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Bubble-free Reactors and Their Development for Continuous Culture with Cell Recycle

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1. BUBBLE-FREE REACTORS—WHY?

In conventional cell-culture reactors, aeration is normally done by bubbling air or pure oxygen through the culture broth. This, of course, in protein-rich cell culture media, causes foaming; and with foam, cell flotation occurs. Phenomena such as wall growth, cell lysis, and uncontrolled release of proteases can be observed in relation to flotation. For these reasons, chemical antifoam agents are often used to prevent foaming. With culture broths containing antifoam difficulties arise in downstream processing procedures, because separation membranes and column chromatography materials are very sensitive to antifoam agents.

These problems cannot be neglected if one has to cultivate genetically manipulated mammalian cells or hybridoma for very long periods of time in continuous or perfusion cultures. Hence, there was a need for the development of a bubble-free reactor system for the continuous cultivation of such new cells both in small and large scale.

2. BUBBLE-FREE AERATION—HOW?

The simplest technique for bubble-free aeration is surface aeration, which is used in culture flasks or in multitray cultivation facilities. But for large-scale systems, surface is better supplied by immersing membranes into the culture broth, as proposed by Miltenburger 1979 (*1*) and others (*2, 3*). It was well known from blood oxygenation that silicone tubing could meet the demand for oxygen transfer capacity for artificial lungs, which have been produced as flat-plate-and-sheet membrane oxygenators or as compact units as spiral-coil membrane oxygenators (*4*). In Invitron's static maintenance reactor, silicone tubes are also used to supply oxygen and to remove CO_2 (*5*).

But since the wall of the silicone tube acts as a diffusion barrier, the surface-related oxygen transfer rate is still small and a tremendous amount of tubing is necessary to supply oxygen in such dense cell-culture systems. This disadvantage of a homogeneous membrane can partly be avoided by the use of porous membranes.

2.1. Aeration with Hydrophobic Microporous Membrane Fibres

Hydrophobic microporous membranes can be used for aeration. In particular, when the pores are filled with gas the oxygen transfer resistance across the membrane wall is less than that of a silicone wall of the same thickness. This is
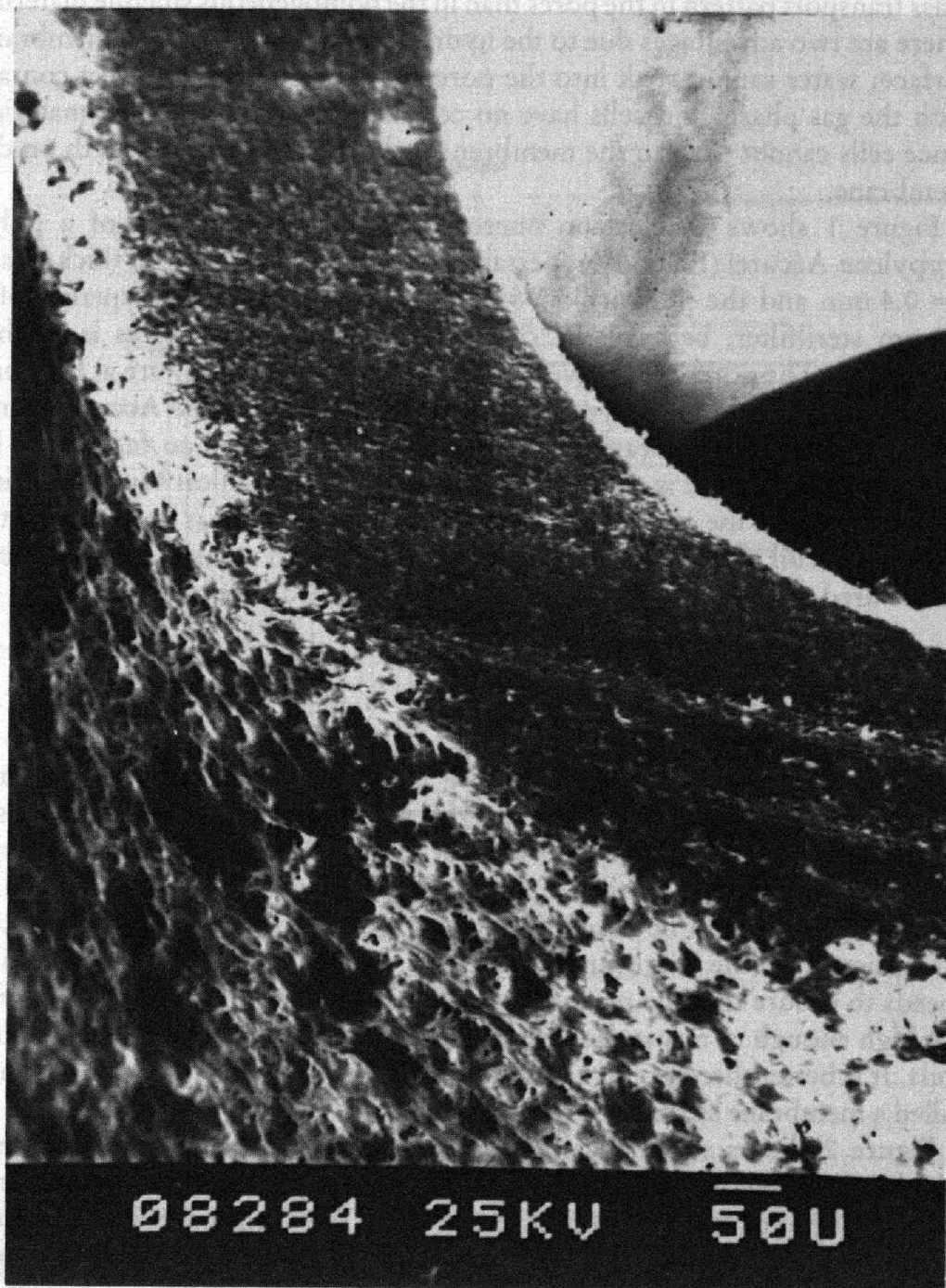
Fig. 1. Electron micrograph of a cross-section of an Accurel membrane fibre wall. Wall thickness $S = 0.4$ mm.

due to the higher diffusivity in the pore-gas-phase and to the more convective mass transport pattern in the pores than in the homogeneous silicone material. There are two advantages due to the hydrophobic property of the membrane surface: water cannot soak into the pores, so that liquid is in direct contact with the gas phase; and cells have no contact with the membrane material. Since cells cannot stick to the membrane wall, there is no cell growth on the membrane.

Figure 1 shows an electron micrograph of a cross-section of a poly-propylene Accurel (Enka, Wuppertal) membrane fibre. The wall thickness is $s = 0.4$ mm and the porosity is 75%. This membrane has the quality of a 0.2 μm sterilfilter, because the connections between the pores is of that magnitude. The pores themselves are relatively wide and they are at least one magnitude greater than the connections between them. The Accurel membrane is the best membrane for aeration purposes found so far. It can be steam-sterilized and it is rigid enough not to need mechanical support during sterilization. On the other hand, the membrane fibre itself is flexible and it is possible to coil it as a spiral coil. Hydrophilized with ethanol, Accurel fibre can be used as a microfiltration membrane (described in Section 3.2).

2.2. The Membrane Stirrer

In homogeneous suspension culture and microcarrier culture systems, gentle mixing is needed to distribute the cells and carriers homogeneously through the liquid volume. Cells and carrier should not be allowed to settle down onto the reactor bottom. On the other hand, the maximum tip speed of a stirrer plate should not exceed 14 cm s^{-1}, otherwise cell damage and cell disruption from the microcarrier can occur. It is difficult at such low stirring speeds to ensure that the reactor is well mixed and that the liquid velocity is not high enough to resuspend cells once they have settled down on the spiral coils. It is better therefore to use the spiral coil itself as stirrer. Such a stirrer is called a membrane stirrer.

Figure 2 shows schematically a reactor vessel with a membrane stirrer which is hanging on elastic silicone tubes on the top plate of the reactor. It is moved without rotation about its own axis. The membrane stirrer gently penetrates the culture broth, performing mixing and providing a uniform introduction of oxygen and desorption of CO_2 without mechanical damage to the cells.

For oxygen supply, the aeration gas streams through the fibre. To avoid bubbling, the reactor head-space has been connected to the gas input tube so that there is automatically a pressure equilibrium between the fibre input and
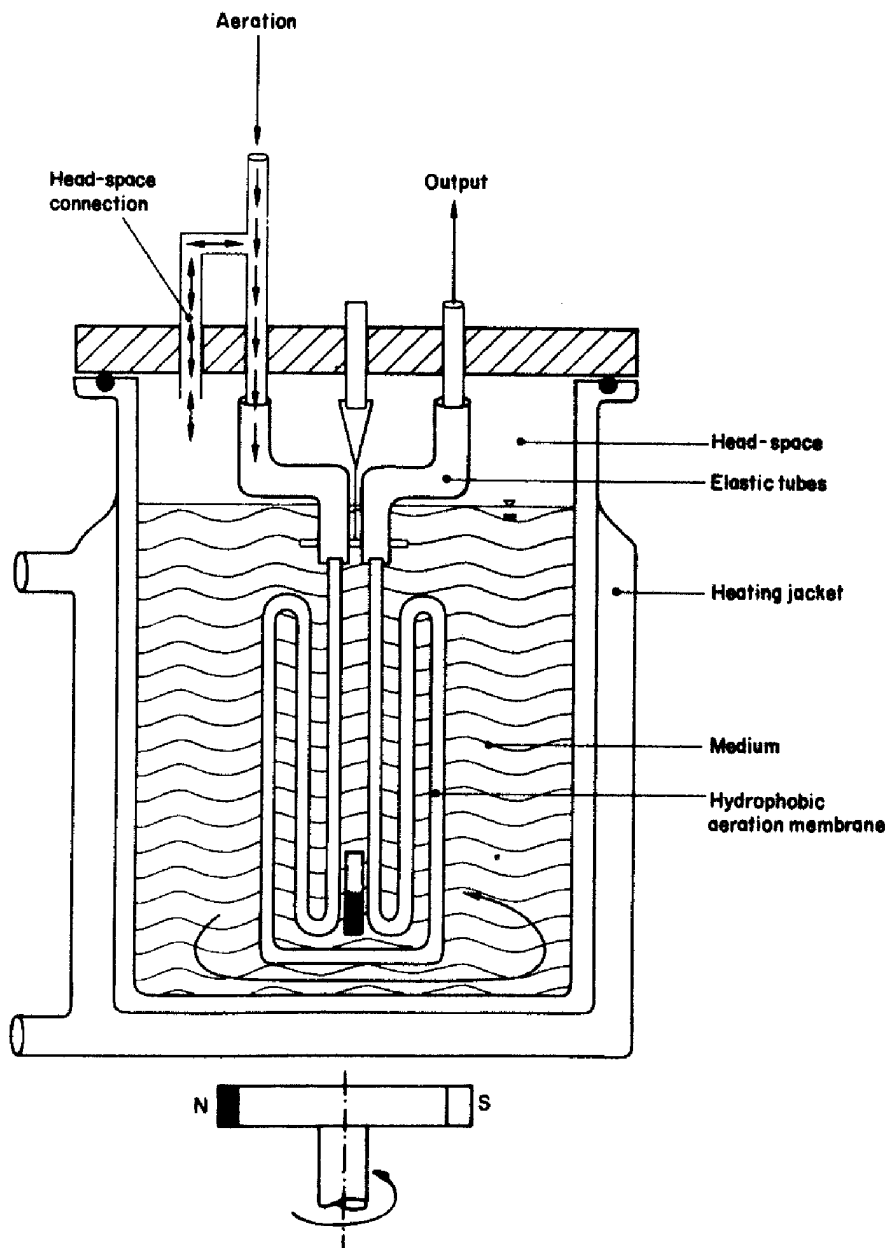
Fig. 2. Membrane stirrer reactor for bubble-free aeration with a porous membrane. The head-space is closed but connected to the gas input to achieve pressure equilibrium between the head-space and membrane. Bubbling cannot occur.
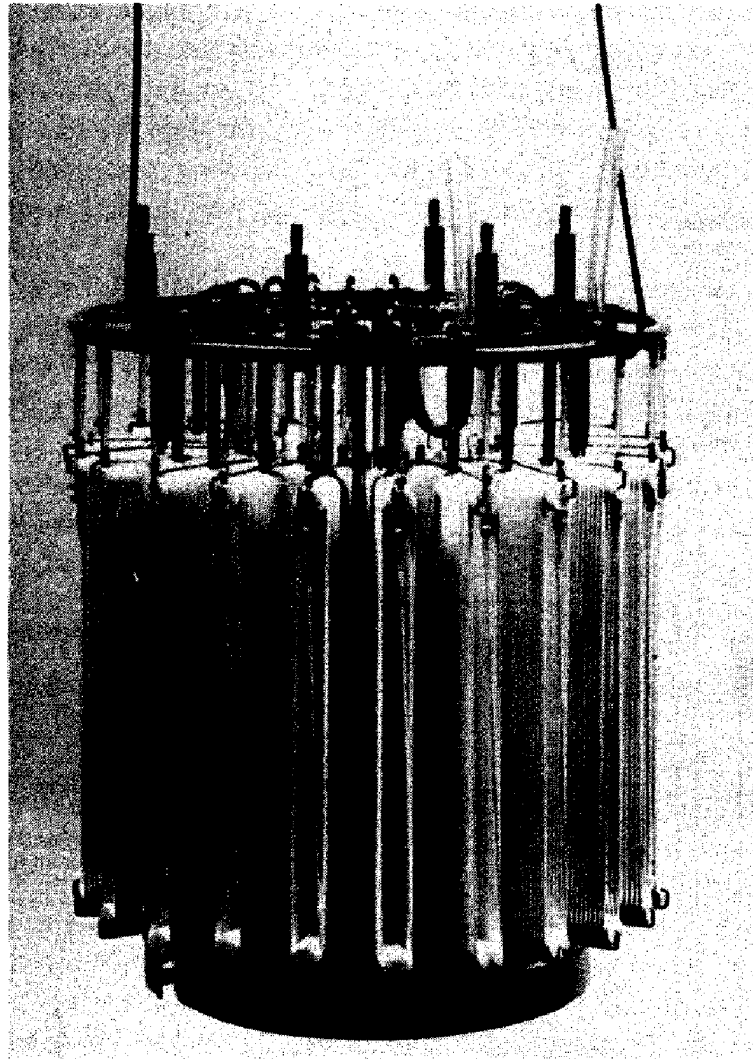
Fig. 3. Membrane-segment stirrer for a 150-litre microcarrier cell-culture reactor.

the head-space. That way the pressure in the liquid is everywhere equal to or greater than the static pressure inside the membrane fibre. Bubbles cannot come out of the pores under such conditions.

In large-scale reactors, the membrane stirrer consists not only of one membrane fibre but rather of several segments which are mounted on one support. The gas is fed parallel throughout the segments. Such a membrane segment stirrer is also moved without rotation about its own axis driven by an eccentric drive while hanging on the coverplate of the reactor. Figure 3 shows a membrane segment stirrer with 22 segments for a 150-litre microcarrier cell-culture reactor.

2.3. Oxygen Transfer Performance of Membrane Stirrers

It is obvious that the oxygen transfer rate is dependent on several parameters. The membrane area per unit volume corresponds to the length of the membrane fibre per unit volume. The gas flow through the segments can be parallel or in series and the speed of membrane movement relative to the surrounding fluid influences the boundary layer renewal.

Oxygen transfer rates have been determined for different membrane speeds, different segment connections, and different gas flow rates. The results for a 20-litre pilot segment membrane stirrer can be seen in Figs 4 and 5. From Fig. 4 one can clearly determine the relation between oxygen transfer rate (OTR) and membrane speed. The OTR is very strongly influenced by the membrane speed, so that it seems to be possible to use the agitation speed of the stirrer for mass transfer control purposes. On the other hand, Fig. 5 shows a saturation curve characteristic between the gas flow rate and OTR. Flow rates greater than 200 ml min^{-1} do not improve the OTR. This means that in the 20-litre

Inset box:
2 m Membrane / litre
Eccentricity = 20 mm
T = 310.15 K
p_K = 1113 mbar
X_{O_2} = 0.2094 mol mol^{-1}
$\dot{V}_M$ = 150 Nml min^{-1}

——— Without a Spiral
········ With a Spiral

Y-axis: OTR$_{max}$ (mg l^{-1} hr^{-1})
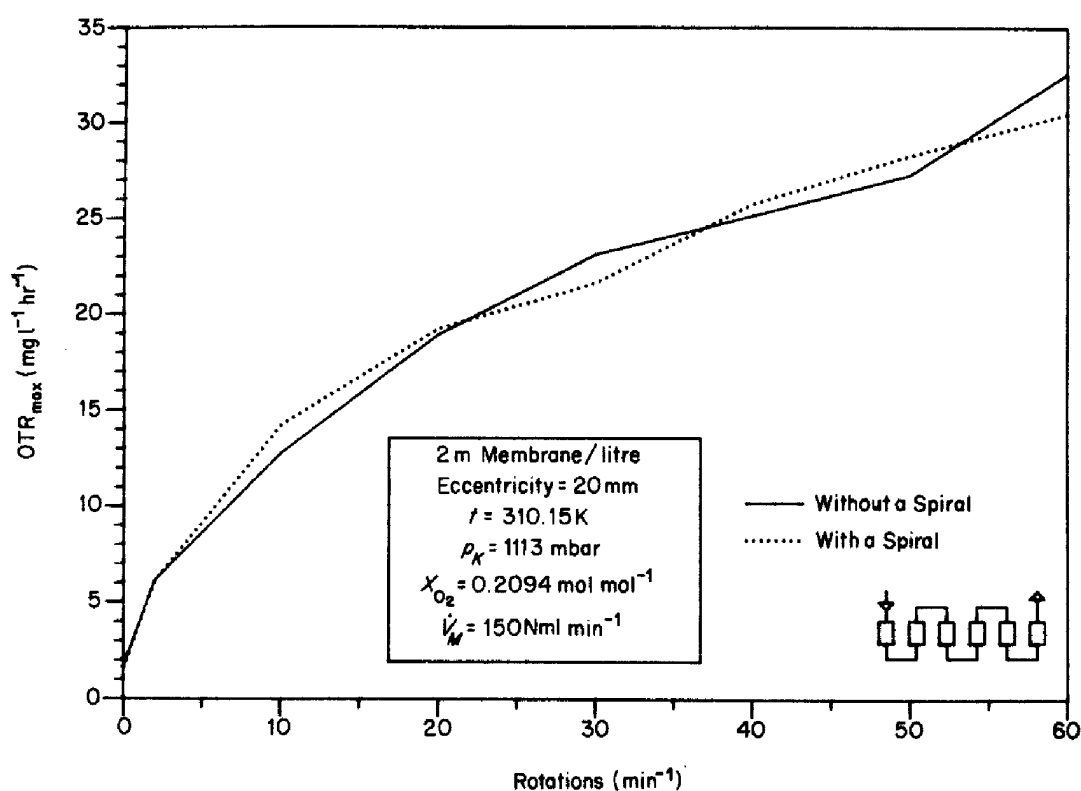X-axis: Rotations (min^{-1})

Fig. 4. Maximum oxygen transfer rate as a function of the membrane stirrer movement for a 20-litre pilot-scale reactor; p_k = head pressure; X_{O_2} = mole fraction of oxygen; V_M = aeration rate.
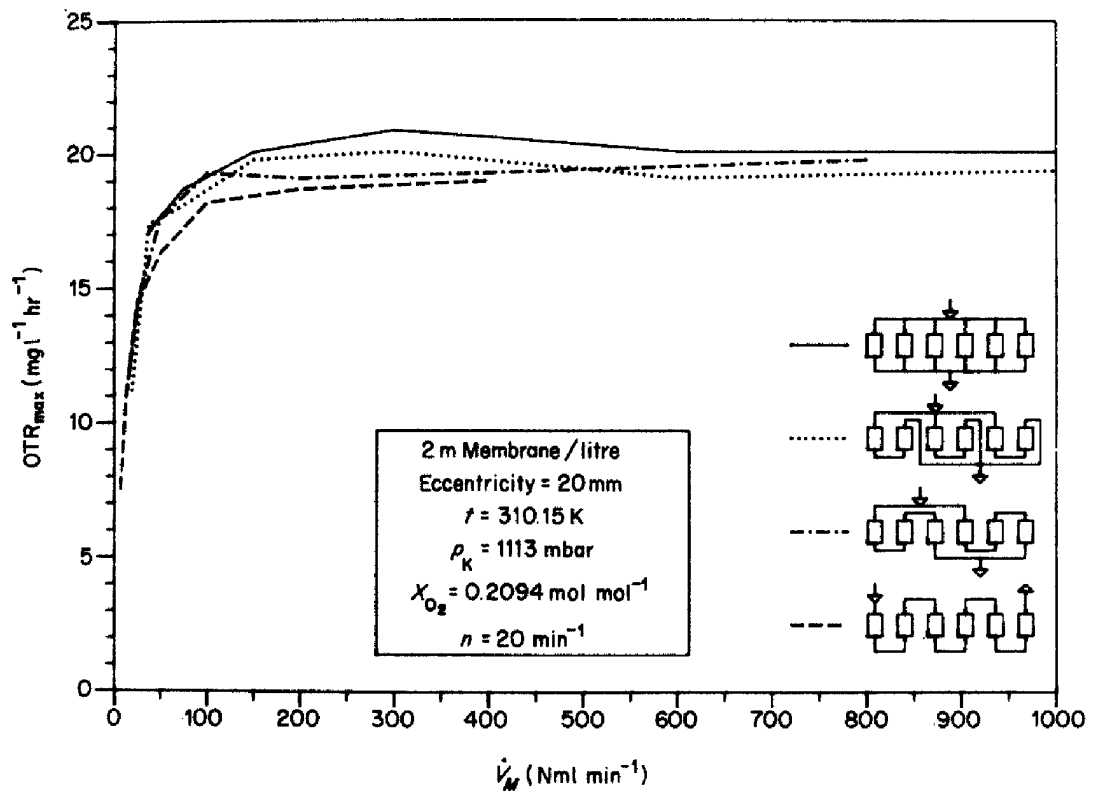
Fig. 5. Maximum oxygen transfer rate for different membrane connections as a function of the inlet gasflow into the membrane stirrer; p_K = head pressure; X_{O_2} = mole fraction of oxygen.

pilot reactor, gas flow rates above 200 ml min^{-1} are uneconomical. The gas flow rate is not a means for controlling the OTR.

The control strategy for DOT control via OTR should be to alter the oxygen concentration in the inlet gas stream.

2.4. Carrier Mixing in Membrane Stirrer Reactors

The membrane stirrer is used not only to aerate the culture broth, but also to mix it and to prevent carrier settling on the bottom of the reactor vessel. As the segment basket does not generate enough axial flow components in the liquid, a turbulence-generating spiral can be mounted beneath the segments (Fig. 6). It moves with the stirrer. The distance between the spiral and the bottom is only a few millimetres, so that strong turbulence vortexes are generated which guarantee adequate carrier mixing even at low stirrer speeds.

The microcarrier distribution has been determined for 20- and 150-litre scale reactors at different membrane stirrer speeds. It can be seen from Fig. 7
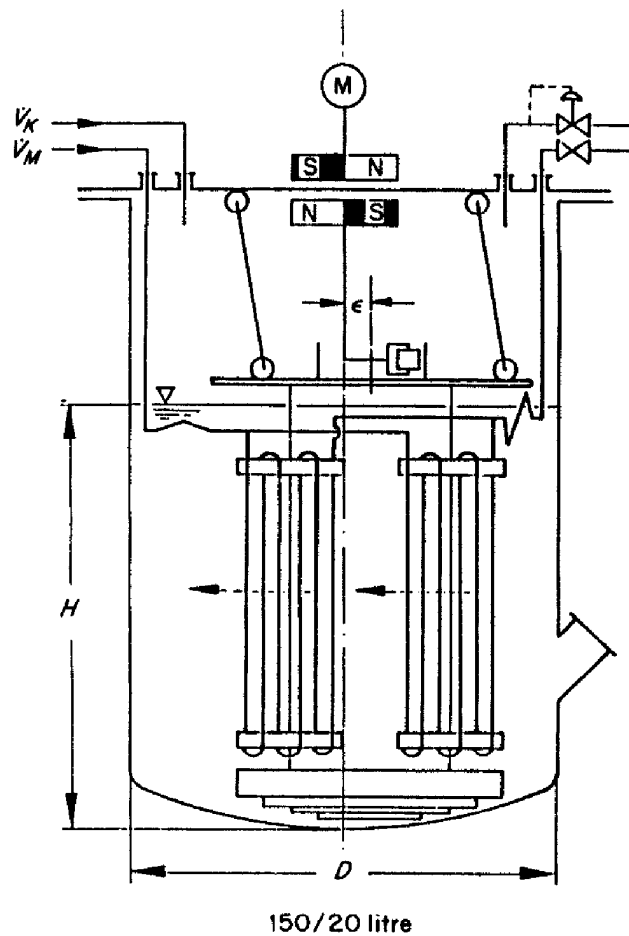
150/20 litre

Fig. 6. Membrane stirrer arrangement with turbulence spiral on the stirrer; ε = eccentricity; $\dot{V}_K$ = head-space aeration rate; $\dot{V}_M$ = membrane aeration rate.

that at stirrer speed u = 8.37 cm s^{-1} the carrier distribution already behaves nearly uniformly.

3. PERFUSION IN BUBBLE-FREE REACTORS

A long-term continuous cultivation can be done with and without cell retention. There are several advantages when cells are retained. Firstly, there is the tendency that the higher the cell density, the higher the productivity which can be achieved; secondly, the self-conditioning ability of cells, i.e. secretion of growth factors and other proteins, seems to increase with the cell density. Therefore, perfusion culture seems to be preferable to chemostat culture, where the cell density cannot be freely extended by the dilution rate.
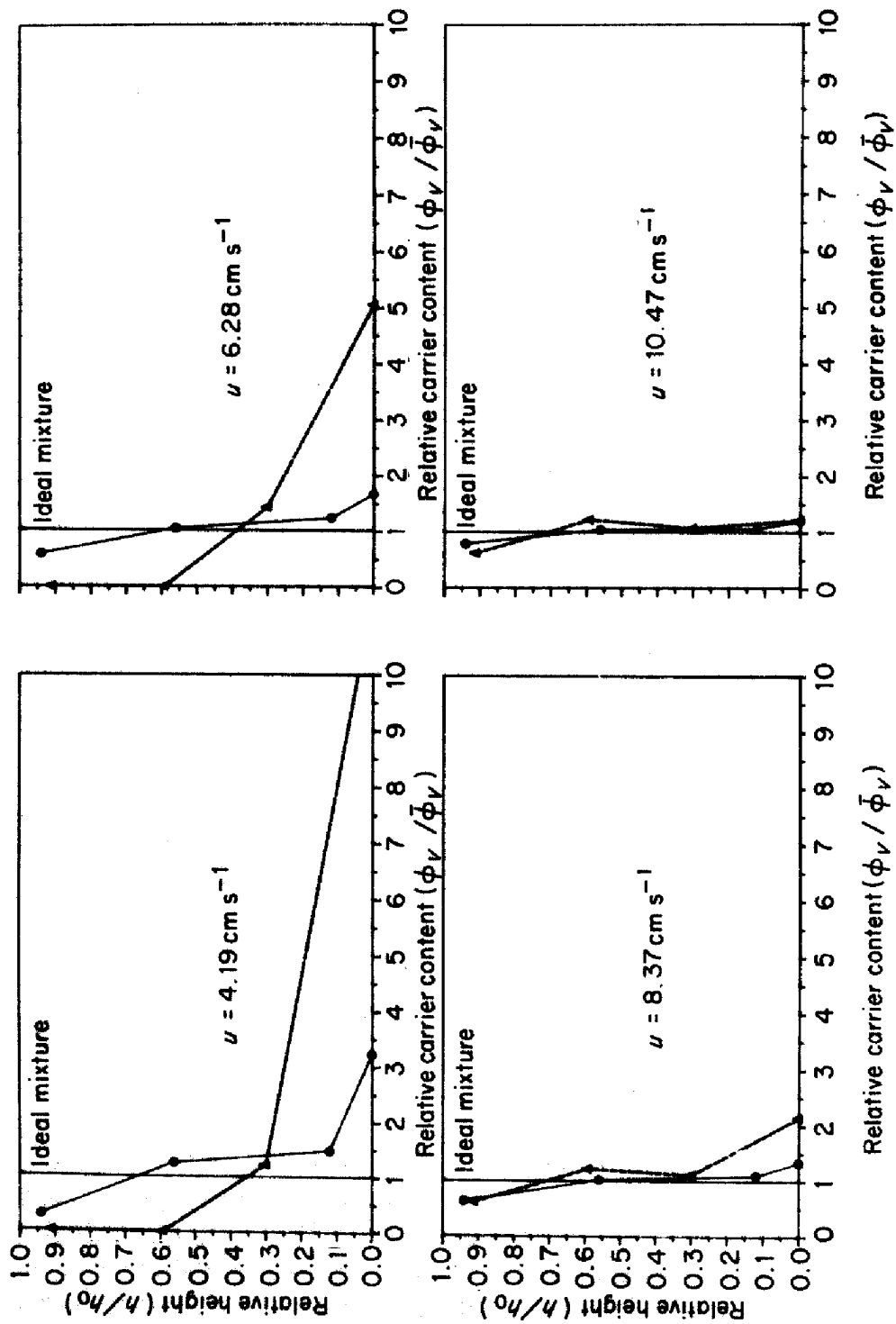
Fig. 7. Microcarrier distribution in membrane-stirrer reactors, for microcarrier Cytodex III.
u = membrane speed; circles, 20-litre Diessel reactor; triangles, 150-litre Diessel reactor.

3.1. Microcarrier Retention with Fluttering Sieve Filter

In bubble-free aeration reactors, microcarriers can be retained using a very simple device—the fluttering sieve filter. Such a filter can be mounted in the centre of a membrane segment stirrer, and because of a very loose attachment of the textile screen on its support, it can flutter. This prevents clogging of the sieve by microcarriers.

As the head-space in large reactors requires a higher pressure than the surrounding, carrier-free but cell-containing broth can easily be withdrawn from the reactor by an aseptic overflow outside the reactor, as shown in Fig. 8. If the head-space pressure is carefully controlled by a back-pressure regulator, no harvesting pump is needed to bring the broth out of the reactor. Such a system has been employed for the successful long-term cultivation of various genetically manipulated cells, e.g. Mouse L, CHO, and BHK. For production of human β-interferon with Mouse L cells, the system has been used continuously for three weeks at 20-litre scale. The dilution rate was about 1 to 2 litres per litre per day (6). Figure 9 shows the time course for such a cultivation. Very high cell density, up to 8×10^6 ml^{-1} was reached, and interferon production could be maintained even without serum in the medium fed.

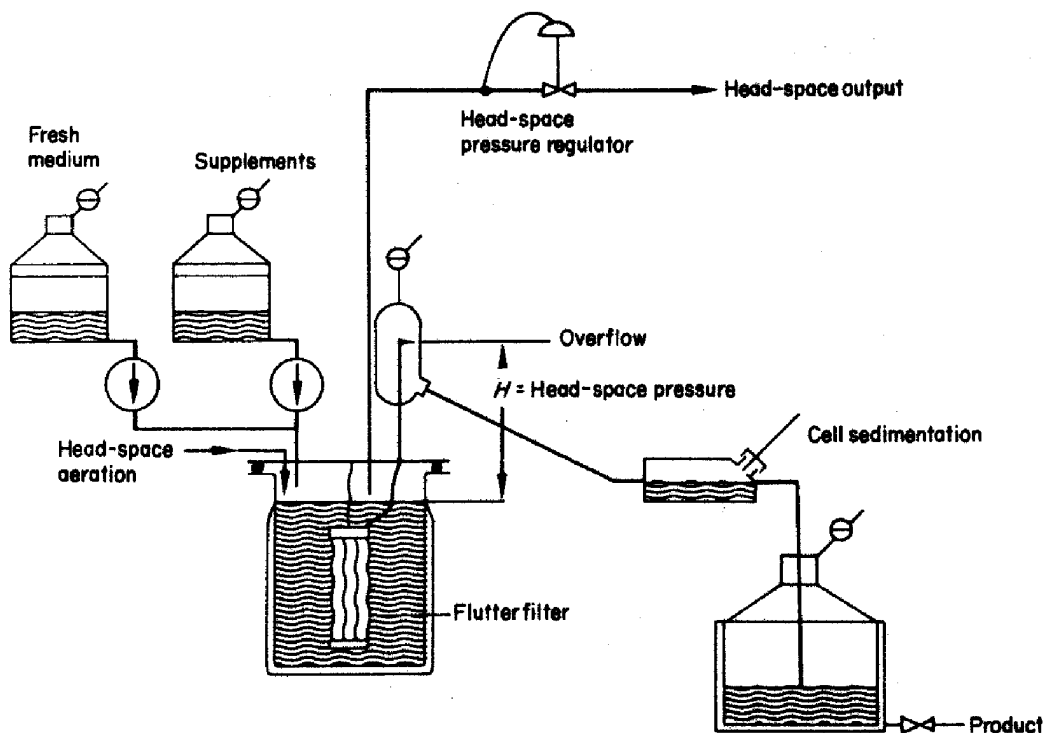**Fig. 8.** Flow scheme of a perfusion reactor system with microcarrier retention using a fluttering sieve filter device.

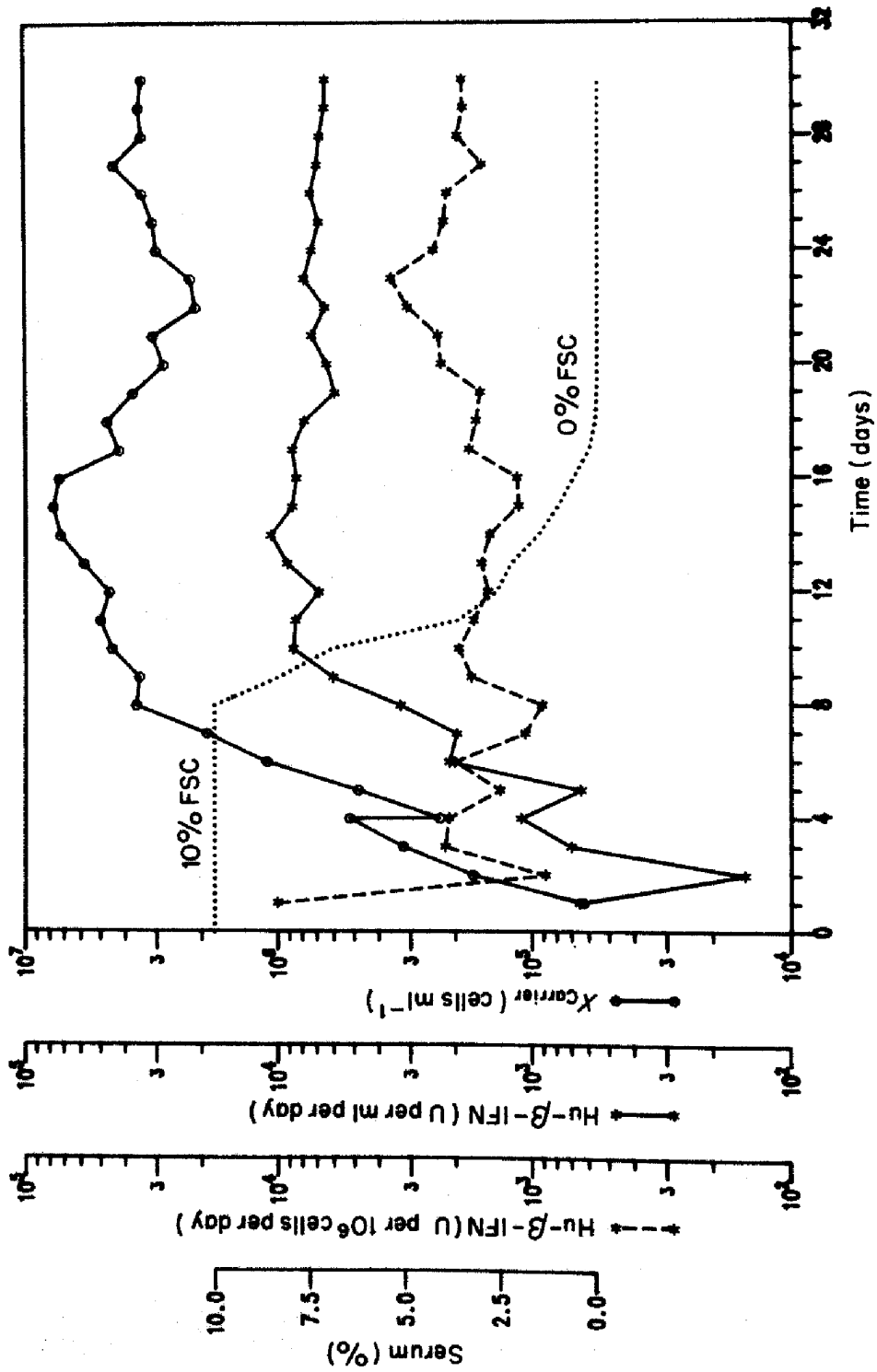**Fig. 9.** Time course of a cultivation of human β-interferon producing Mouse L cells by carrier-free perfusion using a fluttering sieve filter (6).

3.2. Cell Retention with Microporous Membrane Filters

A new technique has been developed for retaining cells in suspension cultures in which a double-membrane stirrer has been employed.

3.2.1. The Double-membrane Stirrer

To the hydrophobic membrane which has been used for aeration only there is added another membrane filter with hydrophilic character, to form the double-membrane stirrer. Figure 10 shows such a double-membrane stirrer in
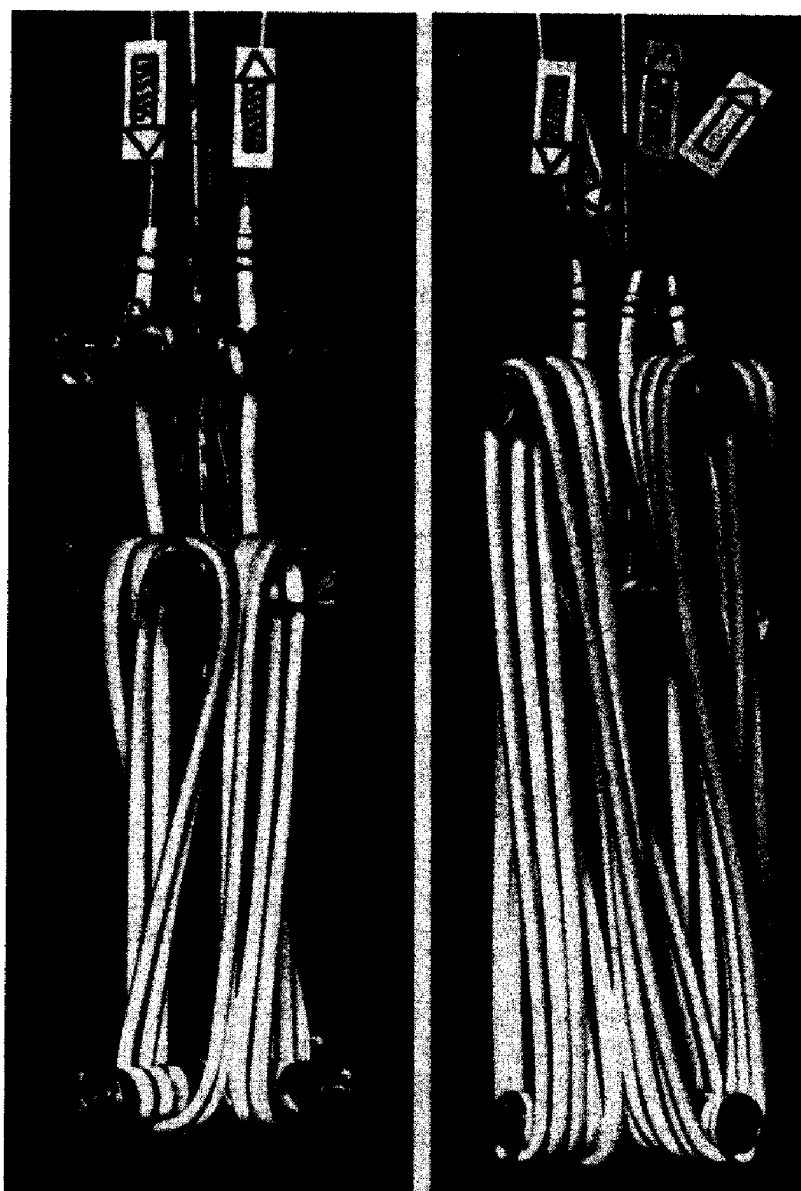

Fig. 10. The double-membrane stirrer for bubble-free aeration and cell retention for a 1-litre suspension culture reactor. Left: hydrophilized microfilter hollow fibre for perfusion. Right: hydrophobic and hydrophilic hollow fibres. ▦▷ medium ⊏▷ gas

which both membranes are made from the polypropylene Accurel hollow fibre. The left-hand side shows the microfiltration membrane which is hydrophilized with ethanol, and the right-hand side shows the stirrer with both membranes. To prevent fouling and clogging of the microfilter membrane by cells, a special procedure for medium supply and harvest, involving the membrane in both steps, has been developed.

3.2.2. The Fresh Medium Backflush Procedure

The microfiltration membrane is used for both uniform medium supply and medium filtration for cell-free harvest (see Fig. 11). During one cycle period the flow direction through the fibre wall is alternated. During the first period, the feeding period, fresh medium is pumped through the membrane into the reactor while the harvesting pump stops the spent medium output. During the second period, the harvesting period, the cell broth is filtered cell-free and withdrawn with the harvesting pump while the feeding pump stops the fresh medium input line.

In this procedure the membrane fibre is filled with fresh medium when the harvesting period commences. To make sure that the portion of fresh medium
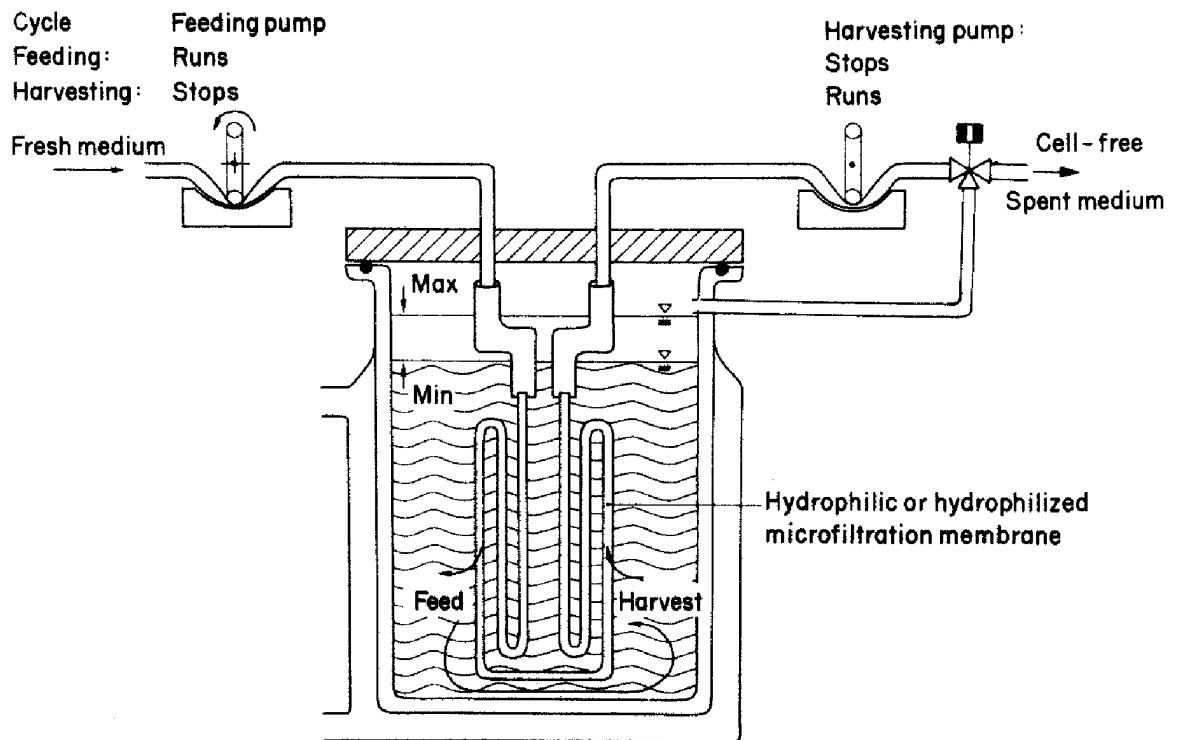

Fig. 11. Medium supply and microfiltration membrane-stirrer segments of a double-membrane stirrer. The pumps allow a two step procedure: feeding—medium is pumped into the reactor while the harvesting pump stops; harvesting—medium is pumped out of the reactor while the feed pump stops.
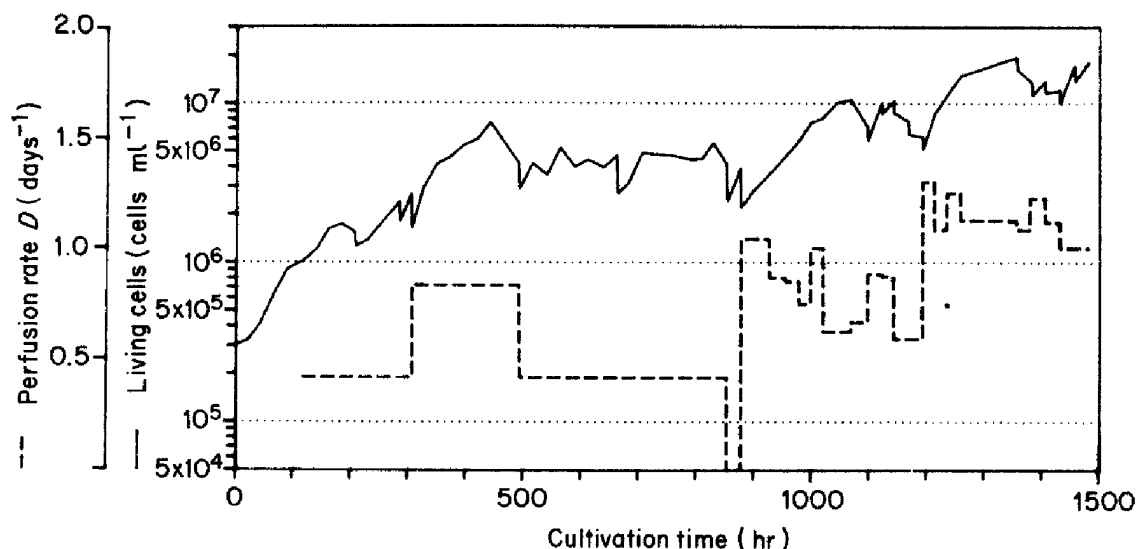
Fig. 12. Time course of a cultivation of a human B-cell line with the double-membrane stirrer for bubble-free aeration and cell-free perfusion of the medium.

is not wasted when harvesting starts, a three-way valve can be installed in the effluent line. This allows recycling of the first part of the spent medium back into the reactor until pure spent medium reaches the position of the valve. The valve is then reswitched and only spent medium will be harvested.

As the microfiltration fibre operates as a sterilization filter, the effluent is completely cell-free. It can be passed directly to any kind of purification device without further treatment.

One metre of Accurel membrane fibre per litre of volume allows a perfusion rate up to 1 litre of fresh medium per litre of broth per day. To guarantee stable long-term operation, that flux should not be extended.

A human B-cell line has been cultivated for a period of two months at cell densities up to 1.5×10^7 cells ml^{-1}, as shown in Fig. 12.

4. SCALING-UP

The new membrane stirrer reactor has been successfully tested for five months semi-continuously in repeated batch mode (7) before scaling-up to 30 litre and 150 litre volumes. For scale-up the height to diameter ratio was kept constant $H/D = 1$, and the amount of membrane surface per litre of broth was also kept constant in all scales.

The mass transfer characteristic as function of Sh (Sherwood number) over Re (Reynolds number) with d the membrane diameter and u the membrane speed has been estimated for 1- and 20-litre scale reactors. In both, we
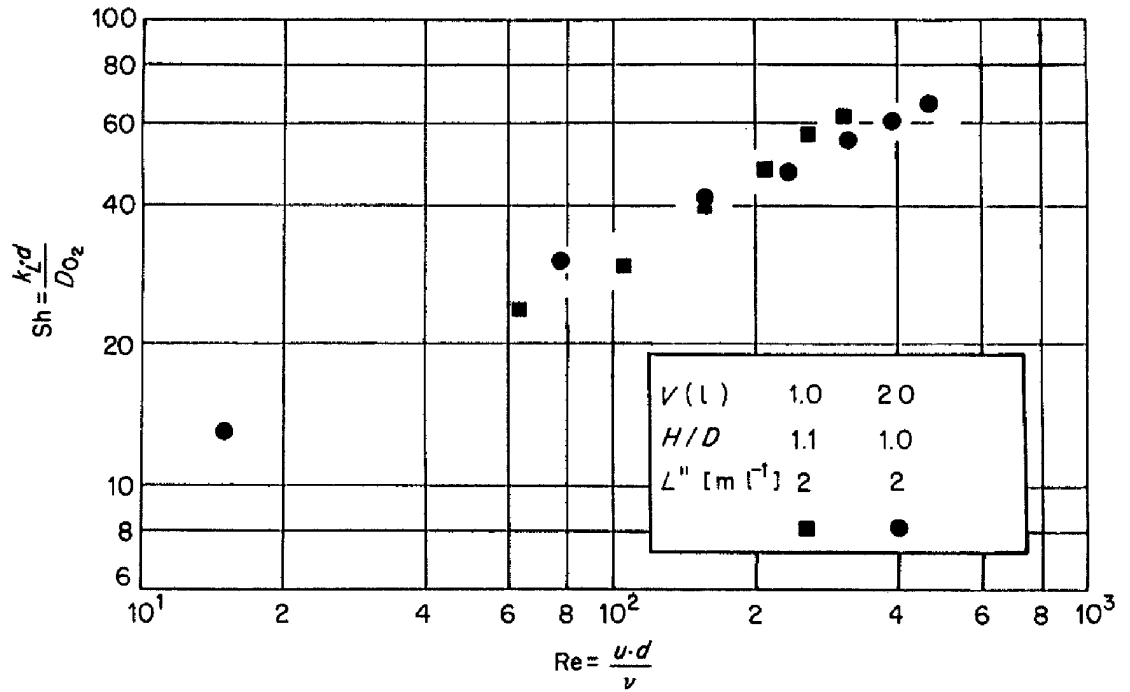
Fig. 13. Oxygen transfer characteristic for membrane-stirrer reactors of 1-litre and 20-litre scale. L^* is the length of membrane fibre per litre of broth.
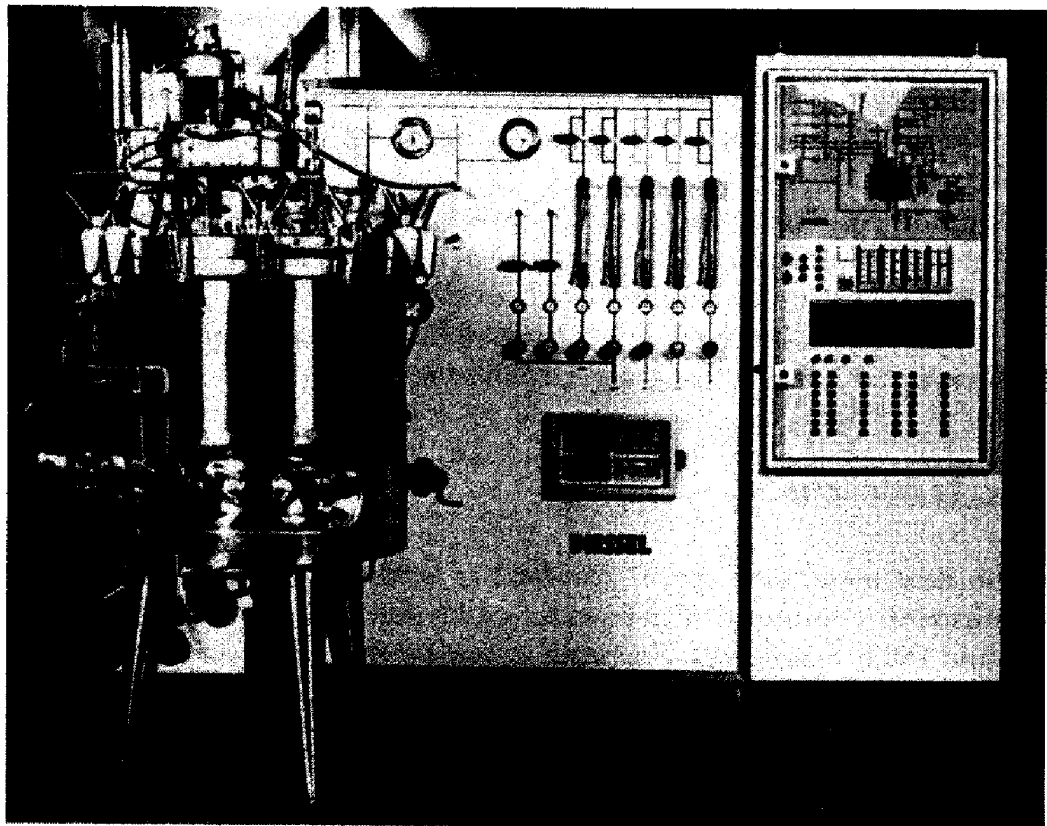

Fig. 14. The 150-litre membrane-stirrer reactor for continuous microcarrier culture.

obtained the same slope as shown in Fig. 13. The largest reactor built so far, a 150-litre reactor equipped with the 22-segment membrane stirrer, is shown in Fig. 14. The oxygen transfer rates obtained in the 150-litre reactor are slightly higher than in the smaller reactors. The reactor has not yet been fully investigated, so that total characteristic cannot be given.

5. CONCLUSIONS

The new membrane-stirrer reactor system seems to have promising qualities for long-term cultivation of genetically manipulated mammalian cells.

The trouble-free application of porous polypropylene Accurel fibres for both bubble-free aeration and perfusion of medium is a new example of successful utilization of membranes in animal cell culture technology.

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