An in vitro study of cellular response to flow with special reference to the cardiovascular system

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An in vitro study of cellular response to flow
with special reference to the cardiovascular system

by

John Warren Krueger

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INTRODUCTION

Review of Incentives for Studying Problem

At first glance, the study of a cellular response to flow appears to be primarily of theoretical concern. But many examples from the in vivo situation can be offered as incentives for conducting such a study. Epithelial cells lining the cardiovascular, respiratory, urinary, and gastro-intestinal systems are repeatedly subjected to gross mechanical flows in the normal course of the systems' functioning. It follows that an understanding of the normal epithelial cell's sessile and exfoliate biology would not be complete until the cellular interaction with the adjacent flow was considered. In actuality, all transport in and between the major organ systems of the body involves exchange across cellular membranes which are subjected to shear stresses, pressure gradients and concentration gradients imposed by mechanical flows. Rarely are epithelial cells considered in this context, although clearly many possibilities for study exist.

Specific incentives for studying a cellular response to flow can be cited from a diverse array of disciplines within the biological and medical sciences. But because of the extensive interest in documenting the mechanics of flow in the cardiovascular system, the foremost incentive for this study results from an interest in relating the biology of the intimal lining of the blood vessels to the adjacent flow conditions. Current interest in biological fluid mechanical studies on flow in the body can be cited for the respiratory, urinary and circulatory systems.
For example, physical studies on the flow characteristics in model bifurcations of the bronchial tree (1,2,3) and the flow geometry of the pulmonary alveolar capillaries (4) have been reported, and a mathematical model of peristaltic, bolus flow common to the urinary ureters has been given (5). However, although venous hemomechanics have received some current attention (6), it must be said that the bulk of the biomedical fluid mechanics' effort resides in arterial hemomechanics research - currently stimulated in large part by a search for a better understanding of arterial disease.

**Arterial disease**

Vascular accidents (i.e. heart attacks, strokes, aneurisms, etc.) have been estimated to be the underlying causes responsible for the majority (55%) of all natural deaths in man (7, p. III-3). Although there are difficulties in attempting a strict definition, the term arteriosclerosis is generally used to refer to any disease of the arterial wall in addition to referring to the specific mineralization in the tunica media (8,9). Arteriosclerotic changes in the media are frequently preceded by an ordered progression of cellular and extracellular, fibrous and fatty accumulations in the arterial intima: This process is known as atherosclerosis (8,10). Progressive atheromatous changes occur in late adolescence (8,9) and they are confined to that avascular part of the arterial wall that is fed by diffusion from the endothelium (9).

In spite of the fact that it has been widely studied, little is known about the exact etiology of atherosclerosis: A multiplicity of
causes is implicated (11-14). What has not been explained to date is why certain sites in the cardiovascular system have a predilection for these lesions (15,16, pp. 50-57, 17). Examples of the site localization and distribution of arteriosclerotic plaques and atheromatous lesions in the larger vessels of the cardiovascular system are offered in Appendix A.

One explanation for the localization of atheroma is that the relationship between the rheology of blood and its clotting behavior may precipitate the aggregation and deposition of microthrombi at the arterial wall (18). Once adherent, these microthrombi may be incorporated into the wall to subsequently form atheromatous lesions. Various vascular geometries predispose given patterns of particle deposition (19). The analogy between the deposition and adherence of microthrombi to endothelium and the distribution of spontaneous atheroma in swine aortas has been reported in support of this concept (20). However, such an 'encrustation' hypothesis of atherogenesis does not serve to explain the commonly seen histological variations in atheromatous lesions. An alternate - but not necessarily exclusive - hypothesis involves the response of the wall itself. A slight but chronic mechanical irritation to the lining of the vascular wall, when superimposed upon biological, hereditary and dietary factors, may predispose given sites to these lesions (16). Mechanical irritation alone may prove to be sufficient, for recently it has been reported that mechanically induced lesions which histologically resemble atheroma can be produced in rabbits without prior elevation of blood cholesterol levels (21). Similar findings have also been reported for the rat (22).
A variety of mechanical factors have been postulated as being important in these arterial changes (16,17,23,24,25,26). For example, a local reduction in the lateral wall pressure resulting from the 'Bernoulli effect' - often erroneously referred to as 'suction' - has been suggested as being an important parameter in atherogenesis (27). (However, the normal differences between systolic and diastolic blood pressures is greater than most reductions in pressure resulting from the Bernoulli relationship. Young observed experimentally that variations in wall pressure are much less than concurrent alterations in wall shear stress through axisymmetric stenoses (28).)

Along with other mechanical stresses, wall shear stress was suggested as being an important causative agent in atherosclerosis as early as 1872 by Rindfleisch (29, p. 212). The distribution of viscous shearing stresses through vascular geometries may have important implications in the distribution of cellular injury. Discretely scoured regions in models of stenoses and at the inner tips of bifurcating tubes have been demonstrated (30). Recently, Fry has demonstrated an acute endothelial injury and cellular mechanical failure when these cells are subjected to high laminar and turbulent shearing stresses (31). Endothelial injury and subsequent changes in wall permeability to large molecules in regions of high shear stresses has been suggested as a factor in atheroma (32). Sudanophilic lesions and intimal cell proliferation have been claimed to occur at entry regions around orifices and branches in hyperlipoproteinemic dogs (33). Wall shear stresses of 80-160 dynes/cm² have been measured
at the inner lips of model arterial bifurcations (34), and these stresses are of the same order of magnitude as the 'critical yield stress' for canine endothelium (i.e. approximately 400 dynes/cm² as defined by Fry (32)). However, the exact magnitude of such parameters must be regarded cautiously, as Fry's experimental technique may have lead to an over-estimation of applied shear stress of up to 100% (35). A wide variety of animals display atheromatous changes (8), and although comparative measurements are not yet available, it does not seem likely that for a given site in the vascular system the wall shear stresses would be of the same magnitude among the species (36, Table 7.4, p. 96). It furthermore seems unlikely that the mechanical properties of structurally, geometrically and cytologically similar cells would be radically different from one species to another. But here again, the comparative mechanical properties of endothelial cells is not available. Also, the effect of other factors such as hypercholesteremia upon the parameters of mechanical failure in the endothelium is not known, and yet this is an obviously important question in atherogenesis. It must also be emphasized that cardiovascular hemomechanics is a complex field of study; the distribution of wall shear stress in the circulatory system is not well documented.

Variations in plaque histology and distribution may be related to the presence of either turbulence or high shearing stresses: It has been suggested that turbulence causes raised fibrous plaques whereas high shear stresses predispose flat, sudanophilic lesions (16). Transient turbulent stresses are conceivable at various points in the vascular
system, including branch points (37), downstream from stenoses (38), and in the proximal ascending aorta beyond the semilunar valves (39,40). The experimental superposition of increased flow throughout arterial segments of hypercholesteremic animals readily leads to atheromatous changes (41). In hypercholesteremic dogs, atheroma will develop downstream from implanted aortic stenoses next to regions of speculated turbulence (42).

Conflicting with these latter views, an alternate and more recent hypothesis contends that atheromas develop preferentially at sites of low wall shear stress (43). A process of shear mediated diffusive efflux of cholesterol synthesized in the wall is held to be responsible for the reported absence of atheroma at sites of speculated high wall shear stress. In support of this viewpoint, it can be argued that the magnitude of wall shear stress might be expected to increase in the daughter tubes of arterial branches, whereas the incidence and severity of atheromatous involvement decreases peripherally (44). (Shear stresses fluctuating from 5 to 60 dynes/cm² might be expected to occur in capillaries as shown by a model analysis (45), whereas average wall shear stresses of 20–40 dynes/cm² occur in the canine aorta (34).) If high shear stresses predilect atheroma, it might be argued that an increase in cardiac output – stimulated by physical exercise – would be deleterious to health: Quite the opposite is generally acknowledged to be true.

Flow separation has been suggested as a critical hemomechanical parameter in atherogenesis (25). Studying flow in models of arterial geometries, investigators have described regions of separation which
might predispose arterial branch points (46,25), curvatures (25), poststenotic regions (25,28,38), and regions downstream from projections (47) to these vascular lesions. Unfortunately, most nonanalytical in vitro model studies on flow in bifurcations and curvatures are conducted in models which have little geometrical similarity to in vivo anatomy and so the description of flow regimes must be made with caution. Recently, separation has been described in vivo (48), but as yet the results have not been confirmed using the finer measurement techniques currently available (49). The manner in which the flow regimes within the regions of separation interact with the arterial wall is unknown. The accumulation and deposition of microthrombi is one possible reaction (50), and the alteration of diffusive flux to the wall next to separated regions might be another (44).

Considering the speculation about the role of wall shear stress in atherogenesis, it seems plausible to the author that, as opposed to solely the magnitude of shear stress, the magnitude of the shear stress gradient (i.e. the variation of the wall shear stress along the length of the artery) may have an important net effect upon the cellular monolayer lining the vessels. Temporal variations in shear stress would affect endothelial cells of adjacent regions relatively equally, whereas spatial gradients - by definition - would not. Large spatial shear stress gradients could exist at any region of changing geometry in the arterial system such as entrance regions and curvatures, and especially in regions of flow separation and reattachment. Spatial variations in shear stress would result in radial shear stress in the arterial wall.
which might be sufficient to locally affect the integrity of the elastic laminae in arteries and to instigate the often observed migration of smooth muscle cells from the tunica media to the tunica intima. (A pertinent question is whether the endothelial cells can in fact transmit shear stresses sufficient to alter the behavior of the subintimal components before mechanically failing.) Endothelium over atheromatous plaques is usually continuous (51), and endothelial mitotic activity occurs at the edges of the plaques rather than the centers (52,6). Thus, a concept of simply the magnitude of wall shear stress seems insufficient to account for the distribution of cellular changes in atherogenesis. The cytological reaction of the arterial wall to a specific mechanical stress distribution is discussed in Appendix B, and the role of cells in atheromatous changes will be discussed in the next section. At present, it must be stated that the adjacent blood flow conditions in the development of arterial disease is still not well established.

Although the gross distribution and variation of atheromatous plaques may result from localized macroscopic flow conditions, the question still remains as to the nature of the actual microscopic flow conditions affecting the individual cells (36, pp. 134-135). The effect of the cellular projections upon the adjacent flow should be experimentally observed, as well as the magnitude of Brownian movement in the fluid with respect to the small characteristic dimensions of the cells themselves.

Considering the cited examples, it seems that an experimental evaluation of the cellular response to flow is necessary before conclusions can be made regarding a mechanical trigger for arterial disease. The
description of cellular responses to such variables as shear stress, velocity gradient, pressure and Reynolds number must be accompanied by an exact definition of the actual flow over the cells at the microscopic level. Furthermore, a knowledge of the mechanical and rheological properties of 'epithelioid' cells seems paramount when cellular injury is predicted as resulting from acutely applied extremes of these flow parameters.

Cellular dynamics in preatheromatous changes and intimal biology

In addition to the early subendothelial lipid accumulation, the involvement of individual cells in early atheroma must be emphasized. But the cellular biology of the intima is not well understood: If hemomechanical factors affect intimal cell behavior, the resulting alterations must be superimposed upon the processes of degeneration and repair in the endothelial lining. Furthermore, controversy undoubtedly stems from the fact that the arterial wall is not simply a partitioned, three-layered conduit. It is in fact a complex organ. Although each layer may possess a characteristic cellular population, the cells of one layer are not found independently of the adjacent tissues. Identifying the source of the cellular elements in preatheroma illustrates these considerations.

The actual source of the cells seen in preatheromatous changes is still a matter of active speculation, but the presence of foam cells may result from the combined effects of hypercholesteremia and the local hemodynamic conditions. Intimal cell proliferation and the subintimal fat accumulation in regions of speculated high shear stress have been
described in hyperlipoproteinemic dogs (33). Elevated intimal D.N.A.
activity has been demonstrated in rabbits (52,53). Similar findings have
been reported for the endothelium of swine (54). The increased rates of
mitoses and elevated nuclear activity reported in these studies indicate
that one possible source of the subendothelial 'foamy lipophages' may be
the intima itself. The accumulation of intracellular sudanophilic
material by the intimal cells has been shown (55-57), and the endothelial
phagocytotic and pinocytotic mechanisms involved have been reviewed
(58-60). It has been suggested that the endothelium may transform di­
rectly into foam cells (61).

Superimposed upon those changes triggered by hypercholesteremic
insult may be endothelial repair processes triggered by chronic hemo­
dynamic mechanical injury. The status of endothelial biology prior to
1954 has been admirably reviewed by Altschul (62). He commented that the
supposed 'little or no' replacement of endothelial cells was surprising
considering the chronic stress to which they are subjected. However,
there seems to be little current doubt about the ability of endothelium
to regenerate adjacent to areas of mechanical trauma (63-66, 22), al­
though endothelial mitoses are reported to be rare in normolipemic animals
(63). Only 0.1% of the endothelium in normolipemic animals showed mitotic
figures, and the mitotic rate in the normal media is even less (i.e. 0.01%)
than in the endothelial layer (66). Endothelial cell replication appears
to be inhibited by the presence of adjacent normal cells: Variation in
mechanical stress on the endothelial monolayer might alter this adjacent
cellular influence. Localized elevations of the mitotic index in the
aortic arch and variations in the level of mitotic activity at vascular branch points recently have been reported (67): A net decrease in endothelial regeneration along with aorta was suggested to be related to blood flow coupled phenomena (67). Canine endothelial cells have been shown to have a varying susceptibility to mechanical damage (31, 68). Because differential selection of a shear stress resistant cell population does not occur, susceptibility might be related to cellular aging.

It has also been suggested that these focal cellular accumulations may be derived from pluripotential myointimal cells (69), perhaps in turn derived from medial smooth muscle cells that have migrated through the internal elastic membrane (70). Recently it has been suggested that the intimal foam cells are in fact derived from two sources - the medial smooth muscle cells and the free macrophages carried in the blood (71).

Whether these focal cellular accumulations and patterns of altered endothelial activity result from a primary intimal change, subadjacent medial changes, or localized mechanical-environmental changes at the luminal aspect is difficult to determine. What must be concluded is that the relationship between the mechanics of blood flow, the physiological integrity and the behavior of the endothelial monolayer, and the presence of the cellular components in atheromatous lesions is an unanswered and yet important question in the biology of the arterial wall. In this regard, an analysis of the endothelial response to well-defined flow parameters would be most rewarding.
There are indications other than localized endothelial injury and repair processes which suggest that the mechanics of blood flow exerts an influence on the normal biology of the intimal lining. For example, it has been suggested that vascular mesenchymal tissues reorganize themselves in response to hemodynamic stresses (72), and in this way the vessels become restructured along hemodynamically predilected geometries. Similarly, hemodynamic phenomena may assist valvelogenesis and the formation of intimal cushions (72), although it is not known by what mechanisms this occurs.

The dynamic behavior of the vascular wall may in part be determined by mechanical parameters of blood flow. It has been suggested that an increase in wall shear stress will cause a vessel to increase its diameter (73). A slight mechanical injury applied to the intima in hyperlipemic rabbits will cause the vessel to dilate within 12 to 24 hours (74). There seems to be some basis for the belief that a local intramural reflex mechanism responding to intimal stimulation is responsible for the arterial dilation. Recent experimental evidence appears to confirm that an unknown intramural reflex mechanism exists in which wall shear stress is sensed by the intima (75).

Histologists have always been intrigued by the fact that normal, young endothelial cells are oriented with their long axis parallel to the direction of the blood vessel (76,77). The reason for this preferred orientation is not altogether clear; it has been tempting to implicate blood flow in predisposing endothelial orientation. Cellular injury and
aging result in a random re-orientation of the endothelium (76,77,62,63). Fibroblasts proliferating in response to intimal injury orient parallel to the endothelial cells (78), whereas subintimal smooth muscle cells are normally oriented perpendicularly (64). The disorientation of the endothelial pattern with age, the presence of longitudinally oriented intimal smooth muscle, and the intravascular local mechanical reflexes raise interesting questions about possible flow-coupled structural interrelationships among the tissues of the vessel wall.

Intercellular and intracellular transport mechanisms in the endothelial lining may be altered by the mechanical parameters in blood flow. For example, the uptake of free cholesterol by intimal cells has been hypothesized to be simple, passive physical-chemical reaction (79). Wall shear stress could influence this process - either by altering the concentration gradients and hence diffusive flux (43), or by affecting the 'strain free energy' involved in the physical chemistry of the surface absorptive mechanism (32). In addition, mechanical stresses in the cell membrane might alter wall permeability and active membrane transport processes such as pinocytosis and phagocytosis. The ultrastructural and physical bases of endothelial transport have been reviewed (59): A variety of cellular properties such as the presence of interendothelial gaps, changes in surface area, and Brownian movement suggest a multitude of ways in which shear stress might affect the transport through the endothelial membrane.

For the present, these comments must be treated as speculative simply because of the lack of the appropriate experimental observations. Cellular
and subcellular behavior in transport processes should be observed in cells subjected to both controlled and defined flow conditions to provide answers to this speculation. Such observations would be simplest to perform in vitro.

The hemodynamic role in endothelial injury and thrombosis

The relationship between hemodynamics and endothelial injury plays a significant role in intravascular thrombosis. The pattern and topography of thrombus formation in extra-corporeal shunted models of vessel configurations has been shown to correspond to the distribution of thrombus-initiated atherosclerotic changes (20), and the atherosclerotic histopathological sequence can be inaugurated in the pig aorta after mechanical trauma and endothelialization of the adhering mural thrombi (80). Platelet accumulations stained with Oil Red 0 can be seen at the lesser curvature of the aortic arch and about the lateral aspects of vessel orifices (20). It is not known why platelets accumulate in these regions, although it has been suggested that wall shear stress may play a role (20). The adhesion of platelet microthrombi in similar patterns on morphologically 'normal' swine endothelium (81) suggests that a possible cellular alteration has occurred and been triggered by local hemodynamics. Postulated mechanisms of the platelet-endothelial adhesive reaction have been reviewed (82). A progression of endothelial changes resulting from mechanical and other stimuli has been described in the living microvasculature (83a,83b): A primary increase in endothelial 'stickiness' occurs, followed by disintegration of the cell with
sufficient stimulation (83a). Thus hemodynamic stresses might predispose early submorphological cellular changes in the endothelium leading to platelet micro-thrombi adhesion. The mechanism of such an interaction is obscure.

Platelet micro-thrombi adhere readily to intimal extracellular tissues exposed by either endothelial gap formation (84) or cellular disintegration and sloughing from the basal attachments (68,85). An elaborate study of mechanisms of endothelial disintegration has been reported (85). One theory maintains that continued stress on the endothelial cell, combined with a weakening of the area of attachment, results in sloughing of the cell's nucleus: The exposed endothelial cytoplasm and ATPase attached to the abluminal membrane of the cell then catalyzes platelet aggregation (85).

Alternatively, endothelial cells may undergo submicroscopic cytoplasmic alterations and yet retain an intact cell membrane (84); this suggestion is reinforced by other observations (81). Mechanical rupture of the cell is not necessary for cellular detachment: Exfoliate studies on vascular endothelium found circulating in the blood suggest that with the appropriate mechanical and/or immunologic stresses endothelium may be sloughed in an apparently viable intact state and that thrombi subsequently form at the sites of desquamation (86,87). Submicroscopic cellular alterations due to vascular injury have been reviewed (88).

Clearly, a study of the cellular and subcellular endothelial responses to hemodynamic parameters would have interesting consequences for speculated endothelial cell mechanisms underlying intravascular thrombosis.
Neointimal linings and the evaluation of their suitability in prosthetic devices

An important property of the endothelial lining is that under normal circumstances it is antithrombogenic and fibrinolytic (65). For this reason the relationship between local hemodynamics and the promotion of adhesion and growth of artificial endothelial linings (i.e. neointimas or pseudo-intimas) in vascular prosthesis is of importance in the prevention of thrombus formation in these implants (89). It appears that neointimal cells lining vascular protheses can be derived by slow ingrowth of the adjacent arterial endothelium (90,91), and these cells will be markedly more fibrinolytic than their precursor cells (92). In an effort to hasten the cellular coating of the prosthetic surface, preplatling with a variety of tissue culture cells has been attempted (93,94,95,96): Some of these cell strains result in an antithrombogenic surface (94).

An alternate approach to that of finding a suitably growing, adhesive and mechanically resistive cell strain is to find an appropriate substrate that will promote a quick and permanent pseudo-intimal formation (97,98). Such a surface should promote rapid cellular adhesion, spreading and subsequent growth by its physical properties and surface chemistry. However, methods of evaluating the mechanical and behavioral interaction of cells with their substrate have never been defined on an adequate analytical basis.

As a measure of cellular spreading and adhesion upon a test surface the cell-substrate contact angle may be of use (99). The contact angle so defined could be influenced by such mechanical parameters as the
following: The internal pressure of the cell with respect to its external pressure (100); the shear and pressure stress distribution over the cell's surface (101); the rheomechanical properties of the cell membrane; and the ratios of the surface tensions of the cell membrane, the substrate, and the surrounding medium (102). It is conceivable that as flow induced shear and pressure stresses are imposed upon an attached cell, the change in its leading and trailing contact angles will provide a quantitative relationship by which the mechanical interaction of the cell with its substrate can be evaluated. Furthermore, the rate of change of the cell-substrate contact angle to step-imposed steady shear stresses may bear some relationship to the rheological properties of the cells.

Although cytomechanics will be discussed in more detail later, it is apparent that a suitable method of testing and analyzing the 'mechanical-behavioral' interaction of cells with artificial substrates would be helpful in prosthetics research. Such a method of testing would evaluate both the suitability of the cell strain used and the properties of the substrate biomaterial in question. However, a foundation must first be established in understanding the cellular response to flow. These studies should correlate both a profile view of the cell and a planar observation of the intracellular detail with defined flow conditions. The possibility of in vivo transformation of tissue cultured cells (103) gives further impetus to a preliminary testing of proposed neointimal cells.

In considering these relationships between hemomechanics and the localized cellular biology of the vascular wall as incentives for studying a
cellular response to flow, the following two points must be kept in mind. First of all, the study of cardiovascular hemomechanics is a very complex field, and the discrete distribution of such flow parameters as wall shear stress is only now beginning to be measured with a meaningful precision. Secondly, the mechanical correlates with arterial disease, intimal cellular biology, and intravascular thrombosis all stress the fact that very little is known about the way in which cells alter their behavior in response to flow induced mechanical stresses. Furthermore, it is not simply the response of individual cells that is important in vivo, but rather the behavior of cells in organized tissues such as the endothelial monolayer that must be considered.

In addition to those examples from cardiovascular biology which motivate a study of the cellular response to flow, it is suggested that there are other situations which would also benefit from such a study. These will be considered next.

Considerations in cytology and cell culture

It is clear from the previous discussion about hemomechanical correlates in the localized biology of the arterial wall that the response of intimal tissues to adjacent blood flow should be discussed in cytological terms. But specific questions arising from these considerations are common to other areas of biological study as well. In this section considerations which motivate the study of a cellular response to a viscous flow - quite apart from the cardiovascular system - will be discussed.
One such important consideration is the manner in which flow induced shear stresses affect cellular structure and behavior. For example, it has been suggested that the surface shear stresses may alter the rheomechanical properties of embryonic cells migrating in vivo (104): As the magnitude of the adjacent shear stress changes, the mechanical properties of the migrating cells are altered so that their distribution within the body is affected (104). In this respect, cellular migration may be considered as a process analogous to a very slow flow of the adjacent tissues. A time dependent, non-Newtonian relationship between surface viscosity and surface shear stress in these cells indicates the feasibility of this concept (104), it being proposed that the cells' migratory ability decreases as the cytoplasmic viscosity increases. Thus, the study of a cellular response to flow may have some bearing upon the behavior of embryological tissues in vivo.

The behavior of endothelial cells in many respects resembles the growth and behavior of monolayered cells in tissue culture. Consideration of observations in cell culture should result in a more precise description of cellular mechanisms in arterial wall biology.

The shapes and behavior of cells have been postulated as being related to variations in their cytomechanical properties (105). This may be especially true in tissue culture, in which case applied surface shearing stresses should elicit differential responses between amoeboid, fibroblastoid and epithelioid cells. Such an observation would demonstrate the validity of the hypothesis, it would also have interesting implications for intimal biology where the origin of the cellular elements in
some areas is uncertain but may be related to the adjacent flow differentially affecting adhering white blood cells, fibroblasts, smooth muscle and endothelium.

Morphological changes of cells in tissue culture may be associated with functional changes as well (106). Similarly, modulation of cellular behavior in cell culture may be accompanied by alterations in cytomechanical parameters, subsequently varying a cell's susceptibility to imposed mechanical stresses. In other words, stresses might conceivably predispose certain cellular morphologies in culture and, conversely, morphological transformations may reflect fundamental alterations of the internal stress distribution within the cell.

Although it is possible to culture cells from the vascular intima, the morphology of the cell types obtained differs from that of their in vivo precursors (107-110, 55,56). The lack of orientation in cultured endothelial cells may result from the fact that the cells have not been cultured under appropriate flow induced mechanical stresses. Biological transformation of primary cells in culture is a possibility (103), but in published photographs of primary cultures of endothelium (107,108) the cells appear normal except for the lack of orientation. Biochemical differences between the in vitro and in vivo environments would be isotropic and as such should not affect orientation.

A preliminary study of the effect of defined flow upon permanent strain cells in tissue culture was reported in 1969 (111) and these results have since been published (112). Briefly, living epithelioid cells
grown as an attached, confluent monolayer were subjected to a steady
uniform laminar flow. Although cellular and subcellular morphological
manifestations of flow were evident at surprisingly low shear stresses,
no cellular orientation was reported. (But with regard to the lack of
cellular orientation with the application of chronic shear stresses, it
must be recalled that the cell type employed in this study had been
cultivated in vitro for many generations, and that its predecessors are
not oriented in vivo.) Endothelial orientation in vivo therefore may be
related to the presence of a unique intracellular mechanism which is
shear sensitive. It would be of fundamental interest to extend the re­
ported studies to other cell types and, in particular, primary cultures of
cells that are normally oriented in vivo with the adjacent shear stresses.
It would be interesting to determine whether or not cultured endothelial
cells could be made to orient by applying directional shear stresses over
the cells. Fundamental relationships between the mechanical properties of
cells, their external morphologies, and the surface shear stresses should
result from such comparative studies.

Endothelial cells lose their orientation with age (76,77). Aging in
these cells cannot be considered as an independent process isolated from
the effects of the constantly imposed physiologic mechanical stresses.
Age related changes will be superimposed upon those behavioral and repair
processes triggered by the continual mechanical stresses. Therefore, a
study of the endothelial response to flow is necessary before more comment
can be made about aging mechanisms related to loss of cellular orientation.
Aging in cells affects functional parameters (113,114) and it is conceivable that the cell's mechanical properties is one of them. Virtually nothing is known about the relationship of the cell's age to its susceptibility to shearing stresses, and yet this is also an important topic for study considering mechanisms of endothelial sloughing in intravascular thrombosis (refer to previous discussions). It is suggested that a study of the cellular response to flow would have fundamental interest in the evaluation of age related changes of cells in culture and in the body.

The effect of mechanical stresses upon cellular transport mechanisms has not been widely studied. The application of very high hydrostatic pressures (100 atmospheres) is necessary to stop pinocytosis (115), but because of the incompressibility of cytoplasm, the requirement of such high pressures is not altogether surprising (116, pp. 145-150). Considering the fluid-like subcortical cytoplasm in the cell (117) and the localization of pinocytosis in the peripheral cell membrane, it is conceivable that shear stresses of much lower magnitudes than the 'critical' hydrostatic pressures would be sufficient to alter the pinocytotic mechanisms. Cessation of pinocytosis at relatively low shear stresses has been reported (112).

When stresses are proposed as causing functional changes in cells - or possibly causing mechanical failure - it would be well to consider the mechanical nature of the cell and its components. Cytomechanical properties have been measured for cytoplasm and cell membranes in unattached, spherical cells (118,119,120) but such studies have not been performed for mammalian cells in an attached, extended geometry such as they occur.
on all biological membranes and in tissue culture. The mechanical nature of the cell may be far different that that predicted from measurements made on spherical cells (112) and the application of such measurements to endothelium (31) must be viewed with caution. Surface viscosities have been measured in multilayered tissue culture cells (104) but these measurements have not been correlated with direct cellular observation. The integrity of the cells during such measurements is therefore open to question. The mechanical properties of the red blood cell membrane have been measured in cells made to adhere to glass (101), but these cells are certainly not epithelioid cells.

A variety of cytomechanical properties have been measured in living cells. The viscosity of protoplasm in attached fibroblasts has been estimated by the application of Stokes' law to observations of the translation of electromagnetically vibrated intracellular particles (121). Similar techniques have recently been employed for unattached cells (122). The surface viscosity of cells has been estimated by the application of oscillatory shear stresses to multilayered tissue culture cells (102). Membrane surface tensions have been measured in free-floating spherical cells (118-120), and estimates of the modulus of elasticity of the cell membrane have been made by using a dimensional analysis in free (123) and attached (101) red blood cells.

It is problematic what such measurements represent for attached cells considering their complex structure and geometry. Furthermore, it has not been determined what relationships surface viscosity, surface tension, and
membrane modulus of elasticity have in determining cellular behavior. Observations on the biochemical alteration of cells' mechanical characteristics \((124,125)\) emphasize the importance of employing a meaningful index of the cell's mechanical properties.

It has been proposed earlier in this manuscript that the measurement of leading and trailing contact angles of attached cells subjected to defined shear stresses would provide some insight into cellular cytomechanic and rheologic properties. Such a measurement represents a parameter of the cell's mechanical nature and its relationship to its substrate and its mechanical environment. A cell culture profile chamber has been briefly mentioned in the literature in which it is possible to observe the cellular contact angle to the substrate \((126)\), but the described chamber does not allow for the application of a perturbing stress to the cells. A cell culture profile flow chamber which has provisions for the simultaneous visualization of attached cell profiles and the application of defined shear stresses is required to evaluate the efficacy of such measurements.

It is not clear how cells in the epithelioid geometry are able to withstand constant shearing stresses: Consequently, it would be interesting to determine the distribution of stress within the cell. Presumably the cell membrane withstands most of the shear induced tension, for cytoplasmic birefringence cannot be seen in epithelioid cells subjected to a steady shear stress \((112)\). Birefringence can be detected in moving cytoplasm in amoebae, and it is thought to represent shear stresses within the protoplasm \((127)\). Therefore it appears that the stresses are generally confined to the cell membrane in epithelioid cells and that
they may be focused at the cell periphery where pinocytosis is localized (112). Birefringence has been described in tissue culture cells and has been postulated as having a mechanical significance (128).

The concept that metastatic cancer cells are less adhesive than normal cells has stimulated interest in the measurement of cellular adhesion. A parallel interest resides in the adhesion of neointimal cells and connective tissue cells to the artificial substrates of implant materials (129,96). Testing procedures for measuring cellular adhesion are most easily performed in vitro. As a means of measuring adhesion, cells in tissue culture have been subjected to acute shearing stresses by rotating a glass disc very close to their surface (130): The percentage of the cells remaining on the glass substrate after shear is used as an index of adhesion. In these studies it was pointed out that the mechanism of cellular adhesion to the substrate is not analogous to the opposite process of cell removal from the substrate (130). Furthermore, since it was not possible to directly observe the cells during the application of the shearing stresses, the mechanisms responsible for the removal of the cells from the glass could not be identified. Two modes of cellular removal from the glass are possible: Either a process of mechanical failure of the cell, or alternatively, a process of biological separation of the intact cell from its substrate could have occurred. Clearly, the time course of application of shear stress to the cells as well as the stress magnitude is important in these processes (130,112). Analogous processes of cell removal may occur in vivo where both sloughing of intact endothelium (86) and cellular failure (31) have been reported. Experiments
consisting of subjecting cells to defined stresses should be made in which direct cellular observation is coupled to a recording of the time course of events. The nature of the cells' mechanical failure should be evaluated to determine whether it is a process of membrane rupturing (131) or of yielding of the cells rheological components.

The cellular response to specific mechanical irritants should be studied. For example, shearing stresses may cause sequential changes common to both cells growing in vitro and in vivo: Sequences of such cellular changes as rounding up, swelling and vacuolization with subsequent separation or mechanical failure can be seen in both endothelium in vivo and permanent strain tissue culture cells subjected to graded shear stresses in vitro (31,112). A similar sequence of cellular changes is often reported in pharmacologic tests involving the perfusion of test substances over cells growing in cell cultures, and yet the effect of the perfusion induced flow upon the cells is not known (132,133). Until recently (112), there were no reports of a suitable cell culture chamber which combined the capacity of direct cellular observation with the ability to calculate the defined flow conditions to which the cells were subjected. The possible use of flow induced shear stresses as a biomechanic testing procedure has been suggested earlier in this manuscript.

The response to flow of individual, attached but isolated cells may differ from that of cells growing as a confluent monolayer (134). The influence of adjacent cells upon their neighbors in flow conditions is not known: This has an interesting parallel in the endothelial growth over denuded areas of the vessel wall.
Finally, the study of a cellular response to flow has application in microbiological technology. Many types of mammalian cells grow and produce metabolic byproducts only when attached to a substrate. Endothelial cells grown in 'batch suspension' cultures do not appear to manufacture stable fibrinolytic and thrombolytic materials (135), whereas attached cells do (94). A common problem in tissue culture perfusion systems is that the flow 'leaches out' valuable compounds from the cell's microenvironment (136,137). The use of cell culture perfusion chambers with a mathematically defined laminar flow would allow the calculation of concentration gradients of critical substances more precisely than in the 'batch' reactor type perfusion systems. Presumably this would lead to the readjustment and replenishment of the proper substances to the cells. The tolerance of cells to flow in microbiologic fermenters is important. Shear sensitive protozoa and algae have been grown in such systems to evaluate the shear stress in fermentation reactors (138,139).

Flow induced shear stresses would be useful as a method of obtaining synchrony in the mammalian cell growth cycle so that the biochemistry of metabolism can be studied more precisely. Cells in the interphase part of the growth cycle spread out and attach more firmly to the glass substrate, whereas cells undergoing mitosis round up and are less adherent to the glass (140). Flow induced shear stresses can then be used to select out cells undergoing mitosis. But here again, the effect of the flow upon the interphase cells is not known. Assuming that the effect is negligible, the advantages of using a mechanical technique rather than chemical or irradiation methods seems obvious.
Summary of incentives for studying the problem

A variety of reasons for studying the cellular response to flow has been reviewed. From these comments, it can be seen that such a study would provide answers to questions arising from basic considerations in such diverse areas of study as cardiovascular biology, cytology, and behavioral aspects of cells in tissue culture. In addition, the results of the study can contribute to research in the cellular interaction with prosthetic implant materials and applied microbiology. The study of an endothelial response to flow may further contribute to thoughts about atherogenesis and intravascular thrombosis. Clearly, the cited examples support the belief that a study of the cellular response to flow will have interesting pure and applied scientific consequences.

Because of the broad interdisciplinary scope of these incentives, the research problem will be more precisely defined in the next section.

Definition of Problem

Since a variety of diverse situations offer incentives for the experimental study of a cellular response to flow, it is necessary to define the problem in more precise terms. The effect of flow upon the physical and chemical environment of the cells should be considered, and a justification for the selected experimental procedure should be given.

The physical and chemical environment

Flow is expected to affect cells in several ways: 1) The fluid moving over the cells will exert a mechanical stress upon the cell
membrane which will then be transmitted throughout the cell and to the adjacent substrate, and 2) due to considerations of mass transport phenomena, the concentration of chemical species in the immediate surroundings of the cell will be altered. It is conceivable that flow will affect other phenomena - such as heat transfer processes - but due to current interest as seen in the biological literature (31,44) only mechanical stresses and mass transport processes will be considered in this thesis. (However, once the mechanical flow regimes are fully defined, the heat transfer coefficient can generally be obtained using equations analogous to those derived for the coefficient of mass transfer.)

Regarding the first of these considerations, the mechanical stress exerted by the flow is arbitrarily resolved into two orthogonal components. Shear stresses result from viscous interaction of the relative motion between neighboring molecules in the moving fluid and the stationary 'solid' phase of the attached cells and these will be exerted tangentially on the cell membrane. Because of the complexity of the adsorbed surface coats of cells, it is impossible to define a strict stationary phase border as that of the cell plasma membrane (141). But regardless of the surface rheologies of these absorbed coatings, it is a fair assumption that the shear stresses in the neighboring fluid phase are transmitted to the cell membrane. The complex geometry of the cell and its peripheral attachments to the substrate and other neighboring cells suggests that shear stresses will be distributed in a complex,
nonuniform fashion over the cell surface. Because of the fluid-like 'sol' phase below the cell membrane (117), the resulting stress distribution will be uniform intracellularly.

For Newtonian fluids (i.e. fluids in which the viscosity is a constant independent of the velocity gradient) the shear stress exerted upon the cells is equal to the product of the viscosity times the velocity gradient where the velocity gradient is evaluated at the cell surface. It has been postulated that endothelial cells may alter the flow in their proximity (141), and for this reason the microscopic flow and velocities over the cells should be experimentally observed for the purpose of this study.

Flow will also exert pressure stresses upon the cells in addition to the tangentially applied shear stresses, and these will be exerted (by definition) normal to the cell membrane. Because of the flexible nature of the cell membrane it is assumed that the pressure is evenly distributed uniformly throughout the cell. A pressure drop in the direction of flow (i.e. pressure gradient) exists in viscous fluids, although this would be negligible for cellular dimensions. Of the two types of stresses resulting from flow, it is expected that pressure will have the least effect upon cellular behavior. Contrary to earlier claims (27) this seems to be a valid assumption considering more recent evidence derived from studies in the arterial system (31,116).

In addition to exerting mechanical stresses, flow will also influence the mass transport between the cell and its surroundings. The relationship
between flow and mass transport may be considered by visualizing three regions in which barriers to diffusion will exist: 1) The cell membrane, 2) an adjacent stationary cellular microenvironment, and 3) the fluid moving adjacent to the stationary microenvironment.

Transport of any chemical specie through the cell membrane may be governed by a combination of physical and biological mechanisms and is therefore too complex to analyze from physical consideration alone. However, the diffusive flux of substances between the stationary cellular microenvironment and the flowing medium will be directly proportional to the appropriate concentration gradients. (i.e. Fick's law states that $\phi = (D/\delta)(C_s - C_m)$, where $\phi$ is the flux of substance, $D$ its diffusivity, $(C_s - C_m)$ is the concentration difference between the cell surface, $C_s$, and the main stream fluid, $C_m$, and $\delta$ is the diffusional boundary layer thickness or that distance over which the concentration has reached 99% of $C_m$.) The concentration gradient is often approximated by the ratio $(C_s - C_m)/\delta$. But as substances diffuse from the cell's microenvironment, high velocity gradients in the adjacent flow will convect them further away, thereby maintaining the driving concentration gradient for diffusion. If the adjacent velocity gradients are low, the balance between the processes of diffusion and convection indicates that the concentration gradients will be lower than the previous case and hence the diffusive efflux from the cell's microenvironment less. (Similar considerations can also apply to heat transfer processes (142, p. 308).) Transport processes in which the overall heat and mass transfer coefficients are related to the adjacent velocity gradient may be termed 'shear mediated' or 'forced
convection transfer processes.

A crucial requirement for a process of shear mediated transport between the cell and its surroundings is that the greatest resistance to diffusion be the diffusional boundary layer between the stationary cellular microenvironment and the flowing medium. If the greatest barrier to diffusion occurs between the stationary microenvironment and the cell membrane then the process of forced convection would have a minimal effect on the overall mass transfer coefficient for a particular diffusible specie. For each substance of physiological importance the resistances to diffusion will differ between the cell membrane, the cellular microenvironment, and the diffusional boundary layer. Thus, the relationship between the processes of diffusion and convection of material to and from cells subjected to flow can be very complex.

Since it has been claimed that the flow of tissue culture medium over cells growing in vitro 'leaches' away vital metabolites from the cellular microenvironment (136,137) it would be best to discuss forced convection transfer in more detail. In this situation the critical substances lost by the cells is thought to be either absent from the surrounding medium or present in insufficient quantities to reimburse the cells during the routine washing procedures to which cells in culture are subjected. In this case, it can be said that the major resistance to diffusion is due to the diffusional boundary layer. Therefore, the relationship between the processes of diffusion and shear mediated convection that tends to leach substances away from attached cultured cells can be visualized by
considering a similar problem - that of forced convection transfer of a substance dissolving from a wall into an adjacent falling film in which fully developed laminar flow occurs. For this case it can be shown that the average mass transfer rate for the dissolving substance is directly proportional to the cube root of the velocity gradient (via the hydrodynamic boundary layer thickness) (142, pp. 551-552).

However, many constraints limit the applicability of such a model to the shear mediated transport from the cells. For example, a requirement of the forced convective transfer model is that the length of the wall segment for which transport processes are occurring must be small with respect to the maximum velocity in the adjacent fluid (142, p. 551). When the cellular dimensions are small with respect to the maximum velocity in the adjacent flow the application of this analogy to the leaching of substances from the cellular microenvironment is justified. Convection normal to the cells due to their surface features imposes another constraint: Ideally it should not occur. An additional constraint is that the diffusional boundary layer must be very small in relation to the hydrodynamic boundary layer. The relationship can be calculated by considering the Schmidt number, $S_c = \mu/\rho D$ and using $\frac{\delta_h}{\delta} = (S_c)^{-1/3}$, where $\mu$ is the absolute viscosity of the fluid, $\rho$ is the density, $D$ the diffusivity, and $\delta_h$ and $\delta$ the hydrodynamic and diffusional boundary layers respectively (44). Thus, although a relationship between the net mass transfer coefficient and the velocity gradient can be expected for a substance being leached from tissue culture cells by an adjacent flow, it is not possible to determine the exact functional relationship.
Similar constraints apply to an analogous model of shear mediated transport processes in a fully developed laminar internal flow for which a relationship between the diffusional boundary layer thickness, $\delta$, and the wall shear stress, $\tau_w$, can be readily obtained and expressed as

$$\delta = A\left(\frac{Dux}{\tau_w}\right)^{1/3},$$

where $x$ is the component of distance in the direction of flow from the point at which the transfer processes initiated and $A$ is a constant (143). In this case it is important to recall that the flux is inversely related to the thickness of the diffusional boundary layer, and as such it is again related to the cube root of the wall shear stress (or velocity gradient). It can also be seen that the local mass flux will be related to position ($x$) as well as to wall shear stress. Again, although it is difficult to establish an exact relationship between the wall shear stress, position, and the diffusional boundary layer thickness for cells growing in a monolayer, it might be expected that a growing diffusional boundary layer does exist for some cellular substances, and that its flux is therefore nonuniform over the monolayer.

From these considerations of the physical effects of the flow upon adherent cells the following points must be emphasized: 1) The shear stress exerted upon the cells will be directly related to the velocity gradient in Newtonian fluids whereas the mass transfer coefficient will be related to some function of the velocity gradient; and 2) in uniform, fully developed laminar flow, the shear stress distribution will be constant spatially whereas the local mass flux need not be. The last point suggests that by applying uniform fully developed laminar flow to a
cellular population there might be occasions in which the effect of flow induced shear stresses and flow induced heat and mass transfer phenomena can be differentiated: A uniform variation of cellular changes over the entire population would be indicative of changes resulting from mechanical stress, whereas a nonuniform variation of cellular characteristics would be related to heat and mass transfer coupled phenomena. The last observation would confirm the presence of a specie in the cellular microenvironment whose chief barrier to diffusion is the diffusional boundary layer.

Experimental procedure and thesis organization

The experimental investigation will consist of subjecting cells in tissue culture to a steady, uniform laminar flow. Because of the fact that any number of species with different diffusivities are involved in cellular transport processes, it will be considered sufficient to define simply the flow characteristics and mechanical stresses exerted upon the cells. The following parameters must be known: The absolute viscosity, \( \mu \), of the fluid surrounding the cell; the velocity gradient at the cell surface; and the Reynolds number \( Re = \frac{\rho DU}{\mu} \), where \( \rho \) is the fluid's density, \( U \) is the average velocity, and \( D \) is the thickness of the flow channel. These quantities can only be accurately controlled in vitro, and can be readily ascertained for steady, laminar flow.

Tissue culture techniques allow cell observation concurrent with accurate control of the physical and chemical environment. However, in spite of the fact that complex uncertainties about the surroundings can
be reduced, it should be recalled that cell culture is, at best, an approximate model of the real system. Primary cell cultures growing in vitro often undergo marked morphological and genetic changes after several generations in vitro (103). Thus, the difference between a response of primary cells and permanent strain cells in culture would be of interest. In addition, the mechanism by which cellular modulations are manifested is important in tissue culture, as it may represent a true cellular alteration, or on the other hand, a selection of a new cell population from a few totipotential individual cells.

In order to study the cellular response to flow it will be necessary to employ a suitable cell culture flow chamber that provides conditions of uniform defined flow and allows concurrent observation of subcellular microscopic alteration. Therefore, the first section of this thesis will be concerned with the development of suitable chambers.

Secondly, the actual microscopic flow over the cells should be considered. Observations of the flow characteristics over cells in the cell culture chambers will be presented.

And thirdly, examples of cellular response to flow from the literature should be reviewed and the cellular response to the observed flow conditions in the chambers presented.
PART I. DEVELOPMENT OF A CELL CULTURE FLOW CHAMBER
REVIEW OF LITERATURE

Requirements of a Cell Culture Flow Chamber For Use in the Study

A chief prerequisite in the study of a cellular response to flow is the ability to 1) quantify the flow induced mechanical stresses and 2) approximate certain flow regimes which govern predictable flow-coupled mass transport phenomena. For this purpose it is necessary to employ a cell culture flow chamber in which the flow can be easily predicted and completely defined mathematically. Furthermore, the cell culture chamber should allow experimental observation and verification of the expected flow regimes in the immediate vicinity of the cells.

In order to fulfill another requirement of the study, the chamber must have provisions for the detailed subcellular microscopic examination of living cells as they are subjected to the predicted flow conditions. Since living cells are optically transparent when viewed with bright field microscopy, phase contrast optics are required, indicating that the chamber must be constructed of two flat plane-parallel surfaces. Other considerations of the chamber design indicate the following: 1) It must be composed of materials which are nontoxic to the cells; 2) it must have provisions for a gas buffering air space; 3) it must be sterilizable as an assembled unit (and stored as such) by either chemicals or by heat treatment; and 4) it must be easily constructed from inexpensive materials, easily assembled, and rugged.

Very early in the study it became apparent that a cell culture perfusion chamber that met all of these requirements simply did not exist.
The trend in cell culture perfusion techniques has been to gain more control over the cellular microenvironment. The development of a cell culture flow chamber in which the flow is precisely defined and easily verified is a logical step towards a more quantitative cell and tissue culture procedure. A review of the literature will emphasize these points.

Cell culture chambers previously described in the literature

Since Burrows' classic experiment (144) a multitude of tissue culture perfusion chambers have been described. These have been reviewed (136, 145) along with certain problems regarding their use and the perfusion technique: Speculated 'leaching' of basic metabolites from the cell's microenvironment seems to have been a problem with the perfusion technique (136,137). The variety of cell culture perfusion chambers that have been described indicates a number of solutions to myriads of technical requirements and problems, not to mention personal preferences, of their users (132,133,137,144-176). The general trend in perfusion chamber design innovations has been toward improvement upon the existing design and the incorporation of new materials, but not towards definition of the flow regimes within the chamber.

The first tissue culture chamber used for continuous perfusion was that of M. T. Burrows in 1912 (144). The chamber was essentially a glass and wax sandwich: Paraffin was used as a sealant and a teased cotton wick was used to assist the flow of tissue culture medium. Leakage is a problem when using paraffin or wax, as is sterilization. The flow in the chamber was not defined. After a relative lull for four decades, interest
in tissue culture and cell culture chambers was renewed by developments in phase contrast microscopy and cinemicrography. With the opportunity of observing cellular behavior in detail for over longer periods made possible by improved tissue culture media, cell culture perfusion chamber designs flourished.

The new perfusion chamber designs basically involved three combinations of materials: Lucite acrylic plastic, glass, and metal were combined in various ways with microscope slides and coverslips. Lucite chambers included those of a simple laminar design (172) held together by beeswax or paraffin and those of more intricate designs which required fairly complex machining (146,149,151,164). Although Lucite is easy to machine, it is not durable, it is fragile, and it tends to warp. Furthermore, Lucite cannot be heat sterilized or repeatedly autoclaved without losing its shape or its optical clarity. Lucite chambers may also stress-crack when sterilized with alcohol or other organic solvents. It is difficult to keep the optical surfaces clear and unscratched.

Glass chambers maintain their optical properties and do not easily scratch. Early glass chambers were usually sealed with a wax or cemented together with an epoxy resin (105,153,159). They are delicate and fragile, and they cannot be heat sterilized or autoclaved as an assembled unit. Some units (150,153) involve drilling through glass slides...a difficult process for many machinists.

Metal chambers were essentially frames and clamps used to compress a sandwich of an O-ring and two glass microscope coverslips (133,154,159,161,165,170,174). A spanner ring was generally used for this task. Some
of the metal and glass assemblies were sealed by wax or epoxy (154, 165). While these metal chambers are durable, they are generally difficult to machine since the chamber design must minimize the contact of the metal used with the tissue culture medium. Since most are constructed of stainless steel or chromium plated brass, they are expensive.

A major improvement in perfusion chamber design was reported by Rose in 1954 with the introduction of thick nontoxic gum rubber gaskets (162). All of the popular cell culture chambers since then have tended to utilize his basic design and materials. The Rose chamber is essentially a sandwich of two steel plates that compress two coverslips separated by a gum rubber gasket. Surface tension holds a volume of tissue culture fluid between the coverslips. Syringe needles can be inserted into the gasket for the purpose of perfusion. The chamber is simple to construct, durable, virtually nontoxic, and can be repeatedly heat sterilized and stored. These qualities have secured its popularity with tissue culture workers.

A problem with the Rose chamber arises from the fact that the syringe needles must be inserted through the rubber gasket. A minimum gasket thickness of about 1/8 inch is required - thinner gaskets can lead to breakage of the coverslips. A relatively large volume of fluid - at least 1.90 ml - is therefore necessary (145), and the optical path is relatively long as it is dictated by the chamber thickness. (A very long optical path in the cell culture fluid tends to disperse and diffract the incident cone of illumination coming from a phase contrast condensor (177a).)
A further disadvantage in so far as the requirements of this study are concerned is the fact that the flow in the Rose chamber cannot be easily characterized. The flow conditions are not uniform since the fluid must enter and leave the chamber at localized regions of relatively high velocity near the orifices of the syringes. Furthermore, air bubbles, which become entrapped in the chamber because of its geometry, disrupt the flow within the chamber.

A modification of Rose's design has dealt with reducing the gap thickness (167) but it unfortunately cannot be used for perfusion. Chamber gap thicknesses have been reduced successfully, but at the cost of reverting back to using a wax seal and employing a rather elaborate construction (145). Further modifications of the Rose chamber include the introduction of new materials (171) and the redesign of the gasket to provide a separated gas phase for the purpose of buffering pH in the flow chamber (168). If rubber gaskets are used in a closed chamber, their relative impermeability to oxygen and carbon dioxide helps to maintain a constant pH within the cell culture vessel: Since silicone rubbers are more permeable to carbon dioxide than other rubbers, chambers must be maintained in a 5% carbon dioxide atmosphere (177b). A redesign of the perfusion chamber such that a small quantity of a carbon dioxide rich gas could be entrapped within the chamber without displacing the fluid or altering the flow would be helpful in improving the Rose chamber.

In an effort to reduce leaching in the perfusion chamber technique, a semipermeable cellophane membrane has been added to the sandwich assembly under which the cells grow separated from the flowing medium (169).
This idea has been extensively developed (137). The cellophane strip technique represents an attempt to more rigorously control the cells' microenvironment. However, in testing the effect of pharmacological substances upon cells, the cellophane which prevents leaching of substances away from the cells may also prevent passage of the test substance to the cells. Clearly, it seems preferable to identify the molecular species absent from the cell culture medium and to replenish these species rather than to resort to the use of artificial barriers to diffusion.

An attempt to quantify tissue surface potentials in culture by the introduction of electrodes into the perfusion system has been reported (152). The chamber geometry did not provide a defined flow to the tissues in this chamber.

Only a few cell culture perfusion chambers described in the literature have geometries for which the flow can be easily defined mathematically. By far the simplest is a capillary tube flow chamber (176). In this device the capillary tube was surrounded by a fluid which has the same refractive index as glass so that optical distortion due to non-parallel surfaces was minimized. Nevertheless, objectionable image distortion and loss of contrast and intracellular detail in the published pictures indicate that this geometry would not be suitable for detailed cellular observation with phase contrast optics. (No attempt was made to match the refractive index of the tissue culture medium to that of the glass capillary tube.)
At low flow rates (i.e. Reynolds numbers) several of the described flat chambers with very narrow gap separations (149,160,172,145) may have predictable flow characteristics in portions of their flow channels. Although the flow may be laminar, it may not be uniform or fully developed, and so different regions of the cellular population could be subjected to different flow conditions. The flow characteristics of these chambers have not been described: Various entry region characteristics may occur due to rapid flow (high velocities) through small bore needles (149,160,172,145) and beyond steps (145) in these chambers.

Flow chambers with defined flow have been developed for biological studies other than tissue culture work (81,101,178,179a). They have been used to study the effects of low stagnation upon thrombus formation in the blood (178,179a) and to apply known shear stresses to red blood cells adhering to a glass surface (101). In general, such chambers are unsuitable for tissue culture work, either due to their optical properties or to the fact that they cannot be easily sterilized.

A suitable cell culture flow chamber in which the flow can be precisely defined and verified experimentally was first described by the author in 1969 (111). The chamber is inexpensive, nontoxic to the cells used, and has excellent optical characteristics when used with an inverted stage phase contrast microscope. This chamber will be described next along with further improvements and alterations upon its basic design.
INVESTIGATIONS

Basic Design and Use of Cell Culture Flow Chamber

Figure 1 shows a diagram of the cell culture flow chamber that was designed to provide steady, uniform laminar flow. The test section of the flow chamber consisted of two closely spaced, parallel plates with a channel width of 18 mm. Channel depths varying between 0.25 mm and 1.0 mm could be obtained by using commercially available silicone rubber membranes*. Tissue culture medium entered and left the parallel plate channel through entrance and exit reservoirs. The reservoirs were elevated for two reasons: 1) to entrap small gas bubbles which can adhere to the glass, coalesce, and disrupt the uniformity of the flow, and 2) to provide a gas reservoir over the culture fluid. As the fluid entered the entrance region it flows through an effective constriction in cross sectional area in order to provide stability of flow conditions. The inlet and outlet ducts were set perpendicular to the flow direction in an attempt to reduce fluid 'jetting' in the entrance region to the channels. Two flow channels were accommodated in each chamber assembly. One of the channels was used as a control to observe the growth of cells under no-flow conditions.

In use, the sterilized cell culture chamber was inoculated with the particular cell strain of interest in such a manner that the dispersed

individual cells attached to the lower transparent wall* of the channels. When the cells attached to the surface, they grew and multiplied, forming a confluent monolayer of cells covering the entire bottom of the flow channel (refer to Figure 2). The process generally took anywhere from 48 to 72 hours depending largely upon the particular cell strain used. Madin Darby Bovine Kidney (M.D.B.K.) permanent strain cells (shown in Figure 3), Pig Kidney permanent strain 15 (P.K.-15) cells (shown in Figure 4), and primary cultures of human umbilical vein endothelial cells have been grown in these flow chambers.

Flow of tissue culture medium over the cells growing on the coverslip approximated flow between infinite parallel plates. A channel width/height (W/H) ratio of greater than 4 or 5:1 is often used to simulate this geometry. In the cell culture channel used in this study W/H ratios ranged between 18 and 72:1 so that the assumption of the infinite parallel plate geometry appears to be justified. With a channel height of 0.5mm and a flow rate of 1.23 cc/min, the Reynolds number, \( R_e = \rho HV/\nu \), was 0.0163 (\( \rho \) is the fluid density and \( V \) is the mean velocity). This value is well below that generally accepted as the critical transition Reynolds number for flow between parallel plates. The cell-height/channel depth ratio for all of the cells used was between 0.01 and 0.02. The fluid mechanics of the infinite parallel plate geometry is reviewed in Appendix C, and the relationships between the flow parameters in the cell

*24mm X 60mm, No. 2 thickness microscope coverslips. Corning Glass Works, Corning, N. Y.
culture flow channels are shown in Table 1 and Table 2.

In the flow chamber with a channel height of 0.5 mm the shear rates (i.e. velocity gradient) at the wall could be varied from much less than one to greater than 36 sec\(^{-1}\). The viscosity of the tissue culture medium used could be increased considerably from its normal value by adding bovine serum albumen. The shear stress applied at the face of the cells could thus be varied while maintaining the same flow rate and velocity gradient at the wall. By decreasing the width of the channel (and sacrificing the large \(w/H\) ratios) shear rates in excess of 1500 sec\(^{-1}\) were produced with the above chamber assembly.

Uniform flow across the width of the channels was verified by injecting India Ink and photographically recording the positions of the horizontal fronts of the dye at given time intervals. The distance between similar positions on the dye fronts did not vary over the length or the width of the flow channel. The dye fronts were never flat due to entrance effects.

A parabolic velocity profile across the channel height was verified by injecting Latex microspheres* into the tissue culture fluid and noting their velocities at selected depths in the channel. A typical velocity profile that was observed is shown in Figure 5 and clearly approximates the parabolic condition. Some jetting was observed near the entrance

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region where this profile was made, as is seen in the figure, but importantly, the variation of the velocity gradients near the wall from that for a parabolic distribution seems negligible. The flow characteristics in the vicinity of the cells was studied by photographing or timing particle translations in a horizontal lamina of a known thickness of fluid at a known depth in the flow channel, as discussed in Part II of this manuscript. The precision of this method for obtaining velocity profiles is directly related to the depth of focus (Δh) of the microscope objective and the magnification of the microscope eyepiece used. For the 40X N.A. 0.65 microscope objective and 15X eyepiece used to obtain this profile, the theoretical depth of focus is between one and two microns (179b, p. 33). For proper depth positioning in the flow channel it was necessary to known the refractive index of the fluid (179b, p. 36). By comparing the angles of incidence and refraction of a light beam projected into a reservoir of tissue culture medium, the refractive index was determined to be 1.35.

Materials Used in Cell Culture Flow Chambers and Variations in Construction

The bottom retaining plate in all of the cell culture flow chambers consisted of 1/8 inch brass with rectangular milled openings for microscopic observations. Similarly, No. 2 thickness microscope coverslips were utilized in all of the chambers constructed: Thinner coverslips tended to bow out objectionably at high rates of flow. Bowing of thin
coverslips could have otherwise caused an appreciable error in the calculation of the velocity gradients in the flow channel. For example, at a predicted shear rate of 0.258 sec$^{-1}$ in Figure 5, the measured shear rate was 0.25 sec$^{-1}$. However, when the flow rate was increased by a factor of three times, a shear rate of 0.83 sec$^{-1}$ was expected whereas a value of 0.67 sec$^{-1}$ was experimentally observed (No. 1 thickness coverslips were used in this instance). In spite of the use of the thicker coverslips, very satisfactory resolution and contrast of intracellular detail were achieved as demonstrated by the photomicrographs in Figures 3 and 4.

A variety of materials and configurations in chamber construction were utilized once the simple fluid mechanics of the basic flow channel design were confirmed. The basic alteration in all of the subsequent chambers essentially consisted only of modifications in the construction and the materials used in the flow chamber's top plate (Figure 6). Three variations in the chamber's top plate construction were made in implementing the basic flow channel design contained within the prototype flow chamber described in 1969 (111).

In the prototype chamber, the top plate consisted of a bonded unit assembly consisting of a 1/4 inch thick Lucite acrylic plastic plate, two glass microscope slides* 1mm in thickness, and four stainless steel inlet ducts made from number 16 gauge syringe needles. The Lucite plate had two

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*Precleaned Goldseal Microscope Slides, tolerance 0.97 to 1.07mm. Clay Adams, Inc. New York, New York.
rectangular openings, over which the microscope slides were mounted such that the bottom of the plate remained flush. A room temperature vulcanizing silicone rubber adhesive* was used to bond the cut slides into milled excavations on the bottom of the plate. The stainless steel tubes used for the entry and exit ducts were mounted with an epoxy cement, and the milled surfaces of the Lucite plate and epoxy exposed to the tissue culture fluid were coated with a thin layer of the silicone rubber adhesive.

In all of the further modifications of the prototype chamber, the entrance and exit ducts permanently bonded into the top plate were discarded in favor of removable hollow stainless steel screws. The screws were drilled hollow with a .055 inch drill. The ends of the screws were milled smooth for 1/4 inch to accept the perfusion tubing (refer to Figure 1c and Figure 6 for detail), the external diameter corresponding to a 15 gauge syringe needle (.080 ± .005 inch) so that the commercially available perfusion tubing** used in these studies fit snugly. By using these stainless steel removable entrance ducts the use of epoxy was eliminated. Silicone rubber washers were used to affect a seal between the screw and the top plate, and these were made from the silicone rubber gasket material cited previously.


**Silastic Medical Grade Tubing, .062 inch I.D. and .125 inch O.D. Dow Corning Corporation, Medical Products Div., Midland, Mich.
In an effort to simplify the machining necessitated by the milling and flush mounting of the microscope slides into the top plate, a simpler chamber design was implemented. It consisted of a simple flat Lucite top plate 1/4 inch thick into which entrance regions had been milled. Holes were drilled to accommodate the removable screw entrance and exit ducts. Because of the optical clarity of the Lucite, the top plate required no provisions for the milled observation ports or glass slides. One of these chambers is shown in Figure 7b. The simple Lucite plate chamber represents the first alteration upon the prototype design.

A second alteration in the prototype chamber was the introduction of 1 cm thick polycarbonate plates* in place of the clear Lucite. Since polycarbonate is not optically clear, microscope observation holes and bonded flush mounted microscope slides had to be reincorporated into the top plate design. Polycarbonate flow chambers are illustrated in Figures 6 and 7c.

Thirdly, chambers were constructed in which the plastic top plates were replaced by a 3 X 1 1/8 inch glass plate that was either 3/16 or 1/4 inch thick**. Holes for the entrance regions were drilled and shaped in both ends of the plate by using diamond tipped dental bits†. An

*Cadco Lexan @ Polycarbonate Plates, 3/8 inch thick. Cadillac Plastics and Chemical Company, Davenport, Iowa.

**Precut Corning Pyrex @ Polished Glass Plates. Fred S. Hickey, Co., Schiller Park, Ill.

†Iowa Dental Supply Co., Des Moines, Iowa.
assembled glass cell culture flow chamber is illustrated in Figure 7a. A second silicone rubber gasket was utilized in these chambers between the glass top plate and the bottom of the upper brass plate that was used to sandwich the chamber components together. This second gasket markedly reduced the incidence of glass breakage in the upper plate during the chamber compression.

Cell Culture Profile Flow Chamber

In the cell culture flow chambers described in the last section, the flow and the cells were viewed from the horizontal planar aspect. In order to study the flow over the cells in more detail and to study the cells' mechanical interaction with their physical environment and substrate, a cell culture profile flow chamber was developed.

An assembled cell culture profile flow chamber is shown in Figure 8a. The infinite parallel plate geometry in this chamber was created by closely approximating the flat edges of two thick Lucite plates. The ends of the flow channel and its depth was determined by two silicone rubber 'end' gaskets 1mm thick which were compressed by the polished edges of the Lucite plates. Microscope slides formed the sides of the flow channel. The 'front' slide had two stainless steel entrance ducts screwed into it as shown in Figure 9. Two silicone rubber gaskets were compressed between the glass microscope slides, the apposed Lucite plates, and the edges of the two 'end' gaskets at the end of the flow channel. Since tissue culture cells do not readily adhere to a Lucite surface, a
A microscope slide cut to the proper dimensions was flush-mounted in the top edge of the bottom Lucite plate (Figure 8b). It is possible to substitute any desired test material in place of the glass substrate in this chamber. Two brass plates held the several components together.

The chamber was assembled by first lightly screwing the brass plates together with the components in their proper position. Two large 'C' clamps were used to uniformly compress the edges of the plastic plates, and the brass side plates were then tightened securely and the 'C' clamps removed. The chamber was sterilized by autoclaving in the lightly-assembled state.

The cells in the cell culture flow profile chamber were viewed from the side rather than from the top. For this purpose the chamber was mounted on a horizontally oriented microscope fitted with a 10X, N.A. 0.25 objective as shown in Figure 10. For the best 'optical' results, the surface of the front microscope slide was oriented perpendicular to the axis of the microscope objective. Since the principle of observation (refer to Figure 11) requires that light from the condensor be reflected from the surface of the glass substrate into the microscope objective, the flow channel was oriented at an angle (viz. 'reflectance angle') with respect to the axis of the microscope objective. Four profile flow chambers were constructed: They represented all the possible combinations between 6mm and 12mm thick plastic plates and 5 and 10 degree reflectance angles. Considering the rhomboidal cross section of the flow channels and the added width due to the Silastic side gaskets, the channel w/H ratios were 7:1 and 13:1 respectively.
In use, the cells were allowed to settle, attach and grow on the glass substrate of the bottom Lucite plate. A 10X microscope objective was used to view the cells from the side. It was found that this objective lens has sufficient working distance - 16mm - to allow focusing on cells in the middle of even the widest of the flow channels used. The theoretical depth of focus for a 10X, N.A. 0.25 objective lens when used with a 15X eyepiece was estimated to be 25 microns (179b, p. 33), so that the microscope focused upon a vertical slice of fluid 25 microns thick and inclined either 5 or 10 degrees with respect to the vertical axis. Since the velocity profiles could be observed directly in this flow channel, it was not considered necessary to be able to accurately predict the velocity gradient.

Examples of profile views of M.D.B.K. cells growing in the cell culture flow profile chamber are shown in Figure 12. Figure 13 shows the profile appearance of the same cells grown in a capillary tube* 'flow chamber'. Noticeable optical distortion was apparent in the latter device.

*Kimax capillary tube, 1.6-1.8 X 100mm. Kimble Products, Glass and Plastic Laboratory Ware, Toledo, Ohio.
GENERAL OBSERVATIONS

Cellular growth of P.K.-15, M.D.B.K., and primary cultures of human endothelial cells flourished in all of the cell culture flow chambers constructed as evidenced by Figures 2, 3, 4, and the control endothelial cultures shown in Part III of this manuscript. Permanent strain tissue culture cells generally became confluent within 48 hours: Variations occurred due to the plating density of the cell suspension. The plating density in these experiments was maintained between 25,000 and 40,000 cells per square centimeter. The endothelial cell cultures showed much more variation in the time required for confluency: In stable cultures confluent outgrowths occurred anywhere from 72 to 96 hours.

Sterility was a major problem in the prototype chamber. As the chamber design was subsequently simplified and the number of materials and bonded junctions reduced, the incidence of contamination became markedly reduced to the point where it was not a problem. Because epoxy was utilized in the construction, the prototype chambers could not be autoclaved but had to be chemically sterilized with alcohol. It is thought that contamination in these chambers resulted from the presence of residual microorganisms sequestered in microscopic cracks at the epoxy-plastic-steel bond sites. In such small cracks microbes could have resided relatively protected from the germicidal action of the biodegradable detergent* used to clean the chambers and from the action

of the alcohol during the sterilization procedure*. Furthermore, the alcohol and other organic solvents used to sterilize and clean the chambers tended to cause stress cracking at the machined surfaces of the plastic thus creating more regions where microscopic organisms could accumulate. The useful lifespan of the prototype chambers was therefore reduced. The incidence of flow chambers contaminated before the inoculation of the cells suspension was markedly reduced when the removable stainless steel screw ducts were introduced and the epoxy bond eliminated.

The internal diameter of the screw entrance ducts was made as wide as structurally feasible (.055 inch), approximating the internal diameter of a 16 gauge hypodermic needle. By maximizing this diameter the velocities at the entrance region were correspondingly reduced. Nevertheless, some jetting was present as seen in Figure 5, but it was not critical to the calculation of the wall shear stress. Furthermore, since a major purpose of the chamber was to apply and study the effect of known shear stresses upon cells, (refer to Part III of manuscript) it is desirable to maximize the internal diameters of the entrance ducts in order to reduce the shear stresses to which the cells in suspension are subjected when they are inoculated into the chamber. (However, no difference in the growth rate of P.K.-15 cells injected into a Petri dish was noted when 16, 18, and 21 gauge hypodermic needles were used to inoculate the suspensions.)

*70% Ethanol for six hours, followed by washing with sterile, deionized water.
Due to the dispersion of the illuminating cone of light from the phase contrast condenser annulus, a loss of contrast in intracellular detail could be discerned when using the thicker Lucite plate chambers. However, the loss in contrast was not objectionable when using the lower power (10X) objective. The ease of machine tooling and unit construction seemed to warrant the slight loss in optical quality when low magnification work was desired. Chambers of 1/4 to 3/8 inch thick Lucite plastic could be autoclaved without warping. Due to its ability to withstand more heat than Lucite, the polycarbonate chambers could be autoclaved repeatedly.

Since autoclaving may result in the deposition of minute oil residues from steam lines it was desirable to reduce this possibility. Those chambers which were autoclaved were first filled with deionized water, so that the flow channel was filled with steam produced from the deionized water rather than the autoclave lines. This procedure seemed to work quite well, as no difference in cellular attachment could be discerned between those chambers that had been autoclaved by this process and those that had been dry-heat sterilized. Because of the confidence in the technique, it was desirable to ultimately be able to dry-heat sterilize* the cell culture flow chambers. In this regard the all-glass chambers proved to be very satisfactory, as the Lucite plastic can not withstand the 160° C temperature required in the dry-heat sterilization process.

*160° Centigrade for 90 minutes.
The glass chambers were best suited for low and medium power magnification work with a separable long working distance condensor.

The tissue culture medium* used in these experiments requires an atmosphere of 5% CO₂ in air since it contains a bicarbonate buffering system. For this reason elevated reservoirs were introduced at the entrance and exit regions of the flow channels. As the gas-sparged M.E.M. entered the flow chamber which was maintained in a 37° C. incubator, the fluid was warmed and the solubility of the gas decreased. The gas bubbles that might have otherwise adhered and coalesced between the parallel plates were instead trapped within the elevated reservoirs. The reservoirs thus performed the dual function of maintaining uniformity of the flow between the parallel plates and providing a gas interface for pH buffering within the flow channel.

The relationships between the fluid mechanical parameters within the chambers are given in Table 1 and Table 2. For all the flow rates employed in this study the Reynolds numbers were relatively low. Laminar flow was therefore expected at all of the flow rates employed, and the equations used to predict the wall shear rates within the flow channels should have been valid.

In using the cell culture profile flow chambers, it was found that the 6mm thick Lucite plates with reflectance angles of 5 and 10 degrees gave the best cellular images (refer to Figure 12). The 10 degree channel

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was the easier to optically align for the best contrast and cellular
definition. This might be expected when the principle of operation is
considered. In the 5° channels the larger number of nondiffracted light
rays reaching the objective lens tend to wash out the cellular detail
created by the rays diffracted by the cells.

Cellular definition was also reduced when the cell population in the
profile flow channels became too large. In order to produce a good image
it was necessary to have clean reflective areas both behind and in front
of the cells being observed. For this reason the cell culture profile
flow chamber is best used for situations in which individual cells or
small cellular colonies are to be studied rather than fully confluent
monolayers. Any substance that has been polished to a suitable level of
reflectance could be substituted for the glass substrate in these cham-
bers. Thus, the cellular profiles and attachment could be observed on
substrates other than glass.

It is interesting to note the differences in profile appearance
between cells grown in the profile flow chamber and the same cells grown
in a simple capillary tube. Optical aberrations undoubtedly occur in
the latter device as is seen in the 'bunched' appearance of the cells in
Figure 13.

In both the cell culture flow chambers and the profile flow chambers
the membrane gasket thickness will influence other experimental parameters.
For example, as seen in Table 1, for a selected wall shear stress thinner
gaskets will provide flow with lower Reynolds numbers. Also, by using
thinner gaskets the width/height (w/H) ratio is elevated and the channels
more closely approximate the infinite parallel plate configuration. At
the same time, however, a thinner gasket will adversely affect the cell
height/channel depth ratio and the plating density of the cell suspension
injected into the chambers. Other considerations must be taken into
account when selecting a particular channel thickness in perfusion
experiments. A thinner gasket will allow maintaining a desired shear
stress for a longer period of time before the fixed volume of an infusion-
withdrawal pump empties; yet the control culture of cells are provided
with a proportionately smaller volume of fluid with which to buffer
environmental changes in a no-flow channel.
COMMENTS AND SUMMARY

A simple laminar design was utilized in the construction of a cell culture flow chamber in which the flow was precisely defined and experimentally verified. The actual flow conditions were seen to agree with those calculated from the appropriate fluid mechanical considerations.

Three chamber constructions were offered.

The simplicity of the laminar design confers a flexibility of the cell culture flow chamber's use. The geometry of the flow channel can be altered simply by cutting new shapes into the silicone rubber gasket: In this way a nonuniform flow conditions may be applied to the cells. For example, by changing the width of the flow channel along its length, an accelerating or decelerating flow situation can be produced such that a gradient of wall shear stress (and pressure) exists within the flow channel. Therefore, a cellular response to a variety of fluid mechanical conditions could be studied using this chamber design.

It is suggested that the simple laminar design of the cell culture flow channel also allows the study of cellular interaction with mass transfer phenomena. The defined fluid mechanics of the flow in this chamber would be the first step in such a study.

Importantly, the chambers also allow surface visualization of the cultured cells and excellent phase contrast microscopy of intracellular detail concurrent with the application of the known flow conditions. Thus, the described cell culture chamber is ideally suited for studying a cellular response to well defined flow conditions.
A cell culture profile flow chamber was described in which the cells could be viewed from the side as they were being subjected to known mechanical stresses. Due to the design of the chamber's bottom plate, it is possible to view the cellular profile as they are attached to any polished foreign substrate. In addition, a catalogue of the cellular profile behavior might be of use in other cell culture chambers with defined flow...such as constrictions in capillary tubes...to study the responses of cells to mechanical stresses when they can not be observed directly without image distortion.
Figure 1. Basic cell culture flow chamber design
A. SIDE VIEW OF FLOW CHANNEL

B. TOP VIEW OF ASSEMBLED CHAMBER

C. DETAIL OF ENTRANCE REGION
Figure 2. Confluent monolayer of M.D.B.K. cells, surface contour, Oblique illumination, 20X objective lens, N.A. 0.45 (total magnification approximately 500 times)

Figure 3. M.D.B.K. cells, (-) Phase contrast, 20X objective lens, N.A. 0.45. Depth of focus 5-8 microns. (Particle streamlines emphasized by dashed lines) (Total magnification approximately 480 times)
Figure 4. P.K.-15 cells, (+) Phase contrast, 40X objective lens, N.A. 0.65. (Total magnification approximately 640 times)
Figure 5. Velocity profile in flow channel ($\Delta h$ equals depth of focus of microscope objective)
<table>
<thead>
<tr>
<th>Gasket thickness</th>
<th>( \tau_w = 10^{-3} )</th>
<th>( \tau_w = 10^{-2} )</th>
<th>( \tau_w = 10^{-1} )</th>
<th>( \tau_w = 1.0 )</th>
<th>( \tau_w = 10 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2h = H</td>
<td>( \gamma = 0.14 )</td>
<td>( \gamma = 1.4 )</td>
<td>( \gamma = 14 )</td>
<td>( \gamma = 140 )</td>
<td>( \gamma = 1400 )</td>
</tr>
<tr>
<td>1. 2h = 1.0 mm (W/H = 18)</td>
<td>Q ( = .025 )</td>
<td>Q ( = .25 )</td>
<td>Q ( = 2.5 )</td>
<td>Q ( = 25 )</td>
<td>Q ( = 250 )</td>
</tr>
<tr>
<td></td>
<td>( R_e = .031 )</td>
<td>( R_e = .31 )</td>
<td>( R_e = 3.1 )</td>
<td>( R_e = 31 )</td>
<td>( R_e = 310 )</td>
</tr>
<tr>
<td>2. 2h = 0.5 mm (W/H = 36)</td>
<td>Q ( = .00635 )</td>
<td>Q ( = .0635 )</td>
<td>Q ( = .635 )</td>
<td>Q ( = 6.35 )</td>
<td>Q ( = 63.5 )</td>
</tr>
<tr>
<td></td>
<td>( R_e = .00794 )</td>
<td>( R_e = .0794 )</td>
<td>( R_e = .794 )</td>
<td>( R_e = 7.94 )</td>
<td>( R_e = 79.4 )</td>
</tr>
<tr>
<td>3. 2h = .25 mm (W/H = 72)</td>
<td>Q ( = .0016 )</td>
<td>Q ( = .016 )</td>
<td>Q ( = .16 )</td>
<td>Q ( = 1.6 )</td>
<td>Q ( = 16 )</td>
</tr>
<tr>
<td></td>
<td>( R_e = .0020 )</td>
<td>( R_e = .020 )</td>
<td>( R_e = .20 )</td>
<td>( R_e = 2.0 )</td>
<td>( R_e = 20 )</td>
</tr>
</tbody>
</table>

**B. Vertical cell culture flow profile chambers: 2h = 1.0 mm**

| 10°, w = 7 mm (W/H = 7) | Q \( = .010 \) | Q \( = .10 \) | Q \( = 1.0 \) | Q \( = 10 \) | Q \( = 100 \) |
|                        | \( R_e = .032 \) | \( R_e = .32 \) | \( R_e = 3.2 \) | \( R_e = 32 \) | \( R_e = 320 \) |
| 5°, w = 13 mm (W/H = 13) | Q \( = .019 \) | Q \( = .19 \) | Q \( = 1.9 \) | Q \( = 19 \) | Q \( = 190 \) |
|                        | \( R_e = .032 \) | \( R_e = .32 \) | \( R_e = 3.2 \) | \( R_e = 32 \) | \( R_e = 320 \) |

*Explanation of symbols: \( \tau_w \), shear stress at wall in dyne/cm²; \( \gamma \), velocity gradient (shear rate) at wall in sec⁻¹; Q, flow rate in cc/min.; \( R_e \), Reynolds number \( \rho DV/\mu = \rho Q/\mu w \). The kinematic viscosity, \( \mu/\rho \) for M.E.M. tissue culture medium was determined to be \( .0074 \text{ cm}^2/\text{sec.} \), and is used for the above calculations. (See Part III) The relationship between \( \tau_w, \gamma, Q, w, \) and \( h \) is reviewed in Appendix C.*
Table 2. Relationship between shear rate ($\dot{\gamma}$) and flow rate ($Q$) in cell culture flow channels*  

<table>
<thead>
<tr>
<th>Cell culture flow chambers</th>
<th>Shear rate (velocity gradient) expected in chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Gasket thickness,</td>
<td>$\dot{\gamma} = 5.5 \text{ (min/sec}\cdot\text{cm}^3) \cdot Q \text{ (cm}^3/\text{min)}$</td>
</tr>
<tr>
<td>$2h = 1.0 \text{ mm} = H$</td>
<td>$w/H = 18$</td>
</tr>
<tr>
<td>2. $2h = 0.5 \text{ mm}$</td>
<td>$\dot{\gamma} = 22 \cdot Q$</td>
</tr>
<tr>
<td>$w/H = 36$</td>
<td></td>
</tr>
<tr>
<td>3. $2h = 0.25 \text{ mm}$</td>
<td>$\dot{\gamma} = 89 \cdot Q$</td>
</tr>
<tr>
<td>$w/H = 72$</td>
<td></td>
</tr>
</tbody>
</table>

| Cell culture profile        |                                                 |
| flow chambers               |                                                 |
| 1. 10° reflectance angle   | $\dot{\gamma} = 14.3 \cdot Q$                  |
| $w/H = 7$                  | $H = 1 \text{ mm}$                             |
| 2. 5° reflectance angle    | $\dot{\gamma} = 7.4 \cdot Q$                  |
| $w/H = 13$                 | $H = 1 \text{ mm}$                             |

*The relationship between the shear rate and the flow rate is given by $\dot{\gamma} = 3Q/(2wh^2)$, as discussed in Appendix C.
Figure 6. Cell culture flow chamber components
(reduced to approximately 0.6 times actual size)
a. silicone rubber gasket
b. stainless steel screw entrance ducts
c. top plate
d. flush-mounted microscope slides
e. outline of coverslip

Figure 7. The three types of flow chambers constructed

b. Lucite acrylic plastic

a. all glass

c. polycarbonate
Figure 8. Cell culture profile flow chamber

a. Front view of assembled chamber

b. Lucite plates, showing flush-mounted glass microscope slide on lower plate

Figure 9. Components of cell culture profile flow chamber: a. assembled chamber; b. Lucite plates with end gaskets; c. front microscope slide with removable screw entrance and exit ducts; d. back gasket with rear microscope slide; e & f. front and rear brass retaining plates

   a.          b.
   c.          d.
   e.          f.
Figure 10. Use of cell culture flow profile chamber

Figure 11. Diagram of illumination in cell culture profile flow chamber, $\alpha$ indicating the reflectance angle - that angle from which the cells are viewed in the microscope objective
silicone rubber
light source
microscope slide
in a glass substrate
Figure 12, a & b. Cells growing in cell culture profile flow chamber. (M.D.B.K.) Lines indicate 100 microns. (Total magnification approximately 200 times)

Figure 13. Profile view of cells indicated by arrows. (Total magnification approximately 200 times)
PART II. VISUALIZATION OF THE MICROSCOPIC FLOW

OVER CELLS IN CHAMBER
REVIEW OF LITERATURE

'Macroscopic' flow conditions have been reviewed in the introduction to this manuscript which may 1) influence the biology of the arterial wall, and 2) induce atherogenesis. However, the nature of the flow must also be considered at the microscopic level when the cellular mechanisms in these processes are discussed. Similarly, in the study of a cellular response to flow, the nature of the flow immediately next to the cells should be described. Translations of particles flowing over tissue culture cells have been described by the author and coworkers (112), but these observations need to be refined. The purpose of this study is to more precisely describe the flow over the cells by using more refined flow visualization techniques.

Little work has been done in studying the flow conditions adjacent to the endothelial cells lining the cardiovascular system (36, Pp. 134-135). It has been postulated that pathologically swollen endothelial cells could disrupt the streamlines in the adjacent blood flow, and that the resulting microscopic flow regimes could induce further cellular changes (180a). It has also been suggested that submicroscopic ridges seen in surface scanning electronmicrographs of endothelium assist in 'rifling' the adjacent blood flow (180a). Endothelial cells round-up in response to irritations (31,83a,83b), but it is not known how their altered geometry affects the neighboring flow. There are no studies about the effect of cellular projections upon the flow with which to evaluate such speculations.
Model studies can be of assistance in analyzing the effect that cells may have upon the adjacent flow. Studies on the effect of wall projections upon steady flow in rigid tubes suggest that the geometric parameters of interest are the height of the cellular projection, the radius of curvature of the cell, and the Reynolds number (47). Unlike that study (47) however, the ratio of the heights of cellular projections to the height of the flow channel (or the diameter of an artery) will be very small. Thus, the total cross sectional area of the flow channel will be minimally restricted by the presence of the cellular projection, and the average velocity in the flow would not be affected by the cells.

Due to the 'no-slip' condition at the boundary, a velocity gradient will exist at the wall. The cells will project into this gradient. Thus, disturbances in the flow created by the cells may be related to both the heights of the cells and the magnitude of the velocity gradient.

It would be of interest to determine how far the disturbance caused by the cellular projections extends into the flow field. One possible use of such observations would be to help assess the amount of convective transport normal to the cells. Attempts should be made to observe the flow over the cells from both the horizontal and the vertical aspects. The pertinent geometrical parameters of the cell should be recorded.
Equipment, Methods, and Procedure

Horizontal flow visualization in cell culture flow chamber

The microscopic flow conditions over the cells were studied from the horizontal aspect in the cell culture flow channel described in Part I of this manuscript. Suspensions of M.D.B.K. cells (250,000 to 400,000 cells per milliliter) were injected into the sterilized chambers, and the cells were allowed to settle and attach to the lower coverslip of the chamber's flow channel. The cells were allowed to grow and replicate until they had formed isolated colonies over the surface of the glass substrate. This generally took between 15 and 20 hours for channels that were 1 mm in depth. The flow of cellular debris and of suspensions (less than 1%) of Latex microspheres in tissue culture medium was then observed with an inverted stage phase contrast microscope*. Although any of the objectives could be used for the visualization of the flow patterns, the use of the 10X, N.A. 0.25 and the 20X, N.A. 0.45 phase contrast objectives gave the best results. The microscope was equipped with a 37°C warm stage constructed from a 1/8 inch thick brass plate and an electric patch heating element ordered to specifications**. The heating element was powered and controlled by using a proportional


control integral heating circuit*. The perfusion system and tissue culture medium that were used are described in more detail in Part III of the manuscript. An infusion-withdrawal pump** was used to insure steady flow.

Three procedures were used to document the flow over the cells: 1) The particle translations of the Latex microspheres were photographed on Panchromatic film†; 2) The flow of the microspheres was recorded on videotape++ and replayed for inspection and analysis; and 3) The position of the microspheres at known time intervals was photographed by using a stroboscopic illuminator# coupled to the phase contrast microscope. The equipment used to videotape-record the flow of the microspheres is described in the next section.

**Vertical flow visualization in cell culture profile flow chamber**

The microscopic flow over the cells was visualized in the vertical aspect by employing the cell culture profile flow chamber described in Part I of the manuscript. M.D.B.K. cells were injected into the sterilized 6mm, 10° reflectance angle profile flow chamber. They were allowed to

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*Precision Temperature Control, General Electric Triac Handbook.


#Strobonac Type 1531-A. General Radio Co.: Concord, Mass.
settle, attach, and grow on the lower glass substrate. During this time the chamber was maintained in a 37°C tissue culture incubator*. Since complete cellular confluency degrades the optical contrast within the profile chamber, the flow over small groups of cells was studied. The flow over the cells was best studied from 10 to 15 hours after inoculation of the cell suspension. At this time the chamber was removed from the incubator and placed on a microscope as shown in Figure 10. Latex microspheres suspended in tissue culture medium were allowed to flow over the cells, again the flow being regulated by an infusion-withdrawal pump.

The experimental setup for collecting and recording the flow visualizations is shown in Figure 14. A television camera** with its lens removed was aligned with the horizontally oriented microscope. A 10X N.A. 0.25 objective lens was used on the microscope as this lens afforded sufficient working distance (about 16mm) with adequate resolving power. A 15X Kellner eyepiece was used to project the image onto the light sensitive grid of the television camera. A 1 inch videotape recorder+ was used to record the flow data onto the videotape. A television monitor coupled to the videotape recorder was used to locate the microscopic field and to focus the system.

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**Model 605 camera. Raytheon Learning Systems Co., Michigan City, Indiana. (Resolution = 300 lines.)

+Sony Videocorder® EV-210 1 inch. Sony Corp., Tokyo, Japan. Distributed by SuperScope, Sun Valley, California.
Since the total magnification through this system is difficult to calculate, a stage micrometer* was positioned on the microscope stage and the image was then projected onto the television screen. The image was then traced onto clear acetate sheets overlaid upon the television screen (Figure 15). To determine magnification when the results were recorded photographically, the micrometer grid was simply photographed along with the rest of the field.

The maximum height of vertical disturbances in particle translation over the cells was determined on videotape playback. In addition, the position of the microspheres at given time intervals was noted by replaying the videotape in a slow motion, sweep-by-sweep mode. The position of the microspheres was recorded on acetate sheets at intervals of four sweeps of the television scan. The time interval per sweep was determined by recording the motion of a stop watch and replaying it in the same fashion.

GENERAL OBSERVATIONS

Small particles of cellular debris near living cells were affected by random Brownian vibration in the no-flow condition. The amplitude of this vibration was seen to be less than one micron. Characteristic cell lengths ranged between 50 and 100 microns. Cellular heights (measured in the cell culture profile flow chamber) ranged between 10 and 20 microns. Therefore, this random vibration was seen to be less than 2% of the characteristic cell length and less than 10% of the characteristic cellular projection height.

When Latex microspheres (6 - 14 microns in diameter) were injected into the medium flowing over the cells it was possible to focus the microscope upon horizontal laminae of these particles. Using a 20X, N.A. 0.25 microscope objective, the effective vertical thickness (Δh) of the layer observed is between five and eight microns (179b, P. 33). As the microscope was focused towards the cells, a limiting height was reached where these horizontal laminae no longer existed. When the 40X objective lens (Δh equal to one to two microns) was focused upon small particles of biological debris in the medium, it was seen that at a shear rate of 90 sec\(^{-1}\) this height is between five and six microns from the cell membrane.

Figure 3 shows a colony of M.D.B.K. cells in which the translation paths of Latex microspheres were photographed. Convective acceleration was seen over the cells at 'A', and a region of abrupt deceleration of the microspheres beyond the colony was seen (at 'B'). This behavior was
particularly noticeable with shear rates exceeding 100 sec$^{-1}$. The depth of focus in Figure 3 was less than ten microns.

Stroboscopic illumination was employed in an attempt to more precisely visualize the particulate flow over the cells (refer to Figures 16 and 17a). At very low shear rates (6.8 sec$^{-1}$) particle acceleration was seen over the colonies of M.D.B.K. as shown in Figure 16a (refer to streamline 'A'). Deceleration was seen beyond the cells as shown by streamline 'B'. Figure 16b shows the effect of doubling the shear rate upon the acceleration and deceleration of the microsphere particles as they flowed over the cellular projections: Little difference was apparent at these low shear rates. In Figure 16c the strobe illuminator was used with a 10X, 0.25 N.A. phase contrast objective in which the effective depth of focus was increased to 25 microns (179b, P. 33). Changes in the horizontal components of the microspheres' velocity were not apparent at this height over the cells.

Darkfield stroboscopic illumination gave a sharper image than phase contrast stroboscopic illumination as shown in Figure 16d. Using a high stroboscopic frequency produced the imagery of 'trains' of particles as they streaked across the microscopic field at the intermediate shear rates (i.e. from 10 to 100 sec$^{-1}$). This is illustrated in Figure 17a.

Vertical deflections of the particles as they flowed over the cells were visualized in the cell culture profile flow chamber. By tape recording the flow over the cells and then replaying the data in the slow motion, sweep-by-sweep mode, it was possible to visualize the positions
of the Latex microspheres at discrete time intervals. The time interval for each sweep of the videotape recording system was determined to be 1/60 second, and so by selecting the particle positions at four sweep intervals a period of .066 second between particle positions was obtained. (Virtually any period can be selected as the velocity of the particles and convenience dictate.) Figure 17b, a, and d illustrate tracings made from acetate overlays of the positions of Latex microspheres flowing over the cells.

In all of the tracings it is apparent that the paths of microspheres flowing within 30 microns of the substrate were affected by the cellular projections. The velocity gradient is apparent in these tracings. Not all of the particles were seen to be interact with the cells. However, it must be recalled that in these studies the orientation of the focal plane is ten degrees with respect to the vertical axis of the cells, and the depth of focus of the focal plane is 25 microns. Thus, particles may be visualized which are not directly 'over' the cells.
COMMENTS AND SUMMARY

When considering the similarity of the microscopic flow observed in this study with that in the circulatory system, the question arises as to the geometric similarity of the cells pictured in Figures 2, 3, and 12 to those which line the arteries. In the larger arteries (i.e., those with large arterial radii with respect to cellular dimensions) and radius of curvature of the bases of individual cells becomes large and the situation approaches that of a monolayer of cells growing on a flattened surface. It should be recognized that endothelial cells are generally more flattened in appearance than those cells shown in Figure 12. However, due to chronic mechanical or biochemical stress they may round-up (31,58,83b) so that their geometry may approximate that of the M.D.B.K. cells in this study. A major factor which complicates the analysis of blood flow at the microscopic level is that blood is a suspension of nonrigid particles*.

*Steady flow of blood in vitro has been shown to be nonuniform and unsteady at the microscopic level (180b), and this microscopic behavior has been ascribed to the interaction of particles flowing in high shear gradients. If the flow of rigid particles is studied, they can be seen to 'dance' in and out of the region adjacent to a smooth wall (181a). The unpredictable particle translations described in these studies indicate that a complex shear stress distribution may be present at the wall of arteries due to the aberrant behavior of the red blood cells. (In smaller vessels a 'plasmatic gap' may exist (181b).) The effect of the wall, projections on the wall, and the interaction of particles suspended in the medium must all be considered important when the microscopic flow next to the endothelium is considered. When flow visualization techniques are used which employ Latex microspheres, the described flow conditions may reflect influences from all of these considerations.
The region of 'disturbed' flow observed downstream from the cellular projections was characterized by a deceleration of the Latex microspheres. It is doubtful that this was due to flow separation. When the cellular dimensions are considered, approximate values for the appropriate dimensionless parameters are much less than those predicting separation beyond smooth wall projections (47). Assuming 15 microns for the cell protuberance height (d), and considering the chamber depths (2h) used in this study, the largest d/h ratio expected would be 0.12. Considering the smallest cell characteristic length as 50 microns, then the smallest radius of curvature (r) would be approximately 30 microns. Thus the ratio of the cellular radius of curvature to the flow channel's half thickness (r/h) would be 0.24. Using these values as conservative estimates for the extreme cases, and consulting published experimental data (47, Figure 6), it can be seen that separation would not be expected even at the highest Reynolds number used in this study (Re = 320, from Table 1).

It is difficult to analyze the flow over cellular projections in the cell culture flow chambers. The height in the fluid at which the particles are first affected by the cells is not easily obtained, and the cellular geometry is not apparent. Cell height can be determined optically in the same fashion as the depth of the particles flowing in laminae of the cells was determined (112), but the uncertain and variable refractive index of the cytoplasm may affect these measurements (179b, P. 36). Visualization of the cellular geometry and the flow of particles over the cells obviated these difficulties.
From the studies in the cell culture profile flow chamber, it appeared that convective acceleration of particles flowing within 10 microns of the cells was caused by displacement of the particles into an adjacent, faster moving lamina of fluid. The Latex microspheres were not neutrally bouyant: Those particles not in the main flow field tended to roll along the lower wall of the flow channel. Thus, the abrupt deceleration of the particles as they were carried beyond the cells was due to displacement down into slower moving laminae nearer the wall.

Most of the observed particle velocity changes occurred within five to eight microns of the cells surface. (This was seen in both the cell culture flow chambers and the cell culture profile flow chambers.) Thus, discrepancies in the microscopic flow of particulate suspensions which were observed in these experiments from the actual flow over the cells may have resulted from two factors. In the first place, the particles are of the same order of magnitude as the cellular protuberance height; secondly, the particles diameter can exceed the depth of focus of the microscope.

It is doubtful that the deflection of the Latex microspheres flowing within 30 microns of the cell surface can be ascribed solely to these facts. Because of the dilute suspensions (less than 1%) used in this study, particle interactions within the flow were thought to be minimal.*

*Furthermore, it has been stated that if the particles are less than 10% of the channel's dimension then Poiseuille flow should be expected (182). The particles' diameter are about 1% of the flow channel's height, and Poiseuille flow was confirmed in Part I of the manuscript. Thus, particle interactions within the flow are minimal.
Therefore the fluid flowing over the cells is disturbed to at least a height of 30 microns by the cellular projection.

Finally, the utility of the television recording system in analyzing the flow over the cells should be emphasized. Without using high speed microcinematography it would have been difficult to photograph particles at the right moment in their traverse across the microscopic field unless some form of continual monitoring and reviewing of data was used. However, the television system cannot adequately register particles that have a high velocity and so its use for observation at high shear rates was limited. The major problem seemed to be the response time of the camera.

In summary, the microscopic flow immediately adjacent to cells growing in vitro has been observed from both the horizontal and the vertical aspects. The flow was visualized by observing the motions of Latex microspheres. From the horizontal aspect acceleration and deceleration of the particles were seen in the microspheres flowing within ten microns of the cell surface. This behavior was thought to be enhanced by the size of particles. Vertical deflections were seen in microspheres flowing within 30 microns of the cell surface.
Figure 14. Experimental setup for flow visualization in cell culture profile flow chamber. Profile view of M.D.B.K. cells as seen through television system.

Figure 15. A stage micrometer has been projected onto the television screen and traced upon a clear acetate sheet. (Total magnification is 340 times)
Figure 16. Stroboscopic visualization of flow over cells in cell culture flow chamber. \( \gamma \) = shear rate (i.e., velocity gradient); F.P.M., flashes per minute of strobe illuminator; lines equal to 100 microns. All flow from left to right

a. M.D.B.K. cells
20X objective, depth of focus 5-8 microns
\( \gamma = 6.8 \text{ sec}^{-1} \)
300 (F.P.M.)
total magnification about 340X

b. M.D.B.K. cells
20X objective, depth of focus 5-8 microns
\( \gamma = 13 \text{ sec}^{-1} \)
1200 F.P.M.

c. P.K.-15 cells
10X phase contrast, depth of focus 20-25 microns
\( \gamma = 42.0 \text{ sec}^{-1} \)
600 F.P.M.
total magnification 120X

d. P.K.-15 cells
10X darkfield, depth of focus 20-25 microns
\( \gamma = 42.0 \text{ sec}^{-1} \)
1200 F.P.M.
Figure 17. Visualization of flow over cells: comparison of flow visualization in cell culture flow chamber and flow profile chamber. $\gamma =$ shear rate; F.P.M., flashes per minute of strobe illuminator; T = period between flashes. Length between small divisions equal to ten microns

a. P.K.-15 cells, darkfield
cell culture flow chamber
10X objective, depth of focus 20-25 microns
flow from right to left
$\gamma = 83.2$ sec$^{-1}$
3000 F.P.M.

b. M.D.B.K. cell tracing
cell culture flow profile chamber (10° reflectance angle)
T = 4/60 sec between particle positions indicated by circles
flow from right to left
$\gamma = 5.5$ sec$^{-1}$
depth of focus 20-25 microns

c. M.D.B.K. cell tracing
cell culture flow profile chamber (10° reflectance angle)
T = 8/60 sec between particle positions indicated by circles, squares
flow from right to left
$\gamma = 5.5$
depth of focus 20-25 microns
d. M.D.B.K. cell tracing
cell culture flow profile chamber (10° reflectance angle)
T = 4/60 sec between particle positions indicated by circles, squares, and triangles
$\gamma = 27$ sec$^{-1}$, flow from left to right
depth of focus 20-25 microns
PART III. CELLULAR RESPONSE TO FLOW
Incentives for studying the cellular response to flow have been offered in the introduction to this manuscript. With the exception of the author's work (112), there have been no reports of studies specifically attempting to document the manner in which mammalian cells are affected by well defined flows. However, studies can be cited from the literature in which flow has been an important variable. Reviewing these studies will give an indication of what might be expected in the study of a cellular response to flow.

Tissue culture cells are subjected to various flow conditions during the course of their existence on glass surfaces. Flow has generally been imposed over tissue cultured cells for at least three reasons: 1) to enhance cellular interaction and aggregation in cell suspensions (183); 2) to apply pharmacological agents to cells in drug testing procedures (132,133,154,171); and 3) to replenish the cell culture fluid so that the essential metabolites are supplied and the cellular waste products are removed (184-186,136). Interest in the last of these reasons is due to the fact that high flow rates may 'leach' vital metabolites from the cellular environment (136). Rate of cell growth has been found to increase up to a maximum and then to decrease as the flow rate is increased still further (185,186). The same phenomenon has been found for cells growing in suspensions (184). However, fluid mechanical parameters such as the velocity gradient and wall shear stress were not measured in these studies. Therefore, it is impossible to extrapolate the results to cell
culture perfusion chambers with different geometries. Technologically, it would be desirable to be able to do so. Furthermore, it is not certain whether the observed effects are due to flow induced mechanical stresses or flow coupled mass transport phenomena.

With several major assumptions, it is possible to estimate the order of magnitude of the velocity gradients and wall shear stresses in two studies in which cells in tissue culture have been subjected to chronic flows. For example, it was found that seven day embryo chick heart explant cells responded optimally to flow rates of 0.15 cc/hour (186). If it is assumed that the chamber geometry approximated the parallel plate configuration, that the gasket thickness was no smaller than the currently available thin silicone rubber sheets, and that the cross sectional area of the chamber was not less than that of the flow chambers described in this manuscript, then the velocity gradient may be estimated from the relationship expressed in Table 1. These are reasonable assumptions, considering the cell culture chamber that these researchers had already described (149). For a flow rate of 0.15 cc/hour, the speculated shear rate used in this study was then no greater than .013 sec⁻¹. If the viscosity of the tissue culture medium employed is assumed to approximate that of water at 37°C, then the wall shear stress was on the order of $10^{-4}$ dyne/cm² in these experiments. Although it must be acknowledged that this is a very gross approximation of what must have been the experimental situation, a wall shear stress of less than $10^{-4}$ to $10^{-3}$ dyne/cm² seems reasonable. The shear stresses in this study of primary
cultures of chick heart fibroblasts must be regarded as very low. Although the fluid mechanical parameters were not described in this study (186), the cell migration rates were recorded; but as far as can be determined, the migration rates do not appear to have been published.

The fluid mechanics can be more accurately predicted in a study in which tumor cells were subjected to steady flow in long (18cm) capillary tubes (176). Neglecting the effect of the cellular projections upon the inside geometry of the capillary tube, the length of these tubes suggests that the cells were probably subjected to fully developed laminar Poiseuille flow. These authors reported that in 250 micron internal diameter capillary tubes some tumor cells (permanent-strain cells) thrived whereas others gradually peeled away when the average fluid velocity past the cell was .0400 cm/sec (176). If the velocity gradient is evaluated at the wall, it can be seen that the attached cells were probably subjected to shear rates of approximately 13 sec⁻¹. Furthermore, if the viscosity of the tissue culture medium approximated that used in this study (0.7 cp @ 37°C) then it can be said that the cells were probably subjected to wall shear stresses of about .09 dyne/cm². These shearing stresses are considerably higher than the estimated stresses which the chick heart fibroblasts had been subjected and found to thrive (186). It is interesting to note that permanent strain tissue culture cells (176) may be more resilient to flow than primary cell cultures (186).

Although there have been no other reports of the response of cells to what might be considered well defined 'chronic' flow conditions, there
has been much interest in the effect of acute, disruptive shear stresses upon both cells growing in vitro (125,130,131,138) and in vivo (31,32). The effect of acute shear stresses upon osmotically sensitized protozoa suspended in a couette flow device has been studied (138). A shear stress of 24 dynes/cm$^2$ caused only slight damage to the freely floating cells. As the flow became turbulent a rapid drop in the cellular viability was reported, but not all cells were affected similarly (138). Shear stresses rather than shear rate were thought to be important in this study.

As a means of measuring cellular adhesion to the substrate, acute shear stresses have been applied to rat fibroblasts growing in petri dishes (125,130,131). (A rotating plate viscometer was used to create the shear in these studies.) The cells were detached from the glass by shear stresses of 7.3 dynes/cm$^2$ applied for 20 seconds (130). It was stated that cell adhesion was ultimately determined by the behavior of the cell periphery, and that a process of 'ductile' rupturing of the cell plasma membrane occurred rather than a process of clean separation from the glass substrate (131). However, the cells were not observed when they were being subjected to the shear stress.

The endothelial cell response to acutely imposed, disruptive shear stresses has been studied in vivo (31,32), although a large error may have existed in the calculation of the actual shear stresses (35). A progressive sequence of cellular changes was described with increasing shear stress (31). Cellular changes such as endothelial migration,
piling-up, and cytoplasmic granulation with cellular swelling were ascribed to increased shear stress (31). At low shear stresses swelling and deformation of the cytoplasm were seen. At higher shear stresses the cytoplasmic border was seen to disappear and fat droplets and inclusion bodies appeared in the cytoplasm (31). Finally, disintegration of the cytoplasm occurred with marked deformation of the remaining cell nuclei. A 'critical yield stress' has been defined for endothelium (about 200 dynes/cm$^2$) below which no histological changes occur (32). A 'critical erosion stress' (about 400 dynes/cm$^2$) above which the cells disintegrate was similarly defined (32). Between these two values lies an 'acute yield stress' at which the cells first deform continuously under the elevated shear stress (32).

In reviewing the work upon the affect of acute flow upon cells fundamental questions arise as to the mechanical nature of the cell. It is interesting to note that from steady flow data (125,130,131) the cell plasma membrane would be expected to mechanically yield at shear stresses of an order of magnitude less than those necessary to disrupt endothelial cell plasma membranes in vivo (32). However, the importance of steady versus nonsteady flow is not known, nor is the 'protective' role of the cell surface coats understood. Thus, the rheological properties of cells might affect the observed response to imposed flows. The effect of biochemical stress (hypercholesteremia) upon the cell's mechanical properties is not clear (31).

In the cited acute, 'disruptive' flow studies it was not possible
to continually monitor cellular behavior or to observe the progression of subcellular structural alterations. Microscopic observations of the responses of endothelium to subacute mechanical stresses have been described in microvascular studies (68,83a,83b,85). The observed responses of injured endothelium in the microvasculature parallel the cellular alterations described in the acute flow studies (31,32); however, the applied stresses in these instances are not defined.

From these considerations questions arise about the cellular response to mechanical irritation. For example, what types of cellular alterations result from chronic mechanical stresses? Similarly, at what stress magnitude do these changes become irreversible and progressive? Clearly, the response of individual cells and confluent cell monolayers to more precisely defined stresses should be evaluated under carefully controlled experimental conditions. The concepts of 'critical' and 'acute yield stresses' and of 'erosion stresses' for cells should be explored.

These comments have dealt with reviewing only those studies in which flow was a pertinent experimental parameter in observed cellular behavior. It is apparent that current and future interest will demand more knowledge about the mechanical properties of attached cells and their response to well defined flow.
INVESTIGATIONS

Equipment

Perfusion system

Figure 18 illustrates the tissue culture perfusion system that was used to provide a steady flow rate to the cell culture flow chambers described in Part I. The perfusion system is shown schematically in Figure 19. A constant (+ 0.1 cm) hydrostatic pressure head was maintained in the system by sparging 5 per cent CO₂ in air* (for buffering pH) through cell culture medium flasks (A) located in a 37°C incubator (Figure 18). The gas was first washed and humidified by bubbling through a water flask, and then it was filtered by passing it through cotton in a sterilized flask. Interposed between the sterile filter flask and the wash flask was a column of water in a graduate cylinder (F) that was used to maintain a constant pressure. The glass tubing entering the filter flask was the last nonsterile point in the perfusion line. Using a greater-than-atmospheric pressure within the perfusion system insured that if small leaks had occurred in the line, sterile air and tissue culture medium would have been forced outward. Thus, the risk of accidental contamination of the sterile perfusion lines was reduced.

A constant rate of flow was obtained by an infusion withdrawal pump** (Figure 18, 19(B)). The pump was used in the withdrawal mode so

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*Matheson Co., Inc. Joliet, Ill.

**Cited previously, page 83.
as to provide a syringe reservoir of steadily increasing volume to receive the used cell culture fluid. (The cell culture fluid was under a hydrostatic pressure head of 50 cm H$_2$O.) It was felt that operating the pump in this mode assisted the steadiness of the flow by reducing the occurrence of pump binding, and it also favored the maintenance of system sterility.

Two cell culture flow chambers (C) were maintained in the incubator, providing a capacity of four flow channels. Two or three channels were used simultaneously for independent flow rate studies while the remaining was reserved for a zero-flow control. The cell culture fluid in the sparging flasks (Figure 18, A) within the incubator was distributed via rubber tubing* to syringes mounted near the microscope. The connection between the rubber tubing, the distribution syringes, and the cell culture flow chamber was made by a pre-sterilized, disposable 4-way plastic stopcock**. The distribution syringes allowed easy manipulation of the fluid and entrapped bubbles in the perfusion lines. The cell culture flow chambers were connected to the distribution syringes and a second stopcock valve on the withdrawal pump by means of silicone rubber tubing^.

Spent gas from the sparging flask was released into the atmosphere after passing it through a second water column pressure regulator.

*Latex rubber, translucent, medium wall tubing, 3/16 X 1/16 inch. Sargent and Welch Co., Chicago, Ill.

**Color course 4 way stopcock, #R62. Tavenol Laboratories, Inc. Morton Grove, Ill.

^Silastic Medical Grade Tubing, .062 inch X .125 inch. V. Mueller and Co. Chicago, Ill.
Frittered glass tipped tubing* was used at all gas-fluid interfaces in the system in which bubbling was to occur (i.e. humidifying wash bottle, water column pressure regulators, and sparging flask). A simple pressure manometer (Figure 19, '2') was placed in the perfusion line at the point where the fluid left the sparging culture flask.

The perfusion system was sterilized by autoclaving its components separately: The system was assembled using sterile technique. Only the disposable 4 way stopcocks were not reused.

The perfusion system was made rigid by employing Pyrex glass tubing wherever possible. This reduced the oscillations of flow unavoidably introduced during manipulation of the chambers on the microscope stage.

Tests

**Viscosity measurements**

The absolute viscosity of the tissue culture medium and its components was determined using a Cannon-Fenske type viscometer**. In this device the kinematic viscosity of an unknown fluid is determined by measuring the time it takes for a given volume of fluid to flow between two points in the capillary channel. The results are then compared to that of a standard fluid - distilled water. The experiments were carried

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*Gas Dispersion Tubes, cylindrical frittered glass tip, Pyrex brand glass (course 12C). Sargent & Welch, Chicago, Ill.

out at 35°C by placing the device inside the tissue culture incubator. Since the density of the fluid must be known to calculate its absolute viscosity from the kinematic viscosity, known volumes of the warmed fluids were weighed. The results of the viscosity determinations are given in Table 3.

A tissue culture medium consisting of M.E.M.*, 5% Calf Serum**, and antibiotic+ was determined to have an absolute viscosity of 0.701 cp, whereas a tissue culture medium consisting of M.E.M., 10% Calf Serum, and antibiotic was determined to have an absolute viscosity of 0.752 cp (both at 35°C).

It was also determined that these tissue culture media were Newtonian fluids. A 20 cm long plastic chamber was constructed which had a flow channel geometry similar to that of the cell culture flow chambers. The pressure drop across a 15 cm segment was measured at steady flow rates comparable to those used in the in vitro cellular flow studies. A linear relation was found between the pressure gradient and the flow rate. Thus the viscosity of the solutions was constant at the shear rates used in these studies.

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*Minimal Essential Medium (M.E.M.). Grand Island Biological Company, Grand Island, N. Y.

**New Born Calf Serum, Grand Island Biological Company, Grand Island, N. Y.

+120 mg Streptomycin and 120,000 units Penicillin G per 400 ml of M.E.M. Grand Island Biological Company, Grand Island, New York.
Experimental Procedure

The response of permanent strain M.D.B.K. cells and primary cultures of human umbilical vein endothelial cells to steady uniform, fully developed laminar flow was studied. The cell culture flow chambers described in Part I were used to apply these flow conditions to the cells. The preparation and cleansing of the chamber components were discussed in Part I.

The basic experimental design was relatively simple. The sterilized cell culture chamber was inoculated with a suspension of either M.D.B.K. or endothelial cells. The cells settled and attached to the lower wall of the chamber within 30 minutes, during which time they were maintained at 37°C in the nonflowing medium. After the cells had attached to the bottom surface of the flow channel they grew and multiplied, spreading to form a confluent monolayer (one cell thick). (This process generally took less than 2 days for the M.D.B.K. cell suspension and less than 4 to 5 days for the endothelial cell culture.) Before the cells had reached confluency cell populations were selected and marked on the glass surface with a diamond tipped marking objective*. The cells were then subjected to a preselected steady flow rate for at least 24 hours...in most cases longer. One flow channel was reserved as a biologic control in which there was no flow. At 24 hour intervals the tissue culture medium in the control culture was changed so as to provide fresh medium

*Diamond-tipped marking objective, Leitz, Wetzlar, Germany.
to these cells. At selected time intervals after the initiation of the flow the chambers were removed from the incubator and examined with the phase contrast microscope. The individually selected and marked cell populations could be repositioned to an accuracy of \( \pm 4 \) microns. The results consisted of observing and recording the appearance of the control and experimental cell populations photographically*. The wall shear stress levels ranged from \( 10^{-3} \) to \( 10^{-1} \) dyne/cm\(^2\) in these studies. Since the cells were subjected to flow for at least 24 hours, the flows were termed 'chronic'.

**Short term, high shear stress flow studies**

In addition to the chronic application of flow to cells, the same cell types were subjected to flows of shorter duration which imposed shear stresses equal to or higher than one dyne/cm\(^2\). These studies were arbitrarily termed 'acute'. In the acute flow studies the time interval between photographic inspection of the cells was necessarily reduced. In several cases these experiments were recorded on videotape and replayed later for analysis of cellular changes that occurred too rapidly to be photographed by standard procedures. Although the cellular detail and resolution in this technique is markedly reduced, the records do serve to document the cellular changes occurring at the higher magnitudes of shear stress.

*The phase contrast microscope and the film used have been cited on page 82 and 83, respectively.*
Special remarks: M.D.B.K. cells

M.D.B.K. cells suspended in M.E.M. were obtained from the Veterinary Diagnostic Laboratory at Iowa State University. In most cases they had been pre-refrigerated, but it is not thought that this affected their final growth and behavior. The cells were inoculated in the flow channels so as to have a plating density between 25,000 to 40,000 cells/cm². Tissue culture medium consisting of M.E.M., 5% calf serum, and antibiotic was used. Since these permanent strain cells become confluent within 48 hours the perfusion was started within 24 hours after injection of the cells into the flow channel.

Special remarks: Endothelial cell cultures

Primary cell cultures of umbilical vein endothelial cells were isolated from freshly obtained human umbilical cords*. Viable endothelial cells were isolated from aseptically refrigerated umbilical cords, usually within 90 minutes, but as long as eight hours, after birth.

The procedure used to isolate the endothelial cells from the umbilical vein is given in Appendix D. This procedure was derived from published techniques (107,108). A slight modification was introduced when it was discovered that the pH of the trypsin solutions used in the procedure varied from 6.4 to 8.1. Since the optimal pH for tryptic activity in vitro is 7.8 (187), a sterile solution of 8.8% NaHCO₃ was used to elevate low pH's to 7.8. Attempts were made to control the plating density of

*Department of Obstetrics, Mary Greeley Hospital, Ames, Ia.
the endothelial cells, but this proved to be very difficult because the cells were often clumped together making cell counting unpredictable. Furthermore, the presence of unwanted red blood cells in the culture also affected the results of the viable cell counting.

A common problem encountered in working with the umbilical cords was vasconstrictive obliteration of the umbilical vein. (This appeared to be related to the amount of tension applied to the cord by the attending physician at delivery.) Obliteration of the vein often resulted in trypsin solution being forced outside of the wall of the vein and into the extravascular tissue. (This was not due to inability to cannulate the lumen.) This is undesirable as it aggravates trying to remove substance from the vein, and it can add unknown cellular species to the cell suspension obtained from the incubated vein.

The presence of erythrocytes in the cell suspension indicated a possible 'contamination' of the pure cultures of endothelium. Multipotential cells circulating in the blood could give rise to cell types other than endothelium in vitro.

Since many cell types can be isolated from living tissue, attempts were made to determine the origin and the identity of the cells obtained from the umbilical cords. The following procedures were used to verify that the cell types isolated were in fact endothelial cells: 1) Umbilical veins were stained before and after trypsinization with a 0.4% solution of silver nitrate after a recommended technique (64). (The staining procedure is given in Appendix D.); 2) the cells in culture were stained
using these same procedures; 3) the umbilical cords were sectioned and stained with a Harris hematoxylin and eosin stain before and after the trypsinization procedure.

The identity of the endothelial cells isolated from the trypsinized umbilical cords was confirmed with the silver nitrate staining procedure. Figure 20 shows a 72 hour primary cell culture of human endothelium that was isolated from the umbilical vein. The characteristic endothelial intercellular cement lines are quite evident as well as the elongated nuclei. The appearance of these cells is similar to published descriptions in vivo (64,76-78); and in vitro (107,108,188). Figure 21 shows the effect of the trypsinization procedure upon the endothelial lining of the umbilical vein. About half of the endothelial cells are still adhering to the vein's wall at this site. Other preparations demonstrated that this was about the maximum yield that could be expected from the technique. The appearance of the subintimal reticular fibers (Figure 21) suggests that they have not been disrupted by the trypsin; therefore smooth muscle cells should not appear in the culture. Histological cross sections of the trypsinized and nontrypsinized cord reaffirmed that the tunica media of the vein was not disrupted by the experimental procedure. It therefore appears that the cell types that were isolated using the procedure outlined in Appendix D were endothelial cells. This supports the results of others (108).

Since primary cell cultures are generally slower growing than permanent strain cells in culture, the perfusion tests were begun after 72
hours of cell growth under no flow conditions. Also, to conform to published recommendations (108) a cell culture medium consisting of M.E.M., 10% calf serum, and antibiotic was used. Thus the culture media used for the studies on M.D.B.K. and endothelial cells are essentially similar except for the increased serum concentrations used for endothelial cells. In addition, Amphotericin B* was added to the growth medium in the summer months to inhibit the growth of contaminating yeast cells.

Special Remarks: Comments about microscopy and differences between M.D.B.K. cells and endothelial cells

Figure 22 illustrates the contour appearance of M.D.B.K. cells that had reached confluence. This optical effect was created by misalignment of the phase contrast condensor to cause oblique illumination of the cells. Figure 23 illustrates a phase contrast view of the same cell population. Comparison of these two photomicrographs shows that many of the surface features apparent in Figure 22 may be artifactual (due to the presence of residual phase contrast). For example, it can not be said conclusively that the cell nucleoli contribute to the surface features of the cells as suggested by Figure 22. Nor can the phase contrast appearance be uncritically accepted: The deep gaps that appear between the M.D.B.K. cells in Figure 23 are not apparent in Figure 22. The amount of contrast seen in phase contrast microscopy (related to product of the specimen thickness and its refractive index) is indeterminate.

*Amphotericin B Fungizone®, Squibb & Sons, E.R. New York, N. Y.
These comments should be recalled when viewing the photographic data presented in the Results.

Comparison of Figure 20 and 23 serves to emphasize fundamental behavioral differences between the endothelial cells and M.D.B.K. cells. Figure 23 illustrates a region of cellular overgrowth in mature 64 hour M.D.B.K. cells: In this region the monolayered condition no longer exist. Although crowded M.D.B.K. cells will grow over each other, it is interesting to note that in the monolayered portions of this colony the adjacent borders of the cells do not overlap. Cellular overlap has been suggested as being the cause of the intercellular cement lines in the endothelium (189). P.K.-15 cells and M.D.B.K. cells did not show the presence of silver cement lines when stained with the same technique used to demonstrate these structures in endothelium. Mature endothelial cells were never observed to grow over each other. This difference in intercellular staining characteristics will undoubtedly be reflected in differences between these cell strains in cell behavior, and consequently, a cellular response to flow. Ideally, both cell strains should be approaching the monolayered conditions for this study, but not be overpopulated.

A difference between M.D.B.K. cells and endothelial cells affected the manner in which experimentally observed changes in the cell populations were described. An ideal way of doing this would have been to use the 'mitotic index', i.e. the percentage of cells undergoing mitosis in a given time interval. However, mitotic figures were not discernible in the endothelial cell cultures. (They undoubtedly occurred.) The lack
of observed mitoses in endothelium may have been due to the fact that they were either 1) very rare, 2) very short termed in duration, or 3) not characterized by the extreme changes in phase contrast that characterized mitoses in M.D.B.K. cells. (Refer to Figure 23 for an example of a mitotic figure in the M.D.B.K. cells.) Therefore, changes in the experimental and control cell populations during any time interval were expressed in terms of the current number of 'viable epithelial' cells with respect to the number of 'viable epithelial' cells at the start of flow. 'Viable' cells were those cells in which a distinct nucleus, nucleoli, and perinuclear cytoplasmic components could be identified. 'Epithelial' cells were those cells which were polygonal in shape with at least 50% of the cell margin attached to the substrate. Cells undergoing mitosis were considered to be both viable and attached.
RESULTS

Response of Cells to Chronic, Steady Low Shear Stress Flows

Table 4 gives the experimental design in the study of the response of cells to long duration (at least 24 hours) flow. These studies were arbitrarily limited to wall shear stresses ranging from $10^{-3}$ dyne/cm$^2$ to $10^{-1}$ dyne/cm$^2$. 26 tests were performed in this range, and a total of 131 cell populations were observed. The counts of viable epithelial cells per square centimeter were averaged for all of the tests at a given wall shear stress level, and these are presented as percentages in Table 5. The number of viable epithelial cells was expressed as a per cent of the viable epithelial cells present at the start of flow (i.e. at time equal to $T_0$). The average cell population density at $T_0$ is also given for each of the shear stresses studied. The data in Table 5 are represented graphically in Figure 24 and 25. Since population densities changed with time, the ratio $(E)/(C)$ of the experimental cells' per cent population of viable epithelial cells $(E)$ to the control cells' per cent population of viable epithelial cells $(C)$ is also given in Table 5. Thus, the viability and attachment of the control and experimental cells may be compared directly at any time during the experiment.

M.D.B.K. cellular response at $10^{-3}$ dyne/cm$^2$

Discrete cellular alterations were observed when M.D.B.K. cells were subjected to flows which imposed wall shear stresses of as little as
Figure 26 illustrates a composite contact print showing the response of one control and four experimental cell populations from one of the tests run at this shear stress level. The shear stress magnitude in this case was \(1.8 \times 10^{-3}\) dyne/cm\(^2\).

Close inspection of the intracellular appearance revealed that a cellular response to flow was reflected in the behavior of intracellular phase-bright granules. In 13 out of 15 experimental cell populations (i.e. 87%) the immediate result of the flow (i.e. within four hours of the interval between observations) was to produce a cell response consisting an intracellular granular condensation at the perinuclear region of the cells. (This can be seen by comparing the differences of cellular appearance in the first and second rows of photographs in Figure 26. Another 'immediate' response to flow was decreased cellular radii. This was accompanied by the presence of intracellular gaps. This occurred in 11 out of the 15 populations studied at shear stresses of \(10^{-3}\) dyne/cm\(^2\).

As the cells adjusted to the flow the perinuclear phase-bright granules redispersed throughout the cytoplasm of the cell. This was seen in all of the experimental cell populations which showed the initial granular condensation with the initiation of flow. Redispersion of the cytoplasmic granules was concurrent with a more rounded cellular appearance in the experimental populations. In all of the control populations the cells appeared much flatter than the experimental cells; the difference in appearance became pronounced as time progressed. (Refer to Figure 27, and compare the 15 and 20 hour experimental cells with their controls.)
As the control cells aged the peripheral cytoplasm became devoid of granules and the perinuclear phase-bright granules condensed. In all cases at this and other shear stresses this behavior was a prelude to cellular detachment from the substrate. The comparative differences in cellular appearance between the experimental and control populations became most evident after 20 hours from the start of flow (refer to Figures 26 and 27).

Another type of intracytoplasmic granule became evident with time in experimental cell populations that did not appear within the control populations. This granule was phase-dark, and it was interspersed among the other cytoplasmic granules. It did not occur independently of phase-bright granules. The presence of this dark granule added to the illusion of a cellular fullness or 'rounded' appearance in the experimental cells. As the population density of the experimental cells increased the intercellular gaps were seen to decrease in size.

Some of these cellular changes can be seen by inspection of Figure 28. Perinuclear accumulation of intracellular phase-bright and dark granules can be seen in the experimental cell populations (B) when compared with their relative absence in the control cell populations (A) of the same age in culture. Intercellular gaps illustrated in the experimental cell population were common to the type seen in the other experimental groups. The rounded appearance of the experimental cells is noted as contrasted to the flattened appearance of the control cells. This latter difference became more apparent with age.
The effect of the flow upon the number of viable, attached M.D.B.K. cells is shown in Table 5. The data are represented graphically in Figure 24. As seen in Figure 24, the number of viable, attached cells in the five control populations decreased markedly (by 70%) after 24 hours, whereas cells subjected to flow exerting $10^{-3}$ dyne/cm$^2$ increased by 60% in the same period. This was also evident by visual inspection of the photographic data (Figures 26 and 27). The decrease in the cell density in the control cell populations was concurrent with the perinuclear granular condensation and the presence of a homogeneous marginal cytoplasm.

Birefringence has been reported in cells exhibiting cytoplasmic motion (127) and intracellular filamentous organelles exerting mechanical forces (128). Thus cells in which the cytoplasm withstood stresses might be expected to exhibit birefringence. Birefringence was not observed in either the experimental or the control cell populations when these cells were observed between crossed Polaroid filters. No cellular deformation was seen with the onset of flow, nor was there any cellular orientation with flow seen at shear stresses of $10^{-3}$ dyne/cm$^2$.

A net cellular migration in the direction of the applied shear stresses is suggested by results shown Figure 26. This can be seen by visualizing the changes in the gross morphology of the cell colonies with time. Such changes were visualized in only two out of the four tests at this shear rate. This was done by noting position of the estimated center of these areas was only 100 microns per 32 hours. Not much significance can be attached to flow induced 'cell migration' at these shear
stress magnitudes, since individual cells moved in and out of the microscopic fields between sampling times. Some cells could be seen to move against the direction of flow as seen by inspection of Figure 24.

M.D.B.K. cell response at $2.5 \times 10^{-2}$ dyne/cm$^2$

Similar to the lower magnitude shear stress flows, perfusion in the range of $10^{-2}$ to $10^{-1}$ dyne/cm$^2$ increased cell viability and attachment in the experimental cell population. This can be seen by inspection of the appearance of the control and the experimental cells in Figure 29. A decrease in population density was seen between these two cell groups. Referring to Table 5, it can be seen that overall, a 21 per cent decrease of viable epithelial cells in the control population was observed after 24 hours, whereas an overall 40 per cent increase occurred in the experimental population subjected to flow for 24 hours.

Close inspection of the experimental cell populations revealed that soon after the onset of flow, the cells showed the presence of dense perinuclear granules described at the lower shear stress flows. Again, these dispersed as perfusion continued. After eight hours of flow, five of the 14 experimental cell populations showed the redispersion of these granules well completed (although the process could be seen starting in all of the other experimental cell populations). After 16 hours, cytoplasmic granular dispersion was complete in all 14 cell populations exposed to flow. Phase-dark granules appeared in the cytoplasm of the stressed cells, as in lower shear stress studies. Figure 23 illustrates these changes in cells subjected to shear stresses of $2.5 \times 10^{-7}$ dyne/cm$^2$ for 24 hours.
Both phase-bright and phase-dark granules were dispersed within the cytoplasm and consequently the cells had a well rounded appearance. Pinocytosis was identified in these cells by referring to published photographs and descriptions of this phenomena (190,191). In M.D.B.K. cells the process of pinocytosis occurred at the active free margins of the cell colonies. Uptake of proteins has been shown to occur first in cells located at the periphery of epithelial monolayers (192) where pinocytotic ruffles occur. Pinocytosis was evident in all of the experimental cell populations at both the free leading and trailing edges at this shear stress.

Control cell populations showed the same sequence of changes as the controls in the lower shear stress flows: Perinuclear granulation preceded cell detachment from the substrate in both cases. The marginal cytoplasm was also devoid of any phase contrast features in the control cell populations.

Flow was first seen to influence cell morphology at this shear stress magnitude. In four out of 14 experimental cell populations some gross morphological rearrangement appeared to be associated with the direction of the applied shear stress. The changes took the form of a preferential development on the downstream, trailing margin of the cell colony. In the remainder of the test populations changes in colony morphology appeared to be random.

Changes in the cellular morphology became evident when small colonies of M.D.B.K. cells were subjected to shear stresses of $10^{-1}$ dyne/cm$^2$. 
Examination of individual cells at higher magnification confirmed that this was due to deformation of the peripheral marginal cytoplasm. This can be seen quite clearly in Figure 30. A small colony of four M.D.B.K. cells is shown in which the appearance of the cells before (A) and after (B) the application of a steady shear stress of $1.40 \times 10^{-1}$ dyne/cm$^2$ was recorded. Pinocytosis could be seen to cease at the leading edges of the cell colonies as shown in Figure 30. Disappearance of pinocytotic ruffles indicates that a mechanism for protein uptake by the marginal cell membrane (192) has been disturbed by the flow. Marked cytoplasmic deformation can be seen in Figure 30. Nuclear rotation can also be seen in the deformed cells by noting the relative positions of the nucleoli in two of the cells. The flow oriented cytoplasm returned to the pre-nonstresses polygonal shape of the cells when the flow was removed. The original position of the cell borders and the cell nuclei in (A) are superimposed upon the stresses cells in (B) as shown. Thus, the net migration of the nuclei in the direction of flow became evident as well as the deformation of the cell borders (refer to Figure 30).

**Endothelial cell response to flow at $10^{-3}$ dynes/cm$^2$**

All of the endothelial cell experimental populations studied (i.e. 16) were adversely affected by the application of low wall shear stress flows of $10^{-3}$ dyne/cm$^2$. This can be seen by inspection of the four experimental cell populations shown in Figure 31. The decrease in cell viability and attachment with flow is visually apparent. The cell population (percent viable attached cells) steadily decreased with the onset of flow.
(Table 5 and Figure 25). Only 68 per cent of the cells remained viable and attached in the experimental cultures after ten hours of flow whereas the control populations showed a net increase of nine per cent in the same time period (Table 5). After 18 hours, the experimental cells were reduced to 41 per cent of their number at the start of the flow whereas the control populations had increased to 22 per cent of their original number during the same time period. This is shown graphically in Figure 25. Intracellular detail of the experimental cells reflected the process of detachment from the glass substrate at these low flow rates.

No mitoses were ever observed in the control or the experimental cell populations in all of the endothelial cell cultures.

**Endothelial response to flow at** $10^{-2}$ dyne/cm$^2$ and $10^{-1}$ dyne/cm$^2$

Increasing the flow from wall shear stress levels of $10^{-3}$ to $10^{-2}$ dyne/cm$^2$ markedly improved the endothelial cell adherence to the glass and the cellular viability (Figure 32 and Figure 33). As can be seen in Table 5, the average number of viable attached cells in the experimental populations began to approach that of the control cell populations. A decrease in the population density of viable cells was still noted after approximately 20 hours of culture under the flow conditions.

A further improvement was noted as the flow was increased to the next higher order of magnitude. For example, the differences in appearance of the experimental and the control cells observed at the lower flow shear stress is minimized in Figure 33.
Details of endothelial cellular changes at the chronic flow rates are shown in Figures 34 and 35. Figure 34 shows the differences in appearance with time (19 hours) between control cell and experimental populations subjected to shear stresses of $10^{-2}$ dyne/cm$^2$. The adverse affect of the flow is demonstrable in Figure 34d: Cellular detachment is readily apparent in this figure. The increased granularity of the cells in the experimental cell population was characteristic of the process of cellular detachment from the substrate. Also demonstrated in this figure is the apparent motility of the endothelial cells in culture. The presence of dead cells is not seen in the experimental population shown in Figure 35d. The migration of the endothelial cells away from the primary cell colony is especially evident in the control cell populations (Figures 35a and 35b).

Table 5 and Figure 25 demonstrate that as the shear stress was elevated, the numbers of viable attached cells in the experimental population approached that of the control.

In addition to the gross overall changes seen in the population densities of attached viable cells, subtle changes occurred in the appearance of the endothelial cells subjected to flow. Figure 36 demonstrates cellular detail of endothelial cells subjected to a shear stress of $10^{-1}$ dyne/cm$^2$ for three hours. The peripheral cytoplasm appears more filamentous and 'thready' in the cells shown in this photograph. An increase in the size of the intercellular gaps occurred as is shown in this illustration. No cellular deformation nor cellular birefringence was detected in any of the endothelial cell populations.
A fundamental difference existed between endothelial cells and M.D.B.K. cells in their response to low wall shear stress, 'chronic' flows. Epithelial cell viability was increased in M.D.B.K. by low wall shear stress flow, whereas it was decreased in endothelial cells. This is shown in Figure 27. In this figure the effect of flow upon the E/C ratio is shown (i.e. the effect of flow upon the ratio of the numbers of viable epithelial cells in the experimental and control cell populations relative to the numbers at the start of flow). E/C ratios are given in Table 5. As the shear stress was elevated the endothelial cell viability increased.

Response of Cells to Short Duration, High Shear Stress Flows

The experimental design for the study of the response of cells to short duration, high shear stress flows is shown in Table 6. A total of 13 tests were conducted upon M.D.B.K. cells and endothelial cells in which the wall shear stress exceeded one dyne/cm^2. The cells were studied by marking cell populations and returning to the selected sites at given time intervals for observation. In four tests the response of the M.D.B.K. cells were videotape recorded as the cell culture flow chamber was maintained in position on the warm stage of the microscope.

Observations on M.D.B.K. cell behavior at shear stresses of greater than 1.0 dyne/cm^2

Cellular deformation and separation from the glass substrate were caused by flow that exerted shear stresses as little as 1.0 dyne/cm^2.
This response behavior appeared to be an extension of the trend described at shear stresses of $10^{-1}$ dyne/cm$^2$ shown in Figure 30.

Figure 38 shows a colony of four M.D.B.K. cells which were subjected to a shear stress of 1.0 dyne/cm$^2$ for 30 minutes. Retraction of the cells occurred at the trailing edge of the colony. As the flow was stopped those cells that had started to detach from the substrate rounded up (this is shown in Figure 38c). Clearly, cell separation could not be considered to be a simple process of 'peeling' of the cell from the substrate. Fine filamentous extensions of the cells could still be seen to be attached to the glass in Figure 38c.

Cellular deformation was also seen to occur at shear stress magnitudes of 1.0 dyne/cm$^2$. This deformation was affected by the intercellular contact of the neighboring cells within a colony. This phenomena is indicated in Figure 38d. In this figure, the outlines of the peripheral borders of the cell colony were traced before (A) and after (B) 30 minutes of flow exerting a shear stress of 0.7 dyne/cm$^2$. The positions of the nuclei before the flow are also shown, and the arrows indicate the direction of their movement as the colony deformed. By inspection, it can be seen that although there was noticeable deformation due to flow, intracellular deformation was not directly related to the direction of the applied shear stresses. Control populations of cells did not exhibit the degree of migratory activity that could have explained the reshaping of the colony morphology within this short time interval. In all seven of the tests performed, a similar pattern of intracellular deformation
occurred when cell colonies deformed under shear stresses of 1.0 dyne/cm$^2$. Because of the indeterminant effect of neighboring cells in a monolayer, the deformation of any particular cell could not be predicted.

It is interesting to note that in M.D.B.K. cells mitoses could occur in spite of shear stresses greater than 1.0 dyne/cm$^2$. Figure 39 shows a mitotic cell in a colony of M.D.B.K. cells being subjected to a shear stress of 1.5 dyne/cm$^2$. Very fine filaments could be seen anchoring the cell to the glass substrate. Since the cell has retracted against the flow toward its neighbors, intercellular bonds must be considered as being important in sustaining cells against the shear stress during mitoses. (Intracellular gap formation occurred between the cells as is indicated in Figure 39.)

Shear stresses of only 10 dyne/cm$^2$ could cause cellular failure in M.D.B.K. Figure 40 shows a colony of M.D.B.K. cells that had been subjected to $1.4 \times 10^1$ dyne/cm$^2$ for 15 minutes. The outlines of the former borders of the cells can still be seen upon careful inspection of Figure 40. The perinuclear region of the cells remained adherent to the glass substrate in the disrupted cells. Cell failure was not seen in all of the test runs and thus 10 dyne/cm$^2$ should be viewed as the lower limit for which cellular failure might be expected to occur for M.D.B.K. cells.

Some of the dynamic M.D.B.K. cell responses which occurred at high shear stress flows can be seen represented in Figures 41, 42 and 43. These figures represent a series of photographs made from the cell images
as they were projected onto the television screen. The pictures were made at critical times during the videotape replay of the experiment. The advantage of this methodology is that events of interest can be selectively studied when they occur.

At 1.0 dyne/cm², immediate cellular withdrawal and deformation with the onset of the flow could be seen (Figure 41). Cell withdrawal from the substrate and points of pinocytotic activity are shown. Cellular withdrawal occurred at the leading edges of the colonies as seen in Figure 41b. It also occurred at the trailing edge (41d) as previously described in Figure 38. Thus a process of total cell retraction occurred at this shear stress superimposed upon cellular deformation.

It is interesting to note that at places where pinocytosis occurred withdrawal of the cell did not as readily occur (Figure 41b, c, e). When the flow was stopped and the cells were allowed to 'rest' for 15 minutes cellular reattachment could be seen at the substrate formerly covered by the cells (Figure 41f). Again, mitosis occurred during the shear stress without detachment of the dividing cell from its neighbors.

Figure 42 illustrates the process of cell separation from the substrate at shear stresses on the order of magnitude of 10 dynes/cm². In these figures the cell-to-substrate bond failed before the intercellular bonds. In many instances that were not recorded, plates of detached cells were seen to freely float by the field of view in the microscope. These detached cells were still bound into the intercellular configuration they possessed before separation from the substrate.
Figure 43 shows the profile view of M.D.B.K. cells as they were subjected to a shear stress of 1.0 dyne/cm$^2$. (They were grown and observed in the cell culture profile flow chamber described in Part I.) Changes in the leading (l") and the trailing (t") contact angles were apparent when the cells were stressed by the flow. Figure 43b also demonstrates a cellular deformation with flow as the central region of the cell was seen to deform about 10 microns in the direction of flow. Cells returned to their normal profile appearance when the flow was stopped (Figure 43d).

These remarks have dealt only with observations on isolated colonies of M.D.B.K. cells. The cell numbers per field of view were averaged for the three tests in which eight experimental populations were observed and tabulated in Table 7.

Most of the separation which occurred at a shear stress greater than 1.0 dyne/cm$^2$ occurred within the first few minutes after the initiation of the flow. This was easily seen by visual interpretation of these results, as whole colonies were seen to detach from the substrate at a given instant.

Observations on endothelial cell behavior at shear stresses greater than 1.0 dyne/cm$^2$

Figure 44 shows the results of one of three tests conducted upon the endothelial cells in which the shear stresses exceeded 1.0 dyne/cm$^2$. The appearance of the cells tested at this shear stress remained quite uniform: There was no noticeable difference in subcellular appearance between cells subjected to 1.0 dyne/cm$^2$ and 10 dynes/cm$^2$ over short periods.
of time. However, shear stresses of 10 dynes/cm$^2$ were more efficient in separating endothelial cells from the substrate than the lower magnitude shear stress flows. This can be seen by inspection of the per cent decrease in population with respect to time shown in Table 7.

No catastrophic cell failure such as shown in Figure 40 was ever seen when working with endothelial cells. Cellular deformation was never apparent. No birefringence was observed in any of the control or experimental cell populations in this study.
Several comments should be made about the experimental procedure before interpreting the results of this study.

A fundamental difficulty resides in the basic experimental design of comparing the experimental cells with cells in a 'no-flow' control. For example, inspection of the control M.D.B.K. cells in Figures 26, 27, and 29 suggests that the control cells were being deprived of essential nutrients in the 'no-flow' state. The decline in the numbers of viable epithelial cells beyond 20 hours in the control populations and the presence of detaching cells emphasizes this point. These changes occurred in spite of daily replenishment of the cell culture medium. (Nor can these changes be divorced from the normal aging of these cells in vitro. For this reason changes resulting from flow were expressed in terms of the E/C ratio in Table 5 and Figure 37.) In retrospect, differences in the biochemical compositions of the control and the experimental cell populations could have been eliminated by redesign of the cell culture flow channel. A flow channel geometry which incorporated a region of stagnation (in which the control cells could grow relatively protected from the adjacent experimental flow conditions) would have obviated this difficulty.

However, during the course of the experimentation it became apparent that the experimental cells could be used as their own controls. Thus, the appearances of experimental cells at different shear stress magnitudes
were compared in addition to being compared with their respective controls.

A green filter was used to minimize the effect of light upon the appearance of cells during microscopic observations (193). If light did affect the cells, it is assumed that the experimental and the control cells were affected in a similar manner.

When comparing the responses of endothelial cells and M.D.B.K. cells to flow it must be recalled that the biology of these 'epithelial' cells is different. As permanent strain cells in tissue culture, M.D.B.K. cells exhibit a stable morphology. These cells are fully adapted to growth in vitro. However, since endothelial cells are primary cell types in culture, morphological transformations occur in adapting to the in vitro environment.

A progression of morphological changes was seen in the endothelial cultures. Reticular outgrowth of cells occurred from attached clumps isolated from the trypsinized umbilical cords (Figure 35a). These cells transformed into elongated epithelioid cells (Figure 35b) which became polygonal as confluency was approached (Figure 20). Differences in behavior of epithelioid and reticular cells in culture have been reviewed (194). The long latent period, the slow growth, and the rarity of mitoses are all expected characteristics of primary epithelioid cell cultures (194). Clearly, one must be cautious when comparing the endothelial cell response at a given shear stress to that observed for the M.D.B.K. cells.

Considering these remarks it must be said that identifying a discrete cellular response to flow, and flow alone, in these studies must
rest upon a very conservative analysis of the observed responses. Nevertheless, interesting features of the cellular alterations with respect to the flow were seen, and these shall be discussed next.

**Cellular alterations related to flow**

Several M.D.B.K. cellular alterations resulting from flow seem clear from this study. The observed responses and the shear stress magnitude at which they were first seen are summarized in Table 8.

In M.D.B.K. cells the decrease in cellular radii of curvature with the application of steady flow represents a source of speculation. Cell 'rounding-up' has been predicted analytically from changes in the surface tensions in the cell membrane (102). It is not known whether the adaptation with time observed in the M.D.B.K. cells represents a change in the fundamental mechanical properties of the cell membrane. Another possible explanation might be derived from the work of A.S.G. Curtis (104) in which he described an initial transient increase in 'surface viscosity' of cells subjected to oscillating shear stresses. However, it is not known how the various mechanical parameters of the cell interrelate in predisposing cell morphological changes.

The appearance of a decreased radius of curvature in the cells may have been enhanced due to illusions created by phase contrast microscopy and the behavior of intracytoplasmic particles. Profile views of cells subjected to flow suggest that M.D.B.K. cells did in fact 'round-up' as seen in the cells pictured in Figure 43d. Cells which had not been subjected to flow appeared flatter from the profile aspect (Figure 12).
More data are required using this profile flow chamber to obtain support for these comments.

Many authors have described cellular injury to nonspecific mechanical stresses in which cell 'rounding-up' occurs (195, 196, 31, 32, 83a, 83b). However, the mechanism for 'rounding-up' is not known. The absence of birefringence in the results suggests that this response was not due to contraction of intracellular fibrils. This is in contradiction to speculated mechanisms for endothelial contraction due to mechanical and biochemical insult (83b).

The lag time between the onset of flow and the increase in numbers of viable cells suggests that a transient adjustment to steady flow occurred in M.D.B.K. cells. The redispersion of intracellular granules with time also emphasizes the transient response of M.D.B.K. cells to the onset of flow.

The occurrence of intercellular gaps, the accumulation and behavior of the intracytoplasmic granules, and the decrease of the radius of curvature of the cell can not be considered to be independent processes. Nor can the progression of these changes with time increase in population density. It is uncertain whether these alterations were due to mechanical parameters mass transfer, or possibly other flow phenomena.

The mechanical response of the M.D.B.K. cells to deforming stresses (i.e. those greater than $10^{-1}$ dynes/cm$^2$) emphasizes the importance of the cell's marginal border in maintaining epithelial cell morphology. Figures 30, 38, and 41 illustrate that at points where pinocytosis
ceased or was initially absent cell retraction from the substrate more readily occurred. Importantly, steady flows exerting shear stresses as low as $1.4 \times 10^{-1}$ dyne/cm$^2$ caused a cessation of pinocytic ruffling at the leading edges of these cells (Figure 30). The function of pinocytosis as an 'anchoring' mechanism has important implications in the growth of epithelial cells over denuded areas under steady shear stresses. Uptake of proteins occurs first in cells located at the peripheral aspects of epithelial monolayers where pinocytotic ruffles occur (192). Therefore, the cessation of pinocytosis at the leading edges of cells subjected to shear stresses could have altered uptake of proteins and amino acids in the stressed cells. Therefore, one possible mechanism for a flow related chronic embarrassment to the cells' metabolic processes is seen from the described behaviors of stressed cells observed in this study. Similarly, a flow related mechanism involving altered cellular metabolism might be postulated for describing the detachment of cells subjected chronic flows in which the shear stresses were not sufficient to 'peel' them from the substrate.

The mechanical properties of the cell and its components were undoubtedly important in predisposing cells to deformation in response to shear stresses (a lower limit of $10^{-1}$ dyne/cm$^2$ was seen for initiation of M.D.B.K. cell deformation in this study). The migration of nuclei in Figures 30 and 38d suggest that intracellular material accumulate at the 'trailing' edge of the cell if other cells downstream impeded their deformation.
The patterns of intracellular deformation emphasize the importance of adhesion at the cell margin and the influence of its neighbors (Figure 30, and 38d). Thus, the patterns of deformation in confluent monolayers will be different than that seen for individual cells. The presence of adjacent cells may therefore affect the ability of cells to withstand shear stresses within the body. It is suggested that an initial injury — by removing the constraining influence of neighboring cells — may predispose cell deformation in the adjacent uninjured cells.

Noticeable cellular detachment was seen in the M.D.B.K. cultures with the onset of flow exerting shear stresses greater than one dyne/cm². It is clear from this study that three possible modes for cell detachment existed. First, examples of mechanical failure of the cells were seen at the highest shear stress employed in this study (1.5X10¹ dynes/cm²). Second, entire cells separated from the substrate without mechanical disruption as shown in Figure 42. Thirdly, cells separated by a process of 'biological' retraction from the substrate as seen in Figure 41. Thus a progression of mechanisms occurs by which the cells separated from the substrate under high shear stresses. The lower shear stresses afford the cells the opportunity to withdraw, whereas the higher shear stresses cause either an immediate failure or a separation of the whole cell from the glass.

The retraction of the cell marginal border from the substrate at shear stresses of 1.0 dyne/cm² appears to represent an extension of the processes of cell 'rounding-up' seen at lower shear stress magnitudes.
The response seems to have been centrally mediated within the cell: It could be seen that at points of pinocytotic activity the membrane still adhered to the substrate. Both leading and trailing edges were seen to retract. Thus cellular separation from the substrate resulted from both deformation due to the shear stress plus a reaction mediated by biological responses within the cell. As seen in these studies, the cells re-attached to preferred cell morphologies when the flow was removed. This is thought to be due to a cellular preconditioning of the glass substrate that favored reattachment rather than the presence of residual stresses within a 'viscoelastic' cytoplasm.

In considering the responses of the endothelial cells to the application of chronic flows, it can not be explained why lower shear stress flows were deleterious to the cultures without having been reflected in the appearance of the control cells. Elevating the shear stresses improved both the appearance and the adhesion of the endothelial cells growing in the experimental cell populations.

Summary

The behavior of permanent strain M.D.B.K. cells and primary cultures of endothelial cells subjected to steady, laminar flow has been described for shear stresses ranging from $10^{-3}$ to 10 dynes/cm$^2$. Fundamental differences between these cells' response to flow were seen. Low shear stress flows of 24 hours' duration increased the numbers of attached viable M.D.B.K. cells, whereas the same flows decreased the numbers of
attached, viable endothelial cells. As the shear stress was increased, the numbers of viable endothelial cells increased.

Cellular alterations occurred in M.D.B.K. cells at shear stresses well below those necessary to disrupt the cell membrane. A transient cellular response to the onset of flow was described for M.D.B.K. cells.

The response of the cells to flow related to many factors. The magnitude of the shear stress, the presence of neighboring cells, and the pinocytotic activity of the cell margin all seemed to be important in determining the manner in which the cells responded to flow.

The role of pinocytosis at the free cellular margins affected the observed behavior at deformable shear stresses. A biological anchoring function has been postulated for pinocytosis at the cellular margins. A biological mechanism for the chronic metabolic embarrassment of the cell resulting from flow induced cessation of pinocytosis has been offered.

Three modes of flow related cellular removal from the substrate were described. Flows exerting greater than one dyne/cm² caused 1) retraction of the free cellular margins and withdrawal from the substrate, 2) separation of intact cells from the substrate, and 3) mechanical disruption of the cell membrane. The pinocytotic activity of the cell's free margin was seen to be an important hindrance to the first of these processes.

As this study demonstrates, the observed responses of cells depends upon the cell type and a complex relationship between the cells adhesion to the substrate, its adhesion to its neighbors, and the mechanical properties of the cells components.
Figure 18. Perfusion system for flow studies
A. cell culture medium flasks
B. infusion withdrawal system
C. cell culture flow chambers
D. inverted stage phase contrast microscope
E. distribution syringes
F. constant pressure regulator
G. 2nd pressure regulator
Figure 19. Schematic representation of perfusion system. Letters indicate components shown in Figure 18; arrows indicate direction of flow; '1', point in perfusion lines beyond which system was sterile; 2, pressure manometer.
THREE-WAY VALVE
GAS INLET 5% CO₂ IN AIR
THREE-WAY VALVE
GAS WASH & HUMIDIFY
PRESSURE REGULATING COLUMN
STERILE FILTER
OUTFLOW PRESSURE REGULATOR
WITHDRAWAL PUMP (B)
DISTRIBUTION SYRINGES
CELL CULTURE FLOW CHAMBER
MEDIA SPARGING FLASK & RESERVOIR
PRESSURE REGULATING COLUMN (F)
STERILE FILTER (A)
Table 3. Physical constants for cell culture media used in the study (at 35°C)

<table>
<thead>
<tr>
<th>Fluid</th>
<th>Absolute viscosity $\mu$ (centipoise)</th>
<th>Kinematic viscosity $v = \mu/\rho$ (centistoke)</th>
<th>Fluid density $\rho$ (gm/cm$^3$)</th>
<th>Specific weight $\gamma$ (dynes/cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>0.7225 cp*</td>
<td>0.728</td>
<td>0.993</td>
<td>973</td>
</tr>
<tr>
<td>M.E.M. + 5% Calf Serum + antibiotic</td>
<td>0.701</td>
<td>0.698</td>
<td>1.005</td>
<td>985</td>
</tr>
<tr>
<td>M.E.M. + 10% Calf Serum + antibiotic</td>
<td>0.752</td>
<td>0.742</td>
<td>1.015</td>
<td>995</td>
</tr>
<tr>
<td>100% Calf Serum</td>
<td>1.700 cps</td>
<td>1.690</td>
<td>1.020</td>
<td>999</td>
</tr>
</tbody>
</table>

Figure 20. 'Intercellular cement lines'
72 hour endothelium,
AgNO₃ stain (refer to Appendix D).
Subjected to 10⁻¹ dyne/cm²
from right to left for 6 hrs.
20 X objective, phase contrast
arrows demonstrate presence of
silver lines between cells
(total magnification about 310 times)

Figure 21. Luminal surface, trypsinized
umbilical vein (trans-illuminated)
AgNO₃ stain
10 X objective, phase contrast
arrows demonstrate presence of inter­
cellular silver lines
fine vertical filaments are
reticular fibers between smooth
muscle cells in tunica media
(total magnification about 76 times)

Figure 22. Surface contour, M.D.B.K. cells
64 hour cells
Subjected to 2.5X10⁻² dyne/cm²
for 24 hours, flow from right to left
20 X objective, 'oblique' illumination
(total magnification 280 times)

Figure 23. Phase contrast view of cells
in Figure 22
10 X objective
'm' indicates mitotic figure
Central portion of photograph
illustrates M.D.B.K. cell over­
growth
(total magnification 280 times)
Table 4. Distribution of tests: experimental design in 'chronic' flow studies

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Wall shear stress magnitude</th>
<th>Number of tests</th>
<th>Distribution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.D.B.K. cells</td>
<td>$10^{-3}$ dynes/cm$^2$</td>
<td>4</td>
<td>15 E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 C</td>
</tr>
<tr>
<td></td>
<td>$2.5 \times 10^{-2}$</td>
<td>4</td>
<td>14 E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 C</td>
</tr>
<tr>
<td></td>
<td>$1.4 \times 10^{-1}$</td>
<td>3</td>
<td>4 E</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>$10^{-3}$</td>
<td>4</td>
<td>16 E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 C</td>
</tr>
<tr>
<td></td>
<td>$10^{-2}$</td>
<td>6</td>
<td>23 E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>14 C</td>
</tr>
<tr>
<td></td>
<td>$10^{-1}$</td>
<td>5</td>
<td>23 E</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5 C</td>
</tr>
</tbody>
</table>

*Experimental cell populations (E), and Control populations (C) selected with diamond tipped marking objective
<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Shear stress magnitude dynes/cm²</th>
<th>Time</th>
<th>Viable, attached cells (per cent of population at start of flow)</th>
<th>Control population (C)</th>
<th>Experimental population (E)</th>
<th>(E)/(C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.D.E.K.</td>
<td>$10^{-3}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o$</td>
<td>41,500 cells/cm²</td>
<td>51,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 10$ hrs</td>
<td>142%</td>
<td>117%</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 15$ hrs</td>
<td>140%</td>
<td>120%</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 24$ hrs</td>
<td>140%</td>
<td>150%</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 32$ hrs</td>
<td>41%</td>
<td>240%</td>
<td>5.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 x $10^{-2}$</td>
<td>$T_o$</td>
<td>36,000 cells/cm²</td>
<td>34,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 8$ hrs</td>
<td>107%</td>
<td>105%</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 16$ hrs</td>
<td>110%</td>
<td>112%</td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 24$ hrs</td>
<td>79%</td>
<td>140%</td>
<td>1.78</td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>$10^{-3}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o$</td>
<td>24,000/cm²</td>
<td>24,000/cm²</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 10$ hrs</td>
<td>109%</td>
<td>68%</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 18$ hrs</td>
<td>122%</td>
<td>41%</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 22$ hrs</td>
<td>115%</td>
<td>2%</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$T_o + 24$ hrs</td>
<td>97%</td>
<td>&lt; 1%</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>
$10^{-2}$

<table>
<thead>
<tr>
<th></th>
<th>$22,000/cm^2$</th>
<th>$22,000/cm^2$</th>
<th>$25,000/cm^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_o$</td>
<td>93%</td>
<td>85%</td>
<td>85%</td>
</tr>
<tr>
<td>$T_o + 10$ hrs</td>
<td>113%</td>
<td>91%</td>
<td>97%</td>
</tr>
<tr>
<td>$T_o + 19$ hrs</td>
<td>112%</td>
<td>45%</td>
<td>70%</td>
</tr>
<tr>
<td>$T_o + 31$ hrs</td>
<td>89%</td>
<td>37%</td>
<td>85%</td>
</tr>
</tbody>
</table>

$10^{-1}$

<table>
<thead>
<tr>
<th></th>
<th>$33,000/cm^2$</th>
<th>$25,000/cm^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_o$</td>
<td>108%</td>
<td>85%</td>
</tr>
<tr>
<td>$T_o + 12$ hrs</td>
<td>97%</td>
<td>70%</td>
</tr>
<tr>
<td>$T_o + 25$ hrs</td>
<td>85%</td>
<td>71%</td>
</tr>
</tbody>
</table>

$T_o$ represent the cellular age at the start of flow.
Figure 24. Changes in number of attached viable M.D.B.K. cells in experimental flow and control cell population.
Figure 25. Changes in number of viable, attached endothelial cells in experimental and control cell populations
ENDOTHELIAL CELLS

CONTROL CELLS

EXPERIMENTAL CELLS

$10^{-3}$ dyne/cm$^2$

$10^{-2}$ dyne/cm$^2$

$10^{-1}$ dyne/cm$^2$

PER CENT OF POPULATION AT START OF FLOW

HOURS AFTER START OF FLOW
Figure 26. M.D.B.K. cellular response to 'chronic' flow. Wall shear stress equal to $1.8 \times 10^{-3}$ dynes/cm$^2$. Refer to text for description. (Total magnification approximately 70 times.)
M.D.B.K.
24 hour cells
300μm

4 hrs.
8 hrs.
18 hrs.
22 hrs.
26 hrs.

(flow, $T_w = 0.018$ dyne/cm²)
Figure 27. M.D.B.K. cellular response to 'chronic' flow. Wall shear stress equal to $10^{-3}$ dyne/cm$^2$.
The control and experimental cell populations in the first row demonstrate the effect of 32 hours stagnation of tissue culture medium. Cell viability and attachment improved upon the initiation and replenishment of the cell culture medium. (Total magnification approximately 65 times.)
\{ \text{flow, } \tau_w = 10^{-3} \text{dyne/cm}^2 \} \quad \text{control cells} \\
\\
M.DBK. \\
32 hour cells \\
300\mu m \\
\\
10 hrs \\
15 hrs \\
20 hrs \\
32 hrs
Figure 28. Detail of control (A) and experimental cell populations in Figure 26. Cells subjected to .0018 dyne/cm$^2$, flow from left to right for four hours. (Total magnification approximately 500 times.)

a. Control (A) cell population  
28 hour cells

b. Experimental (B) cell population  
28 hour cells
'b' indicates perinuclear granular accumulation
Figure 29. M.D.B.K. cellular response to 'chronic' flow. Wall shear stress equal to $2.5 \times 10^{-2}$ dyne/cm². Refer to text for description. (Total magnification approximately 37 times.)
flow, \( \tau_w = 2.5 \times 10^{-2} \text{dyne/cm}^2 \)
Figure 30. M.D.B.K. cells subjected to deformable shear stresses. Cells (A) before and during (B) flow from left to right (shear stress magnitude equal to $1.40 \times 10^{-1}$ dyne/cm$^2$ and was applied for 45 minutes before B was taken). (Total magnification 500 times.)

a. Before flow (A) (note pinocytosis occurring at P')

b. During flow (B) note cessation of pinocytosis, and the original outlines of colony A, with former position of nuclei indicated. Arrows show direction of nuclear movement (nuclear rotation indicated at nucleus n and n').
Figure 31. Endothelial cell response to 'chronic' flow. Wall shear stress equal to $10^{-3}$ dyne/cm$^2$. Refer to text for description. (Total magnification approximately 30 times.)
control cells

{flow, $\tau_w = 10^{-3}$ dyne/cm$^2$}

ENDOTHELIAL CELLS

48 hour cells
300 $\mu$m
10 hours
14 hours
18 hours
22 hours
Figure 32. Endothelial cell response to 'chronic' flows. Wall shear stress equal to $10^{-2}$ dyne/cm$^2$. Refer to text for description. (Total magnification approximately 63 times.)
(flow, $\tau_w = 10^{-2}$ dyne/cm$^2$) control cells

- 72 hour cells
- 300μm
- 9.5 hrs
- 15 hrs
- 19 hrs
- 31 hrs

ENDOTHELIAL CELLS
Figure 33. Endothelial cell response to 'chronic' flows. Wall shear stress equal to $10^{-1}$ dyne/cm$^2$. Refer to text for description. (Total magnification approximately 63 times.)
ENDOTHELIAL CELLS

\{\text{flow, } \tau_w = 10^1 \text{ dyne/cm}^2\} \quad \text{control cells}

- 48 hour cells
- 300\mu m
- 12 hrs
- 19 hrs
- 25 hrs
Figure 34. Detail of endothelial cell response to low wall shear stress, 'chronic' flow. Flow exerted shear stress of $10^{-2}$ dyne/cm$^2$ in 'd'. Populations repositioned to $\pm$ 4 micron accuracy. (Total magnification equal to 270 times.)

a. Control cell population
   72 hour cells

b. Control cell population
   91 hour cells
   (note migration of cells in a)

c. Experimental cell population
   72 hour cells, no flow

d. Experimental cell population
   91 hour cells
   ($10^{-2}$ dyne/cm$^2$ for 19 hours, flow from left to right)
   'e' indicates detailing cells, 'f' indicates presence of dead cells
Figure 35. Detail of endothelial cell response to low wall shear stress 'chronic' flows. Flow exerted shear stress of $10^{-1}$ dyne/cm$^2$ in 'd'. Populations re-positioned to $\pm 4$ micron accuracy. (Total magnification equal to 270 times.)

a. Control cell population
   48 hour cells

b. Control cell population
   72 hour cells
   (note migration of cells in a)

c. Experimental cell population
   48 hour cells, no flow
   (10$^{-1}$ dyne/cm$^2$ for 24 hours, flow from left to right)
   'e' indicates detaching cells
Figure 36. Subcellular changes in endothelial cells subjected to $10^{-1}$ dyne/cm². (Total magnification approximately 380 times.)

a. 72 hour endothelium
   no flow

b. Endothelial cells after three hours of flow
   'g' indicates region of intercellular gap
   formation and cellular pulling apart;
   'S' indicates filamentous, thready appearance
   of cytoplasm; numbers (1,1',2,2'...) indicate
   same cell in control (1) and experimental (1')
   population; (an increase in perinuclear
   granularity can be seen in these cells)
Figure 37. Comparison of M.D.B.K. and endothelial cell viability and attachment with flow. \((E)/(C)\) is the ratio of the experimental (E) to control (C) cell percentage viability and attachment (with respect to given cultures at start of flow).
M.D.B.K

1. = $10^{-3}$ dyne/cm$^2$
2. = $2.5 \times 10^2$ dyne/cm$^2$

ENDOTHELIUM

3. = $10^3$ dyne/cm$^2$
4. = $10^2$ dyne/cm$^2$
5. = $10^1$ dyne/cm$^2$

HOURS AFTER START OF FLOW
Table 6. Distribution of tests: Experimental design in short duration, high shear stress flows

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Wall shear stress magnitude</th>
<th>Number of tests</th>
<th>Number of colonies studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.D.B.K.</td>
<td>1.0 dyne/cm²</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>10 dynes/cm²</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>1.0 dyne/cm²</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10 dynes/cm²</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>
Figure 38. M.D.B.K. cells subjected to separating (a-c) and deformable (d) flows

a. 20 hour cells
   no flow
   (total magnification 380X)

b. Flow from left to right for 30 minutes, wall shear stress equal to 1.0 dyne/cm². Arrows indicate cellular retraction

c. Flow stopped; arrows indicate cellular retraction (fine filamentous extensions of cells are still attached to glass in region of arrows)

d. 81 hour M.D.B.K. cells subjected to deformable flow (0.7 dyne/cm² from left to right) (total magnification 200 times)
   A. outline of colony and nuclear positions before flow
   B. new position of colony after 30 minutes of flow (arrows indicate nuclear movement)
Figure 39. Cellular division during high shear stress flow (M.D.B.K.). Successful mitosis is shown at 1.5 dynes/cm², flow from left to right. 'm' indicates mitotic cell and 'g' indicates intercellular gap formation. (Total magnification approximately 200 times.)

Figure 40. Mechanical failure of cells subjected to high shear stresses (M.D.B.K.). Flow from left to right, exerting shear stress of 15.4 dynes/cm². (Total magnification approximately 80 times. 'p.n.' indicates perinuclear regions of cells; 'e.b.' indicates original cell margins; and 's' indicates streaking of adhering cytoplasm in direction of flow.)
Figure 41. M.D.B.K. cells subjected to deformable shear stresses. Flow from left to right as indicated on television screen. Shear stress magnitude 1.0 dyne/cm². (Total magnification equal to 175 times.)

- **a.** Cells at $T_0$, no flow
  - 'm' indicates mitotic figure

- **b.** Cells at $T_0 + 10$ minutes
  - Note cellular withdrawal and deformation at arrows (1')
  - Line indicates 200 microns

- **c.** Cells at $T_0 + 17.5$ minutes
  - Note successful mitoses, 'm'
  - Tight adhesion at pinocytotic edges of cells, (2')
  - Disturbed flow around dividing cells

- **d.** Cells at $T_0 + 25$ minutes
  - Arrows show further cell retraction (1''), as compared to (1')

- **e.** Cells at $T_0 + 30$ minutes
  - Arrows (3') show retraction of nonpinocytic borders

- **f.** Cells after flow stopped for 15 minutes
  - Note reattachment of nonpinocytic borders to preconditioned glass substrate (4')
Figure 42. Biological separation of intact cells. M.D.B.K. cells subjected to 10 dynes/cm² in direction shown on television screen. (Total magnification equal to 175 times)

a. Cell colonies at T₀ (no flow)

b. Cells at T₀ + 10 minutes.
   Cell (1') at top of lower left hand colony separating from glass. Note attachments remain to neighbors

c. Cells at T₀ + 11 minutes.
   Outline of 1' indicated by dashed lines

d. Cells at T₀ + 11.5 minutes.
   Cells (2') at bottom of upper right hand colony beginning to separate from glass, while other cell (1') still adheres lower colony

e. Cells at T₀ + 13.5 minutes.
   Both cells swept way by shear stress of 10 dynes/cm²
Figure 43. Profile views of cells under flow (total magnification equal to 230 times)
(The contact angles 1 and t have been reversed in labeling these figures.)

a. M.D.B.K. cells
   no flow
   dashed lines approximate contact angles of cell 1

b. Flow from right to left, 10 minutes
   1.0 dyne/cm^2 (note 10 micron movement of central body of cell 1' as shown and difference between leading 1" and trailing t" contact angles)

c. New cell grouping.
   1.0 dyne/cm^2 from left to right (note contact angle differences)

d. M.D.B.K. cells after flow
Table 7. Comparison of M.D.B.K. cell and endothelial cell response to short term, high shear stress flows

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Shear stress magnitude</th>
<th>Per cent cell in population relative to population at start of flow ($T_0$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.D.B.K.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 dynes/cm$^2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$T_0 + 15$ minutes</td>
<td>41%</td>
</tr>
<tr>
<td></td>
<td>$T_0 + 30$ minutes</td>
<td>39%</td>
</tr>
<tr>
<td></td>
<td>$T_0 + 45$ minutes</td>
<td>37%</td>
</tr>
<tr>
<td></td>
<td>$T_0 + 60$ minutes</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td>$T_0 + 75$ minutes</td>
<td>35%</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 dynes/cm$^2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$T_0 + 1$ hour</td>
<td>93%</td>
</tr>
<tr>
<td></td>
<td>$T_0 + 3$ hours</td>
<td>87.5%</td>
</tr>
<tr>
<td></td>
<td>$T_0 + 6$ hours</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>10 dynes/cm$^2$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$T_0 + 6$ minutes</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>$T_0 + 12$ minutes</td>
<td>89%</td>
</tr>
<tr>
<td></td>
<td>$T_0 + 18$ minutes</td>
<td>81%</td>
</tr>
</tbody>
</table>
Figure 44. Endothelial cells subjected to short term, high shear stress flow. Wall shear stress equal to 10 dynes/cm². (Very little change in cellular detail was noticeable in endothelial cells subjected to shear stresses of this magnitude.) (Total magnification approximately 33 times)
$\tau_w = 10$ dynes/cm$^2$
<table>
<thead>
<tr>
<th>Order of magnitude of wall shear stress (dynes/cm²) at which alteration first noted</th>
<th>Cellular alterations due to steady, laminar flow (M.D.B.K. cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-3}$</td>
<td>Transient gap formation between cells</td>
</tr>
<tr>
<td></td>
<td>Perinuclear granules appear and subsequently disperse in cytoplasm</td>
</tr>
<tr>
<td></td>
<td>Decrease in cellular radius of curvature</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>Flow influences net colony growth patterns (net colony migration)</td>
</tr>
<tr>
<td></td>
<td>Cytoplasmic deformation</td>
</tr>
<tr>
<td></td>
<td>Cessation of pinocytosis at leading margins of cells</td>
</tr>
<tr>
<td>1.0</td>
<td>Cellular retraction from substrate</td>
</tr>
<tr>
<td>10</td>
<td>Withdrawal of nonpinocytotic margins</td>
</tr>
<tr>
<td></td>
<td>Cellular separation from substrate</td>
</tr>
<tr>
<td></td>
<td>Mechanical disruption of cells</td>
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</tbody>
</table>
LITERATURE CITED


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APPENDIX A: LOCALIZATION AND DISTRIBUTION OF ARTERIAL LESIONS

Examples of the localization and distribution of arteriosclerotic and atherosclerotic lesions are shown in Figures A-1 and A-2 respectively.

Figure A-1 represents a radiograph demonstrating the distribution and severity of calcified lesions in the iliac bifurcation and the aortic arch in an elderly human male*. When the arterial wall becomes sufficiently mineralized, the location of these arteriosclerotic lesions can be seen with X-rays. The distribution of the radio-opaque areas is of particular interest to the study of hemomechanics, for there appears to be a mechanical predilection for site localization of these lesions. That the flow of blood is in some way involved in the pathogenesis of these lesions is suggested by noting the following: 1) the bilaterally symmetrical distribution at the iliac bifurcation; 2) the alternating distribution at the lesser curvatures of the common iliac arteries; and, 3) the involvement of the lesser curvature of the aortic arch. As stated in the introduction, very little is known about the in vivo hemomechanics or the role by which the physical parameters in the blood flow actually interact with the biological processes in the adjacent arterial wall.

Figure A-2 demonstrates the distribution of spontaneous atheromatous plaques in a swine aorta**. Both from the aspect of plaque distribution

*Specimen courtesy of the Department of Anatomy, University of Wisconsin Medical School, Madison, Wisconsin.

**Specimen courtesy of Dr. Bernard Skold and the Department of Veterinary Anatomy gerontology project, Iowa State University. Pig # 25, 600 pound boar, 5 years.
and the cellular composition of the lesions, spontaneous atheroma in swine has been shown to be a good model of human atherosclerosis (197, 198). This aorta has been stained with Sudan IV to visualize the intimal lipid deposits. Significantly, there are three variations of plaquation present: 1) The curved aortic arch shows discrete linear atheromas, and in the thoracic aorta these discrete plaques are confined to the entrances of the small intercostal arteries; 2) Beyond the diaphragm a more diffuse, severe type of plaquation is present; 3) The iliac bifurcations show a discrete, heavy plaquation at the entrance lip and along the lateral sides of the branching.

The similarity of the winging morphology of the lesions at the entrance regions indicated (a') is interesting. Localization of these lesions at vascular branches has been attributed to the presence of vasa vasorum at the orifices in the larger arteries of the bovine (199). However, capillaries are not normally seen in the inner one-third of the human aorta, except when pre-existing, age related, intimal changes also occur (200). Thus, intimal capillaries may simply represent a normal vascular reaction to a pre-existing irritant.

Local hemomechanical flow conditions could provoke such an irritation. Mechanical shear stresses (32) and alterations of diffusive transport through the avascular portion of the arterial wall (44) may represent an adequate stimulus. Both Figures A-1 and A-2 suggest that site distribution in the arterial tree and spread of lesions within the arterial wall are related to a coupling of hemomechanical factors, flow mediated transport in the arterial wall, and pre-existing anatomical considerations.
Figure A-1. Distribution of arteriosclerotic lesions. X-ray of human iliac bifurcation (left) and aortic arch (right). Reduced to approximately one-half actual size.

Figure A-2. Distribution of atherosclerotic lesions. Swine thoracic and abdominal aorta stained with Sudan IV. Reduced to approximately one-half life size.
APPENDIX B: REACTION OF THE ARTERIAL WALL
TO A SPECIFIC MECHANICAL IRRITANT

Observations on aging phenomena in the arterial intima (76,77) and speculation about the role of the intimal cells in the localization of atherogenesis (21,22) both stress the need for more fundamental knowledge about the normal cellular biological reaction of the arterial wall to specific mechanical irritants.

The predictable distribution of mechanical stresses that results from flow through a stenosis can be used to analyze the response of the arterial intima to variations in wall shear stresses. If an artery is surgically banded such that its luminal cross sectional area is chronically reduced, the increased velocity of the blood flowing through the narrowed region will exert greater than normal wall shear stresses upon the adjacent intima. The bending and resultant compressive and tensile stresses at the stenosis can be expected to be confined within the arterial media (32). If the stenosis represents a sufficient reduction in cross sectional area a complex distribution of wall shear stresses will occur through the stenosis as a result of the phenomena of flow separation and poststenotic turbulence (28,38).

Figure B-1 represents a longitudinal section of a canine common carotid artery in which a chronic stenosis, estimated to represent approximately an 85% reduction in cross sectional area, was induced. The artery was removed three weeks from the date of the implantation of the stenosis. With such a geometry, at the estimated physiological flow rates, the
lateral wall pressure might be expected to decrease approximately four times whereas the wall shear stress would be expected to increase 19 times. With an 85% reduction in cross sectional area, and at these flow rates, flow separation might be expected to develop. The point where the wall shear stress is effectively zero would occur slightly distal to the point of maximum stenosis (28). This indicates that a high shear stress spatial gradient existed in this region. Of particular interest in Figure B-1 is the localization of a circumscribed intimal cushion just distal to the point of maximum narrowing.

Figure B-2 is a photomicrograph of the cellular elements within the circumscribed intimal hyperplasia. Of particular interest is the fact that the endothelium overlying the region appears to be continuous and intact, although some swelling may be noted by careful inspection of the nuclei (a silver nitrate stain and a surface view would have removed any ambiguities about the continuity of the endothelial membrane). It is unknown whether shear stresses and gradients of shear stress through this nonphysiological stenosis exist at other normal vascular geometries. The origin of the subendothelial cellular elements in this region of intimal hyperplasia is uncertain. They may have been derived from smooth muscle cells migrating from the stresses media, from the endothelial cells replicating in response to the mechanical stresses, or from pre-existing subintimal cell types such as fibroblasts. The nuclei do not appear fibroblastic, however.

The artery shown in Figure B-2 represents one of four such chronic stenoses implanted in dogs. Although it remained in situ for the longest
time, it was the only one in which the arterial lumen was patent. The results reiterate the fundamental problem of *in vivo* experiments in which the arterial geometry is altered to produce changes in blood flow: It is difficult to differentiate between intimal responses to altered fluid mechanics and arterial responses to the implant and surgical manipulation. *In vitro* analysis can eliminate many of these potential ambiguities when dealing with a relatively complex organ such as the arterial wall.
Figure B-1. Diagram of implanted stenosis in canine common carotid artery

Figure B-2, a&b. Cellular elements in stenotic intimal hyperplasia, Hematoxylin and Eosin stain (total magnification approximately 400 times)
flow

0.3 cm

100 microns
APPENDIX C: FLOW BETWEEN INFINITE PARALLEL PLATES

Since the flow of tissue culture medium in the cell culture flow channels used in this research approximates flow between infinite parallel plates, the appropriate fluid mechanics of flow in this geometry will be reviewed as follows:

A. Demonstration of parabolic velocity profile

The geometry of the flow channels can be diagrammed as below with the appropriate coordinate system as shown.

\[ u = u(x, y, z) \]

The Navier-Stokes equations which completely describe flow can be written in rectangular coordinates as follows:

For the X direction

\[
\frac{\delta u}{\delta t} + u \frac{\delta u}{\delta x} + v \frac{\delta u}{\delta y} + w \frac{\delta u}{\delta z} = - \frac{1}{\rho} \frac{\delta P}{\delta x} + X + \mu/\rho \left[ \frac{\delta^2 u}{\delta x^2} + \frac{\delta^2 u}{\delta y^2} + \frac{\delta^2 u}{\delta z^2} \right] \]

C-1

For the Y direction

\[
\frac{\delta v}{\delta t} + u \frac{\delta v}{\delta x} + v \frac{\delta v}{\delta y} + w \frac{\delta v}{\delta z} = - \frac{1}{\rho} \frac{\delta P}{\delta y} + Y + \mu/\rho \left[ \frac{\delta^2 v}{\delta x^2} + \frac{\delta^2 v}{\delta y^2} + \frac{\delta^2 v}{\delta z^2} \right] \]

C-2
For the Z direction

\[
\frac{\delta w}{\delta t} + u \frac{\delta w}{\delta x} + v \frac{\delta w}{\delta y} + w \frac{\delta w}{\delta z} = - \frac{1}{\rho} \overline{\frac{\delta P}{\delta z}} + Z + \mu \rho \left[ \frac{\delta^2 w}{\delta x^2} + \frac{\delta^2 w}{\delta y^2} + \frac{\delta^2 w}{\delta z^2} \right]
\]

C-3

where \( u, v, \) and \( w \) are the fluid velocities in the \( x, y, \) and \( z \) coordinate directions respectively; \( \rho \) is the density of the fluid; \( \overline{P} \) the pressure; \( t \) is time, and \( X, Y, \) and \( Z \) are the body forces in the direction of respective coordinates.

If it is considered that the flow is steady the accelerative terms \( \frac{\delta u}{\delta t}, \frac{\delta v}{\delta t}, \) and \( \frac{\delta w}{\delta t} \) are equivalent to zero. Furthermore, if it is assumed that the plates are very large (infinite) such that the boundaries at \( z \) and \( x \) do not influence the flow and that the plates are perfectly parallel, then \( v \) and \( w \) are zero and the flow is by definition uniform. Since \( v \) and \( w \) are equal to zero, all the terms in C-1, C-2, and C-3 containing \( v \) and \( w \) are similarly zero.

From the differential form of the continuity equation written in rectangular coordinates

i.e. \( \frac{\delta u}{\delta x} + \frac{\delta v}{\delta y} + \frac{\delta w}{\delta z} = 0 \)

It is seen that \( \frac{\delta u}{\delta x} \) must also be zero.

A further assumption is made that the fluid at the boundary adheres to the boundary so that at \( y = \pm h, \) \( u = 0. \) From these considerations it can be seen that \( u \) is only a function of \( y, \) (i.e. \( u = f(y) \)). The only body force is due to gravity (i.e. \( Y = -g \) and \( X = Z = 0 ). \)
Therefore, with the assumption of steady, uniform, laminar flow in infinite parallel plates with no slip at the boundary, equations C-1, C-2, C-3 can be rewritten as follows:

For the X direction

$$0 = -\frac{1}{\rho} \frac{dP}{dx} + \frac{\mu}{\rho} \frac{d^2u}{dy^2}$$

C-4

For the Y direction

$$0 = -\frac{1}{\rho} \frac{dP}{dy} - g$$

C-5

For the Z direction

$$0 = -\frac{1}{\rho} \frac{dP}{dz}$$

C-6

From equation C-4 and C-6 it can be seen that the pressure, P, is only a function of x and y. Integrating equation C-5 yields the expression

$$-P = \rho(y) + f(x)$$

so that at a given y, \( \frac{dP}{dx} \) is at best only a function of x and is actually equal to a constant.

Equation C-4 can be integrated twice with respect to y as follows:

$$\frac{d^2u}{dy^2} = -\frac{1}{\mu} \frac{dP}{dx}$$

C-4

$$\int \left( \frac{du}{dy} \right) dy = -\int_{\mu} \frac{dP}{dx} dy$$
\[
\frac{du}{dy} = -\frac{1}{\mu} \frac{dP}{dx} y + C_1
\]

\[
u = -\frac{1}{\mu} \frac{dP}{dx} \frac{y^2}{2} + C_1 y + C_2 \tag{C-7}
\]

The constants \( C_1 \) and \( C_2 \) are then evaluated.

Assuming a symmetrical velocity distribution, at \( y = 0 \), \( \frac{du}{dy} = 0 \).

\[
\frac{du}{dy} = -\frac{1}{\mu} \frac{dP}{dx} (0) + C_1 = 0
\]

and at \( y = h \), \( u = 0 \) due to no slip condition at the wall,

\[
0 = -\frac{1}{\mu} \frac{dP}{dx} \frac{h^2}{2} + C_2 \]

\[
C_2 = -\frac{1}{\mu} \frac{dP}{dx} \frac{h^2}{2} \tag{C-8}
\]

Therefore, combining equations C-7 and C-8, it can be seen that for steady, laminar flow of a Newtonian fluid between infinite parallel plates a parabolic velocity distribution is expected and can be expressed as

\[
u = \frac{1}{2\mu} \frac{dP}{dx} [y^2 - h^2] \tag{C-9}
\]

B. Expression of wall shear stress in terms of the experimental parameters used in study

The flow rate, \( Q \), per unit width of the flow channel can be expressed as

\[
\eta / \nu = -h \int_{h}^{y} u \, dy \tag{C-10}
\]
Combining equation C-9 with C-10 and performing the integration yields
\[ Q/w = \frac{2}{3\mu} \frac{dP}{dx} h^3 \]  
C-11

Solving equation C-11 for \( \frac{dP}{dx} \) and substituting into equation C-9 yields the desired expression of velocity, \( u \), in terms of position, chamber dimensions, and flow rate:
\[ u = \frac{3}{4} \frac{Q}{w} \frac{y^2}{h^3} - \frac{3}{4} \frac{Q}{wh} \]  
C-12

For a Newtonian fluid, the shear stress, \( \tau \), in a parallel plate channel is given by the following expression:
\[ \tau = \mu \frac{du}{dy} \]  
C-13

Differentiating equation C-12 with respect to \( y \), and evaluating the resulting expression for the shear stress at the wall (\( \tau_w \) at \( y = h \)) gives the desired relationship between the experimental variables as shown in C-14.
\[ \frac{du}{dy} = (2) \frac{3}{4} \frac{Q}{w} (y) \frac{1}{h^3} \]
\[ \frac{du}{dy} \bigg|_{h} = \frac{3}{2} \frac{Q}{wh^2} \]
\[ \tau_w = \mu \frac{du}{dy} = \mu \frac{3}{2} \frac{Q}{wh^2} \]
\[ \tau_w = \mu \frac{3}{2} \frac{Q}{wh^2} \]  
C-14
APPENDIX D: METHODS

Umbilical Cord Endothelial Cell Culture Monolayer*

I. Equipment required for method.

<table>
<thead>
<tr>
<th>Sterile</th>
<th>Nonsterile</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 clamps-hemostats</td>
<td>arterial clamp</td>
</tr>
<tr>
<td>2 sterile sponges (gauze)</td>
<td>umbilical tape</td>
</tr>
<tr>
<td>scissors</td>
<td></td>
</tr>
<tr>
<td>infusion needle</td>
<td></td>
</tr>
<tr>
<td>4 long 15 gauge needles</td>
<td></td>
</tr>
<tr>
<td>100 ml syringe</td>
<td></td>
</tr>
<tr>
<td>2 30 cc disposable syringes</td>
<td></td>
</tr>
<tr>
<td>50 cc syringe</td>
<td></td>
</tr>
<tr>
<td>2 centrifuge tubes</td>
<td></td>
</tr>
</tbody>
</table>

Solutions (pre warmed to 37°C)

- 100 ml sterile C.M.F.-P.B.S.
- 10-15 ml sterile 0.25% Trypsin in CMF-PBS
- 100 ml Hanks solution
- 20 ml growth medium, sterile
  (M. E. M., 20% Calf Serum, Antibiotics)

II. Procedure for harvesting endothelial cells from vein.

A. The cord was stored aseptically at 4°C no longer than 4 hours.

B. An end was secured with a sterile clamp and hung on a support.

C. All blood was sponge-wiped clean, and the clamped ends were cut and discarded with sterile scissors. The vein was reclamped at the proximal, freshly cut end with a sterile hemostat. The cord was resuspended vertically on the support.

D. Using sterile forceps, the infusion needle was inserted into the umbilical vein with a sterile three-way valve and momentarily secured with artery clamp. Umbilical tape ligatures were then placed above and below the protrusion on the infusion needle.

E. The distal end of cord was cut, all venous clots were gently expressed. The vein was not squeezed closed such that the opposing sides stuck together and rendered infusion difficult.

*Procedure adapted from reference 108.
F. 100 ml CMF-PBS was slowly perfused through the vein to wash out remaining blood. It was easiest to clamp the 100 cc syringe to the support and suspend the cord in this manner. The CMF-PBS was drained through the distal, open end.

G. The distal effluent end of the cord was clamped off or, preferably, tied off with umbilical tape.

H. 10-15 ml of 0.25% Trypsin solution was syringed into proximal end, the valve closed and recapped.

I. The cord was placed in the original 8 ounce container, covered with Hank's solution, and incubated for 45 minutes at 37°C.

J. With a sterile 50 cc syringe loaded with 20 ml of growth medium, the contents of the vein were emptied through the inlet valve. The cell suspension obtained was equally distributed into two sterile centrifuge tubes after depositing a drop on a hemocytometer for counting. The centrifuge tubes were recapped.

K. The cell suspension was spun at 500 rpm for 15 minutes, and the supernatant was decanted and discarded.

L. The cells were resuspended by repipetting with the appropriate volume of growth medium into a sterile syringe.

M. The cell suspension was injected into the chambers.

For confluency in a chamber of 1 mm in depth, a volume sufficient to bring final cell suspension concentration to $4 - 6 \times 10^6$ cells/ml was used.

**Comments**

It is claimed that the endothelial cell population should become confluent in 12 to 24 hours, that confluency is not accomplished by cell replication, and that the cells should adhere within three hours (108). But these claims were never met in experience of this study where much longer periods were recorded for these events.
Reagents

I. Calcium & Magnesium Free Phosphate Buffered Saline. (CMF-PBS)*

A. 10 X Stock Sol'n

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na Cl</td>
<td>80.0 gm</td>
<td>Dissolve in 1000 ml distilled H₂O.</td>
</tr>
<tr>
<td>K Cl</td>
<td>3.0 gm</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.73 gm</td>
<td></td>
</tr>
<tr>
<td>K H₂PO₄</td>
<td>0.20 gm</td>
<td></td>
</tr>
<tr>
<td>Glucose (Dextrose)</td>
<td>20.0 gm</td>
<td></td>
</tr>
</tbody>
</table>

B. Working Solution

Dilute 1 in 10 with distilled H₂O, (1 ml 10 X CMF-PBS + 9 ml distilled H₂O)

C. (1-X) 150 ml aliquots are dispensed and autoclaved for shelf storage.

II. Trypsin Solution. (0.25%)

A. Place 2.5 gm trypsin** in clean beaker. Add 2 – 3 ml of 1 X CMF-PBS and make into a paste.

B. Dissolve in 900 ml of 1 X CMF-PBS stir continuously until essentially all of trypsin is dissolved.

C. Make up to a final volume of 1000 ml with 1 X CMF-PBS and filter to sterilize.

*Modified from reference 201, p. 240.

** Trypsin 1:250. Difco Laboratories, Detroit, Michigan.
III. Hanks Balanced Salt Solution. (HBSS)

A. Premixed*

5 X solution stock:

1. Add package contents to 1 liter sterile distilled H₂O.
2. Refrigerate, or autoclave.
3. For use dilute 5 X stock 1 parts to 4 parts distilled H₂O; dispense and autoclave or refrigerate.
4. Add 1 tube sterile NaHCO₃ solution to 500 ml 1 X solution. Color turns red at pH = 7.4.
5. Use

B. OR, Home made. See reference 201, p. 214.

IV. Silver nitrate stain for intercellular cement**.

The following staining procedure was used to demonstrate the presence of intercellular cement once the cell cultures or the umbilical cords had been washed with saline and then 5% glucose.

A. 0.4% AgNO₃ (Silver Nitrate) for 2 minutes
B. 5% Glucose, three changes
C. 3% Cobalt Bromide + 1% Ammonium Bromide for one minute
D. 5% Glucose, three changes
E. The cells were then fixed with 4% formaldehyde solution and exposed to a bright light for 8 hours to develop the silver lines.

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**Adapted from reference 64.