SHEAR STRESS INDUCED STIMULATION OF MAMMALIAN CELL METABOLISM

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Abstract

A flow apparatus has been developed for the study of the metabolic response of anchorage-dependent cells to a wide range of steady and pulsatile shear stresses under well-controlled conditions. Human umbilical vein endothelial cell monolayers were subjected to steady shear stresses of up to 24 dynes/cm^2, and the production of prostacyclin was determined. The onset of flow led to a burst in prostacyclin production which decayed to a long-term steady state rate (SSR). The SSR of cells exposed to flow was greater than the basal release level, and increased linearly with increasing shear stress. This study demonstrates that shear stress in certain ranges may not be detrimental to mammalian cell metabolism. In fact, throughout the range of shear stresses studied, metabolite production is maximized by maximizing shear stress.

Introduction and Approach

One of the major challenges to the scale-up of mammalian cell culture is often stated to be the detrimental effects of shear stress on cell viability. However, mammalian cells exhibit a wide range of shear sensitivity with respect to the stress level required to cause overt lysis and death. Some recent evidence suggests, that at lower levels, shear stress may have beneficial effects on cellular metabolism (1) and possibly viability. Since cells in most bioreactor configurations will invariably be subjected to some degree of fluid mechanical stresses, a clear understanding of the effects of shear stress on cellular metabolism will be required for optimal design and operation of mammalian cell reactors. This problem may be best approached by studying cell metabolism under conditions of well-characterized shear stress. To this end, we have developed a flow apparatus capable of subjecting cultured anchorage-dependent cells to a wide range of steady and pulsatile shear stresses for long time periods.

The cells used in this study are primary human umbilical vein endothelial cells. Endothelial cells form a multifunctional lining of the intimal surface of blood vessels. This lining is continuously subjected to both steady and oscillatory fluid shear stresses, resulting from the flow of blood in the circulation. Since they are the only anchorage-dependent cells that are normally physiologically exposed to shear stress, they may provide a useful model for cells in a bioreactor.

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One function of endothelial cells that appears to be modulated by shear stress is the metabolism of arachidonic acid (1, 2). Arachidonic acid is an essential polyunsaturated fatty acid stored in its esterified form in the cell membrane phospholipids. It is liberated from phospholipids by the action of a phospholipase (Figure 1). In umbilical vein endothelial cells, the major metabolite of arachidonic acid is prostaglandin I_2 (PGI$_2$) or prostacyclin, which is produced by the cyclooxygenase pathway (Figure 1). Prostacyclin is a strong vasodilator and the most potent endogenously generated inhibitor of platelet aggregation. It has important biological activities in nanamolar concentrations.

Previously, we had demonstrated that shear stresses of 10 dynes/cm^2 stimulate the production of prostacyclin in cultured endothelial cells (1). Oscillatory shear stress (one Hertz) with the same mean value enhanced the production rate even further. In the present study, the flow apparatus we have developed for the study of the response of cultured anchorage-dependent cells to fluid shear stress is described in detail. In addition, the earlier data (1) are extended to demonstrate the effects of a wide range of steady shear stress on the production of prostacyclin by primary human umbilical vein endothelial cells.

Human umbilical vein endothelial cells were harvested from umbilical cords by means of culture procedures adapted from Gimbrone (3). For removal of the endothelial cells, the veins were cannulated, rinsed with 100 ml of phosphate-buffered saline (PBS; Gibco, Grand Island, NY), filled with 0.03% collagenase enzyme (Cooper Biomedical, Malvern, PA) in Medium 199 (Gibco, Grand Island, NY), and incubated for 30 minutes at room temperature. After incubation, the enzyme solution was flushed through the vessel with 100 ml of PBS, and the effluent was collected and centrifuged at 650 x g for 10 minutes. The cell pellet was resuspended in Medium 199 supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 200 U/ml penicillin, 200 µg/ml streptomycin (Gibco, Chagrin Falls, OH), and 300 µg/ml glutamine (Gibco). The cell suspension was plated onto glass slides (75 x 38 mm; Fisher Scientific, Pittsburgh, PA). The glass slides were pretreated with 0.5M NaOH for two to three hours and rinsed with distilled water, thereby enhancing cell adhesion by conferring a charge on the glass surface. Two slides were seeded per cord (5.0 x 10^4 to 1.0 x 10^5 cells per slide). Cultures became confluent after three or four days and flow loop experiments were run three days after the cultures reached confluency.

The apparatus consists of two reservoirs, situated one above the other, with a parallel-plate flow chamber positioned between them (Figure 2). Flow is driven through the chamber by the hydrostatic pressure head created by the vertical distance between the upper and lower reservoirs. The pressure head is maintained constant by continuous pumping of culture medium from the lower to upper reservoirs at rates in excess of that flowing through the chamber. The excess drains down the glass overflow manifold (O.D. 19mm, Pyrex, Corning Glass Works, Corning, NY), which also serves to facilitate gas exchange with the medium. The upper and lower reservoirs are blown of glass, while the interconnecting tubing consists of Teflon PFE (O.D. 0.125 in., Cole Parmer, Chicago, IL), except for the section through the roller pump which is silicone (Masterflex, Cole Parmer, Chicago, IL). Silicone

collars join the reservoirs to the manifold and tubing. The relatively inert and gas impermeable teflon tubing prevents water and gas loss and minimizes adsorption of cell metabolites.

The flow chamber consists of a machine-milled polycarbonate plate, a rectangular Silastic (size 0.020 in., Dow Corning, Midland, MI) gasket, and the glass slide with the attached endothelial cell monolayer (Figure 3). These were held together by a vacuum maintained at the periphery of the slide, forming a channel of parallel-plate geometry. The use of vacuum insured an uniform channel depth. The depth of the channel formed was normally 220 μm, and the area of cells exposed to shear was 16 cm². For studies of cell metabolism, it is usually important to minimize loop fluid volume and maximize cell surface area. The polycarbonate plate has two manifolds through which medium enters and exists the channel. The entry port is larger than the exit port and serves as a bubble trap. A valve opposite the entry port allows for the removal of the bubbles.

All loop parts are washed in Haemo-sol (Baltimore, MD), rinsed in deionized water, and oven-dried, then autoclaved. The loop parts are allowed to cool, assembled under a laminar flow hood, and reautoclaved. Medium is added to the top reservoir (10 - 20 ml), filling the bottom reservoir as well, and flooding the chamber. Then the slide with the cultured cells is inverted over the flooded chamber, and clamped. Care must be taken to avoid entrapment of air bubbles in flow channel.

During an experiment, the circuit components are placed in a 37°C air curtain incubator system and the reservoirs are connected to a gassing system that maintains a humidified atmosphere of 95% air and 5% CO_2.

The wall shear stress on the cell monolayer in the flow chamber may be calculated using the momentum balance for a Newtonian fluid and assuming parallel-plate geometry:

$$\tau = \frac{6Q\mu}{bh^2} \qquad (1)$$

where

Q = flow rate cm^3/sec

μ = viscosity, about 0.01 dyne-sec/cm^2

h = channel height, 0.022 cm

b = slit width, 2.5 cm

τ = wall shear stress, dynes/cm^2

The mean residence time of medium in the flow chamber and the tubing between reservoirs for the experiments performed ranged from 5-30 seconds. Therefore, in time course studies of metabolite production, sampling from the lower reservoir contributes a temporal error of the order of less than one minute.

The flow rate was controlled by adjusting the relative distance between the two reservoirs by changing the length of the overlow manifold tubing. The flow rates were monitored by an electromagnetic flow probe (Zepeda Instruments, Seattle, WA). The Reynolds number of the flow through the chamber is given by:

$$Re = \frac{Uh\rho}{\mu}$$

$$= \frac{Q\rho}{\mu b} \qquad (2)$$

where U is the characteristic or <u>mean</u> average flow velocity, ρ is the density of the medium, and μ is the viscosity of the medium. Combining equations (1) and (2),

$$Re = \tau(\frac{h^2\rho}{6\mu^2}) \qquad (3)$$

The value of the quantity in the parenthesis for the apparatus is 0.807. For the range of shear stresses used in the present study, the Reynolds number varied from 0 to 20, indicating that fluid flow through the chamber was laminar. Because of the large aspect ratio (b/h) and low Reynolds number found in the flow chamber, the above equation is valid for nearly all of the monolayer surface except very near the lateral edges of the flow chamber.

Schlichting (4) gives an estimate of the entry length (ℓ) for plane Poiseuille flow as:

$$\ell = .04 \text{ (h) Re} \qquad (4)$$

For our maximum Reynolds number of 20 and a channel height of .022 cm, the entry length is approximately .018 cm. Since the channel length was 6.4 cm, the entry length was negligible for these experiments.

In the present study, cell monolayers were subjected to shear stress for seven to eight hours. Samples were withdrawn very 5 to 30 minutes, and the flow circuit was simultaneously replenished with fresh medium to maintain a constant circulating volume of 20 ml. Sample and replenishing volumes were both 1 ml. At the end of each experiment, the monolayers were photographed and cells were harvested and counted with a hemocytometer to determine the total count. Cells were harvested enzymatically by treating the attached cells with trypsin-EDTA (1 mg/ml porcine trypsin, 0.2 mg/ml EDTA without Ca^{2+} and Mg^{2+}; Gibco, Grand Island, NY) for three to five minutes, centrifuging the cell suspension at 650 x g for 10 minutes, and resuspending the pellet in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution (Irvine Scientific, Santa Ana, CA). Viability was determined by the ability of cells to exclude trypan blue dye (Gibco, Grand Island, NY). The viable count was used to normalize the prostacyclin production rates with respect to cell number. To eliminate variations due to individual donors and monolayer age, replicate cultures were used. Here, cell monolayers were grown from cells seeded at identical density from the same pool of donor cords and utilized concurrently. Cell monolayers were exposed to steady shear

stresses of 6, 16, and 24 dynes/cm^2 and sampling was done simultaneously. This covers the range of physiological interest for time average wall shear stresses in arterial vessels. In experiments designed to control for the flow chamber conditions, cell monolayers were perfused by a syringe pump at a near-zero shear stress (0.016 dynes/cm^2). The outflow medium was collected for several hours to determine the basal release rate.

Prostacyclin production in the experiments was assayed in samples by measuring levels of immunoreactivity to the stable nonenzymatic breakdown product of prostacyclin, 6-keto-prostaglandin $F_{1\alpha}$ (Figure 1). Before assaying, the samples were incubated at 37°C for half to one hour, to ensure hydrolysis of all prostacyclin to 6-keto-prostaglandin $F_{1\alpha}$. The assay sensitivity was 5 pg/0.1 ml, and cross-reactivity of the antibody with other culture media components and prostaglandins was negligible.

The radioimmunoassay buffer used for dilutions of antibody and tracer, and for the charcoal suspension, consisted of 0.05 M Tris-HCl, pH 7.5, and 0.1% gelatin (Sigma Chemical Co., St. Louis, MO). The solution was heated slightly to dissolve the gelatin. The radioimmunoassay was performed by incubating 0.1 ml of 6-keto-prostaglandin $F_{1\alpha}$ standards (Sigma Chemical Co. St. Louis, MO) or samples with 0.2 ml of diluted antibody and 0.1 ml of ^{3}H -labelled 6-keto-prostaglandin $F_{1\alpha}$ (10,000 cpm) (New England Nuclear, Boston, MA), in polypropylene tubes (Sarstedt, W. Germany) and vortexing. The standard curve was constructed from standards ranging in concentration from 5 pg/0.1 ml to 500 pg/0.1 ml, which were diluted in radioimmunoassay buffer. The efficiency of adsorbtion of free antigen by charcoal was determined by the blank tubes, which contained 0.1 ml tracer and 0.3 ml buffer. Each standard and sample was run in triplicate. Quality control was monitored by placing known standards (100 pg/0.1 ml) of 6-keto-PGF$_{1\alpha}$ at intermediate points amongst the samples to be assayed. The incubation was carried out at 4°C for 16 hours. Separation of the bound and free antigen was achieved using gamma-globulin-coated charcoal. The charcoal suspension was prepared by dissolving 1 gm of bovine gamma-globulin (Sigma Chemical Co., St. Louis, MO) and 3 gm of Norit A charcoal (Sigma Chemical Co., St. Louis, MO) in 100 ml of radioimmunoassay buffer. The supernatant obtained after centrifugation was counted in a liquid scintillation counter, in the presence of 10 ml liquid scintillation fluid (Hydrofluor, National Diagnostics).

Cumulative production of prostacyclin was determined by performing a mass balance over the flow loop, taking into account samples withdrawn and medium replenished. By means of a Marquardt nonlinear regression analysis utilizing the statistical software package SAS, measurements of cumulative production of prostacyclin were fitted to a four-parameter equation:

$$P_c = A + B.(1 - \exp(-T/C)) + D.T \qquad (5)$$

where P_c is the cumulative production of prostacyclin, T is time, and A, B, C, and D are the parameters to be determined. By taking the time

derivative of Equation (5), a smooth expression for the rate of production (P) as a function of time is obtained:

$$P = B/C \exp (-T/C) + D \qquad (6)$$

The parameters calculated by the regression analysis are related to the kinetics of the rate of production. The parameter D represents the steady-state or long-term production rate (SSR), and C was a time constant for the rapidity of decay of the initial burst in production of prostacyclin. Furthermore, given the length of the sampling interval, the peak production rate can be expressed as the rate of production at time zero (B/C + D).

Results and Discussion

Cell monolayers perfused at a near-zero shear stress of 0.016 dynes/cm^2 produced 11 $\pm$ 0.5 pg PGI$_2$/10^6 cells/min (n=3). This production rate of prostacyclin is essentially the same as that seen for tissue culture (no flow) controls of human umbilical vein endothelial cells. Figure 4 depicts the cumulative production and production rate of a typical set of experiments where replicate endothelial cell monolayers were exposed to shear stresses of 6, 16, and 24 dynes/cm^2. The onset of flow led to a sudden increase in production of prostacyclin which decreased to a constant or steady state rate (SSR) within several minutes (Figure 4). The SSR increased with increasing shear stress (Table 1). The SSR of cells subjected to 24 dynes/cm^2 were significantly greater than the SSR of cells subjected to 6 dynes/cm^2 (p < 0.01; two-tailed t-test). All values of the SSR are greatly different from the tissue culture (low or no flow) controls. In Figure 5, SSR is plotted as a function of the steady shear stress imposed. The SSR/stress response is essentially linear in this range of shear stress, with a slope of 4.9 pg/10^6 cells/min/dyne/cm^2 (corr = 0.93). It should be noted that this range of shear stresses covers the time average estimates of shear stresses normally seen in the human vascular system.

Both the peak production rate (B/C + D) and the time constant for decay of the initial burst of PGI$_2$ production (C) were not significantly affected by the magnitude of steady shear stress exposure over the range studied (up to 24 dynes/cm^2). This is shown graphically in Figure 4.

The flow apparatus described above provides a simple and inexpensive method for exposing anchorage-dependent cells to uniform wall shear stress, and has several advantages over other devices used to evaluate the effect of mechanical stress on cell function. Flow through the chamber is laminar and well characterized (5), and allows for in situ superposition of pulsaltile flow. Furthermore, the flow chamber can be mounted on an inverted microscope, allowing for continuous visualization using video microscopy.

The flow system is well-suited for analysis of the effects of shear stress on the metabolism of attached cells. This system has been used

to subject endothelial cells to shear for times in excess of five days. The excellent cell-to-volume ratio (10^6 cells/10 ml) facilitates the study of the production of metabolites by cells. Furthermore, sampling of the medium is possible without cessation or disturbance to the flow in the cell-lined parallel plate flow chamber. Since most of the apparatus is assembled of glass and teflon, there is no loss of medium due to permeation through the tubing or evaporation. This avoids problems associated with changes in the osmotic balance of the medium.

Several groups have studied shear stress effects on cultured endothelial cells using a modified cone-and-plate viscometer (6, 7). Such an apparatus gives nearby uniform shear fields when operated at moderate to low speeds. These devices can be adapted to allow continuous microscopic monitoring, though this is not easy. The disadvantages of the cone-and-plate device as currently operated are that it has a smaller cell-to-volume ratio, does not permit continuous sampling of the cell incubating medium, and has significant tissue culture medium evaporation which requires continuous infusion of fresh medium for purposes of adjusting the osmolarity.

Others (8) have used flow loop devices qualitatively similar to the one described herein. However, the fluid volumes necessary to operate these apparati are typically of the order of 100 ml, and the surface area of cells exposed to defined shear in the flow chambers has been approximately 1 cm^2. The low cell-to-volume ratio make these devices inadequate for use in most metabolic studies, since many metabolites of interest are produced at very low rates, yet have extremely important biological effects.

The data demonstrate that physiological levels of steady shear stress and the onset of shear stress dramatically stimulate prostacyclin production in cultured human endothelial cells. While step increases in shear stress led to a burst in production that leveled off within several minutes, shear stress itself exerted a sustained stimulation which lasted several hours (the duration of the experiments). This longer term stimulation of prostacyclin production rate varied linearly with increasing shear stress. Previously, we have shown that the degree of stimulation of production is a function of the pulsatility of the wall shear stress as well as its mean value (1).

The ability of cells to respond to external stimuli involves the transduction of a signal across the cell plasma membrane. One such external stimulus appears to be fluid shear stress.

Shear stress and the time-variance in shear stress presumably act on cells by perturbing the cell cytoskeleton and its membrane morphology. Time-varying or fluctuating shear stress, such as pulsatile shear and step-changes in shear, probably perturb the anchorage-dependent cell as a whole, causing membrane perturbations at the points of attachment to the cytoskeleton and the substratum. On the other hand, steady shear stress may act by amplifying the natural thermal or Brownian fluttering or rippling of the membrane (9). This flutttering has been observed in red blood cell membranes and phospholipid bilayers (9). Our earlier work (1) has shown that pulsatile stress (1 Hertz, amplitude 20% of the

mean) can lead to significantly greater production rates of PGI_2 when compared to steady stress exposure at the same mean value.

Previously, it has been demonstrated that shear-induced production of prostacyclin may be blocked by ibuprofen (1). This would indicate that the point of action of stress stimulation is at the cyclooxygenation of arachidonic acid or in preceding steps. Since it is generally believed that arachidonic acid metabolism is rate-limited by its intracellular availability (10), it is possible that shear stress acts by increasing the rate of liberation of arachidonic acid. Active secretion of prostacyclin is not likely since there are no preformed intracellular stores of prostaglandins (11).

There are several possible mechanisms by which shear stress-induced membrane perturbations could mimic a hormone/receptor interaction to stimulate cell metabolism intracellularly. Shear stress may induce increased phospholipase C activity, caused by translocation of the enzyme, increased substrate (arachidonic acid) pool availability to phospholipase C (particularly from that stored in phosphoinositols) due to shear-induced membrane movements or changes in membrane fluidity, direct activation of calcium-activated phospholipase A_2 by increased calcium ion permeability, or most probably a combination of these mechanisms.

Levels of shear stress in the range of 5 - 20 dynes/cm^2 can be seen in mammalial cell bioreactor environments (12). Release of various arachidonic acid metabolites stimulated by this stress exposure can greatly modify reactor performance. It has been shown that cell-cell communication via arachidonic acid metabolites can alter cell function, leading to degranulation and aggregation in a shear field (13). The endothelial cells used in this study are unusual for tissue cells in that they normally do live in an environment which includes fluid mechanical force exposure. Because of this, the results presented above may be limited to this cell type, and extrapolation of these findings to the metabolic response of other mammalian cells to shear stress exposure should be done with care.

Hollis and co-workers (14, 15) have observed in both in vivo and in vitro studies that shear stress stimulates increased histamine forming capacity in bovine aortic endothelial cells. If shear stress stimulates protein synthesis, there may be possible applications in mammalian cell bioreactor design. Giard and co-workers (16) observed that human fibroblasts secrete up to 30 fold greater amounts of interferon when maintained on microcarriers in spinner flasks compared to cells in roller bottles. Since the shear stresses that cells are exposed to in the spinner flasks are much higher than those in roller bottles, the increased production may be attributable to shear stress-induced stimulation of interferon synthesis.

By choosing an appropriate reactor configuration, cells may be subjected to moderate shear stresses that would stimulate synthesis of proteins, yet not cause cell detachment and subsequent loss of viability. In fact, the strength of adhesion to the substrate, in and of itself, may be increased if cells are subjected to shear stress.

Preliminary studies on endothelial cells subjected to shear in serum-free medium suggested that sheared cells exhibit increased strength of attachment and viability compared to control cultures (our laboratory, unpublished observations). Studies of these phenomena should include investigation of the effects of shear stress on the synthesis and release of adhesive proteins such as fibronection and collagen.

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Table 1: Effect of Steady Shear Stress on the Steady-State Production Rate of Replicate Cell Monolayers

Experiment	Shear	Stress	
	6 dynes/cm^2*	16 dynes/cm^2	24 dynes/cm^2
1	40	118	134
2	66	88	127
3	71	85	120
Mean ± S.D.	59 ± 16*	97 ± 18	127 ± 7*

* $p < 0.01$; two-tailed t-test

Production rates are given as pg PGI_2/10^6 cells/min. Note the very low flow or tissue culture production rate of PGI_2 was 11 pg/10^6 cells/min.

Figure Legends

Figure 1: Arachidonic acid cascade via the cyclooxygenase pathway. Arachidonic acid is liberated from cell membrane phospholipids via the action of a phospholipase. This is metabolized by a series of enzymes (complex) to the unstable endoperoxide PGH_2. In umbilical vein endothelial cells, the major pathway from PGH_2 is to PGI_2 via prostacyclin synthetase. PGI_2 is unstable and in buffers at pH 7.4 is hydrolized to 6-keto-$PGF_{1\alpha}$ with a half life of approximately 3 minutes.

Figure 2: Drawing of small volume flow loop. (1) upper reservoir, (2) lower reservoir, (3) overlow manifold, (4) filtered humidified 95% air + 5% CO_2 input, (5) gas outlet, (6) flow chamber, (7) gasket, (8) slide with cell monolayer, (9) microscope objective, (10) vacuum, (11) sampling port, (12) roller pump, (13) PFA teflon tubing, (14) constant pressure head, and (15) flow probe.

Figure 3: Parallel plate flow chamber. The polycarbonate plate, the gasket (G), and the glass slide (H) with the attached cells are held together by a vacuum (C), forming a channel of parallel plate geometry. Medium enters at entry port (A), through slit (E), into the channel, and exits through slit (F), and exit port (B). Entry port (A) also serves as a trap for bubbles, which can be removed through valve (D).

Figure 4: Effect of steady shear stress on prostacyclin production – representative set of experiments. Replicate cell monolayers were exposed to shear stresses of 6, 16, and 24 dynes/cm^2. (A) The cumulative production of prostacyclin; (B) The production rate, computed as the derivative of the fitted curves in (A).

Figure 5: Effect of steady shear stress on prostacyclin production. The steady state production rates plotted against steady shear stress. The values 6, 16, 24 dynes/cm^2 represent averaged values of 3 replicate sets of experiments (Table 1). The values for 0.016 and 10 dynes/cm^2 were obtained from data presented elsewhere (1). Errors represent the SEM.

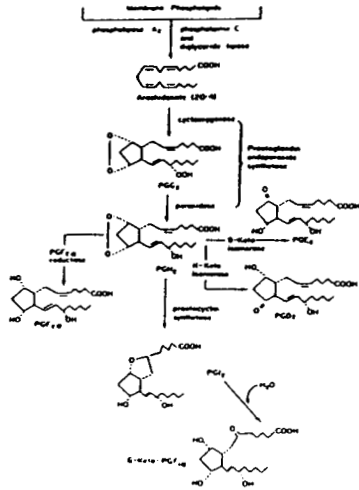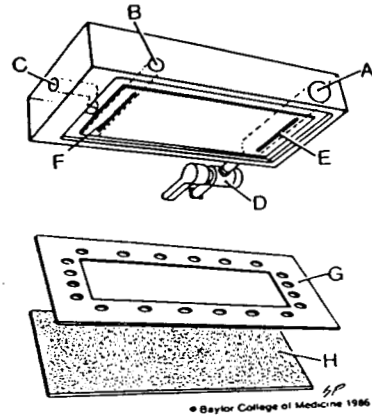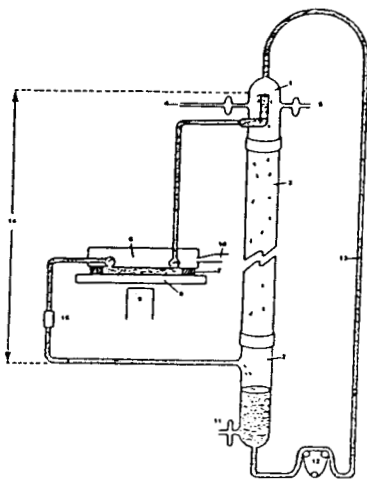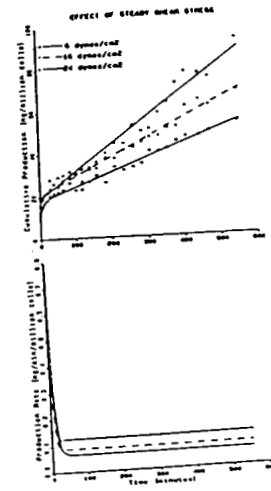# SHEAR STRESS INDUCED STIMULATION OF MAMMALIAN CELL METABOLISM

Figure 1

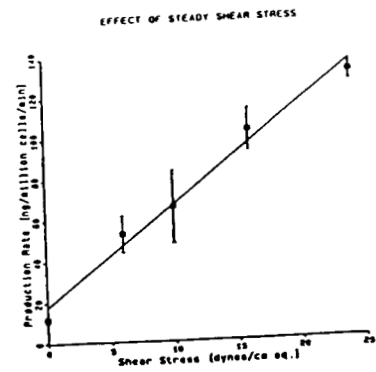Figure 3

Figure 2

Figure 4

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Figure 5