene-styrene rubbers and acrylonitrile-butadiene-styrene (ABS) resins are, in principle, capable of the same surface modifications, though we haven't yet attempted them.

In addition to the DNFB-polymer mentioned above, Manecke (11) has also produced a copolymer of methacrylic acid and methacrylic acid m-fluoroanilide, which upon nitration yields a polymer containing the DNFB grouping. This one is a bit complex for our knowledge of polymer chemistry so we are having a graduate student in chemical engineering attempt it.

Diisopropylfluorophosphate (DFP) is an extremely toxic and reactive member of a class of organophosphorous compounds which irreversibly inhibit esterase enzymes (Fig. 4).

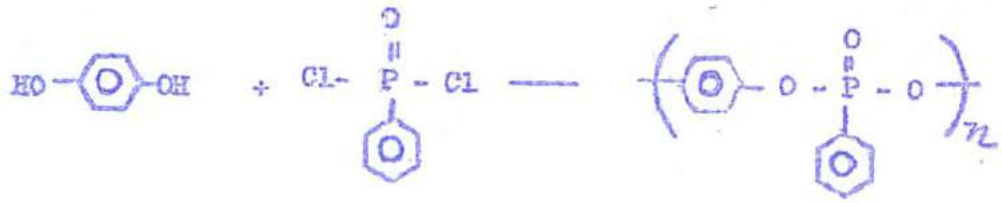


Organophosphorous Inhibitors DFP

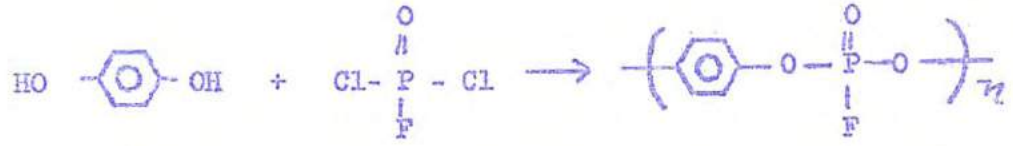
Fig. 4: Organophosphorous Inhibitors and DFP.

DFP has been shown to inhibit Hageman Factor by Schoenmakers (4), though the results of others indicate the inhibition may not affect clotting activity (5). In any event, such a compound is capable of irreversibly binding esterases and, if produced on a solid surface, should form a proteinated surface.

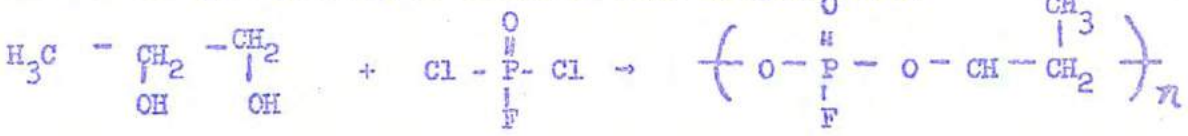
Sorenson's book (14) on polymer chemistry contains the reaction:



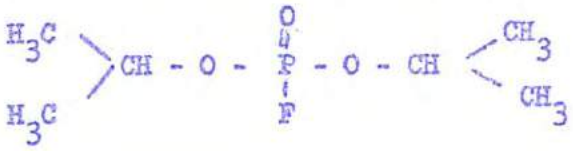
If this reaction is performed with phosphorous oxydichloro fluoride instead of with phenyl phosphonyl dichloride, we get:



Such a polymer contains the reactive heart of the organofluorophosphate inhibitors. If now the polymerization is produced using propylene glycol instead of hydroquinone, we should be able to synthesize:



Such a polymer has a frightening resemblance to DFP:



Molecular models of the polymer show that conformations exist wherein the P-F bond is directed up and out from the surface. Such a surface should have a very high reactivity for enzymes. The phosphorous oxydichlorofluoride is presently being synthesized by Mr. Herb Yen; he will attempt the polymerization as soon as the phosphorous-containing monomer is ready. By proper choice of the glycol we can vary the R-groups on the organofluorophosphate, which should vary its enzyme reactivity.

We are in the process of preparing the DNFB-polyaminostyrene polymer by the surface modification of polystyrene and the DFP-like organophosphorous polymer. We do not plan to produce any of the others at this time because of time limitations. These polymers are all enzyme-reactive, but not necessarily specific for Hageman Factor or even the coagulation proteins. They may just react with nearly everything in sight, thus producing a proteinated surface. This may be good, as Copley (15) has shown that protein surfaces significantly increase in vitro clotting times. It may also mean that Hageman Factor might be activated on the proteinated surface (9). We will have to find out.

There are many other surfaces which might be chemically convertible so that they are protein-reactive (20). Cellulose has long been a favorite carrier. We have limited ourselves to the polystyrene derivatives and the organophosphates primarily due to time limitations.

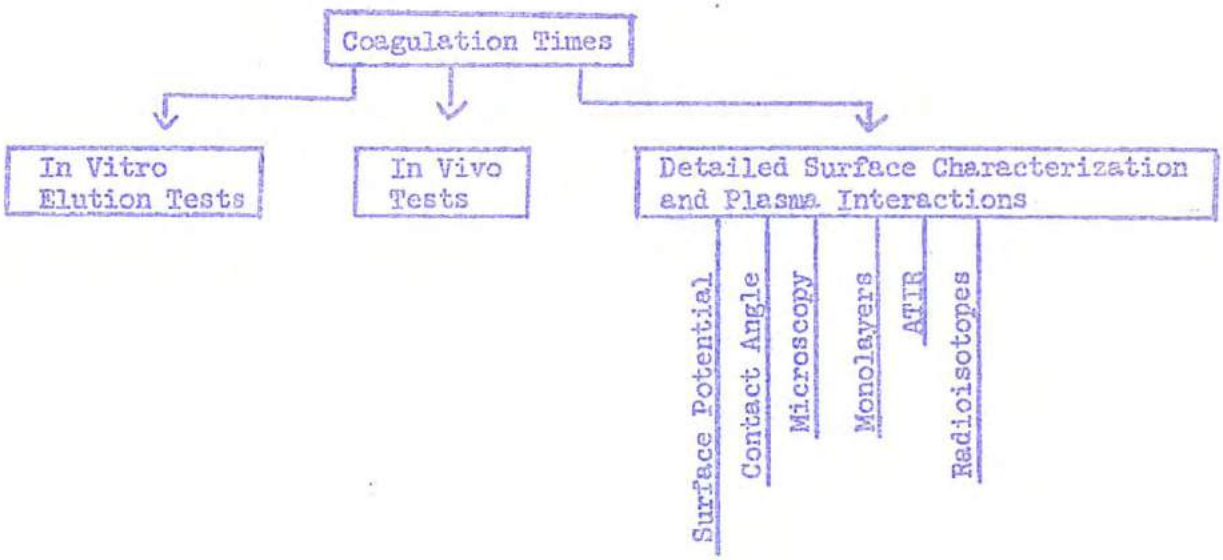
Thus the chemical approach has many problems. Until we know exactly what Hageman Factor looks like, and until we are sure that it is Hageman Factor that is responsible for contact activation, we cannot design surfaces containing groups to specifically inhibit it.

Many of the limitations of the chemical approach are bypassed in the geometric approach. In this approach we are using polyamino styrene surfaces for a pilot study. We are binding short-chain acid-terminated polyesters to polyaminostyrene hoping to produce a surface "rough" on the 100-200 Angstrom scale, as I discussed earlier. (Fig. 1) The number of variables to contend with is large. Such a surface may not be encumbered by many of the problems and limitations we noted for the chemically reactive surface. It is possible that large proteins, e.g., fibrinogen, could completely coat and smooth out such a surface, perhaps destroying its effectiveness. We will cross that bridge a bit later in the project. We are particularly intrigued by this geometric approach and will devote much effort to it in the near future.

We are also studying the effect of surface energy (16) and morphology on the clotting behavior of surfaces of polyethylene, as it is structurally so variable, and isotactic polystyrene, as it would correlate closely with what we are doing.

EVALUATION:

Our evaluation plan is outlined below:



All of the surfaces we prepare are characterized by clotting times. Those that appear promising will be singled out for more extensive evaluation. This will include in vitro elution tests and in vivo implantation tests at the Halsted Laboratory for Experimental Surgery of the University of Colorado Medical Center by Dr. Bruce Paton. In order to characterize the surface and possibly optimize its effect, we also study it by the following techniques:

Contact Angle -- which given an indication of the polar nature and surface energy, the importance of which has been shown by Lyman (16).

Phase- and interference-contrast microscopy -- which shows us the morphology and the actual formation of fibrin with respect to the surface.

Surface Potential -- which tells the surface potential differences which arise through the adsorption or bonding of species to the surface.

Monolayer Techniques -- used to calibrate the surface potential apparatus and can also give information about protein bonding and proteinated surfaces.

Radioisotopes -- will be used in the elution and in vivo studies to determine surface stability.

The above techniques enable the surface to be characterized with respect to surface energy, surface structure, surface potential, dipole orientation of surface species, and will enable studies of protein adsorption. In addition, the chemical nature of the surface is known from attenuated total internal reflection (ATIR) infra-red spectroscopy, a technique used to monitor the preparation of the surfaces.

The coagulation time is measured on a turbidity apparatus, based on the increased light absorption of plasma as fibrin forms. This technique has been well documented in the literature (17). The major difference between our instrument and those previously described is that clotting times of opaque surfaces can be measured (Fig. 5). We use platelet-rich recalcified ACD blood-bank plasma for our room temperature in vitro clotting tests.

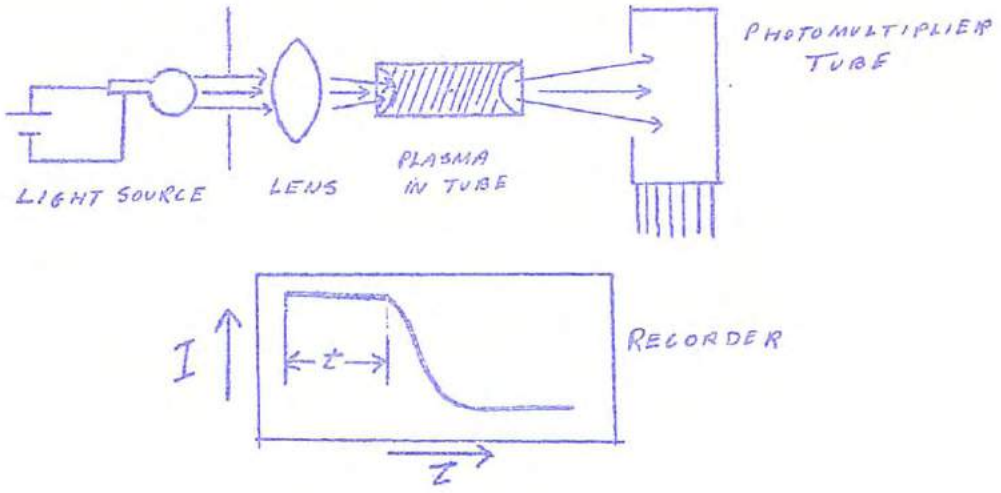


Fig. 5: Apparatus for Clotting Time Determinations By means of Turbidity Measurements.

PROGRESS:

In our original research plan we proposed to spend the first six months preparing the special polymer surfaces and establishing the techniques for their evaluation. The remaining six months are to be used to evaluate, characterize, and optimize the surfaces. We are adhering to this schedule, though the organophosphorous polymers may be a little late.

I have been mentioning our work and progress on polymer preparation throughout the talk.

We have very little clotting data at this time as we are concentrating on preparing the surfaces. The clotting data will be obtained in the very near future when we are ready to evaluate these surfaces.

The microscopy technique is proving very exciting. Up to now we have been using a Rube-Goldberg set up and the results are not worthy of presentation. We can see fibrin formation on different surfaces and note the difference in clot initiation and morphology as a function of the type of surface. We are using platelet-deficient plasma to enable us to view fibrin formation slowly and unencumbered by cellular influences. We have observed its formation in dark-field and phase-contrast. Now that a new research microscope is available to us, we will be using interference-contrast shortly. The technique is as follows: Polymer films are deposited from solution onto glass slides (18) and covers lips, recalcified plasma is applied, and the coverslip sealed with hot paraffin to minimize evaporation and denaturation effects at the air-plasma interface. We then view fibrin formation on a relatively large surface area of sample.

COMMENTS:

Yesterday we noted a great deal of discussion on the differences between in vivo and in vitro tests and discussion on the effect of air bubbles on clotting tests. If we again assume intrinsic clotting is initiated by the activation of a protein through surface-induced changes, then let us consider the air/water interface.

It is fairly well established that proteins are denatured at air/water interfaces (19), resulting in exposure of the hydrophobic residues on the molecule into the air phase and retention of the hydrophilic portions by the water or plasma phase. Thus the air/water interface may just appear as a hydrophilic surface due to the denatured protein monolayer. Hageman Factor might be activatable on such a hydrophilic surface. Vroman has deduced a model for clotting based on the denaturation of Hageman Factor at a hydrophilic surface (9), the subsequent reactions then being a series of hydrophobic-hydrophobic and hydrophilic-hydrophilic bonds.

Thus air denaturation effects may be important, particularly if the solid surface under study is not a strong activator of coagulation. We are observing clot formation at air/plasma interfaces. Though we cannot yet conclude anything, the next slide demonstrates that air/plasma interface denaturation effects may be important. (Fig. 6 is a diagrammatic picture of the slide).

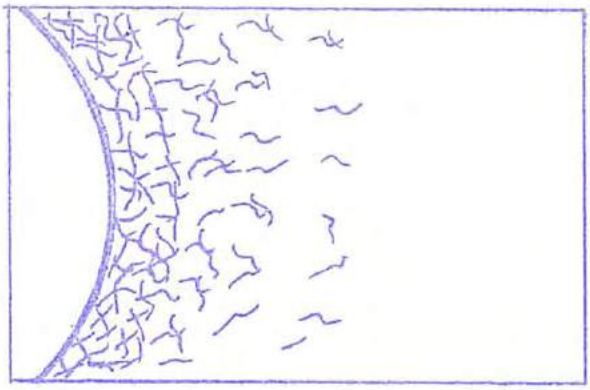


Fig. 6: Diagramatic Representation of Micrograph Showing Fibrin Formation at Surface of a Trapped Air Bubble.

Another observation is that Don Lyman noted yesterday that low energy surfaces adsorb phospholipids. A low energy surface containing adsorbed phospholipid may present a polar or hydrophilic surface to the solution; such a surface might be amenable to Hageman Factor activation.

Finally, I'd like to show that our geometric approach on the inhibition of contact activation is not incompatible with some of the results presented here.

The polyelectrolyte and hydrogel surfaces, with their high water content and possible ease of protein penetration, may appear rough on the molecular scale and thus prevent Hageman Factor activation.

Also Dick Falb has noted that Hageman Factor is adsorbed by both heparinized and non-heparinized surfaces, but that it is not activated at a hepanized surface. If heparin is bonded at one end with the rest of the molecule sticking out into the solution, and if its length is of the order of 200-400 Angstroms, which is not unreasonable, then the heparin surface might be a surface which is rough on the scale of proteins.

We are intrigued by the possible properties of geometrically rough surfaces and will be devoting a great deal of effort to this approach in the remaining seven months of the project.

That fairly well covers our work up to the present. We plan to have most of the surfaces ready for evaluatio several months; then the extensive evaluation and testing can begin.

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